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Advances in amplification-free detection of nucleic acid: CRISPR/Cas system as a powerful tool

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ABSTRACT

Amplification technologies such as polymerase chain reaction (PCR) play an important role in nucleic acid detection. However, they require bulky and sophisticated thermal cycling instrument, as well as are prone to get false-positive results due to amplicon contamination. Currently, CRISPR/Cas system has become an increasingly popular diagnostic tool for nucleic acid with the discovery of its *trans*-cleavage activity which can degrade single-stranded DNA or RNA at a very high turnover rate. This inherent signal amplification capability allows CRISPR/Cas system to detect unamplified nucleic acids. Here, we reviewed the recent advances of CRISPR-based amplification-free methods for nucleic acid detection. With the assistance of various signal enhancement strategies, the detection sensitivity could be comparable to that of amplification-based methods. We then presented the pros and cons of these methods. And the subsistent challenges including sample preparation, off-target effect, sequences limit, quantitative and multiplex detection were further discussed in this review. It is probable for CRISPR-powered detection methods to pave the road for rapid, cheap, highly sensitive and specific on-site detection without amplification.

1. Introduction

CRISPR is an important defense system that protects bacteria and archaea from the infection of viruses and plasmids [1], which integrates a short segment of foreign nucleic acids into CRISPR locus at the first invasion [2]. In the occurrence of the infection by the same invader again, CRISPR-derived RNAs (crRNAs), transcribed and processed by the CRISPR, assemble with Cas proteins (or Cas effectors) to form CRISPR-associated ribonucleoprotein (RNP) complexes that can recognize, hybridize and cleave invading nucleic acids [3]. CRISPR/Cas systems have been divided into 2 classes, 6 types and many subtypes [4,5]. Class 2 including type II, type V and type VI [6–8], which depends on single effectors rather than multi-effector complexes like class 1, is widely employed due to its simplicity. In addition to the well-known application of genome editing and disease treatment [9], with the discovery of *trans* cleavage activities of Cas13a on single-stranded RNA [7] and Cas12a on single-stranded DNA (ssDNA) [10], the capability of CRISPR/Cas systems for nucleic acid detection has attracted considerable attention. The first such detection system termed as SHERLOCK was released at 2017 [11]. Subsequently, many nucleic acid detection platforms have been developed which employ CRISPR/Cas systems for pathogen surveillance, tumor diagnosis, and field deployment, like DETECTR system [12], HOLMES system [13], etc. They have the excellent capability to achieve a detection sensitivity of at least fM or mostly aM concentration of target nucleic acids, and a high specificity to identify single-base variation. Meanwhile, these techniques are programmable, rapid, and cost-efficient.

However, the majority of these methods require an inevitable step, nucleic acid amplification, prior to CRISPR/Cas sensing. Amplification approach such as polymerase chain reaction (PCR) requires sophisticated thermal cycling procedures, therefore relies on specialized and bulky experimental equipment, leading this procedure to be time-

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consuming and costly. And there is also a potential risk for PCR procedure to obtain false-positive signals due to inappropriate primer design or amplicon cross-contamination [14]. Isothermal amplification techniques including loop-mediated isothermal amplification (LAMP) [15], rolling circle amplification (RCA) [16], nicking enzyme-assisted amplification (NEAA) [17], etc. Avoid the thermal cycling process. However, the amplification procedure is still time consuming.

The collateral cleavage activity of CRISPR/Cas proteins occurs at a very high turnover rate. On average, Cas13a cleaves ~10^4 nonspecific RNA within cleavage of a target RNA [18], resulting in vast signal amplification. When combined with other technologies, like electrochemical, optical and colorimetric technologies, CRISPR/Cas systems could be harnessed to build amplification-free detection methods. Here, we review the recent advances of CRISPR/Cas system-based amplification-free methods for nucleic acid detection. We first summarize the characteristic of five frequently used Cas effectors and detection principle of CRISPR/Cas system-based methods. And we further present advances on nucleic acid amplification-free detection technologies combining CRISPR/Cas systems, and their advantages and disadvantages. Finally, the current challenges and perspectives will be discussed.

2. Brief introduction of five commonly used Cas effectors

CRISPR arrays consist of a series of repeats sequence (approximately 20–50 base pairs) and spacer sequences often neighboring conserved sets of Cas genes [19]. Spacer sequences were formed from invading nucleic acid fragments that possess a protospacer adjacent motif (PAM) [2]. An effective CRISPR/Cas system needs two compositions, crRNAs or single-guide RNA (sgRNA) [20], and Cas effectors, to form RNP complexes [3]. The crRNA is programmable to recognize and bind multitudinous target nucleic acids [21]. Cas effectors which are expressed from Cas genes have cleavage activities and have extensive diversification [22]. Cas9, dCas9, Cas12a, Cas13a and Cas14a are attractive and frequently used in nucleic-acid-based detection among the diverse Cas effectors.

Cas9 effector is capable of recognizing and cleaving double-strand DNA (dsDNA) with the assistance of crRNA and *trans*-activating crRNA (tracrRNA) [20]. In some circumstance, sgRNA as an alternative functionally equivalent to original two-RNA complex has been engineered by combining with crRNA and tracrRNA [23]. When dsDNA targets appear, Cas9 recognizes and binds with them guided by sgRNA which is complementary to target segment and PAM sequence on the target site to unwind the dsDNA helix [24]. The site-specific cleavage of target sequences, which is also called *cis* cleavage, takes place at a locus that three base pairs upstream of the PAM [25,26]. The *cis* cleavage is conducted by HNH and RuvC nuclease domains which cut targeted and non-targeted strands respectively [27]. Different from Cas9, nuclease-deactivated Cas9 (dCas9) cannot cleave the dsDNA because of inactivated nuclease domains of RuvC1 and HNH, but retains the binding ability [28]. Highly specific binding capability allows dCas9 become powerful recognition receptor, which could generate signals for sensitive detection [29,30].

Cas12a effector targets to dsDNA and ssDNA. crRNA is employed to recognize target sequence by a short PAM sequence [31]. After the Cas12a/crRNA complex binding with target sequence, the *cis* cleavage for both strands of dsDNA target occurs that only RuvC domain is required. Compared with Cas9, Cas12a effector possesses non-specific cleavage (*trans* cleavage or collateral cleavage) activity that can cleave non-specific ssDNA upon the formation of a ternary complex comprise of Cas12a, crRNA and target sequence [12]. PAM sequence is essential for dsDNA recognition and cleavage but not for target ssDNA [12,32]. Cas13a also has *trans* cleavage activity, but the target of non-specific cleavage is RNA [33]. And the crRNA recognizes complementary target RNA by protospacer-flanking sequence (PFS) sequence [34]. The cleavage activity of Cas13a is conducted by two HEPN domains [35]. Cas 14 proteins are exceptionally small RNA-guided nucleases which belong to a subset of Cas12f proteins [36]. Cas14a effector can cleave target ssDNA without PAM sequence, and triggers indiscriminate cleavage of ssDNA [36]. However, recent research has proved that a 5' T- or C-rich PAM sequence is capable of triggering dsDNA target cleavage in at least some Cas14a effectors [8]. A summary of five commonly used Cas effectors is presented in Table 1. Cas9 and Cas14a effectors have been demonstrated to be sensitive to discriminate one nucleotide mismatch which could be employed to single nucleotide polymorphism (SNP) detection [37,38].

