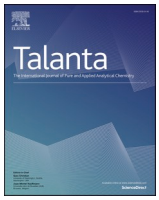
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Non-canonical CRISPR/Cas12a-based technology: A novel horizon for biosensing in nucleic acid detection

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ABSTRACT

Nucleic acids are essential biomarkers in molecular diagnostics. The CRISPR/Cas system has been widely used for nucleic acid detection. Moreover, canonical CRISPR/Cas12a based biosensors can specifically recognize and cleave target DNA, as well as single-strand DNA serving as reporter probe, which have become a super star in recent years in the field of nucleic acid detection due to its high specificity, universal programmability and simple operation. However, canonical CRISPR/Cas12a based biosensors are hard to meet the requirements of higher sensitivity, higher specificity, higher efficiency, larger target scope, easier operation, multiplexing, low cost and diversified signal reading. Then, advanced non-canonical CRISPR/Cas12a based biosensors emerge. In this review, applications of non-canonical CRISPR/Cas12a-based biosensors in nucleic acid detection are summarized. And the principles, peculiarities, performances and perspectives of these non-canonical CRISPR/Cas12a based biosensors are also discussed.

1. Introduction

Nucleic acids carry rich genetic information and are typical biomarkers for disease identification. Nucleic acid testing is widely used for the diagnosis of causal factors, food safety and environmental monitoring [1,2]. The most popular nucleic acid detection technology is polymerase chain reaction (PCR), which is considered as the gold standard, e.g., coronavirus disease 2019 (COVID-19) detection [3]. However, PCR-based nucleic acid detection techniques still suffer from several limitations, including time-consuming, expensive, use of bulky and sophisticated instruments, and the operation of professional personnel [4,5]. Therefore, attention has been drawn by a variety of strategies with high sensitivity and specificity, efficiency, flexibility of use and low cost for nucleic acid detection.

The cluster regularly interspaced short palindromic repeats (CRISPR) and its associated proteins (Cas) is a significant adaptative immune strategy in prokaryotes [6]. Based on the organization of the effector proteins, the CRISPR system can be classified into two main classes, named Class 1 and Class 2, and further subdivided into different types. The Class 2 systems include three types, e.g., type II (Cas9), type V (Cas12a, Cas14a), type IV (Cas13a) [7], which are showed in Table 1. Among the CRISPR-associated biosensors reported in recent years, Cas12a based biosensors are more common due to the unique advantages of Cas12a including specially recognizing target DNA and its strong activity of *trans*-cleavage [8]. To date, a large number of nucleic acid detection platforms have been developed based on the CRISPR/Cas12a system, e.g., DETECTR and HOLMS, which are canonical Cas12a-based methods [9–11]. The canonical CRISPR/Cas12a system mainly includes target single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA), CRISPR RNA (crRNA), Cas12a nuclease, non-targeted ssDNA reporter and its reaction buffer. Firstly, target of dsDNA or ssDNA with protospacer adjacent motif (PAM), a short sequence TTTV necessary for identification dsDNA target, is recognized specifically by Cas12a-crRNA complex. Then, a ternary complex is formed to cleave the adjacent ssDNA reporter non-specifically. However, the canonical CRISPR/Cas12a based biosensors are hard to meet the requirements of higher sensitivity, higher specificity, higher efficiency, larger target scope, easier operation, multiplexing, low cost and diversified signal reading. Consequently, non-canonical CRISPR/Cas12a biosensors have been developed to achieve desirable performance above through altering the key elements of canonical CRISPR/Cas12a system. Here, we mainly summarize the non-canonical CRISPR/Cas12a-based technologies that have been reported in recent years for the detection of nucleic acid targets.

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2. Canonical CRISPR/Cas12a based biosensors

2.1. Key elements of the canonical Cas12a

Canonical CRISPR/Cas12a system is composed of Cas12a protein, target DNA, crRNA, reporter, and reaction buffer. Cas12a with the RuvC catalytic activity is a crRNA guided endonuclease in the Class 2 CRISPR systems (Fig. 1A) [8]. CrRNA consists of a 19 nt repeat sequence and a 23–25 nt spacer sequence [8,12]. The repeat sequence will bind to the inactivate Cas12a to form a ribonucleoprotein complex. The spacer sequence is complementary to a specific DNA target [13], which can be ssDNA or dsDNA with PAM [14]. Generally, the reporter is short ssDNA labeled with fluorophore and quencher at both ends, which could be non-specifically *trans*-cleaved into pieces by activated Cas12a to generate fluorescence signal [15]. In particular, the ssRNA can be also used as a substrate for Cas12a *trans*-cleavage [16]. The reaction buffer is a solution that provides optimal reaction condition for CRISPR/Cas12a system. For example, the *trans*-cleavage activity of Cas12a nuclease was influenced by monovalent salt K^+ and Na^+ concentration [16]. As the salt K^+ and Na^+ concentration increase, both the *cis*- and *trans*-cleavages of degradation rate of Cas12a decreased. And both bovine serum albumin (BSA) and L-proline could enhance the detection sensitivity of CRISPR/Cas12a system [17].

2.2. Canonical Cas12a biosensors

Having understood the important elements of the Cas12a system, we next discuss the application to canonical Cas12a. Canonical CRISPR/Cas12a based nucleic acid detection consists of three key steps including nucleic acid amplification, identification of specific nucleic acid sequences and readout of detection results. For instance, a canonical method HOLMES could be able to use to detect target DNA directly on the basis of Cas12a (Fig. 1B) [10]. This method introduced PCR amplification to obtain large amount of target DNA for CRISPR/Cas12a system in order to further improve the sensitivity. In the existence of target DNA, the Cas12a-crRNA binary complex formed a ternary complex with the target DNA, thus cleaving unrelated and non-targeted ssDNA reporter in the system and illuminating the HEX fluorescence (or any other fluorescence).

However, some canonical Cas12a-based biosensors are hard to meet the requirements of higher sensitivity, higher specificity, higher efficiency, larger target scope, easier operation, multiplexing, low cost and diversified signal reading. For example, without isothermal amplification or PCR, the canonical CRISPR/Cas12a biosensors are only able to detect targets low to the pM level [9,10]. And the detection efficiency of Cas12a is often restricted by its *trans*-cleavage enzyme kinetics in the detection of target at low concentrations [18]. When introducing isothermal amplification techniques into the detection system, the specificity and accuracy may be affected by potential introduced carryover contamination [19]. Besides, if both DNA and RNA targets are present in the detection system, canonical CRISPR/Cas12a system could not identify and discriminate them simultaneously. In addition, the application of the canonical CRISPR/Cas12a system is limited due to the requirement of PAM [20–22]. This aforementioned issue may be addressed by the modified Cas12a system. The systems with modifications on the target sequence, crRNA, Cas12a nuclease, reporter, and reaction conditions are collectively referred to as non-canonical CRISPR/Cas12a system, all of which are all described in detail in Section 3.

3. Non-canonical CRISPR/Cas12a based biosensors

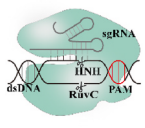
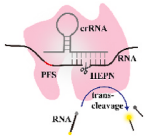
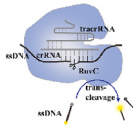
To further avoid the shortcomings of canonical biosensors, a large number of non-canonical Cas12a based biosensors have emerged. We next discuss six aspects of non-canonical strategies, including PAM, target sequence, crRNA, Cas nucleases, reporter, and reaction conditions, which performance have been summarized in Table 2.

3.1. Non-canonical PAM sequence in DNA

PAM is essential to dsDNA recognition in the target sequence. The PAM sequence in target dsDNA is precisely recognized by Cas nuclease under the guidance of crRNA. The canonical PAM for Cas12a contains 5′-TTTV-3′ where V can be A, C and G. Study has shown that the variations in PAM sequence played an important role in the activity of *trans*-cleavage of Cas12a. For example, PAM sequence changing from "TTTC" to "TTT", "TTC", "TT" or "T" decreases the fluorescence signal generated by Cas12a system [23]. In contrast, non-canonical PAM sequence "VTTV" increases the fluorescence signal [24]. As a result, the PAM sequence can be optimized to improve detection sensitivity and to enlarge the scope of detectable targets.

A called sPAMC test employing suboptimal PAM was developed to obtain a rapid, specific and sensitive one-pot detection of SARS-CoV-2 (Fig. 2A) [24]. In this study, Lu et al. for the first time designed multiple crRNAs for different SARS-CoV-2 genes and used them in a one-pot detection where amplification and CRISPR/Cas12a reactions were combined into single step. Some crRNAs for target DNAs with non-canonical PAM were found to exhibit faster fluorescence signal dynamics than others. Thus, those non-canonical PAMs with better performance were referred to as the suboptimal PAM of Cas12a. Finally, Lu et al. demonstrated that the VTTV sequence was considered as the best suboptimal PAM by PAM variation screening. And TCTV was also a good suboptimal PAM candidate for one-pot detection. Compared to canonical PAM in one-pot detection of SARS-CoV-2, the efficiency of sPAMC was 2- to 3-fold faster (only 15 min). The limit of detection of sPAMC was 1 cp/μL. The sensitivity was 94.2 %. The specificity was

Table 1
Characteristics of different Cas proteins in class 2 CRISPR system.

Cas effector	Cas9	Cas12a	Cas13a	Cas14a
Canonical schematic				
Type	II	V	VI	V
PAM/PFS	NGG	TTTV	A/T/C	no
Targets	dsDNA	dsDNA, ssDNA	RNA	ssDNA
Guide RNA	sgRNA	crRNA	crRNA	crRNA
Trans-cleavage	no	ssDNA, RNA	RNA	ssDNA

Abbreviation: crRNA, CRISPR RNA; dsDNA, double-stranded DNA; PAM, protospacer adjacent motif; PFS, protospacer flanking site; ssDNA, single-stranded DNA.

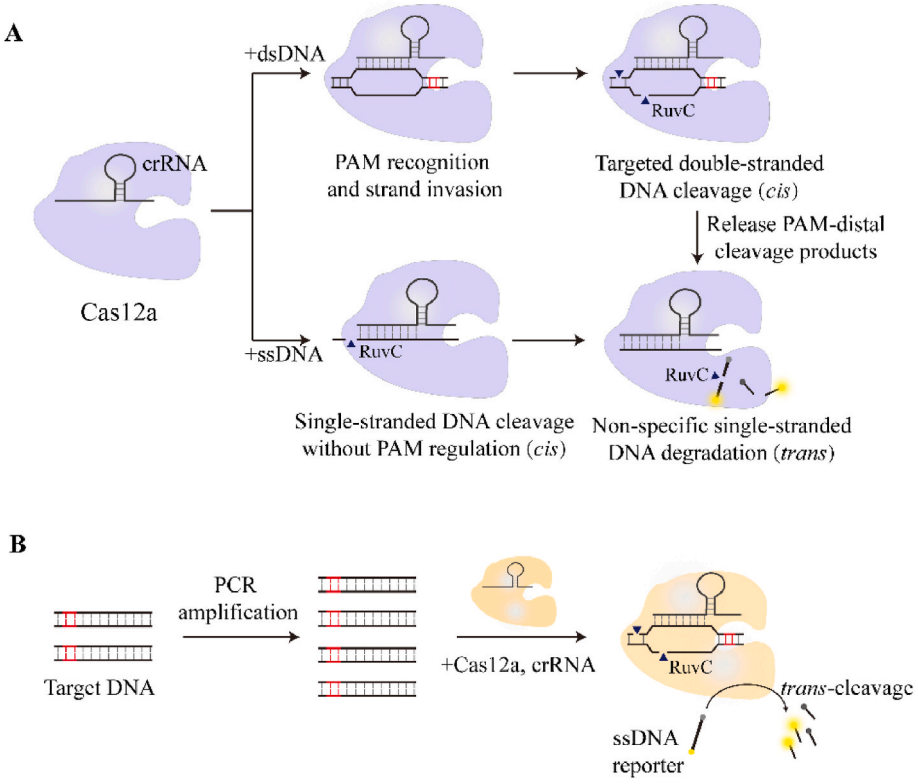


Fig. 1. Schematic of canonical CRISPR/Cas12a system. (A) Schematic of *cis*- and *trans*-cleavage of CRISPR/Cas12a using target dsDNA or ssDNA activating. (B) Schematic of a canonical method HOLMES. The amplification of target DNA would be first performed. Then, the amplicon was formed a ternary complex with Cas12a/crRNA complex to cleave the quenched fluorescent ssDNA reporter, illuminating the fluorescence.

100.0 % with the comparison of RT-qPCR. A possible explanation for the good performance is that Cas12a-based detection with enhanced stable fluorescence signal has sufficient applications due to the weaker binding affinity between Cas12a and target DNA with suboptimal PAM.

Despite using suboptimal PAM assay can have enhanced efficiency and sensitivity of detection, PAM assistance is still necessary in Cas12a-based systems. To eliminate the strict PAM preference of CRISPR/Cas12a system on the DNA substrate, a generalized PAM-less conditional DNA (pcDNA) substrate with unwound seed region was developed to activate the *trans*-cleavage activity of Cas12a based on the fact that unwound DNA could be processed by Cas12a/crRNA complex even if PAM was absent (Fig. 2B) [25,26]. This pcDNA substrate could be applied for detections of various intracellular biomolecules via stimulus-responsive switch mechanism. In the detection of miRNA-21, there was a linear relationship between miRNA-21 concentration and fluorescence response in the range of 1–250 nM, with a detection limit of 0.25 nM. This work shows great potential for the detection of different types of biomolecules in living cells and demonstrates a novel form of CRISPR/Cas sensing system.

3.2. Targets of ssDNA and RNA

Although the application of the non-canonical PAMs is useful in the nucleic acid detection, the PAM identification is severely limited for CRISPR/Cas12a applications. The ssDNA is more widely used in the CRISPR/Cas12a system because it does not be restricted by PAM sequence. However, Cas12a is fault tolerant of single-base mismatch recognition in detection of ssDNA. Hence, Wu et al. developed a novel CRISPR/Cas12a PAM-unrestricted and ultra-specific target toehold-activator (TOA), which was a dsDNA target with toehold ssDNA (Fig. 3A) [28]. The flexible toehold ssDNA first bound to the crRNA and a DNA-crRNA complex was subsequently formed on the basis of toehold-mediated strand displacement reaction to activate Cas12a. In practical sample detection, EGFR T790 M and L858R mutations could be detected in samples with extremely low abundance. Therefore, TOA may have specific, stable, general, and flexible advantages over canonical ssDNA activators.

Although it is well known ssDNA is the *trans*-cleavage substrate of Cas12a, its activation mechanism and specificity have not been fully elucidated. Li et al. discovered a “synergistic activator effect” that indicated two short ssDNA activators could support a synergistic incorporation mechanism and promote the *trans*-cleavage of CRISPR/Cas12a (Fig. 3B) [29]. And this synergistic activator-triggered CRISPR/Cas12a system has been successfully utilized in the AND logic operation and the discrimination of single-nucleotide variants (SNV). The detection limit of SNV was 0.2 %. And a stick-end dsDNA, as an ultra-specific activator of CRISPR/Cas12a system, was also developed into the detection of single-base mismatches with detection limits of 0.005–0.01 % synthesized DNA [30]. This stick-end dsDNA could form a triplex flap structure with the crRNA, which was more sensitive to mismatches than canonical dsDNA. This finding provides deeper insight into CRISPR/Cas12a and broadens its application scenarios.

Except ssDNA, CRISPR/Cas12a could be also applied to detect miRNA directly. Luo et al. reported that the *trans*-cleavage activity of Cas12a could be triggered straight by miRNA as miRNA would hybridize with crRNA (Fig. 3C) [32]. With the help of fluorescence probe immobilized on the surface of AuNPs, CRISPR/Cas12a system was used to detect miRNA-21. The limit of detection was as low as 10 fM. It is evidenced that biosensor based on CRISPR/Cas12a possessed well potential in miRNA detection, which can be applied into clinical diagnosis.

3.3. Chimeric cas protein

Due to the non-specific *trans*-cleavage activity of Cas12a, it is difficult to develop biosensors based on CRISPR/Cas12a to detect multiple targets simultaneously. At present, Cas12a and Cas13a proteins are

Table 2
Major characteristics of classified non-canonical CRISPR/Cas12a-based detection strategies.

Methods	Detection strategies	Nucleic acid targets	Signal read-out	Limit of detection	Detection time (min)[a]	Refs.
Non-canonical PAM sequence in DNA	nicked PAM	e14a2 mRNA	Fluorescence	100 pM	>60	[23]
	Suboptimal PAM	SARS-CoV-2 RNA	Fluorescence/ Colorimetric	1 cpμl $^{-1}$	15	[24]
	PAM-less	MicroRNA-21	Fluorescence	0.25 nM	>120	[25]
	PAM-less	MicroRNAs	Fluorescence	50 fM	50	[27]
Targets of ssDNA and RNA	Toehold-activator	EGFR T790 M and L858R mutations	Fluorescence	0.05 %	24	[28]
	Two ssDNA Activators	DNA mutations	Fluorescence	0.2 %	90	[29]
	A sticky-end dsDNA activator	EGFR T790 M	Fluorescence	0.01–0.05 %	45	[30]
	MicroRNA activator	MicroRNA	Fluorescence	10 pM	–	[31]
	MicroRNA activator	MicroRNA	Fluorescence	10 fM	35	[32]
Chimeric Cas protein	Chimeric Cas12a and Cas13a protein	SARS-CoV-2	Fluorescence	10 copies	–	[33]
Modified crRNA	Extended crRNA	SARS-CoV-2 RNA	Fluorescence	1 pM	40–60	[34]
	Multiplex crRNA	African swine fever virus	Fluorescence	1 pM	90	[35]
	Dual-crRNA	*Salmonella*	Fluorescence	0.2 cells	30	[36]
	Photocontrolled crRNA	SARS-CoV-2 RNA	Fluorescence	10 copie	>90	[37]
	Photocontrolled crRNA	SARS-CoV-2 RNA	Fluorescence	1 copy	30–40	[38]
	RNA/DNA circuit	SARS-CoV-2 RNA	Fluorescence	544 fM	60	[39]
	Self-recruiting of crRNA	MicroRNA-21	Fluorescence	1 aM	55	[40]
	Autocatalysis-driven feedback amplification network	Hepatitis B virus	Fluorescence	3 copies	>30	[41]
Non-canonical signal reporter	Extended reporters	*Helicobacter pylori*	Fluorescence	43 aM/96 aM	40	[42]
	Hairpin DNA reporters	SARS-CoV-2 RNA	Fluorescence	10 pM	>100	[43]
	A dsDNA reporter	Tobacco curly shoot virus	Fluorescence	10 pM	32.5	[44]
	Double-Stranded λ DNA reporter	ssDNA	Non-optical	250 fM	30	[45]
	DNA-Functionalized AuNPs reporter	Breast cancer gene-1	Fluorescence/ Colorimetric	0.34 fM	30	[46]
	Spherical nucleic acid reporter	MicroRNA	Fluorescence	10 fM	35	[32]
	G-Quadruplex reporter	*Leishmania donovani*	Fluorescence	1 pM	140	[47]
	G-Quadruplex reporter	*Salmonella enterica*	Fluorescence	20 CFU	30	[48]
	G-Quadruplex reporter	SARS-CoV-2 RNA	Fluorescence	0.02 nM	30	[49]
	G-Quadruplex reporter	*Streptococci*	Fluorescence/ Colorimetric	1 aM	30	[50]
	G-triplex reporter	HPV	Fluorescence	50 pM	10	[51]
	Ratiometric fluorescent probe	ssDNA	Fluorescence	3 pM	50	[52]
	Quantum dot–nucleic acid hairpin (QD-HP) molecular beacons	ssDNA	Fluorescence	1 pM	60	[53]
	Noble metal nanoclusters reporter	*Salmonella typhimurium*	Fluorescence	1 CFU	45	[54]
	A novel half-blocking dsDNA reporter	SARS-CoV-2 RNA	Colorimetric	10 copies	60	[55]
	G-Quadruplex reporter	*Salmonella*	Colorimetric/ Photothermal	1 CFU	90	[56]
	Linker ssDNA with DNA-Functionalized AuNPs reporter	*Salmonella*	Colorimetric/ Fluorescence	1 CFU	90	[56]
	Qdots-reporter	African swine fever virus	Colorimetric	0.5 nM/1.25 nM	>120	[57]
	Urease-Conjugated Single-Stranded Oligodeoxynucleotide reporter	African swine fever virus	Colorimetric	0.34 pM	150	[58]
	DNA−PtNP conjugates reporter	Single nucleotide polymorphisms	Colorimetric	0.01 %	60	[59]
	Hairpin transducer	SARS-CoV-2 RNA	Colorimetric	75 copies	45	[60]
	Methylene blue (MB)-ssDNA reporter	HPV-16	electrochemistry	pM	60	[61]
	MB-ssDNA reporter	SARS-CoV-2 RNA	electrochemistry	50 fM	45	[62]
	MB-hairpin DNA reporter	Target DNA	electrochemistry	30 pM	60	[63]
Non-canonical reaction conditions	Mn^{2+}	Target dsDNA	Fluorescence	0.45 μM	15	[64]
	MnO_2 nanosheets	mRNA	Fluorescence	67 pM	15	[65]

[a] "-" means that the detection time is not mentioned by the authors.

widely applied to detect DNA and RNA, respectively. A novel chimeric protein composed of Cas12a and Cas13a was constructed to simultaneously detect DNA and RNA in one reaction tube [33]. First, streptococcus pyogenes Tag (SpyTag) was coupled with LwCas13a to obtain LwCas13a-SpyTag. Then, the LbCas12a was fused with SpyCatcher to form LbCas12a-SpyCatcher. Subsequently, the LwCas13aSpyTag and LbCas12a-SpyCatcher were mixed to form a chimeric protein with the activities of both Cas12a and Cas13a. This chimeric protein could recognize target DNA and RNA to trigger their *trans*-cleavage activities simultaneously. In the analysis of wild type (WT) and N502Y SARS-CoV-2, Cas12a-crRNA was designed to be complementary to WT SARS-CoV-2 and Cas13a-crRNA was to N502Y SARS-CoV-2, respectively. The detection limits of both RNA were 10 copies/test in the range of 10-10^5 copies. This novel chimeric protein could be applied to design innovative detection tool for biomarkers and infective pathogens, which would be one-pot, one-step, rapid and accurate.

3.4. Modified crRNA

Length of crRNA. The off-target effect of Cas12a would lead to low accuracy, high background and false positives. This issue could be addressed by extending the length of the rigorously screened crRNAs in the Cas12a system [66]. Nguyen et al. demonstrated that both specificity and sensitivity of Cas12a for nucleic acid detection could be enhanced by extending 7-mer DNA at the 3' end of crRNA [34]. The highest activity of Cas12a, 3.5 folds higher than that of the canonical crRNA, was observed when it was extended with TA-rich DNA. In the detection of SARS-CoV-2 with this modified crRNA, the detection could be

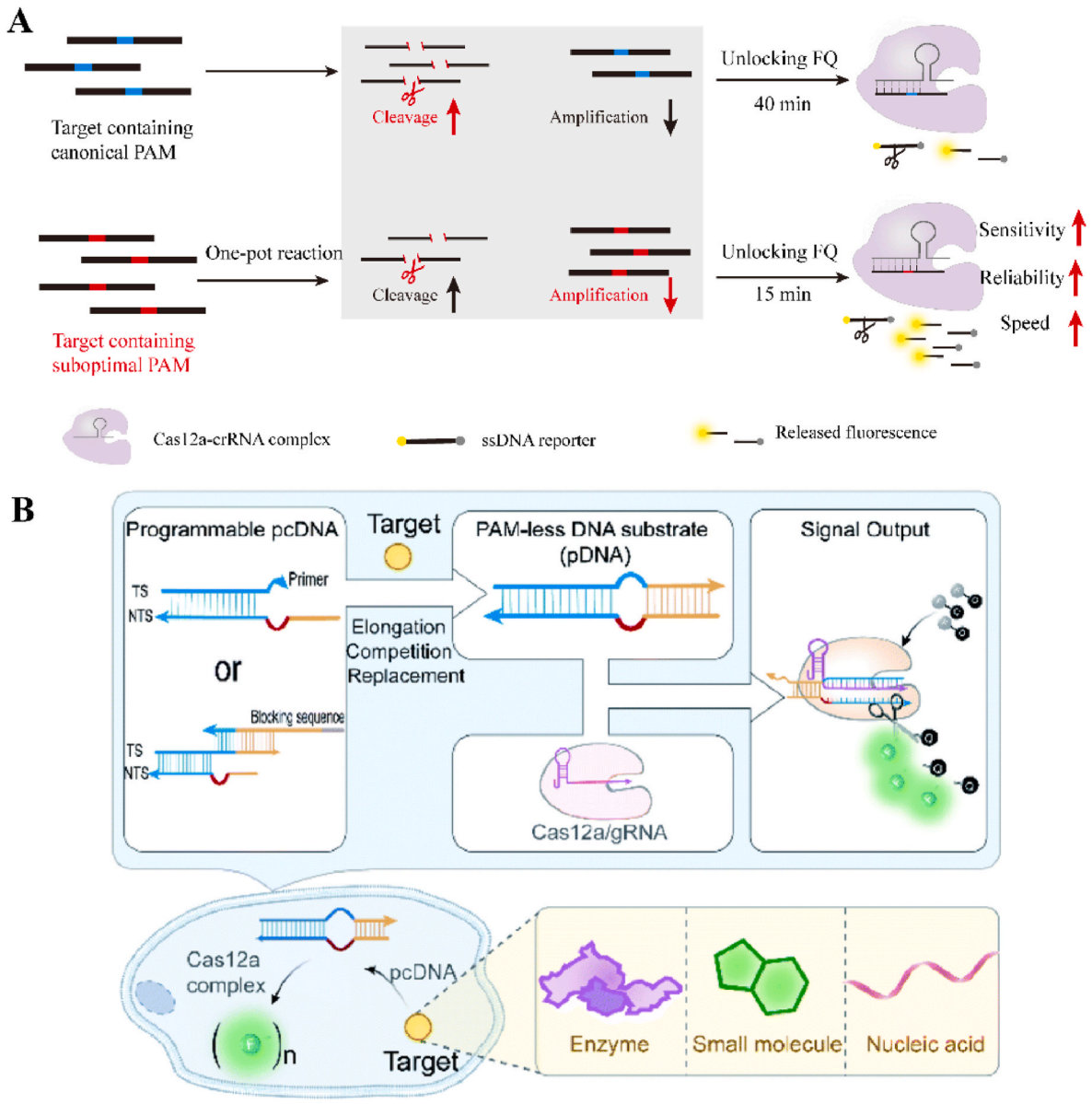


Fig. 2. Modified and non-canonical PAM based CRISPR/Cas12a biosensors for nucleic acid detection. (A) Schematic of a Cas12a-based and using suboptimal PAM assay for SARS-CoV-2 detection in one-pot reactions. When the target DNA contained suboptimal PAM, faster and stronger fluorescence signal production was stimulated due to amplification accumulation. (B) Schematic of Cas12a system based on PAM-less conditional DNA substrates (pcDNAs) (Reproduced with permission from Ref. [25]. Copyright 2022, Royal Society of Chemistry). Different types of targets converted the pcDNA into a PAM-less dsDNA substrate (pDNA), which could activate the nuclease activities of Cas12a.

completed within 40–60 min.

Besides, it was reported that extending the length of crRNA at the 5' end could enhance gene editing efficiency of Cas12a [67]. It was found that the Cas12a nuclease activity relied on the secondary structure of crRNA scaffold, as well as the length of crRNA [68]. The *trans*-cleavage activity of Cas12a disappeared when the length of crRNA that was complementary to the target DNA was less than 20 bases. Thus, not only could the data on the split crRNA assist in understanding the molecular mechanisms of Cas12a, but also help to facilitate the application of Cas12a in vitro diagnostics.

Number of crRNA. In addition to the application of a single crRNA, multiple crRNAs for the same target DNA have been put forward to greatly improve the detection efficiency [35,36]. Different Cas12a-crRNA complexes could be directed to different sites of the same target DNA. This multiplex-crRNA strategy enabled the avoidance of cumbersome, time-consuming, and cross-contamination in the following steps of transferring pre-amplification products into the CRISPR/Cas12a system. Biosensor with this strategy was used to detect African swine fever virus (ASFV) (Fig. 4A) [35]. The detection limit was as low as 1 pM without amplification, which was 64-fold lower than the canonical single-crRNA system. Moreover, the reaction rate of multiplex crRNA system was 4-fold faster than the canonical single-crRNA system. If lateral flow assay, a portable fluorescence detector, or other strategies are integrated into this system in the future, the results can be visualized directly, which is widely applied in field test.