3. CRISPR/Cas-based detection for unamplified nucleic acids

To date, CRISPR/Cas-based detection of nucleic acids has achieved an excellent sensitivity of aM level. However, the required pre-amplification steps limited its application. Some groups have explored the amplification-free detection methods based on CRISPR/Cas system. These detection methods combined CRISPR/Cas systems with various signal readout technologies, such as electrical, electrochemical, fluorescence and nanoparticle technology, to perform sensitive and highly sequence-specific detection.

3.1. (d)Cas9 effector-based approaches

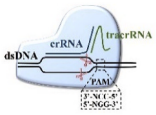
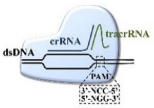
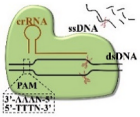
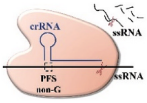
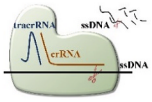
The *cis*-cleavage activity of Cas9 effector and binding activity of dCas9 effector are employed for nucleic acid detection. For detection methods which employ cleavage activity, CRISPR/Cas9 system is used to cleave target dsDNA labelling with electrochemical tags to produce signals which indicate the presence of targets. Xu et al. constructed an electrochemical biosensor which utilized the cleavage activity of Cas9 effector to detect Parvovirus B19 (PB-19) [39] (Fig. 1A). The target ssDNA could hybridize with a ssDNA reporter which was conjugated with methylene blue (MB) at the electrode surface to form a dsDNA. Once the reorganization of that dsDNA, CRISPR/Cas9 system could cut it to produce an electrochemical signal change. The limit of detection (LOD) was 100 fM and a dynamic range crossing 7 orders of magnitude was achieved.

For detection methods which employ binding activity, CRISPR/dCas9 serves as a binding element to form a complex of dCas9 effector, sgRNA and target sequence. The complex can be immobilized on the surface of electrode or magnetic particles for further electrochemical, optical or electrical detection. Uygun et al. employed CRISPR/dCas9 system and electrochemical impedance spectroscopy (EIS) to detect circulating tumor DNA (ctDNA). dCas9/sgRNA complex pre-immobilized on graphene oxide screen printed electrodes could specifically recognize and bind with ctDNA. This biological recognition event would produce a change of surface electron transfer resistance which could be measured by EIS [40]. The detection was rapid, taking 40 s. However, its sensitivity was relatively poor, only 0.65 nM.

Surface-enhanced Raman scattering (SERS) and fluorescence in situ hybridization (FISH) as the optical readout technologies were employed in the amplification-free detection based on the binding activity of dCas9 effectors. Kim et al. developed a CRISPR/dCas9 system-mediated SERS detection approach to detect multidrug-resistant (MDR) bacteria including *staphylococcus aureus, acinetobacter baumannii, and klebsiella pneumoniae* [41]. In this method, bacterial gene could be recognized by dCas9/sgRNA which was combined with Au-coated magnetic nanoparticles (Au MNPs). After target binding, the complex could be easily separated from unbound sequences by external magnet and detected by SERS (Fig. 1B). The LOD for tissue samples was in the range of fM level. Guk et al. utilized CRISPR/dCas9 system to detect methicillin-resistant *staphylococcus aureus* (MRSA) by FISH [42]. The principle of this approach is similar to the above-mentioned approach based on SERS. The only difference is that Guk et al. employs SYBR green I (SG) for dsDNA staining instead of MB used by Kim et al. The detection could be completed in 30 min with a LOD of 10 CFU/mL.

Graphene-based field-effect transistors (gFET) and solid-state

Table 1
A brief introduction of five commonly used Cas effectors.

Cas effectors	cas9	dcas9	cas12a	cas13a	cas14a
					
spacer length	~30 nt	~30 nt	18-25 nt	22-30 nt	20-40 nt
nuclease domains	HNH, RuvC	HNH, RuvC containing two mutations	RuvC	2 x HEPN	RuvC
guide RNA	crRNA and tracrRNA or sgRNA	crRNA and tracrRNA or sgRNA	crRNA	crRNA	crRNA and tracrRNA or sgRNA
PAM/PFS[a] sequence	3′ GC-rich PAM	3′ GC-rich PAM	5′ T-rich PAM	3′ PFS: non-G	5′ T- or C-rich PAM
target type	dsDNA	dsDNA	dsDNA; ssDNA	RNA	dsDNA (partly effectors); ssDNA
cis cleavage	Blunt ends	None	Staggered, 5′-overhangs	Cleavage at uracil sites	Staggered, 5′-overhangs
Trans cleavage	None	None	Complete cleavage of non-specific ssDNA	Complete cleavage of non-specific RNA	Complete cleavage of non-specific ssDNA

[a] PAM, protospacer adjacent motif; PFS, protospacer-flanking sequence.