Activation of crRNA. Generally, an amplification is made to obtain more target DNA for CRISPR/Cas12a detection. The amplification could be suppressed by the *cis*- and *trans*-cleavage activities of Cas12a, leading to a reduced detection efficiency. To address this issue, a robust method was developed based on optochemical control crRNA (Fig. 4B) [37]. In this method, all the components were mixed into a fully closed test tube. In the beginning, the crRNA was inactivated temporarily by a protective

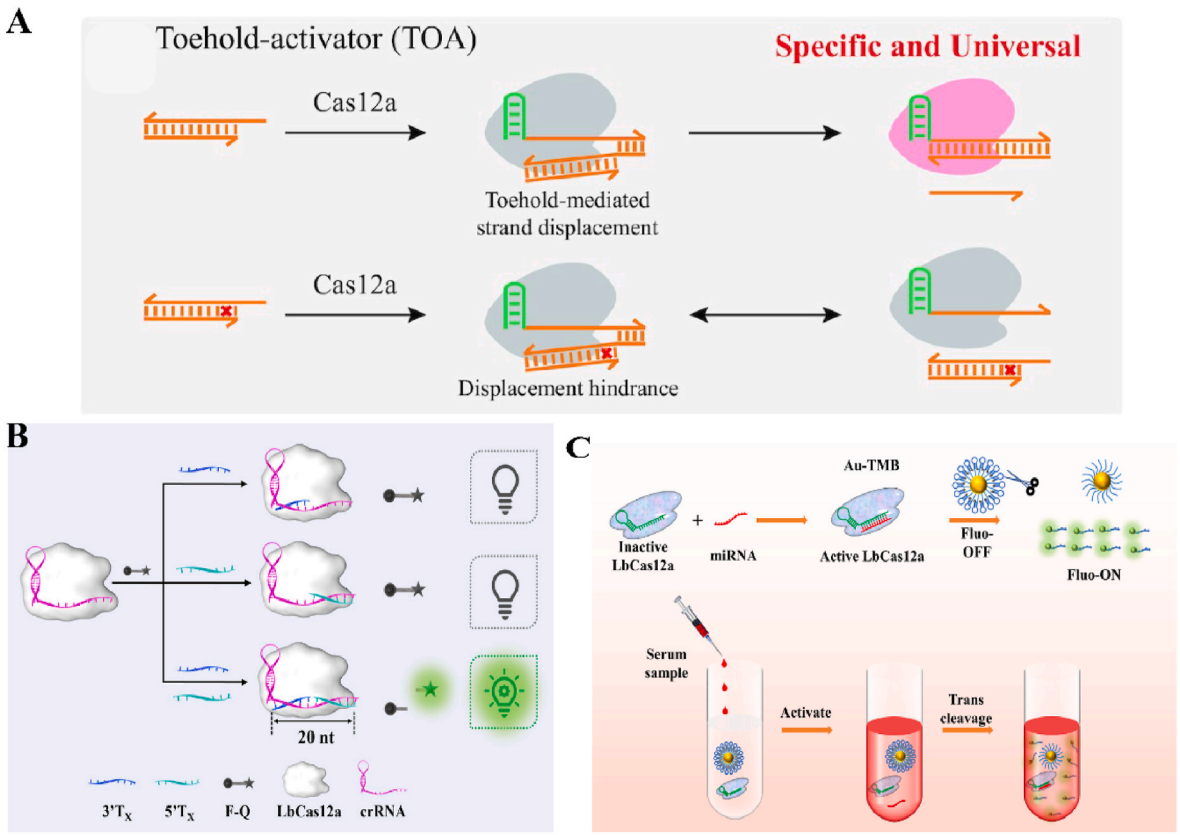


Fig. 3. Detection of nucleic acids by the Cas12a biosensor was based on a modified target. (A) Schematic of toehold-activator of CRISPR/Cas12a (Reproduced with permission from Ref. [28]. Copyright 2022, Oxford University Press). Toehold-activator specifically activated Cas12a by strict toehold-mediated strand displacement reaction without PAM sequence. (B) The short ssDNA synergistic trigger CRISPR/Cas12a system (Reproduced with permission from Ref. [29]. Copyright 2023, American Chemical Society). Two ssDNA short activators could activate the *trans*-cleavage of CRISPR/Cas12a. (C) Schematic of a CRISPR/Cas12a-based biosensors to detection miRNA directly. (Reproduced with permission from Ref. [32]. Copyright 2022, American Chemical Society). The miRNA could be recognized by Cas12a/crRNA complex directly, thus illuminating the fluorescence.

oligo. This protective oligo could be degraded under rapid 365-nm light irradiation to activate the CRISPR/Cas12a system after the amplification step was completed. This photocontrolled crRNA strategy presented a 100 folds improvement in sensitivity compared to the canonical one-pot assay. In detection of SARS-CoV-2 RNA from clinical samples, this strategy achieved a 100 % sensitivity for the ORF1ab gene and 95.6 % for the N gene. Although the detection efficiency and accuracy of this strategy were satisfactory, the design of photocontrolled crRNA was complicated. Hence, another light-start CRISPR/Cas12a reaction by employing caged crRNA was developed [38]. This light-start caged crRNA was obtained by modification of nitropiperonyloxymethyl-caged thymidine, which could be decaged rapidly under 365 nm UV radiation. This photocontrolled one-pot assay was 50-fold more sensitive than conventional assays. Because this caged crRNA could be chemically synthesized directly as regular crRNA, the CRISPR/Cas12a system was simpler than that of light-start protective oligo. This advance in CRISPR diagnostics will facilitate a wide range of detection applications in biomedicine, agriculture, and food safety.

Amplification of crRNA. While the literature above is concerned with enhancing the detection efficiency by altering the sequence, amount and activation of crRNA, the following literature relates nucleic acid circuits to CRISPR systems. A CRISPR/Cas-only amplification network (CONAN) assay was constructed by introducing a special switchable-caged guide RNA (scgRNA) with self-reporting capability via a positive feedback circuit (Fig. 4C) [41]. First, the target DNA 1 was bound by Cas12a-crRNA complex, which activated Cas12a to cleave the intermediate scgRNA and to release the crRNA for the target DNA 2. Again, the *trans*-cleavage of Cas12a was triggered by the released crRNA and target DNA 2 to cleave the scgRNA, which constructed a positive feedback circuit to amplify the fluorescence signal. Notably, this CONAN assay achieved one-enzyme (Cas12a) and one-step, real-time detection of target DNA with ultra-sensitivity. Compared to the canonical CRISPR/Cas12a system, the CONAN assay was 7.2–10.4 times more specific in identifying single-nucleotide mutations. And the lowest detectable concentration of the CONAN system could reach the 5.0 aM level, which was superior to canonical CRISPR detection system. The feature of this CONAN system was amplification-free. Compared with canonical CRISPR/Cas12a system, CONAN system has the advantage to develop biosensors with high-speed, low-cost and simple-operation.

3.5. Non-canonical signal reporter

Typically, a short ssDNA reporter with modification of fluorophore and quencher at each end, inhibiting the fluorescence emission [70], is applied as signal transducer of CRISPR/Cas12a detection system [71]. This canonical reporter would be cleaved into pieces by Cas12a to produce fluorescence signal when target DNA was detected. Currently, non-canonical signal transducers have mushroomed to meet the requirement of different detection, which further extends the application of CRISPR/Cas12a system. Here, we describe different signal reporters from three perspectives: fluorescence, colorimetry, and electrochemistry.

3.5.1. Fluorescence-based reporting element

Extending length of ssDNA reporter. The main reporter for Cas12a-based biosensors is the canonical reporter probe, TTATT, which

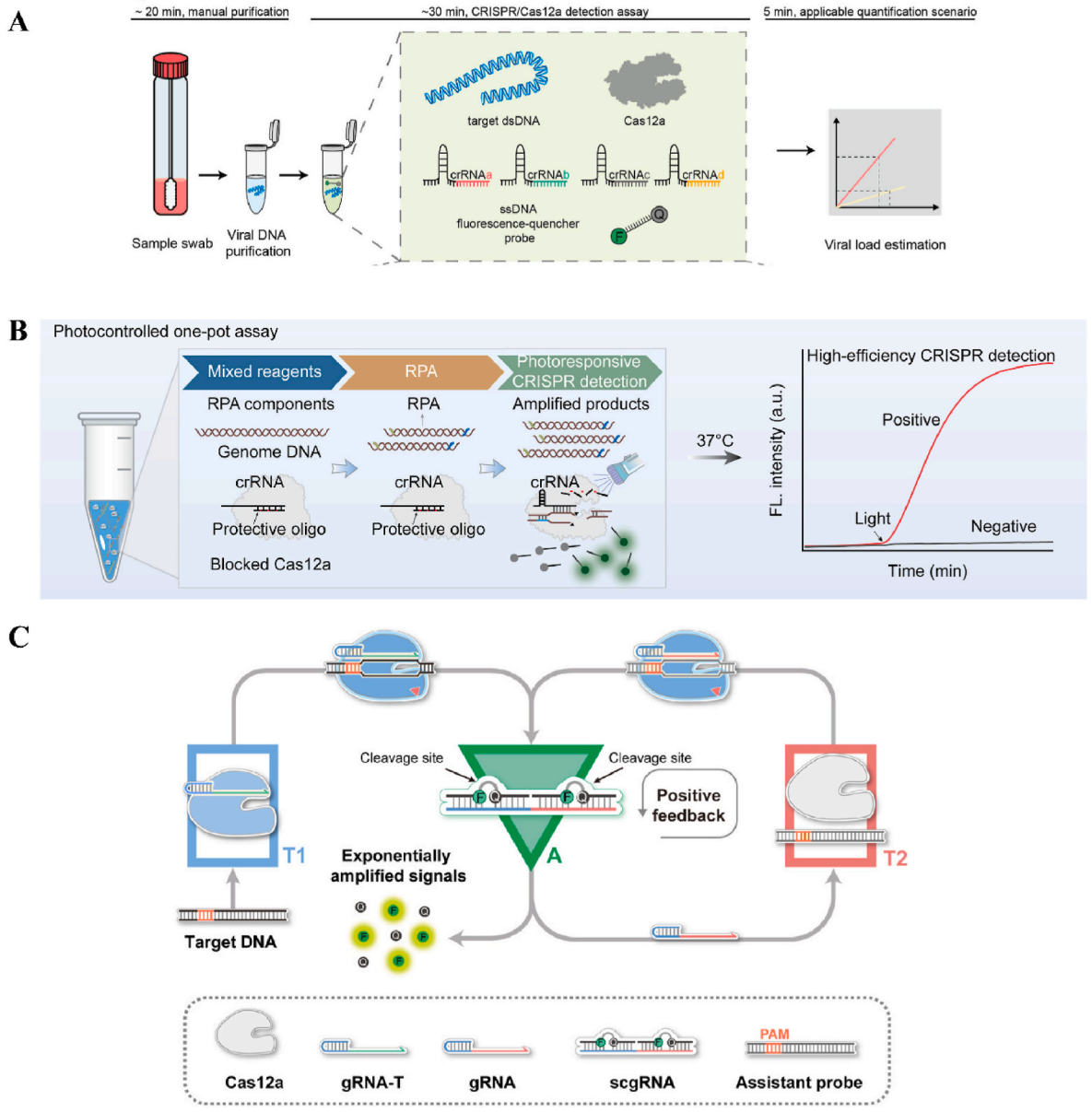


Fig. 4. Modified crRNA assisted CRISPR/Cas12a biosensors for nucleic acid detection. (A) Multiplex Cas12a-crRNA complexes would be directed to a different site on the same target dsDNA (Reproduced with permission from Ref. [35]. Copyright 2022, American Chemical Society). Multiple sequences of crRNA could hybridize target different regions of the same target dsDNA in the same CRISPR/Cas12a system. (B) Schematic of a photocontrolled one-pot CRISPR/Cas12a DNA assay (Reproduced with permission from Ref. [69]. Copyright 2022, PNAS). Under ultraviolet (UV) light at 365 nm, the PC linker of crRNA protective oligo would break and the crRNA was in free state. Then, the function of Cas12a was restored. (C) Schematic of a CRISPR/Cas12a autocatalysis-driven feedback amplification network (Reproduced with permission from Ref. [41]. Copyright 2021, American Association for the Advancement of Science). A special switchable-caged guide RNA (scgRNA) with self-reporting capability drove signal amplification via a positive feedback circuit.

is capable of producing a detectable *trans*-cleavage signal. It was reported that ssDNA reporter composed of 10 thymine (10-T) showed a higher signal-to-background ratio that the canonical one. Its application to detect *Helicobacter pylori (HP)* indicated that 10-T reporter achieve higher cleavage speed and detection sensitivity than the canonical reporter [42]. The limits of detection for the CagA and VacA levels of HP were as low as 43 aM and 96 aM, respectively. Thus, the length of the ssDNA reporter plays a role in the performance of the CRISPR/Cas12a detection system, which can identify and differentiate weak signals, reduce false positives, shorten turnaround time, and improve sensitivity.