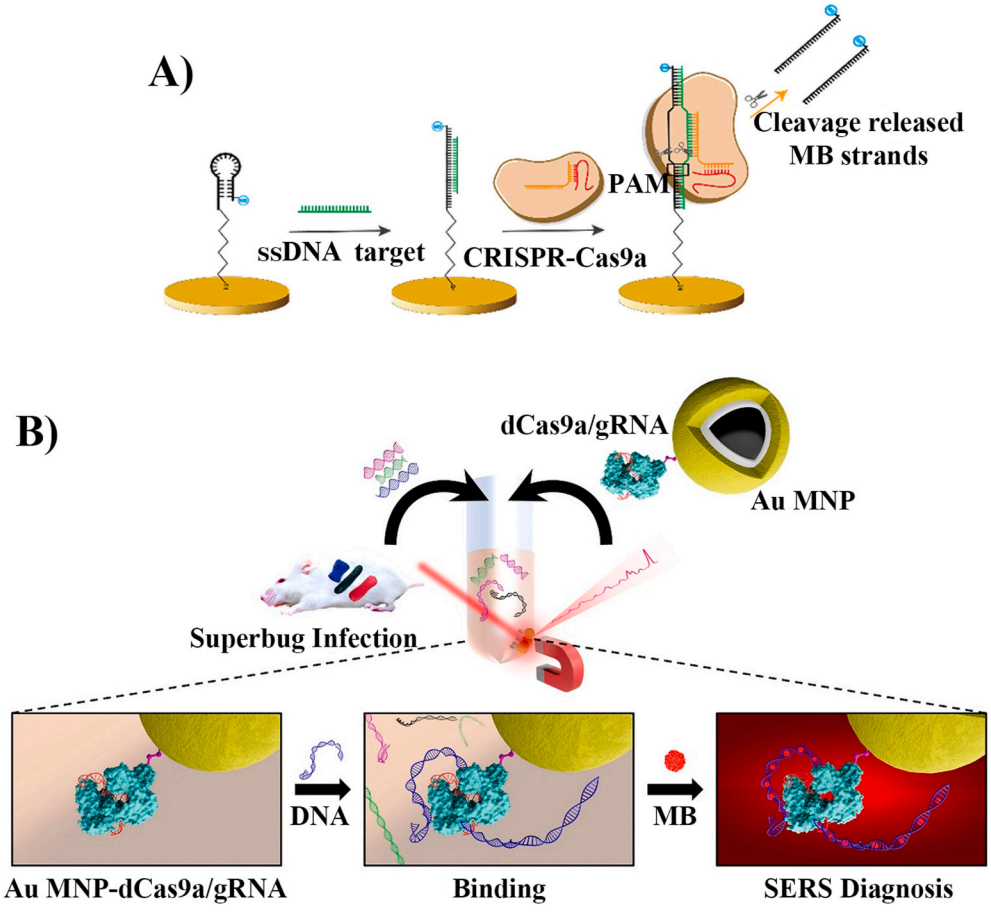


Fig. 1. Amplification-free detection methods based on CRISPR/(d)Cas9 systems combing with electrochemical and optical signals readout. (A) Cas9-enhanced electrochemical DNA sensor. The detection was based on the target induced variation of the electron transfer rate of the electrochemical tag. An enhanced signal change could be obtained with the introduction of CRISPR/Cas9 *cis*-cleavage activity. Reprinted from Ref. [39] with the permission from Elsevier. (B) Surface-enhanced Raman scattering-mediated superbug detection. The dCas9/sgRNA complex which was combined with Au-coated magnetic nanoparticle (Au MNP) could sequence-specifically recognize bacteria gene target. After Raman dye incorporation and magnetic separation, the SERS signal could be obtained from Au MNP-dCas9/sgRNA-target complex to check the presence of targets. Reprinted from Ref. [41] with the permission from American Chemical Society.

nanopore technologies are employed to read out electrical signal based on dCas9 effectors. For gFET, its detection signal can be acquired by the current change between the source and drain electrodes due to the recognition of target sequence and biological receptor immobilized on the transistor [43–45]. Hajian et al. employed dCas9/sgRNA as the biological receptor to directly detect two mutations in dystrophin gene [46] (Fig. 2A). The detection took 15 min and the LOD was 1.7 fM. Balderston et al. incorporated different CRISPR/dCas9 variants to discriminate two SNPs [47]. The detection sensitivity could be 10 ng/μL in 60 min. By spatial separation of different detection system, Bruch et al. presented a gFETs-based biosensor which could perform multiplex detection [48]. Another electrical detection method is solid-state nanopore which has single molecule sensitivity [49]. The current decreases when targets transfer through the nanopore and temporarily plug up the ions passage. Obviously, the specificity of this method is absent. CRISPR/dCas9 system can compensate this drawback by serving as sequence-specific recognition element. Yang et al. developed the first dCas9-assisted solid-state nanopores detection platform for unamplified DNA [50]. Weckman et al. further designed a platform to detect multiple targets [51]. It employed several dCas9/sgRNA complexes to bind with

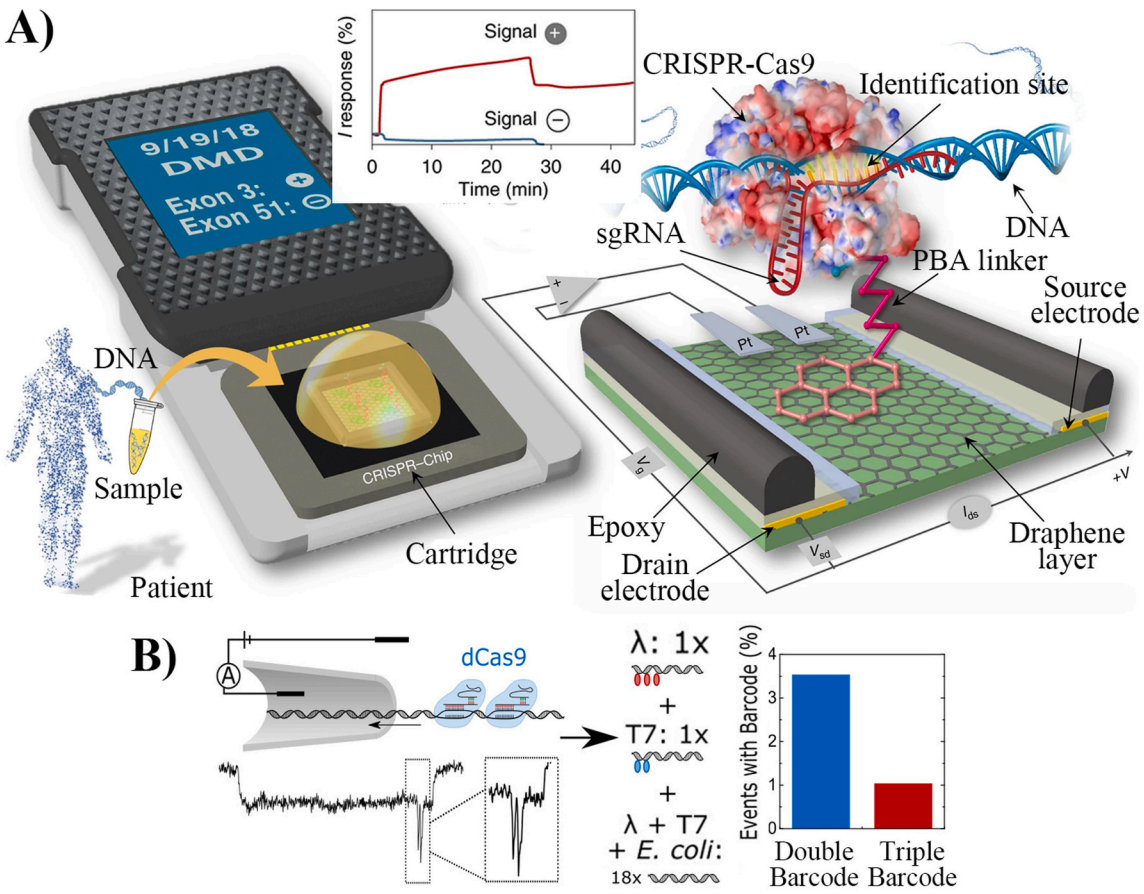


Fig. 2. Amplification-free detection methods based on CRISPR/dCas9 systems combing with electrical signals readout. (A) Detection of gene target from the whole genomic sample based on graphene field-effect-transistor (gFET). dCas9/sgRNA complex immobilized on the surface of the graphene of gFET could bind with target DNA, resulting in the modulation of gFET electrical characteristics which provided an electrical signal change. Reprinted from Ref. [46] with the permission from Springer Nature. (B) Multiplexed solid-state-nanopores sensing achieved by binding double and triple dCas9 probes which produced two and three peaks. Reprinted from Ref. [51] with the permission from American Chemical Society.

different target sequences which could produce double or triple peaks for targets detection (Fig. 2B). However, this detection took several hours. And the multiple dCas9/sgRNA complexes increased the off-target risk and might cause cross-talk in multi-peaks.

3.2. *Cas13 effector-based approaches*

CRISPR/Cas13a was the first system developed for nucleic acid detection [11]. The detection strategies are based on *trans*-cleavage activity of Cas13a effector which could cleave the RNA reporters to produce electrochemical or optical signals.

3.2.1. *Electrochemical assays*

Since Cas13a effector takes RNA as substrates, it could be employed for the detection of microRNA (miRNA) whose abnormal expression may hold a clue to cancer disease [52]. Moreover, miRNAs are suitable targets for amplification-free detection because it is difficult to perform amplification procedure due to their short length. Bruch et al. developed a Cas13a-mediated electrochemical microfluidic chip for miRNAs detection with a LOD of 10 pM. RNA reporter which was linked with glucose oxidase (GOx) and biotin on each side was employed as the *trans*-cleavage target. On the recognition of miRNAs, the activated Cas13a effector would degrade RNA reporters. Only reporters that were not degraded could fix the GOx on the chip surface which had been immobilized with streptavidin. Subsequently, the glucose was oxidized by GOx to produce H_2O_2 that could be detected. The generated amperometric signal was inversely proportional to the concentration of miRNAs. The detection could be completed in 4 h including nucleic acid purification process [53,54]. With the similar approach, Bruch et al. developed a multiplex detection microfluidic chip [55]. The channel in the chip was divided into multiple reaction areas. They found that the pattern of 4 channel sections without spacers provided the best result with a sensitive of 2–18 pM. The shortages of this approach were the complexity of chip-fabrication, multi-washing steps, and etc.

Cui et al. constructed a Cas13a-based electrochemical detection for miRNA-21 [56]. They utilized catalytic hairpin assembly (CHA) reaction to enhance the sensitivity. In the CHA reaction, hairpin DNA 0 (H0) with RNA fragment was cleaved by activated Cas13a effector to release the second target DNA (ST). ST sequences were used as the initiator of CHA which would first hybridize with hairpin DNA 1 (H1) on the surface of Au electrode. Then the opened H1 would hybridize with the MB-labeled hairpin DNA 2 (H2) to release ST strands for the next cycle. The MB closed to the Au electrode could generate electrochemical signals for the detection of miRNA-21 (Fig. 3A). This method obtained a higher sensitivity of 2.6 fM and a broad linear detection relationship range from 10 fM to 1 nM. However, it relied on complicated probes design which limited its widespread application.

3.2.2. *Colorimetric readout assays*

Colorimetric methods detect targets by measuring transparency or color change due to the targets. These approaches are popular because of direct visualized detection of targets and low costs [57].

Spoelstra et al. proposed a turbidity measurement method based on liquid-liquid phase separation (LLPS) phenomenon [58]. The solution