Hairpin DNA reporter. The *trans*-cleavage activity of Cas12a is affected by the secondary structure of the signal reporter. Then, signal reporters with different secondary structures have been reported. A hairpin DNA reporter with fluorophore and quencher at each end was developed for nucleic acid detection (Fig. 5A) [43]. When the *trans*-cleavage activity of Cas12a was activated by the target, the hairpin DNA reporter would be cleaved at the hairpin loop, generating a fluorescence signal. Compared to the canonical signal reporters, hairpin reporters with 10-T stem-loop were capable of generating faster and stronger fluorescence signal, improving the sensitivity and the specificity of CRISPR/Cas12a based detection. The limit of detection for SARS-CoV-2 was as low as 10 pM in a two-pot reaction assay. Furthermore, this hairpin ssDNA reporter had the advantage of possessing a faster cleavage rate by Cas12a compared to canonical ssDNA reporters. And this hairpin ssDNA reporter had been shown to improve sensitivity and enhance specificity in distinguishing single- and double-point mutations of dsDNA amplicons. Therefore, the design of ssDNA reporter with different secondary structure was a feasible strategy to develop

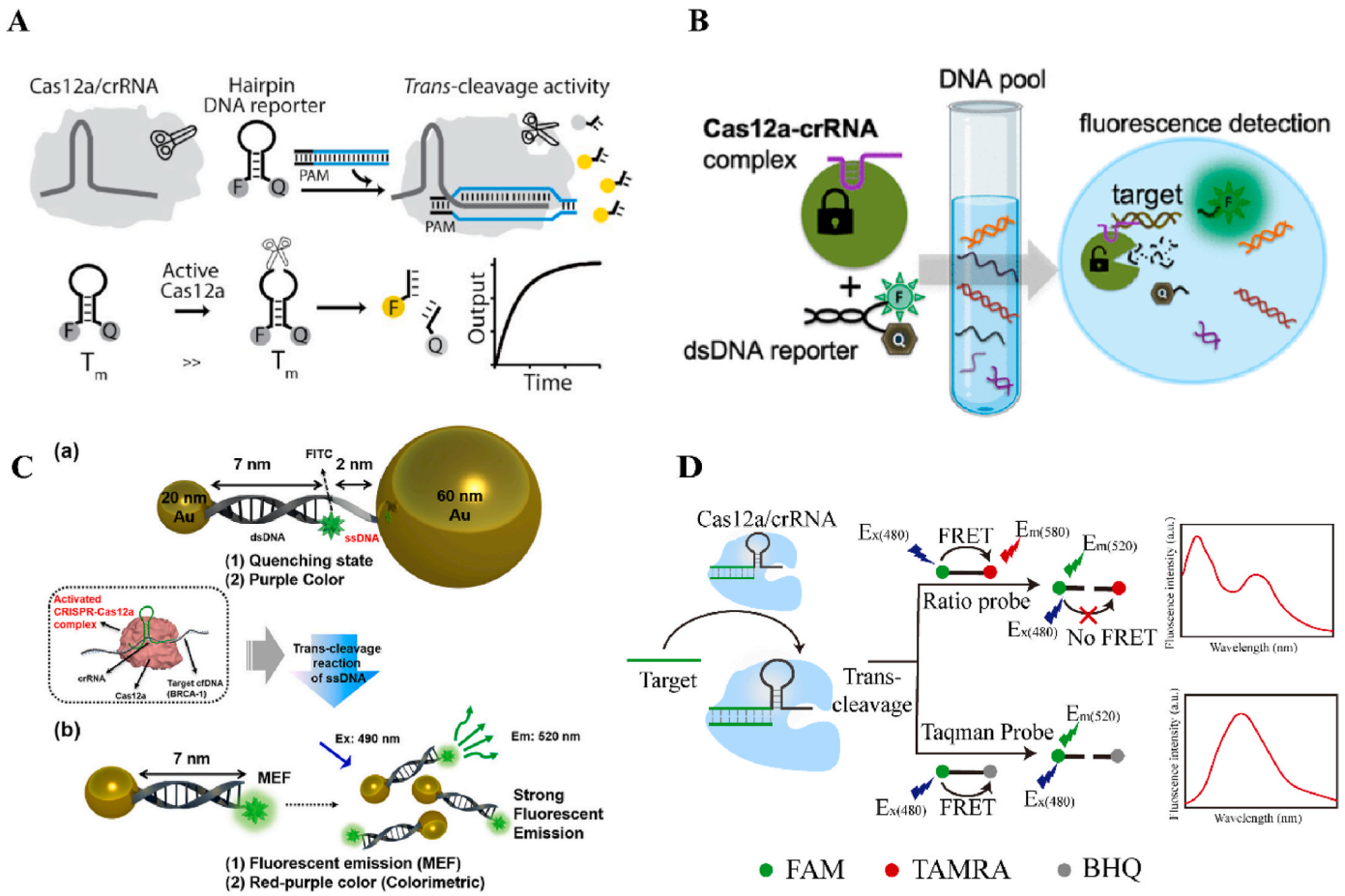


Fig. 5. In addition to canonical reporter, other types of fluorescent probes are present in the Cas12a biosensor. (A) Hairpin DNA reporters were used as substrate of Cas12a *trans*-cleavage (Reproduced with permission from Ref. [43]. Copyright 2022, Oxford University Press). When Cas12a/crRNA complex was activated by target dsDNA, the stem-loop of hairpin DNA reporters was able to *trans*-cleaved to produce fluorescence signal. (B) The dsDNA reporter was used as the substrate of Cas12a *trans*-cleavage (Reproduced with permission from Ref. [44]. Copyright 2021, American Chemical Society). When Cas12a/crRNA complex was activated by target dsDNA, the dsDNA reporters was *trans*-cleaved to produce fluorescence signal. (C) The ssDNA was degraded between 20- and 60-AuNPs via the *trans*-cleavage effect, thus emitting a strong fluorescence signal at 520 nm (Reproduced with permission from Ref. [46]. Copyright 2020, American Chemical Society). When target DNA was added, the ssDNA of the DNA-functionalized AuNP was cleaved by Cas12a to dissociate the 60 nm AuNP from FITC, resulting in fluorescence signal enhanced by 20 nm AuNP. (D) A ssDNA was labeled by fluorescein (FAM) and tetramethylrhodamine (TAMRA) to generate ratiometric fluorescence. When the targets were present, the reporter was cleaved by Cas12a to eliminate the FRET effect, resulting in an increase fluorescence at 520 nm and a decrease fluorescence at 580 nm.

CRISPR/Cas12a based biosensors with better performance.

Double-strand DNA reporters. Except ssDNA reporters, dsDNA reporters have also been reported as substrates for Cas12a *trans*-cleavage activity. A dsDNA reporter labelling fluorophore and quencher at the same end of each strand (Fig. 5B) [44]. Compared to canonical ssDNA reporters, dsDNA reporters had comparable limits of detection but more stable background signal and higher synthesis yield. The background fluorescence signal of canonical ssDNA reporter increased steadily over time. In contrast, the background fluorescence signal of this dsDNA reporter did not fluctuate significantly over time. A possible explanation for the stable background signal was due to the different fluorescence quenching modes, including ssDNA quenched by fluorescence resonance energy transfer (FRET) distance and dsDNA quenched by contact [72]. Therefore, the non-canonical dsDNA reporter existed stable background signal. The synthesis yield of reporter labeled with fluorophore and quencher was determined by the label efficiency. The synthesis of dual-labeling ssDNA was more challenging than that of mono-labeling, leading to lower synthesis yield. Canonical ssDNA reporter needed to label fluorophore and quencher at both ends. While dsDNA reporter required only to label fluorophore or quencher to each of the two complementary ssDNA. Thus, non-canonical dsDNA reporter had higher synthesis yield than canonical ssDNA reporter. Notably, the reaction rate of dsDNA reporter was slower than that of ssDNA reporter, which may lead to longer detection times. In addition, λ DNA was also reported to serve as reporter of CRISPR/Cas12a based detection platform, which was inexpensive, non-optical, simple and sensitive [45]. Thus, dsDNA could be an alternative reporter for CRISPR/Cas12a system to overcome the shortcomings of the ssDNA reporter.

Reporters based on Au nanoparticle. Nanomaterial such as Au nanoparticle (AuNP) was introduced into the signal reporters of CRISPR/Cas12a based biosensors to increase the diversity. Spherical nucleic acid (SNA) reporter could be obtained by immobilizing ssDNA or harpin reporters on the surface of AuNP via thiolate-Au bond [32,73]. This SNA reporter could achieve a much lower limit of detection than the canonical ssDNA reporter system. Furthermore, the signal intensity of fluorophore near AuNP could be enhanced by the surface plasma resonance property. However, the fluorescence would be quenched if the distance between fluorophore and AuNP was less than 2 nm. Based on this phenomenon, reporters using DNA-functionalized AuNP were designed for CRISPR/Cas12a system (Fig. 5C) [46]. Two AuNPs of different sizes (20 nm and 60 nm) were linked by a dsDNA (7 nm long) and a ssDNA (2 nm long). Fluorescein isothiocyanate (FITC) was labeled at the end of dsDNA with a distance of 7 nm–20 nm AuNP and 2 nm–60 nm AuNP. Without target DNA, the fluorophore was quenched by the 60

nm AuNP. When target DNA was added, the ssDNA of the DNA-functionalized AuNP was cleaved by Cas12a to dissociate the 60 nm AuNP from FITC, resulting in fluorescence signal enhanced by 20 nm AuNP. Its application detects cell-free DNA with high sensitivity, less detection time and a detection limit of 0.34 fM, which was about 3 orders of magnitude than the canonical ssDNA reporter system.

G4/G3 DNA reporters. All of the aforementioned reporters for the CRISPR/Cas12a system require fluorophore and quencher labeling on the ssDNA or dsDNA ends. So far, label-free G-quadruplex (G4) and G-triplex (G3) had been applied as reporter probes for CRISPR/Cas12a system to reduce complexity of probe synthesis and cost. Therefore, G4 reporters have drawn scientists' attention to the construction of CRISPR/Cas12a biosensors [47–49,74–76]. G4 was the most studied non-canonical DNA secondary structures, which has the advantage of easily synthesis, small size, low cost and good thermal stability [77,78]. It was reported that G4 and fluorescence dye, *N*-methyl mesoporphyrin IX (NMM), were applied as reporter system for CRISPR/Cas12a biosensor to detect *Salmonella enterica* [48]. The NMM emitted weak fluorescence under the free state. G4 could specifically bind with NMM to enhance the fluorescence intensity. When the target was present, the G4 would be cleaved into pieces, resulting in the loss of the ability to bind to the NMM. Therefore, the fluorescence signal for targets was much weaker than the negative control. The limit of detection for *Salmonella enterica* was as low as 20 CFU. Except for G4, G3 could also serve as reporter probe for CRISPR/Cas12a biosensors [51]. The limit of detection for non-preamplification and preamplification strategies could be as low as 50 pM and 0.1 aM, respectively. Compared to the canonical reporter, this sensitivity was improved by a factor of 20.

Reporter based on ratiometric fluorescent probe. In canonical reporter, fluorophore and quencher are labeled at different ends of short ssDNA, which are susceptible to a variety of confounding factors, including variations in reporter concentration, instrument environment (e.g., excitation and emission source fluctuation, changes in detector working distance, changes in angle of detection, etc.), thus disturbing the intensity of fluorescence signal [79]. To address this issue, two kinds of fluorophores are labeled at both ends of short ssDNA to obtain

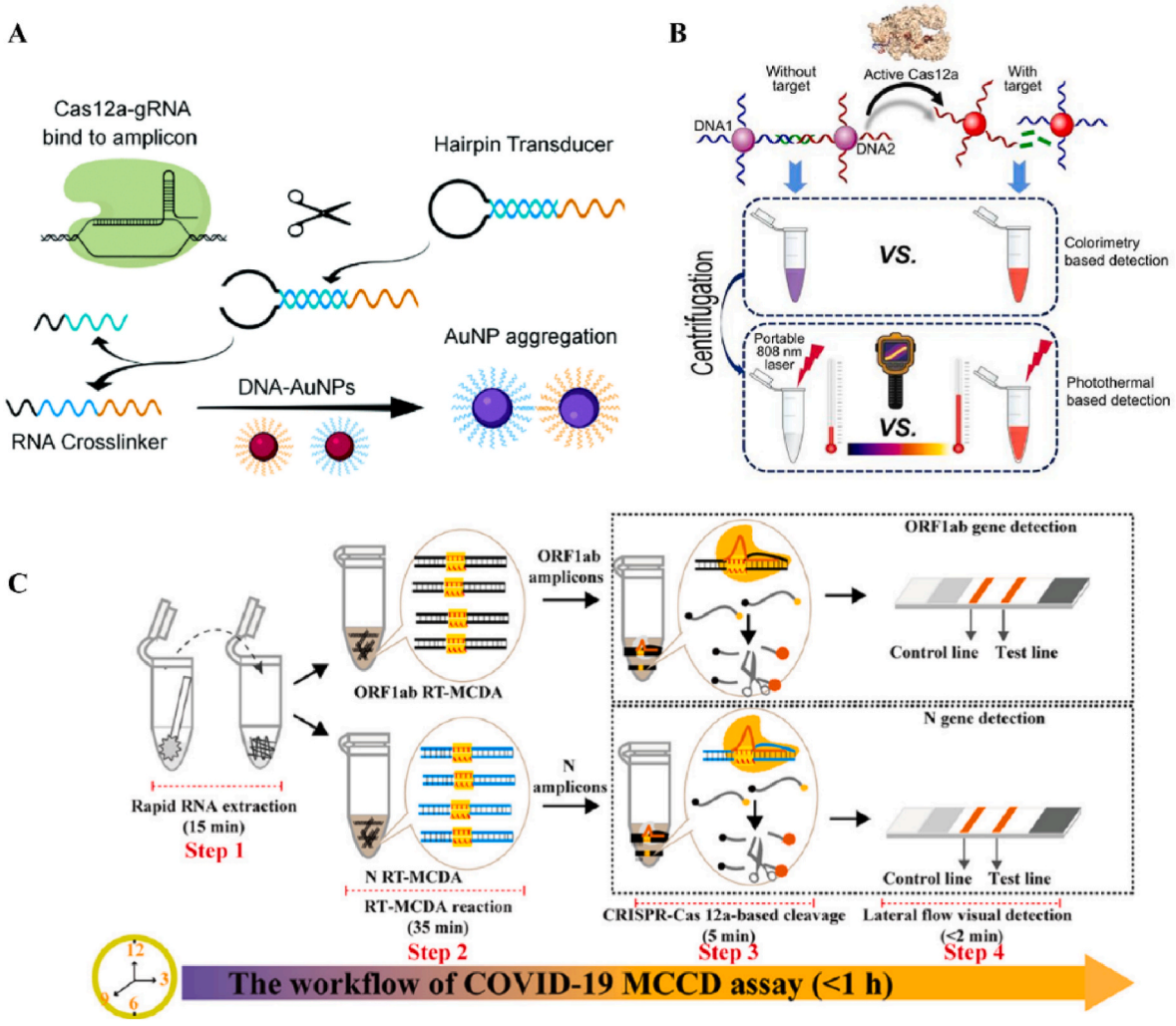


Fig. 6. CRISPR/Cas12a system are based on gold nanoparticle to develop colorimetric biosensors. (A) A hairpin transducer based on gold nanoparticle aggregation were applied in the CRISPR/Cas12a system. (Reproduced with permission from Ref. [60]. Copyright 2021, Royal Society of Chemistry). The DNA loop of the hairpin transducer was *trans*-cleaved by activated Cas12a, which released the RNA crosslinker. This RNA crosslinker could hybrid the ssDNAs on AuNPs. Then, the corresponding solution changed in color from red to purple. (B) AuNPs-DNA1 and AuNPs-DNA2 pairs were cross-linked in the CRISPR/Cas12a system (Reproduced with permission from Ref. [56]. Copyright 2021, American Chemical Society). When target was present, this linker ssDNA (green) was cleaved by Cas12a to make the AuNPs change from aggregated to dispersive and the color change from purple to red.(C) A lateral flow assay was based on gold nanoparticle and Cas12a system (Reproduced with permission from Ref. [83]. Copyright 2021, American Chemical Society). Target amplicons could induce the Cas12a-crRNA complex to nonspecifically cleave a ssDNA reporter molecule modified with biotin and FITC at each terminus. Once the ssDNA reporter was cleaved, the biotin was captured at the test line, indicating a positive readout.

ratiometric reporter. For example, two different fluorescence dyes, fluorescein (FAM) and tetramethylrhodamine (TAMRA), were labeled at the 5′ and 3' ends of ssDNA (Fig. 5D) [52]. Without targets, the fluorescence of TAMRA at 580 nm was strong while the fluorescence of FAM at 520 nm was weak due to the effect of FRET. When the targets were present, the reporter was cleaved by Cas12a to eliminate the FRET effect, resulting in an increase fluorescence at 520 nm and a decrease fluorescence at 580 nm. The ratio between the fluorescence signal at 520 nm and at 580 nm could be applied to quantify the level of target. This limit of detection based on ratiometric reporter could be low to 3 pM without preamplification of target, which was three folds lower than that of canonical reporter. Besides, noble metal nanoclusters could be also employed to construct ratiometric reporter for CRISPR/Cas12a biosensor, achieving detect limit for *Salmonella typhimurium* as low as 1 CFU/mL [54]. And the quantum dots immobilized fluorescently-labeled DNA hairpins could be also designed a ratiometric reporter [53,80]. Therefore, the ratiometric reporter could be applied to improve the sensitivity and to differentiate false-positive signals.