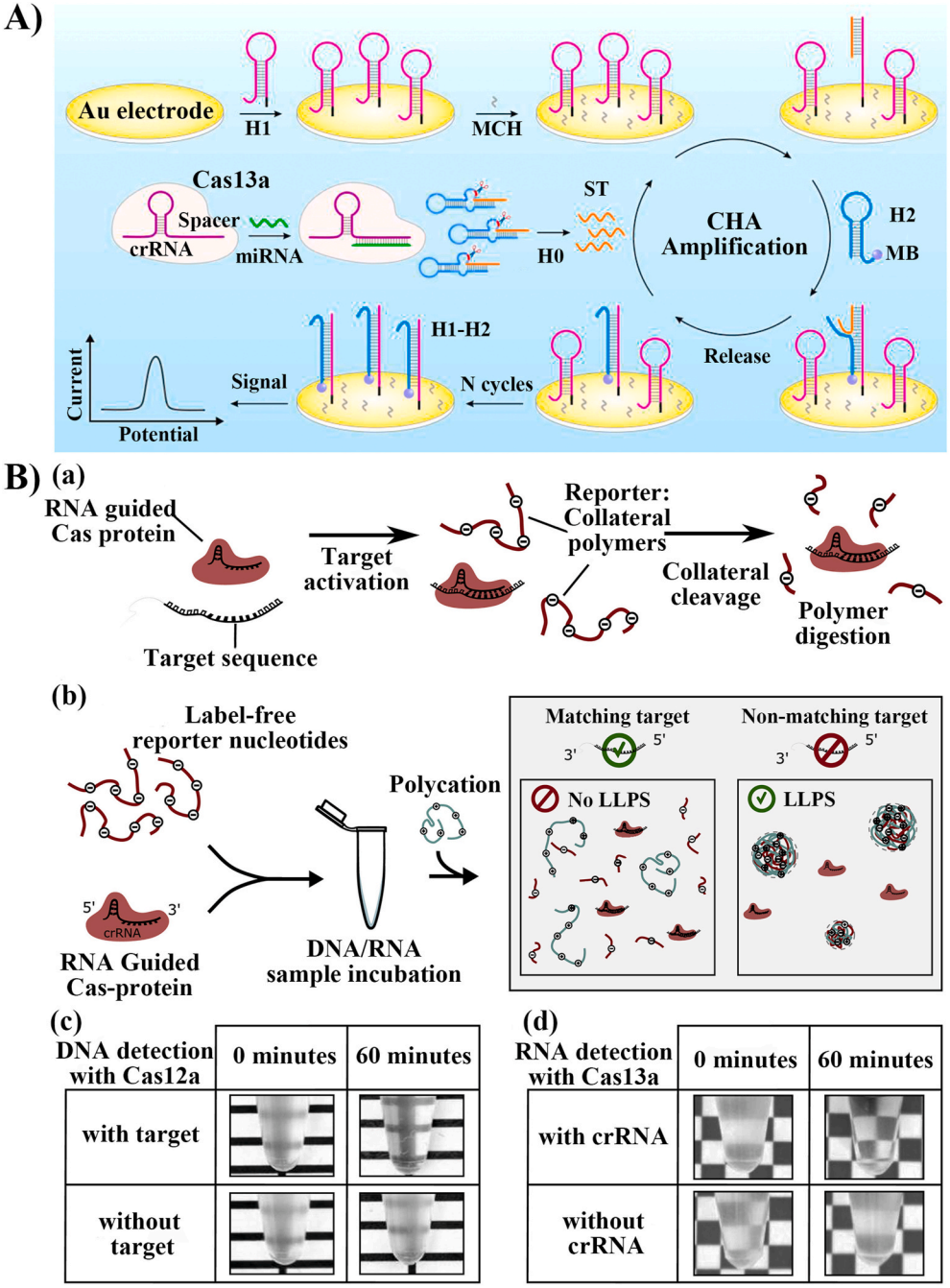


Fig. 3. (A) Catalytic hairpin assembly (CHA)-based electrochemical detection of miRNA. Once miRNA-21 appears, Cas13a/crRNA complex would bind with miRNA-21 target and activate its cleavage activity, resulting in the release of initiators of CHA to generate cascade amplified electrochemical signals. Reprinted from Ref. [56] with the permission from Elsevier. (B) The CRISPR-assisted detection method employing solution turbidity readout caused by liquid-liquid phase separation (LLPS). (a) The *trans*-cleavage activity was activated by targets to digest collateral polymers. (b) The turbidity-based detection mechanism was based on LLPS. In the absence of targets, the solution would be turbid because of LLPS of collateral polymer and polycation. While, in the presence of targets, the solution would be transparent because there was no LLPS due to the degradation of collateral polymers. (c) and (d) The activity of CRISPR/Cas systems was proofed by experiments results. Reprinted from Ref. [58] with the permission from Elsevier.

was turbid in the absence of target RNA because of LLPS which was created by long nucleic acid sequences and positively charged polyelectrolytes in the same solution. While the solution would become transparent in the presence of target RNA because activated Cas13a effector could digest positively charged poly(U) reporters (Fig. 3B). This method has a simple procedure but takes a long time. Its LOD is at nM level.

Aggregation and dispersion of gold nanoparticle (AuNPs) would cause their color changing from purple to red, which could be identified visually [59]. Based on distance-dependent optical characteristic of AuNPs, Yuan et al. presented a Cas13a-based colorimetric detection for miRNA-17 [60]. Target miRNA-17 triggered *trans*-cleavage activity of Cas13a led to the degradation of linker RNA which initiated dispersion behavior of AuNPs. Therefore, the solution would turn into red when miRNA-17 was present (Fig. 4A). This method could achieve a detection sensitivity of ~500 fM within 1 h. Likewise, the method allowed Cas12a effector to replace Cas13a for detection of African swine fever virus (ASFV) with a LOD of 200 copies of genome DNA [60].

3.2.3. *Fluorescence readout assays*

Even though colorimetric readout is cost-efficiency, simple and allow direct visualize detection, its LOD is not as good as that of fluorescence assays. In the detection based on fluorescence readout, RNA reporters labeled with fluorophore can be degraded by target-activated Cas13a effector to produce fluorescence signals. Qin et al. designed a fluorimeter which collected fluorescence by an off-axis parabolic mirror and developed an automated microfluidic chip for CRISPR reaction [18]. The entire detection can be completed in 5 min with a LOD of ~50 fM for Ebola RNA. To further improve the sensitivity, Zhang et al. presented a strategy for viable *Bacillus cereus* detection which employed light-up

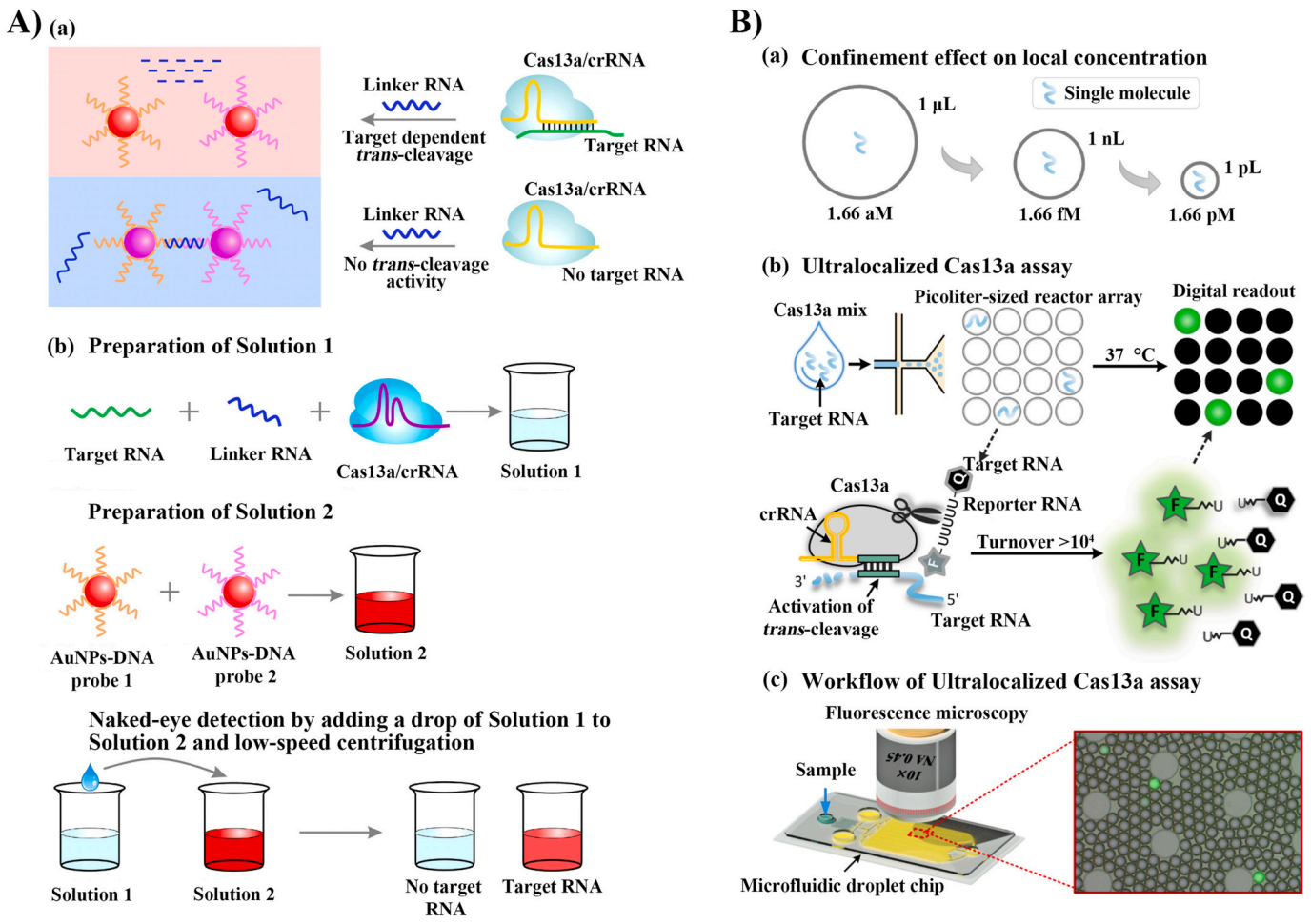


Fig. 4. (A) CRISPR/Cas13a system coupled with colorimetric signals readout for amplification-free detection. Targets-activated *trans*-cleavage activities of CRISPR system triggered an aggregation behavior for designed gold nanoparticles (AuNPs)-DNA probes, resulting in a color change which enabled naked-eye detection. (a) Schematic of CRISPR/Cas system-based colorimetric detection. (b) Workflow of AuNPs-mediated colorimetric readout detection. Reprinted from Ref. [60] with the permission from American Chemical Society. (B) CRISPR/Cas13a system coupled with fluorescence signals readout enabled absolute digital single-molecule quantitation. (a) Local concentration increasing resulting from volume decreasing. (b) Principle of absolute digital quantification detection based on fluorescence readout. CRISPR/Cas13a, RNA targets and reporters were confined in the cell-like-sized reactors by droplet microfluidics to enhance their local concentrations. The reporters were degraded by targets-activated Cas13a effectors to produce fluorescence signals which indicated the presence of targets. Each reactor with fluorescence represented one target in sample. (c) Workflow of droplet-microfluidics chip. Reprinted from Ref. [63] with the permission from American Chemical Society.

RNA reporters labeled with DFHBI-1T dyes [61]. This strategy could detect 10 CFU *Bacillus cereus* and allow to be performed in real food samples. But the free DFHBI-1T dyes may emit a weak fluorescence which would degrade the detection sensitivity. Fozouni et al. employed multiple crRNAs to recognize and bind single SARS-CoV-2 target. When two crRNAs were used, the LOD would be 100 copies/μL in 30 min [62].

In order to enhance its sensitivity and achieve absolute quantification, digital detection approach, such as droplet microfluidics and micro-chamber techniques, has been introduced. It enhances the local target concentration by loading solution into millions of femtoliter microchambers or cell-like-sized droplets. Though the amount of nucleic acids in single chamber is small, the concentration can be high. By controlling the input concentration of targets, each droplet or micro-chamber might contain either one RNA molecule or none. Tian et al. found that, in such small volume, single target triggered CRISPR degradation of fluorescent reporters could generate detectable signals [63]. Therefore, absolute digital quantitation can be accomplished by counting positive and negative droplets. Shinoda et al. proposed a quantification detection platform for SARS-CoV-2, termed as SATORI. It has a sensitivity of ~10 fM and detection time of 5 min. When using three crRNAs to simultaneously bind SARS-CoV-2 RNA, the LOD could be 5.7 ± 2.2 fM (~3.4 × 10^3 copies/μL) [64]. Tian et al. employed droplet microfluidics combined with CRISPR/Cas13a system to detect a variety of RNAs (miRNAs, 16S rRNAs, and SARS-CoV-2). The reaction solution was added into cell-like-sized reactors. After incubating at 37 °C for 60 min, the mixtures were detected using fluorescence microscopy (Fig. 4B). This assay had a more than 10,000-fold increase in sensitivity compared with conventional bulk volume Cas13a-based strategies [63].

3.3. *Cas12 effector-based approaches*

The detection methods based on Cas13a effectors could reach a sensitivity of aM level. However, Cas13a/crRNA only specifically recognizes an RNA target which limits its widespread application. Detection of DNA targets requires CRISPR/Cas12 system. In this section, we introduced some sensitive signal transduction technologies which can combine with CRISPR/Cas12 system to perform amplification-free detection.

3.3.1. *Electrochemical assays*

The amplification-free detection assisted by CRISPR/Cas12a system with electrochemical signals readout was based on the cleavage activity of Cas12a effector. Dai et al. proposed an electrochemical biosensor based on Cas12a effector for HPV-16 and PB-19 detection with a

sensitivity of pM level in 4 h [65]. However, this biosensor utilized linear ssDNA as signal reporter. The distance between redox labels and the electrode surface of linear probes was longer than that of hairpin probes. The long-distance reduced electron transfer efficiency which decreased the current signal gain. In addition, the steric hindrance effect between Cas12a and linear ssDNA might result in low cleavage efficiency. Zhang et al. developed an assay that took advantage of hairpin DNA probes and used differential pulse voltammetry (DPV) to detect Human papillomavirus (HPV). It obtained an improved sensitivity of 30 pM and a wider linear range from 50 pM to 100 nM [66]. Xu et al. presented a similar electrochemical biosensor using square wave voltammetry (SWV) for PB-19 detection in human serum sample. This method was more sensitive, with a LOD of 10 fM [39]. This kind of electrochemical method based on cleavage activity is also applicable for CRISPR type III, V, and VI systems. MB-conjugated AuNPs (MB-AuNPs) can be employed as electrochemical tag to substitute MB. And multiple MBs are capable to simultaneously conjugate on a AuNP. Lee et al. developed a Cas12a-based detection strategy which employed MB-AuNPs to generate enhanced electrochemical signal (Fig. 5A). It could detect dengue virus (DENV) and got a LOD of 100 fM within 30 min [67].

Elelctrochemiluminescence (ECL) can also be used to detect unamplified targets combined with CRISPR/Cas12a system. ECL is chemiluminescence which was triggered by electrochemical method. It employs electron-transfer reactions to form excited states that emit light on electrode surfaces which could be actively controlled rather than photoluminescence with external light sources. The combination of electrochemistry and chemiluminescence for ECL provides several potential advantages detection advantages such as low background signal, broad dynamic range, and simple device requirement [68]. Liu et al. proposed a Cas12a-based ECL assay without amplification which employed cost-effective materials, L-Methionine immobilized gold nanoclusters (Met-AuNCs), as the ECL emitters. Ferrocene-tagged thiolated ssDNA (SH-ssDNA-Fc) was applied to quench the ECL signal generated by Met-AuNCs attached on the surface of electrode. When the sample with human papilloma virus subtype (HPV-16) DNA was added, SH-ssDNA-Fc would be degraded by activated Cas12a effector to produce ECL signal (Fig. 5B). The difference between the two ECL signals could indicate the concentration of targets [69]. The LOD was 0.48 pM in undiluted human blood samples. However, a lack of multiplexing analysis, and long detection time (70min) are the main limitations of this assay.