3.5.2. Colorimetric-based reporters

The fluorescent-based reporters enable CRISPR/Cas12a based biosensors to possess good performance. Alternatively, CRISPR/Cas12a based biosensors with colorimetric-based reporters have also been developed owing to its advantage of visualization with the naked eye, especially for fast qualitative analysis on the spot [60,81,82]. In this respect, AuNP was the most common material applied to construct colorimetric-based reporter. Here, we summarize the advance of CRISPR/Cas12a based biosensors with AuNP as a colorimetric reporter.

Cao et al. reported a colorimetric reporter based on AuNP (Fig. 6A) [60]. Two AuNPs were functionalized with two different thiolated DNA sequences, respectively. Then, a hairpin transducer (HT) was designed including three functional domains: an RNA crosslinker, a DNA loop and a DNA lock. RNA crosslinker was composed of two different sequences that were complementary to the two different functional DNA on the surface of AuNPs, which conferred the ability to facilitate the aggregation of AuNPs. The DNA lock sequence was complementary to a part of the RNA crosslinker to form a hairpin structure, inhibiting the aggregation of AuNPs caused by HT. The DNA loop could serve as a substrate of the *trans*-cleavage of Cas12a to release the RNA crosslinker. When targets were present, HT was cleaved, resulting in a recognizable color change from red to purple within 1 min. This colorimetric reporter has been used to detect SARS-CoV-2 with a sensitivity and specificity of 92.6 % and 100 %, respectively.

In order to develop CRISPR/Cas12a biosensors with colorimetric reporter based on AuNP for quantitative analysis, the absorbance change of AuNP caused by different target concentrations were determined. Then the target level can be recalled by the concentration-absorbance relationship [56,84,85]. For example, Ma et al. developed a CRISPR/Cas12a-powered biosensor for colorimetric detection and photothermal detection (Fig. 6B) [56]. Two AuNPs were functionalized with two different thiolated ssDNA sequences, respectively. Part of the two different ssDNA was complementary to a linker ssDNA, by which the two different functional AuNPs could be linked via annealing to display a purple color. When target was present, this linker was cleaved by Cas12a to make the AuNPs change from aggregated to dispersive, displaying a red color. In addition, aggregated and dispersed AuNPs have different temperature responses to the exposure of near-infrared radiation. Thus, the CRISPR/Cas12a biosensor with functional AuNPs reporter was also a photothermal sensors. The sensor had a good linear range from 10° to 10^{8} CFU/mL *Salmonella*, with a detection limit of 1 CFU/mL in the both colorimetric and photothermal signal modes.

Besides, lateral flow assay (LFA) assisted by CRISPR/Cas12a was also developed for virus detection due to its rapid, cheap and convenient operation [83,86–88]. For example, Zhu et al. reported an LFA for detection of SARS-CoV-2 based on the CRISPR/Cas12a system with sensitivity and specificity of 100 % (Fig. 6C) [86]. In this strategy, the ssDNA reporter for CRISPR/Cas12a system was modified with biotin and fluorescein isothiocyanate (FITC) at the both ends. Streptavidin-immobilized AuNPs (SA-GNPs) which would be deposited on the conjugate pad of the LFA was prepared. The capture reagents including anti-FITC and biotinylated bovine serum albumin (B-BSA) were distributed onto the control line and test line, respectively. When the target was absent, the SA-GNPs that could bind to ssDNA reporters with FITC and biotin would be all captured by anti-FITC antibodies at the control line, displaying a red color. When target was present, the ssDNA reporter was cleaved by activated Cas12a to separate FITC and biotin. Part or all of the SA-GNPs would be captured by the B-BSA at the test line, displaying a red color. The LFA results could be read out within 2 min. Therefore, the LFA based on the CRISPR/Cas12a system has a great potential in clinical, field and resource-poor settings.

3.5.3. Reporters based on electrochemical signal

Electrochemical biosensors have become increasingly popular in medical detection due to the advantages of lower cost, energy efficiency and speed of electrochemical methods [89]. Based on electrochemical platform and CRISPR/Cas12a system (E-CRISPR), a ssDNA reporter normally labeled a methylate blue (MB) tag to develop CRISPR/Cas12a assisted electrochemical biosensors [61,62,90]. This ssDNA reporter was immobilized on the surface of gold electrode and was cleaved by activated Cas12a to generate electrochemical current signal (Fig. 7A) [61]. Without any enzymatic amplification, the detection limit of this electrochemical biosensor could be as low as the pM level. To further enhance the sensing performance with the help of AuNPs, the detection limit of CRISPR/Cas12a assisted electrochemical biosensor for target DNA was as low as 50 fM [62]. Notably, the MB-ssDNA reporter also had limitation that the long distance between the MB and the surface of the electrode decreased the transfer rate of electron. To overcome this limitation, a hairpin DNA (hpDNA) modified with MB tag was designed to sever as a signal reporter (Fig. 7B) [63]. Thus, the electrochemical biosensor could achieve good performance with the assistance of the CRISPR/Cas12a system.

3.6. Improve trans-cleavage activity of Cas12a through reaction conditions

The buffer component of reaction affects the *trans*-cleavage activity of Cas12a [17,34,51,91]. One of study results also demonstrated a similar phenomenon that the concentration of Mg^{2+} was very crucial for the work of CRISPR/Cas12a system [92]. For example, the extra unwinding of the target DNA in the distal PAM region was allosterically stabilized by Mg^{2+} ions [93]. And the best optimized concentration of Mg^{2+} was ranged from 10 mM to 12 mM [34,92]. When the concentration of Mg^{2+} reached 15 mM, the fluorescence intensity began to decrease and the reaction time of highest fluorescent would lengthen in this study. Besides, the fluorescence intensity of study increased after using a buffer containing a concentration of Mg^{2+} to pretreat the fiber nanoprobe, which has been cleaved by activated CRISPR/Cas12a [94]. However, the CRISPR/Cas12a system has been widely applied to in vitro biosensing, but rarely to biosensing in living cells. One reason is the lack of a suitable carry to deliver all factors of the CRISPR/Cas12a system into living cells. Hence, some studies provided Mn^{2+} as a stronger accelerator to facilitate CRISPR/Cas12a based biosensors of intracellular targets [64,65,95,96]. Wang et al. used MnO_2 nanosheets to provide a better carrier for CRISPR/Cas12a system and realize live-cell biosensing analysis rapidly and sensitively of various intracellular targets [65]. The application of the cofactor Mn^{2+} may provide a dependable imaging platform in living cells and promote the CRISPR development in the biosensing field.

4. Perspectives

In the last few years, CRISPR/Cas12a-based systems have been

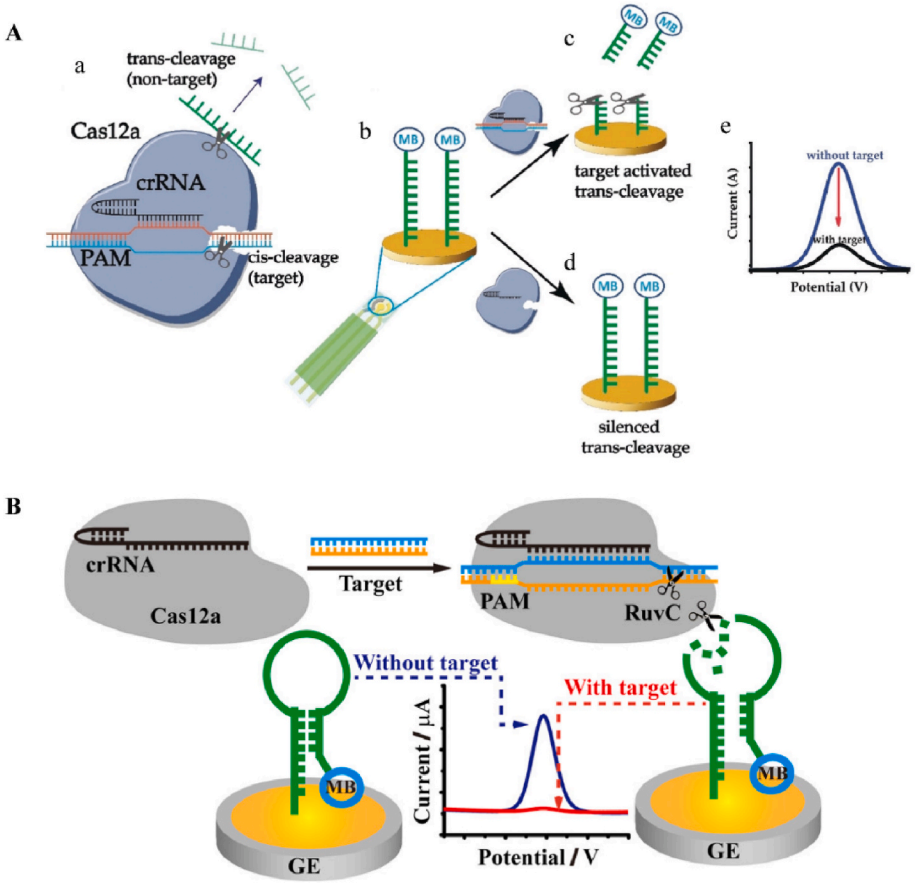


Fig. 7. Cas12a biosensors are based on electrochemical methods. (A) Applying ssDNA to achieve the CRISPR/Cas12a-based electrochemical biosensing platform (Reproduced with permission from Ref. [61]. Copyright 2021, Royal Society of Chemistry). Nonspecific ssDNA reporter with methylene blue (MB) tag could be *trans*-cleaved by activated Cas12a in the presence of target dsDNA, resulting a low electrochemical current. (B) Harnessing hairpin reporters to construct Cas12a-based electrochemical biosensors (Reproduced with permission from Ref. [63]. Copyright 2020, American Chemical Society). Hairpin ssDNA reporter linked with MB tag could be *trans*-cleaved by activated Cas12a in the presence of target dsDNA, resulting a low electrochemical current.

transformed into a widely accessible and pervasive method, which have emerged as the most influential and powerful class of molecular scissors for biosensing applications. To date, a variety of CRISPR/Cas biosensors have been developed based on the *cis*- and *trans*-cleavage activity of Cas12a. The *cis*-cleavage of Cas12a is only single turnover. But the *trans*-cleavage could reach up to a dozen turnovers per second. Therefore, the *trans*-cleavage of Cas12a was frequently applied to cleave the reporter probe in order to amplify the fluorescence signal. To improve the detect performance, modifications of PAM, target, crRNA, Cas nuclease, reporter, and reaction conditions have been introduced in the CRISPR/Cas12a detection system. However, work is still needed in future to enhance detection sensitivity, to reduce background signal, to enlarge target scope, to achieve intracellular imaging, as well as to develop point-of-care (POC) testing platforms.

4.1. Enhance detection sensitivity

The sensitivity of CRISPR/Cas12a based biosensors needs to be further improved. Without isothermal amplification, the canonical CRISPR/Cas12a biosensors can be able to detect targets low to the pM level, which could not meet the detection requirements for low-abundance targets [9,10]. Therefore, one or more amplification strategies including PCR, recombinase polymerase amplification (RPA), rolling circle amplification (RCA) and loop mediated isothermal amplification (LAMP) etc. could be applied to enhance the sensitivity of CRISPR/Cas12a biosensors [97,98]. In this case, the detection limit could reach aM [9]. Besides, other techniques could also be incorporated into CRISPR/Cas12a system [53,99–101]. For example, a glycerol additive promoted 100-fold sensitivity enhancement for one-pot RPA--CRISPR/Cas12a assay for the detection of ASFV and SARS-CoV-2 [100]. Consequently, techniques or strategies should be developed to improve the sensitivity of the CRISPR/Cas system for trace detection in the future.

4.2. Overcome high background signal

The background signal of CRISPR/Cas12a system could be generated by non-specific recognition of enzymes to substrates, non-specific amplification of primers or templates and imperfectly quenched of reporter probes [102]. Among them, non-specific amplification is the main reason for the high background signal, which would influence the accuracy of the detection results [102–105]. Therefore, reducing background signal will definitely boost the signal-to-noise ratio, thus improving the sensing performance. Some efforts have been devoted to solving this problem [105–112]. For example, Niu et al. proposed a strategy that integrating two-templates into the CRISPR/Cas12a system to reduce non-specific amplification in the exponential amplification reactions, whose signal-to-background ratio was improved from 1.3 to 15.4 [105]. However, the background generation mechanism is still poorly understood. Therefore, we should be able to further develop other novel strategies or methods to overcome the background interference of CRISPR/Cas12a biosensors.