Besides electrochemical and ECL methods, electrical detection is also used. Nouri et al. developed a Cas12a-assisted platform based on solid-state nanopores to detect HIV-1. Once the HIV-1 appeared, Cas12a effectors would indiscriminately digest the intact circular ssDNA reporters because of its *trans*-cleavage activity. The initial concentration of circular ssDNA was known as 100 pM. After *trans*-cleavage reaction, the effective concentration of the circular ssDNA was decreased. The remaining intact circular ssDNA was detected by nanopores to check whether HIV-1 was present or not. The detection could complete within 1 h and got a LOD of 10 nM [70].

3.3.2. *Fluorescence readout assays*

The fluorescence signal readout detection methods are based on the *trans*-cleavage activity of Cas12a effector. ssDNA reporters can be labeled with fluorophore or Qdots to produce fluorescence signal after being degraded. He et al. developed an automated and integrated Cas12a-based device with custom designed fluorescence sensing unit for on-site ASFV detection. The fluorescence sensing unit possessed a parabolic mirror that provided a fluorescence collection efficiency as high as a 40× objective lens and the price was ~10× cheaper than standard objective lens. Their device could obtain a sensitivity of 1 pM in 2 h. When the detection time was extended to 24 h, 100 fM level sensitivity could be achieved [71]. Through optimizing the reaction conditions such as the source of Cas12a enzyme, the type of reaction buffer, the kind and concentration of substrate label, and the reaction temperature, Hsieh et al. accelerated the detection time to 46 min and achieved the same sensitivity of 100 fM [72]. However, fluorophore attached to the ssDNA probe has a background signal. Its intensity is similar to that of the samples containing 10 fM ASFV. To further improve the detection sensitivity, Qdots could be employed to replace fluorophore and reduce fluorescence background signal [73].

Precise quantitative detection of nucleic acid is crucial for disease diagnosis, genetic variation quantification, viral load quantification, etc. [74]. Absolute quantification can be achieved by digital PCR technology which requires nucleic acid amplification procedure [75]. Similar to the digital quantification technology, a microdroplet quantification detection assay for ASFV and Hepatitis B virus was developed by Yue et al. Their work showed that optimized reaction conditions (enhanced buffer and 45 °C of reaction temperature) and dual-crRNAs strategy could significantly improve the detection efficiency to achieve single-molecule quantification and a LOD of 100 fM [74]. This method could be compatible with raw samples but took longer reaction time than Cas/13a-based detection.

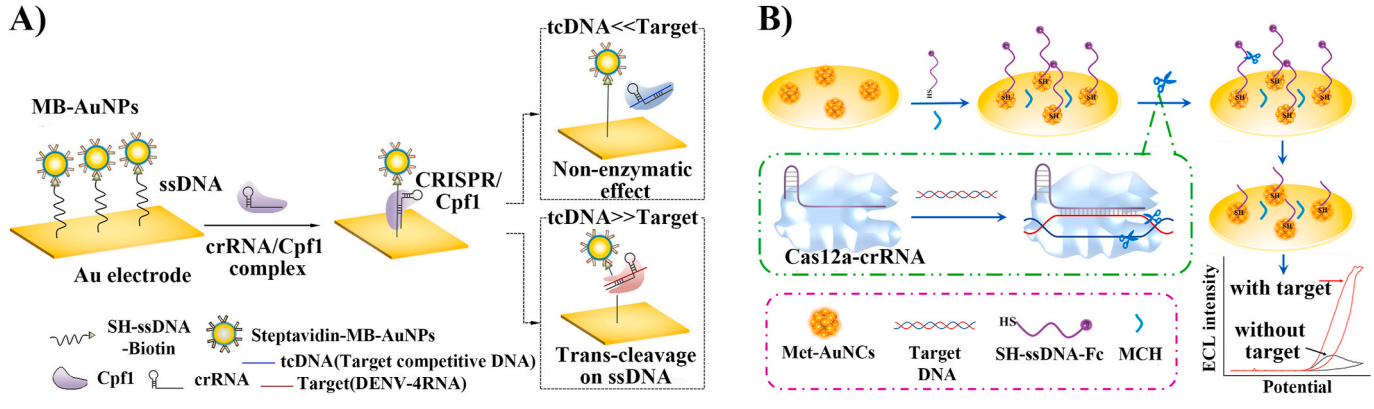


Fig. 5. Amplification-free detection of Cas12a effectors with electrochemical readout. (A) Electrochemical biosensor employing methylene blue-conjugated Au nanoparticles (MB-AuNPs) to produce electrochemical signal change. In the presence of targets, electrochemical signal from MB-AuNPs would be decreased due to the degradation of SH-ssDNA-biotin by activated CRISPR/Cas12a complex. Reprinted from Ref. [67] with the permission from Elsevier. (B) Electrochemiluminescence (ECL) detection for HPV DNA. In the presence of HPV-16 DNA target, Ferrocene-tagged thiolated single-strand DNA (SH-ssDNA-Fc) would be degraded by target-activated Cas12a and L-Methionine stabilized gold nanoclusters (Met-AuNCs) employed as ECL emitters would produce ECL signals. Reprinted from Ref. [69] with the permission from Elsevier. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3.3. Colorimetric readout assays

In addition to AuNPs, which are often employed for colorimetric assay [76], Platinum nanoreporter (PtNP) also could work as colorimetric signal inducer. The ssDNA linked up with PtNP and streptavidin-coated magnetic bead can act as nonspecific reporter. Employing this reporter, Shao et al. developed a Cas12a-based microfluidic chip with a volumetric bar-chart which realized volumetric quantification [77]. The assay was capable to perform multiple detection of 0.01% liver cancer mutations in pure DNA samples with a LOD of 10 pM in 60 min. As shown in Fig. 6A, in the first chamber, PtNPs were transferred to the second chamber with an external magnet after ssDNA reporters were degraded by target-activated Cas12a effectors. In the second chamber, PtNPs initiated catalytic reaction of H_2O_2 to produce O_2. The accumulation of O_2 resulted in volume growth of red ink bars which was showed in the readout channel. The amount of O_2 represented the amount of released PtNPs which could indicate targets DNA.