4.3. Enlarge target scope

The CRISPR/Cas12a system is extremely mature for the detection of nucleic acid targets, yet it is mostly limited to the detection of non-nucleic acid targets. The greatest challenge in detecting non-nucleic acid targets is how to convert non-nucleic acid signals into nucleic acid signals via biological transduction elements, which can be identified by the CRISPR/Cas12a system. These bio-transduction elements primarily consist of aptamers, DNAzymes, antigen-antibodies, transcription factors, etc [113]. For example, a method using an allosteric inhibitor aptamer had been developed to detect adenosine triphosphate (ATP) [114]. And Wu et al. demonstrated a versatile biosensing strategy that uniquely integrated CRISPR/Cas12a with RNA-cleaving nucleic acid enzymes for ATP, *Escherichia coli* and *Klebsiella pneumoniae* detection [115]. Future work should consider incorporating or searching for new biological transduction elements to further broaden the target scope.

4.4. Achieve intracellular imaging

To date, the catalytically inactive protein called dead Cas9 (dCas9) was frequently employed for intracellular imaging [116]. However, reports about the application of CRISPR/Cas12a bioimaging were limited. In living cells, the detection efficiency of the CRISPR/Cas12a system is limited due to the restricted diffusion-mediated intermolecular interactions in the cytoplasm [117,118]. A possible solution is to hold the components of the sensing system in a confined space, by which concentrating reactants can significantly speed up the reaction catalyzed by CRISPR/Cas12a system. Besides, the physiological conditions of live cells also must be carefully considered, which influences the *trans*--cleavage activity of Cas12a [119]. For example, how to deliver the reporter and the substrate and how much concentrations of activators. Therefore, further research is needed to search for appropriate reaction conditions for the development of novel CRISPR/Cas12a based biosensors in living cells.

4.5. Develop point-of-care testing

The point-of-care testing is integrating sample pretreatment, target recognition, and signal sensing into a single device [120]. Point-of-care (POC) testing based on CRISPR/Cas12a system is a newly developed diagnostic tool with sensitivity, specificity and flexibility, which is suitable for on-site testing. Notably, a variety of CRISPR/Cas12a based POC sensing methods for SARS-CoV-2, including microfluidic chips, paper-based sensors, and even single-tube reactions, have been developed in the COVID-19 outbreak. Portable microfluidic cartridges or other miniaturized sensors have great potential for POC testing. For example, Xing et al. developed a finger-actuated microfluidic CRISPR/Cas12a biosensor, which was easy to use, operate, and could be used for POC testing without the help of professional equipment and suitable for setting of constrained resource [121]. Further portable devices can be considered to introduce the CRISPR/Cas12a system and thus construct more convenient POC sensing.

5. Conclusion

In conclusion, nucleic acid detection is of great importance for the diagnosis of various diseases. Therefore, there is an urgent need to develop nucleic acid detection with high sensitivity, high specificity, high efficiency, and low cost. The CRISPR/Cas12a system with advantages of specific target recognition and high *trans*-cleavage activity, is drawing people's attention. Over the past few years, nucleic acid detection based on the CRISPR/Cas12a system has become a broadly accessible and popularized technology. However, the canonical CRISPR/Cas12a is slowly struggling to meet the demand for detection with higher sensitivity, higher specificity, higher efficiency, larger target scope, easier operation, multiplexing, low cost and diversified signal reading. Fortunately, these could be improved by non-canonical CRISPR/Cas12a biosensors via modification of the elements of CRISPR/Cas12a system, including PAM sequence, crRNA, Cas nuclease, reporter and reaction buffer. Thus, the detection performance of CRISPR/Cas12a system could be greatly enhanced by non-canonical modification, which would increase its detection capability and accelerate its practical application. Nevertheless, efforts are still needed to better understand the mechanism of CRISPR/Cas12a system, to further broaden the target scope, to achieve intracellular detection, and to develop point-of-care testing. With the advancement of the Cas12a system, it is believed that the CRISPR/Cas12a system will make more achievements in the field of nucleic acid detection.

CRediT authorship contribution statement

Xueying Lei: Writing - original draft, Visualization, Methodology, Investigation, Conceptualization. **Shengnan Cao:** Writing - review & editing, Visualization. **Tao Liu:** Writing - review & editing, Methodology, Conceptualization. **Yongjun Wu:** Supervision. **Songcheng Yu:** Writing - review & editing, Supervision, Project administration, 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.

Data availability

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

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References

[1] M.M. Kaminski, O.O. Abudayyeh, J.S. Gootenberg, F. Zhang, J.J. Collins, CRISPR-based diagnostics, Nat. Biomed. Eng. 5 (7) (2021) 643–656.
[2] S. Yang, R.E. Rothman, PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings, Lancet Infect. Dis. 4 (6) (2004) 337–348.
[3] X. Dong, L. Liu, Y. Tu, J. Zhang, G. Miao, L. Zhang, S. Ge, N. Xia, D. Yu, X. Qiu, Rapid PCR powered by microfluidics: a quick review under the background of COVID-19 pandemic, Trends Anal. Chem. 143 (2021) 116377.
[4] C.B.F. Vogels, A.F. Brito, A.L. Wyllie, J.R. Fauver, I.M. Ott, C.C. Kalinich, M. E. Petrone, A. Casanovas-Massana, M. Catherine Muenker, A.J. Moore, J. Klein, P. Lu, A. Lu-Culligan, X. Jiang, D.J. Kim, E. Kudo, T. Mao, M. Moriyama, J.E. Oh, A. Park, J. Silva, E. Song, T. Takahashi, M. Taura, M. Tokuyama, A. Venkataraman, O.E. Weizman, P. Wong, Y. Yang, N.R. Cheemarla, E.B. White, S. Lapidus, R. Earnest, B. Geng, P. Vijayakumar, C. Odio, J. Fournier, S. Bermejo, S. Farhadian, C.S. Dela Cruz, A. Iwasaki, A.I. Ko, M.L. Landry, E.F. Foxman, N. D. Grubaugh, Analytical sensitivity and efficiency comparisons of SARS-CoV-2 RT-qPCR primer-probe sets, Nat. Microbiol. 5 (10) (2020) 1299–1305.
[5] V.M. Corman, O. Landt, M. Kaiser, R. Molenkamp, A. Meijer, D.K. Chu, T. Bleicker, S. Brünink, J. Schneider, M.L. Schmidt, D.G. Mulders, B.L. Haagmans, B. van der Veer, S. van den Brink, L. Wijsman, G. Goderski, J.L. Romette, J. Ellis, M. Zambon, M. Peiris, H. Goossens, C. Reusken, M.P. Koopmans, C. Drosten, Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR, Euro Surveill. 25 (3) (2020).
[6] K.S. Makarova, Y.I. Wolf, O.S. Alkhnbashi, F. Costa, S.A. Shah, S.J. Saunders, R. Barrangou, S.J. Brouns, E. Charpentier, D.H. Haft, P. Horvath, S. Moineau, F. J. Mojica, R.M. Terns, M.P. Terns, M.F. White, A.F. Yakunin, R.A. Garrett, J. van der Oost, R. Backofen, E.V. Koonin, An updated evolutionary classification of CRISPR-Cas systems, Nat. Rev. Microbiol. 13 (11) (2015) 722–736.
[7] E.V. Koonin, K.S. Makarova, F. Zhang, Diversity, classification and evolution of CRISPR-Cas systems, Curr. Opin. Microbiol. 37 (2017) 67–78.

[8] B. Zetsche, J.S. Gootenberg, O.O. Abudayyeh, I.M. Slaymaker, K.S. Makarova, P. Essletzbichler, S.E. Volz, J. Joung, J. van der Oost, A. Regev, E.V. Koonin, F. Zhang, Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system, Cell 163 (3) (2015) 759–771.

[9] J.S. Chen, E. Ma, L.B. Harrington, M. Da Costa, X. Tian, J.M. Palefsky, J. A. Doudna, CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity, Science 360 (6387) (2018) 436–439.

[10] S.Y. Li, Q.X. Cheng, J.M. Wang, X.Y. Li, Z.L. Zhang, S. Gao, R.B. Cao, G.P. Zhao, J. Wang, CRISPR-Cas12a-assisted nucleic acid detection, Cell Discov. 4 (2018) 20.

[11] Q. Chen, I. Gul, C. Liu, Z. Lei, X. Li, M.A. Raheem, Q. He, Z. Haihui, E. Leeansyah, C.Y. Zhang, V. Pandey, K. Du, P. Qin, CRISPR-Cas12-based field-deployable system for rapid detection of synthetic DNA sequence of the monkeypox virus genome, J. Med. Virol. 95 (1) (2023) e28385.

[12] F. Teng, J. Li, T. Cui, K. Xu, L. Guo, Q. Gao, G. Feng, C. Chen, D. Han, Q. Zhou, W. Li, Enhanced mammalian genome editing by new Cas12a orthologs with optimized crRNA scaffolds, Genome Biol. 20 (1) (2019) 15.

[13] P. Horvath, R. Barrangou, CRISPR/Cas, the immune system of bacteria and archaea, Science 327 (5962) (2010) 167–170.

[14] B. Paul, G. Montoya, CRISPR-Cas12a: functional overview and applications, Biomed. J. 43 (1) (2020) 8–17.

[15] S.Y. Li, Q.X. Cheng, J.K. Liu, X.Q. Nie, G.P. Zhao, J. Wang, CRISPR-Cas12a has both cis- and trans-cleavage activities on single-stranded DNA, Cell Res. 28 (4) (2018) 491–493.

[16] J.C. Ryan, T. Fuchs, Megumu Mabuchi, Yourik Paul, G. Brett Robb, Cas12a Trans-cleavage Can Be Modulated in Vitro and Is Active on ssDNA, dsDNA, and RNA, bioRxiv Preprint, 2019.

[17] Z. Li, W. Zhao, S. Ma, Z. Li, Y. Yao, T. Fei, A chemical-enhanced system for CRISPR-Based nucleic acid detection, Biosens. Bioelectron. 192 (2021) 113493.

[18] A. Ramachandran, J.G. Santiago, CRISPR enzyme kinetics for molecular diagnostics, Anal. Chem. 93 (20) (2021) 7456–7464.

[19] L. Li, S. Li, N. Wu, J. Wu, G. Wang, G. Zhao, J. Wang, HOLMESv2: a CRISPR-cas12b-assisted platform for nucleic acid detection and DNA methylation quantitation, ACS Synth. Biol. 8 (10) (2019) 2228–2237.

[20] D. Dong, K. Ren, X. Qiu, J. Zheng, M. Guo, X. Guan, H. Liu, N. Li, B. Zhang, D. Yang, C. Ma, S. Wang, D. Wu, Y. Ma, S. Fan, J. Wang, N. Gao, Z. Huang, The crystal structure of Cpf1 in complex with CRISPR RNA, Nature 532 (7600) (2016) 522–526.

[21] S. Stella, P. Mesa, J. Thomsen, B. Paul, P. Alcón, S.B. Jensen, B. Saligram, M. E. Moses, N.S. Hatzakis, G. Montoya, Conformational activation promotes CRISPR-cas12a catalysis and resetting of the endonuclease activity, Cell 175 (7) (2018), 1856-1871.e21.

[22] D.C. Swarts, M. Jinek, Mechanistic insights into the cis- and trans-acting DNase activities of Cas12a, Mol. Cell 73 (3) (2019) 589–600.e4.

[23] D.C. Zhang, Y.R. Yan, X.X. Cheng, T.T. Yang, X.R. Li, S.J. Ding, X.M. Zhang, W. Cheng, Controlling the trans-cleavage of CRISPR-Cas12a with nicked PAM: universal platform for biosensing, Sensor Actuat. B-Chem. 353 (2022).

[24] S. Lu, X. Tong, Y. Han, K. Zhang, Y. Zhang, Q. Chen, J. Duan, X. Lei, M. Huang, Y. Qiu, D.Y. Zhang, X. Zhou, Y. Zhang, H. Yin, Fast and sensitive detection of SARS-CoV-2 RNA using suboptimal protospacer adjacent motifs for Cas12a, Nat. Biomed. Eng. 6 (3) (2022) 286–297.

[25] S. Chen, R. Wang, S. Peng, S. Xie, C. Lei, Y. Huang, Z. Nie, PAM-less conditional DNA substrates leverage trans-cleavage of CRISPR-Cas12a for versatile live-cell biosensing, Chem. Sci. 13 (7) (2022) 2011–2020.

[26] Y. Jeon, Y.H. Choi, Y. Jang, J. Yu, J. Goo, G. Lee, Y.K. Jeong, S.H. Lee, I.S. Kim, J. S. Kim, C. Jeong, S. Lee, S. Bae, Direct observation of DNA target searching and cleavage by CRISPR-Cas12a, Nat. Commun. 9 (1) (2018) 2777.

[27] M. Zhong, K. Chen, W. Sun, X. Li, S. Huang, Q. Meng, B. Sun, X. Huang, X. Wang, X. Ma, P. Ma, PCDetection: PolyA-CRISPR/Cas12a-based miRNA detection without PAM restriction, Biosens. Bioelectron. 214 (2022) 114497.

[28] Y. Wu, W. Luo, Z. Weng, Y. Guo, H. Yu, R. Zhao, L. Zhang, J. Zhao, D. Bai, X. Zhou, L. Song, K. Chen, J. Li, Y. Yang, G. Xie, A PAM-free CRISPR/Cas12a ultra-specific activation mode based on toehold-mediated strand displacement and branch migration, Nucleic Acids Res. 50 (20) (2022) 11727–11737.

[29] Q. Li, Z.L. Song, Y. Zhang, L. Zhu, Q. Yang, X. Liu, X. Sun, X. Chen, R. Kong, G. C. Fan, X. Luo, Synergistic incorporation of two ssDNA activators enhances the trans-cleavage of CRISPR/Cas12a, Anal. Chem. 95 (23) (2023) 8879–8888.

[30] W. Zhang, Y. Mu, K. Dong, L. Zhang, B. Yan, H. Hu, Y. Liao, R. Zhao, W. Shu, Z. Ye, Y. Lu, C. Wan, Q. Sun, L. Li, H. Wang, X. Xiao, PAM-independent ultra-specific activation of CRISPR-Cas12a via sticky-end dsDNA, Nucleic Acids Res. 50 (22) (2022) 12674–12688.

[31] X. Fu, Y. Yin, M. Zhang, F. Peng, Y. Shi, Y. Liu, Y. Tan, Z. Zhao, X. Yin, J. Song, G. Ke, X.B. Zhang, Size-selective DNA nanocage-based activatable CRISPR-Cas12a for sensitive and accurate detection of mature microRNA, Chem. Commun. 57 (26) (2021) 3291–3294.

[32] T. Luo, J. Li, Y. He, H. Liu, Z. Deng, X. Long, Q. Wan, J. Ding, Z. Gong, Y. Yang, S. Zhong, Designing a CRISPR/Cas12a- and Au-Nanobeacon-Based diagnostic biosensor enabling direct, rapid, and sensitive miRNA detection, Anal. Chem. 94 (17) (2022) 6566–6573.

[33] K. Guk, S. Yi, H. Kim, Y. Bae, D. Yong, S. Kim, K.S. Lee, E.K. Lim, T. Kang, J. Jung, Hybrid CRISPR/Cas protein for one-pot detection of DNA and RNA, Biosens. Bioelectron. 219 (2022) 114819.

[34] L.T. Nguyen, B.M. Smith, P.K. Jain, Enhancement of trans-cleavage activity of Cas12a with engineered crRNA enables amplified nucleic acid detection, Nat. Commun. 11 (1) (2020) 4906.

[35] M. Zeng, Y. Ke, Z. Zhuang, C. Qin, L.Y. Li, G. Sheng, Z. Li, H. Meng, X. Ding, Harnessing multiplex crRNA in the CRISPR/Cas12a system enables an amplification-free DNA diagnostic platform for ASFV detection, Anal. Chem. 94 (30) (2022) 10805–10812.

[36] L. Xia, J. Yin, J. Zhuang, W. Yin, Z. Zou, Y. Mu, Adsorption-free self-priming direct digital dual-crRNA CRISPR/Cas12a-Assisted chip for ultrasensitive detection of pathogens, Anal. Chem. 95 (10) (2023) 4744–4752.

[37] M. Hu, Z. Qiu, Z. Bi, T. Tian, Y. Jiang, X. Zhou, Photocontrolled crRNA activation enables robust CRISPR-Cas12a diagnostics, Proc. Natl. Acad. Sci. U. S. A. 119 (26) (2022) e2202034119.

[38] M. Hu, R. Liu, Z. Qiu, F. Cao, T. Tian, Y. Lu, Y. Jiang, X. Zhou, Light-start CRISPR-cas12a reaction with caged crRNA enables rapid and sensitive nucleic acid detection, Angew Chem. Int. Ed. Engl. (2023) e202300663.

[39] D. Li, W. Cheng, Z. Hou, C. Duan, Y. Yao, Y. Chen, G. Yang, Z. Cheng, Y. Xiang, A functional RNA/DNA circuit for one-pot detection of SARS-CoV-2 RNA, Chem. Commun. 58 (97) (2022) 13475–13478.

[40] G. Wang, W. Tian, X. Liu, W. Ren, C. Liu, New CRISPR-derived microRNA sensing mechanism based on Cas12a self-powered and rolling circle transcription-unleashed real-time crRNA recruiting, Anal. Chem. 92 (9) (2020) 6702–6708.

[41] K. Shi, S. Xie, R. Tian, S. Wang, Q. Lu, D. Gao, C. Lei, H. Zhu, Z. Nie, A CRISPR-Cas autocatalysis-driven feedback amplification network for supersensitive DNA diagnostics, Sci. Adv. 7 (5) (2021).

[42] J.D. Habimana, O. Mukama, G. Chen, M. Chen, O.B. Amissah, L. Wang, Y. Liu, Y. Sun, A.L. Li, S. Deng, J. Huang, X.X. Yan, T. Rutaganda, D. Mutangana, L. P. Wu, R. Huang, Z. Li, Harnessing enhanced CRISPR/Cas12a trans-cleavage activity with extended reporters and reductants for early diagnosis of Helicobacter pylori, the causative agent of peptic ulcers and stomach cancer, Biosens. Bioelectron. 222 (2023) 114939.

[43] M. Rossetti, R. Merlo, N. Bagheri, D. Moscone, A. Valenti, A. Saha, P.R. Arantes, R. Ippodrino, F. Ricci, I. Treglia, E. Delibato, J. van der Oost, G. Palermo, G. Perugino, A. Porchetta, Enhancement of CRISPR/Cas12a trans-cleavage activity using hairpin DNA reporters, Nucleic Acids Res. 50 (14) (2022) 8377–8391.

[44] C.W. Smith, M.J. Kachwala, N. Nandu, M.V. Yigit, Recognition of DNA target formulations by CRISPR-cas12a using a dsDNA reporter, ACS Synth. Biol. 10 (7) (2021) 1785–1791.

[45] N. Mohammad, S.S. Katkam, Q. Wei, A sensitive and nonoptical CRISPR detection mechanism by sizing double-stranded λ DNA reporter, Angew Chem. Int. Ed. Engl. 61 (50) (2022) e202213920.

[46] J.H. Choi, J. Lim, M. Shin, S.H. Paek, J.W. Choi, CRISPR-Cas12a-Based nucleic acid amplification-free DNA biosensor via Au nanoparticle-assisted metal-enhanced fluorescence and colorimetric analysis, Nano Lett. 21 (1) (2021) 693–699.

[47] H. Gao, M. Feng, F. Li, K. Zhang, T. Zhang, Z. Zhang, C. Yang, R. Deng, J. Zhang, P. Jiang, G-quadruplex DNAzyme-substrated CRISPR/Cas12 assay for label-free detection of single-celled parasitic infection, ACS Sens. 7 (10) (2022) 2968–2977.

[48] X. Xia, B. Ma, T. Zhang, Y. Lu, M.R. Khan, Y. Hu, C. Lei, S. Deng, Q. He, G. He, K. Zhang, R. Deng, G-Quadruplex-Probing CRISPR-cas12 assay for label-free analysis of foodborne pathogens and their colonization in vivo, ACS Sens. 6 (9) (2021) 3295–3302.

[49] Y. Li, T. Li, B.F. Liu, R. Hu, J. Zhu, T. He, X. Zhou, C. Li, Y. Yang, M. Liu, CRISPR-Cas12a trans-cleaves DNA G-quadruplexes, Chem. Commun. 56 (83) (2020) 12526–12529.

[50] T. Yang, J. Li, D. Zhang, X. Cheng, J. Li, X. Huang, S. Ding, B.Z. Tang, W. Cheng, Pre-folded G-quadruplex as a tunable reporter to facilitate CRISPR/Cas12a-Based visual nucleic acid diagnosis, ACS Sens. 7 (12) (2022) 3710–3719.

[51] T. Li, R. Hu, J. Xia, Z. Xu, D. Chen, J. Xi, B.F. Liu, J. Zhu, Y. Li, Y. Yang, M. Liu, G.- triplex, A new type of CRISPR-Cas12a reporter enabling highly sensitive nucleic acid detection, Biosens. Bioelectron. 187 (2021) 113292.

[52] Q. Liu, M. Liu, Y. Jin, B. Li, Ratiometric fluorescent probe: a sensitive and reliable reporter for the CRISPR/Cas12a-based biosensing platform, Analyst 147 (11) (2022) 2567–2574.

[53] C.M. Green, J. Spangler, K. Susumu, D.A. Stenger, I.L. Medintz, S.A. Díaz, Quantum dot-based molecular beacons for quantitative detection of nucleic acids with CRISPR/Cas(N) nucleases, ACS Nano 16 (12) (2022) 20693–20704.

[54] L. Ma, J. Wang, Y. Li, D. Liao, W. Zhang, X. Han, S. Man, A ratiometric fluorescent biosensing platform for ultrasensitive detection of Salmonella typhimurium via CRISPR/Cas12a and silver nanoclusters, J. Hazard Mater. 443 (Pt B) (2023) 130234.

[55] G. Su, M. Zhu, D. Li, M. Xu, Y. Zhu, Y. Zhang, H. Zhu, F. Li, Y. Yu, Multiplexed lateral flow assay integrated with orthogonal CRISPR-Cas system for SARS-CoV-2 detection, Sensor. Actuator. B Chem. 371 (2022) 132537.

[56] L. Ma, L. Peng, L. Yin, G. Liu, S. Man, CRISPR-Cas12a-Powered dual-mode biosensor for ultrasensitive and cross-validating detection of pathogenic bacteria, ACS Sens. 6 (8) (2021) 2920–2927.

[57] M. Bao, E. Jensen, Y. Chang, G. Korensky, K. Du, Magnetic bead-quantum dot (MB-Qdot) clustered regularly interspaced short palindromic repeat assay for simple viral DNA detection, ACS Appl. Mater. Interfaces 12 (39) (2020) 43435–43443.

[58] J. Ki, H.K. Na, S.W. Yoon, V.P. Le, T.G. Lee, E.K. Lim, CRISPR/Cas-Assisted colorimetric biosensor for point-of-use testing for african swine fever virus, ACS Sens. 7 (12) (2022) 3940–3946.

[59] N. Shao, X. Han, Y. Song, P. Zhang, L. Qin, CRISPR-Cas12a coupled with platinum nanoreporter for visual quantification of SNVs on a volumetric bar-chart chip, Anal. Chem. 91 (19) (2019) 12384–12391.

[60] Y. Cao, J. Wu, B. Pang, H. Zhang, X.C. Le, CRISPR/Cas12a-mediated gold nanoparticle aggregation for colorimetric detection of SARS-CoV-2, Chem. Commun. 57 (56) (2021) 6871–6874.

[61] Y. Dai, R.A. Somoza, L. Wang, J.F. Welter, Y. Li, A.I. Caplan, C.C. Liu, Exploring the trans-cleavage activity of CRISPR-cas12a (cpf1) for the development of a universal electrochemical biosensor, Angew Chem. Int. Ed. Engl. 58 (48) (2019) 17399–17405.
[62] C. Wu, Z. Chen, C. Li, Y. Hao, Y. Tang, Y. Yuan, L. Chai, T. Fan, J. Yu, X. Ma, O. A. Al-Hartomy, S. Wageh, A.G. Al-Sehemi, Z. Luo, Y. He, J. Li, Z. Xie, H. Zhang, CRISPR-Cas12a-Empowered electrochemical biosensor for rapid and ultrasensitive detection of SARS-CoV-2 delta variant, Nano-Micro Lett. 14 (1) (2022).
[63] D. Zhang, Y. Yan, H. Que, T. Yang, X. Cheng, S. Ding, X. Zhang, W. Cheng, CRISPR/Cas12a-Mediated interfacial cleaving of hairpin DNA reporter for electrochemical nucleic acid sensing, ACS Sens. 5 (2) (2020) 557–562.
[64] S. Xie, B. Xu, R. Tang, S. Chen, C. Lei, Z. Nie, Kinetics accelerated CRISPR-cas12a enabling live-cell monitoring of Mn(2+) homeostasis, Anal. Chem. 94 (28) (2022) 10159–10167.
[65] D.X. Wang, Y.X. Wang, J. Wang, J.Y. Ma, B. Liu, A.N. Tang, D.M. Kong, MnO(2) nanosheets as a carrier and accelerator for improved live-cell biosensing application of CRISPR/Cas12a, Chem. Sci. 13 (15) (2022) 4364–4371.
[66] J.R. Spangler, T.A. Leski, Z. Schultzhaus, Z. Wang, D.A. Stenger, Large scale screening of CRISPR guide RNAs using an optimized high throughput robotics system, Sci. Rep. 12 (1) (2022) 13953.
[67] H.M. Park, H. Liu, J. Wu, A. Chong, V. Mackley, C. Fellmann, A. Rao, F. Jiang, H. Chu, N. Murthy, K. Lee, Extension of the crRNA enhances Cpf1 gene editing in vitro and in vivo, Nat. Commun. 9 (1) (2018) 3313.
[68] R. Shebanova, N. Nikitchina, N. Shebanov, V. Mekler, K. Kuznedelov, E. Ulashchik, R. Vasilev, O. Sharko, V. Shmanai, I. Tarassov, K. Severinov, N. Entelis, I. Mazunin, Efficient target cleavage by Type V Cas12a effectors programmed with split CRISPR RNA, Nucleic Acids Res. 50 (2) (2022) 1162–1173.
[69] M. Hu, Z. Qiu, Z. Bi, T. Tian, Y. Jiang, X. Zhou, Photocontrolled crRNA activation enables robust CRISPR-Cas12a diagnostics, Proc. Natl. Acad. Sci. U.S.A. 119 (26) (2022).
[70] S. Zadran, S. Standley, K. Wong, E. Otiniano, A. Amighi, M. Baudry, Fluorescence resonance energy transfer (FRET)-based biosensors: visualizing cellular dynamics and bioenergetics, Appl. Microbiol. Biotechnol. 96 (4) (2012) 895–902.
[71] H. Yue, B. Shu, T. Tian, E. Xiong, M. Huang, D. Zhu, J. Sun, Q. Liu, S. Wang, Y. Li, X. Zhou, Droplet Cas12a assay enables DNA quantification from unamplified samples at the single-molecule level, Nano Lett. 21 (11) (2021) 4643–4653.
[72] S.A. Marras, F.R. Kramer, S. Tyagi, Efficiencies of fluorescence resonance energy transfer and contact-mediated quenching in oligonucleotide probes, Nucleic Acids Res. 30 (21) (2002) e122.
[73] X. Fu, Y. Shi, F. Peng, M. Zhou, Y. Yin, Y. Tan, M. Chen, X. Yin, G. Ke, X.B. Zhang, Exploring the trans-cleavage activity of CRISPR/Cas12a on gold nanoparticles for stable and sensitive biosensing, Anal. Chem. 93 (11) (2021) 4967–4974.
[74] X. Chen, L. Wang, F. He, G. Chen, L. Bai, K. He, F. Zhang, X. Xu, Label-free colorimetric method for detection of Vibrio parahaemolyticus by trimming the G-quadruplex DNAzyme with CRISPR/Cas12a, Anal. Chem. 93 (42) (2021) 14300–14306.
[75] D. Wang, G. Chen, Y. Lyu, E. Feng, L. Zhu, C. Pan, W. Zhang, X. Liu, H. Wang, A CRISPR/Cas12a-based DNAzyme visualization system for rapid, non-electrically dependent detection of Bacillus anthracis, Emerg. Microb. Infect. 11 (1) (2022) 428–437.
[76] L. Yin, N. Duan, S. Chen, Y. Yao, J. Liu, L. Ma, Ultrasensitive pathogenic bacteria detection by a smartphone-read G-quadruplex-based CRISPR-Cas12a bioassay, Sensor Actuat. B-Chem. 347 (2021).
[77] G. Miglietta, M. Russo, G. Capranico, G-quadruplex-R-loop interactions and the mechanism of anticancer G-quadruplex binders, Nucleic Acids Res. 48 (21) (2020) 11942–11957.
[78] S. Takahashi, A. Kotar, H. Tateishi-Karimata, S. Bhowmik, Z.F. Wang, T.C. Chang, S. Sato, S. Takenaka, J. Plavec, N. Sugimoto, Chemical modulation of DNA replication along G-quadruplex based on topology-dependent ligand binding, J. Am. Chem. Soc. 143 (40) (2021) 16458–16469.
[79] L. Yuan, W. Lin, K. Zheng, S. Zhu, FRET-based small-molecule fluorescent probes: rational design and bioimaging applications, Acc. Chem. Res. 46 (7) (2013) 1462–1473.
[80] D.A.H. Christopher, M. Green, Kimihiro Susumu, Spangler Joseph, David A. Stenger, Igor L. Medintz, A. Sebastián, Díaz Passivating quantum dots against histag-displaying enzymes using blocking peptides: salient considerations for self-assembling quantum dot biosensors, Sensors and Diagnostica 2 (2023) 1521–1530.
[81] R. Zhou, Y. Li, T. Dong, Y. Tang, F. Li, A sequence-specific plasmonic loop-mediated isothermal amplification assay with orthogonal color readouts enabled by CRISPR Cas12a, Chem. Commun. 56 (24) (2020) 3536–3538.
[82] M. Cheng, E. Xiong, T. Tian, D. Zhu, H.Q. Ju, X. Zhou, A CRISPR-driven colorimetric code platform for highly accurate telomerase activity assay, Biosens. Bioelectron. 172 (2021) 112749.
[83] Y. Sun, L. Yu, C. Liu, S. Ye, W. Chen, D. Li, W. Huang, One-tube SARS-CoV-2 detection platform based on RT-RPA and CRISPR/Cas12a, J. Transl. Med. 19 (1) (2021) 74.
[84] W.S. Zhang, J. Pan, F. Li, M. Zhu, M. Xu, H. Zhu, Y. Yu, G. Su, Reverse transcription recombinase polymerase amplification coupled with CRISPR-cas12a for facile and highly sensitive colorimetric SARS-CoV-2 detection, Anal. Chem. 93 (8) (2021) 4126–4133.
[85] J. Jiao, K. Kong, J. Han, S. Song, T. Bai, C. Song, M. Wang, Z. Yan, H. Zhang, R. Zhang, J. Feng, X. Zheng, Field detection of multiple RNA viruses/viroids in apple using a CRISPR/Cas12a-based visual assay, Plant Biotechnol. J. 19 (2) (2021) 394–405.
[86] X. Zhu, X. Wang, S. Li, W. Luo, X. Zhang, C. Wang, Q. Chen, S. Yu, J. Tai, Y. Wang, Rapid, ultrasensitive, and highly specific diagnosis of COVID-19 by CRISPR-based detection, ACS Sens. 6 (3) (2021) 881–888.
[87] O. Mukama, J. Wu, Z. Li, Q. Liang, Z. Yi, X. Lu, Y. Liu, Y. Liu, M. Hussain, G. G. Makafe, J. Liu, N. Xu, L. Zeng, An ultrasensitive and specific point-of-care CRISPR/Cas12 based lateral flow biosensor for the rapid detection of nucleic acids, Biosens. Bioelectron. 159 (2020) 112143.
[88] O. Mukama, T. Yuan, Z. He, Z. Li, J.d.D. Habimana, M. Hussain, W. Li, Z. Yi, Q. Liang, L. Zeng, A high fidelity CRISPR/Cas12a based lateral flow biosensor for the detection of HPV16 and HPV18, Sensor Actuat. B-Chem. 316 (2020).
[89] G. Maduraiveeran, M. Sasidharan, V. Ganesan, Electrochemical sensor and biosensor platforms based on advanced nanomaterials for biological and biomedical applications, Biosens. Bioelectron. 103 (2018) 113–129.
[90] F. Li, Q. Ye, M. Chen, B. Zhou, J. Zhang, R. Pang, L. Xue, J. Wang, H. Zeng, S. Wu, Y. Zhang, Y. Ding, Q. Wu, An ultrasensitive CRISPR/Cas12a based electrochemical biosensor for Listeria monocytogenes detection, Biosens. Bioelectron. 179 (2021).
[91] P. Ma, Q. Meng, B. Sun, B. Zhao, L. Dang, M. Zhong, S. Liu, H. Xu, H. Mei, J. Liu, T. Chi, G. Yang, M. Liu, X. Huang, X. Wang, MeCas12a, a highly sensitive and specific system for COVID-19 detection, Adv. Sci. 7 (20) (2020) 2001300.
[92] H. Wu, J.S. He, F. Zhang, J. Ping, J. Wu, Contamination-free visual detection of CaMV35S promoter amplicon using CRISPR/Cas12a coupled with a designed reaction vessel: rapid, specific and sensitive, Anal. Chim. Acta 1096 (2020) 130–137.
[93] H. Son, J. Park, I. Hwang, Y. Jung, S. Bae, S. Lee, Mg(2+)-dependent conformational rearrangements of CRISPR-Cas12a R-loop complex are mandatory for complete double-stranded DNA cleavage, Proc. Natl. Acad. Sci. U. S. A. 118 (49) (2021).
[94] D. Song, W. Xu, X. Han, H. Wang, Y. Zhuo, J. Liu, A. Zhu, F. Long, CRISPR/Cas12a-powered evanescent wave fluorescence nanobiosensing platform for nucleic acid amplification-free detection of Staphylococcus aureus with multiple signal enhancements, Biosens. Bioelectron. 225 (2023) 115109.
[95] J.X. Liu, X.M. Sun, D. Liu, Y.H. Liu, C.Y. Li, Smart NIR light-gated CRISPR/Cas12a fluorescent biosensor with boosted biological delivery and trans-cleavage activity for high-performance in vivo operation, Biosens. Bioelectron. 216 (2022) 114646.
[96] B. Li, J. Yan, Y. Zhang, W. Li, C. Zeng, W. Zhao, X. Hou, C. Zhang, Y. Dong, CRISPR-Cas12a possesses unconventional DNase activity that can Be inactivated by synthetic oligonucleotides, Mol. Ther. Nucleic Acids 19 (2020) 1043–1052.
[97] W. Feng, A.M. Newbigging, J. Tao, Y. Cao, H. Peng, C. Le, J. Wu, B. Pang, J. Li, D. L. Tyrrell, H. Zhang, X.C. Le, CRISPR technology incorporating amplification strategies: molecular assays for nucleic acids, proteins, and small molecules, Chem. Sci. 12 (13) (2021).
[98] Y. Liu, L. Ma, W. Liu, L. Xie, Q. Wu, Y. Wang, Y. Zhou, Y. Zhang, B. Jiao, Y. He, RPA-CRISPR/Cas12a combined with rolling circle amplification- enriched DNAzyme: a homogeneous photothermal sensing strategy for plant pathogens, J. Agric. Food Chem. 71 (11) (2023) 4736–4744.
[99] Y. Du, S. Ji, Q. Dong, J. Wang, D. Han, Z. Gao, Amplification-free detection of HBV DNA mediated by CRISPR-Cas12a using surface-enhanced Raman spectroscopy, Anal. Chim. Acta 1245 (2023) 340864.
[100] M. Lin, H. Yue, T. Tian, E. Xiong, D. Zhu, Y. Jiang, X. Zhou, Glycerol additive boosts 100-fold sensitivity enhancement for one-pot RPA-CRISPR/Cas12a assay, Anal. Chem. 94 (23) (2022) 8277–8284.
[101] S. Lee, D. Nam, J.S. Park, S. Kim, E.S. Lee, B.S. Cha, K.S. Park, Highly efficient DNA reporter for CRISPR/Cas12a-Based specific and sensitive biosensor, Biochip J 16 (4) (2022) 463–470.
[102] Y.C. Du, S.Y. Wang, Y.X. Wang, J.Y. Ma, D.X. Wang, A.N. Tang, D.M. Kong, Terminal deoxynucleotidyl transferase combined CRISPR-Cas12a amplification strategy for ultrasensitive detection of uracil-DNA glycosylase with zero background, Biosens. Bioelectron. 171 (2021) 112734.
[103] K. Dong, W. Shu, J. Zhang, S. Cheng, J. Zhang, R. Zhao, T. Hua, W. Zhang, H. Wang, Ultra-sensitive biosensor based on CRISPR-Cas12a and Endo IV coupled DNA hybridization reaction for uracil DNA glycosylase detection and intracellular imaging, Biosens. Bioelectron. 226 (2023) 115118.
[104] S.Y. Wang, Y.C. Du, D.X. Wang, J.Y. Ma, A.N. Tang, D.M. Kong, Signal amplification and output of CRISPR/Cas-based biosensing systems: a review, Anal. Chim. Acta 1185 (2021) 338882.
[105] C. Niu, J. Liu, X. Xing, C. Zhang, CRISPR-Cas12a-assisted elimination of the non-specific signal from non-specific amplification in the Exponential Amplification Reaction, Anal. Chim. Acta 1251 (2023) 340998.
[106] W. Tian, X. Liu, G. Wang, C. Liu, A hyperbranched transcription-activated CRISPR-Cas12a signal amplification strategy for sensitive microRNA sensing, Chem. Commun. 56 (87) (2020) 13445–13448.
[107] K. Dong, W. Shu, J. Zhang, S. Cheng, J. Zhang, R. Zhao, T. Hua, W. Zhang, H. Wang, Ultra-sensitive biosensor based on CRISPR-Cas12a and Endo IV coupled DNA hybridization reaction for uracil DNA glycosylase detection and intracellular imaging, Biosens. Bioelectron. 226 (2023).
[108] Y.-C. Du, S.-Y. Wang, Y.-X. Wang, J.-Y. Ma, D.-X. Wang, A.-N. Tang, D.-M. Kong, Terminal deoxynucleotidyl transferase combined CRISPR-Cas12a amplification strategy for ultrasensitive detection of uracil-DNA glycosylase with zero background, Biosens. Bioelectron. 171 (2021).
[109] W. Tian, X. Liu, G. Wang, C. Liu, A hyperbranched transcription-activated CRISPR-Cas12a signal amplification strategy for sensitive microRNA sensing, Chem. Commun. 56 (87) (2020) 13445–13448.

[110] J. Zhang, R. Zhao, K. Dong, W. Shu, S. Wei, Y. Yao, J. Zhang, S. Liu, W. Zhang, H. Wang, An ultra-sensitive and specific UCBiosensor via CRISPR-Cas12a and UDG-mediated polymerase chain reaction, Sensor Actuat. B-Chem. 386 (2023).
[111] S. Zhou, H. Sun, D. Huo, X. Wang, N. Qi, L. Peng, M. Yang, P. Lu, C. Hou, A novel methyl-dependent DNA endonuclease GlaI coupling with double cascaded strand displacement amplification and CRISPR/Cas12a for ultra-sensitive detection of DNA methylation, Anal. Chim. Acta 1212 (2022).
[112] X. Wang, F. Wang, J. Wang, Y. Liu, C. Gao, S. Ge, J. Yu, Catalytic hairpin assembly-assisted CRISPR/Cas12a mediated photoelectrochemical biosensor for sensitive detection of miRNA-122, Sensor Actuat. B-Chem. 370 (2022).
[113] X. Cheng, Y. Li, J. Kou, D. Liao, W. Zhang, L. Yin, S. Man, L. Ma, Novel non-nucleic acid targets detection strategies based on CRISPR/Cas toolboxes: a review, Biosens. Bioelectron. 215 (2022) 114559.
[114] P. Qin, P. Chen, N. Deng, L. Tan, B.-C. Yin, B.-C. Ye, Switching the activity of CRISPR/Cas12a using an allosteric inhibitory aptamer for biosensing, Anal. Chem. 94 (45) (2022) 15908–15914.
[115] Y. Wu, D. Chang, Y. Chang, Q. Zhang, Y. Liu, J.D. Brennan, Y. Li, M. Liu, Nucleic Acid Enzyme-Activated CRISPR-Cas12a with Circular CRISPR RNA for Biose, nsing, Small, 2023 e2303007.
[116] C. Van Tricht, T. Voet, J. Lammertyn, D. Spasic, Imaging the unimaginable: leveraging signal generation of CRISPR-Cas for sensitive genome imaging, Trends Biotechnol. 41 (6) (2022) 769–784.
[117] K. Dong, W. Shu, J. Zhang, S. Cheng, J. Zhang, R. Zhao, T. Hua, W. Zhang, H. Wang, Ultra-sensitive biosensor based on CRISPR-Cas12a and Endo IV coupled DNA hybridization reaction for uracil DNA glycosylase detection and intracellular imaging, Biosens. Bioelectron. 226 (2023), 115118-115118.
[118] X. Xia, B. Ma, W. Zhou, H. Yang, C. Lei, Y. Dong, J. Zhang, Q. He, R. Deng, Direct visualization of single-cell non-repetitive genes by in situ activation of collateral activity of CRISPR/Cas12a inside cells, Chem. Eng. J. 451 (2023).
[119] W. Feng, H. Zhang, X.C. Le, Signal amplification by the trans-cleavage activity of CRISPR-cas systems: kinetics and performance, Anal. Chem. 95 (1) (2023) 206–217.
[120] J.E. van Dongen, J.T.W. Berendsen, R.D.M. Steenbergen, R.M.F. Wolthuis, J.C. T. Eijkel, L.I. Segerink, Point-of-care CRISPR/Cas nucleic acid detection: recent advances, challenges and opportunities, Biosens. Bioelectron. 166 (2020) 112445.
[121] G. Xing, Y. Shang, X. Wang, H. Lin, S. Chen, Q. Pu, L. Lin, Multiplexed detection of foodborne pathogens using one-pot CRISPR/Cas12a combined with recombinase aided amplification on a finger-actuated microfluidic biosensor, Biosens. Bioelectron. 220 (2023).