3.4. Multiple CRISPR/Cas systems cascade approaches

The cleavage efficiency of single Cas effector is limited. By combining multiple Cas effectors, Sha et al. developed a cascade CRISPR/cas system for miRNA detection. The successive usage of Cas13a and Cas14a effectors produced a quadratic signals amplification. In this system, a specially designed hairpin DNA containing unpaired rU, named as ST-HP, was served as the *trans*-cleavage target. The unpaired rU part was cleaved by miRNA-activated Cas13a effector to obtain the first signal amplification. Subsequently, the collateral cleavage ability of Cas14a effector was activated by cleaved ST-HP to indiscriminately degrade ssDNA reporter resulting the second fluorescence signal amplification (Fig. 6B). They compared the different combination of Cas13a-Cas12a and Cas13a-Cas14a, and found that the latter had a better LOD of 1.33 fM [78]. However, the design of a feasible ST-HP probe is relatively difficult.

3.5. Other Cas effector-based approaches

Type V and type VI CRISPR-based assays have been developed for amplification-free detection which depended primarily on their collateral cleavage activity. These types of Cas effectors require PAM/PFS sequence on the target nucleic acids. Santiago-Frangos et al. proposed an approach of polymerase activity-based type III CRISPR/Cas system for directly detecting SARS-CoV-2 [79]. The Cas10 effector of Csm complex could be triggered an analogous polymerase activity upon binding with RNA target. Then it would produce cyclic nucleotides, protons and pyrophosphates which could be detected by colorimetric or fluorescence methods in 30 min. They also designed 10 guide RNAs to improve the sensitivity to 10^6 copies/μL. Though the detection efficiency is not as high as that of assays relying on *trans*-cleavage activity, it provides a new approach for CRISPR/Cas system-based detection.

4. Challenges

Nucleic acid extraction is the first step of molecular detection based on nucleic acids. Though sample preparation procedure can provide high purity and concentration of nucleic acids, it is time- and labor-consuming. For CRISPR/Cas-based direct detection which eliminates the amplification step, it may have the potential to detect nucleic acids in unpurified raw samples. However, to date, only some detection approaches based on (d)Cas9 and Cas12a effectors have been reported that their systems could directly work in raw specimens without purification steps. In those cases, blood samples and swabs specimens were mainly used. And it is more difficult for Cas13a-based approaches to be compatible with complex samples because RNA reporters are easily degraded by RNases which are ubiquitous in the environment.

Another challenge of CRISPR/Cas-based detection is the off-target effect of CRISPR/Cas system. The off-target effects refer to Cas effectors binding to unexpected regions and cleaving them. The non-target sequences activated Cas effectors to produce unwished signal [80]. The off-target effect is influenced by multiple factors. In order to avoid off-target effect, it is recommended to optimize the sequence and structure of sgRNA or crRNA and select the Cas effectors with high accuracy. For example, Cas12a effector has a greater specificity than Cas9. Some groups have found that Cas12 b [81] and Cas13 d [82] are capable to improve the on-target activity. However, the practical application of these Cas effectors needs to be further investigated.

In addition, the potential target sequences are limited because CRISPR/Cas systems depend on PAM sequences to recognize targets. Thus, it is difficult to discriminate certain SNPs which are not proximal to PAM. The detection strategies which utilize multi-crRNAs to recognize and hybridize with targets may not easy to be implemented. Some

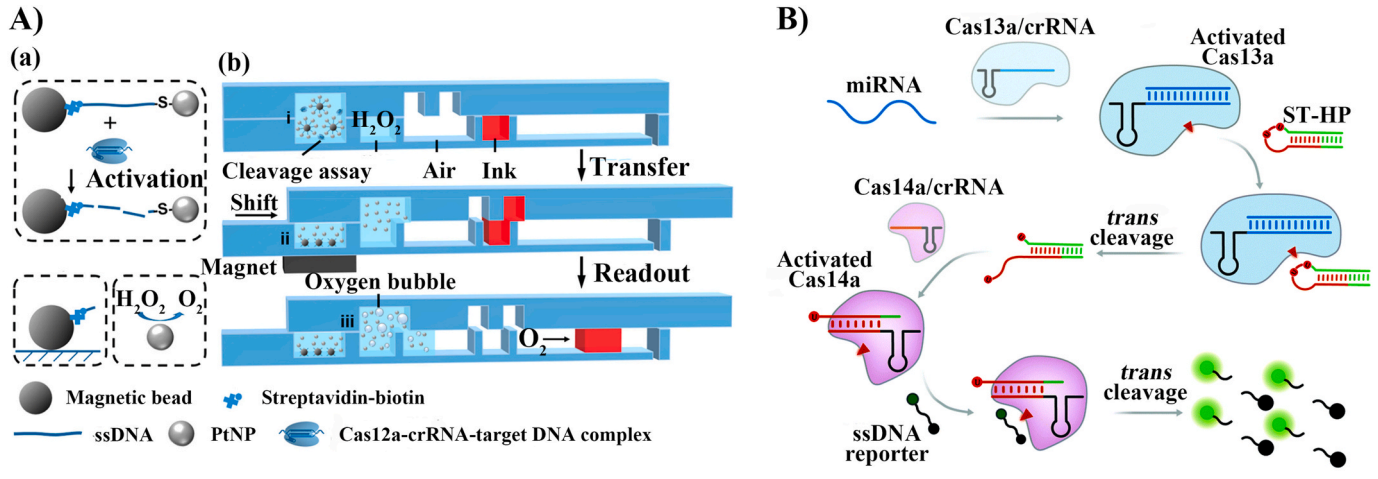


Fig. 6. (A) (a) Platinum Nano (PtNP) reporter assisted detection process. (b) Workflow of the Cas12a-based a volumetric bar-chart detection chip. Once target DNA presented, the activated Cas12a could degrade DNA linker in magnetic-bead-ssDNA-PtNP (BDNP) to unleash PtNPs. BDNPs and unleashed PtNPs would be separated by external magnet. Then, PtNPs initiated the reaction between catalase/PtNP and hydrogen peroxide (H_2O_2) to produce oxygen (O_2) which could move the red ink and realize visual detection. Reprinted from Ref. [77] with the permission from American Chemical Society. (B) Cascade Cas13a and Cas14a system for fluorescence detection. In the presence of target miRNA, the activated Cas13a would cleave the phosphodiester bond next to rU of the designed hairpin DNA (ST-HP). This product could activate the *trans*-cleavage activity of Cas14a to degrade DNA reporter which released fluorescence signal after being cleaved. Reprinted from Ref. [78] with the permission from Royal Society of Chemistry.

approaches such as HOLMES [13] employed specific designed primers to introduce PAM during amplification procedure, which could eliminate the sequences limit but cannot be applied to amplification-free detection.

Quantitative nucleic acid detection is crucial for disease diagnosis, genetic variation quantification, viral load quantification, and etc. However, most detection methods based on CRISPR/Cas system are hard to obtain the quantified results because *trans*-cleavage reaction is a kind of enzymatic reaction. The number of degraded ssDNA/RNA reporters increases over time so that it is difficult to relate with target copies. With the application of droplet microfluidics and micro-chamber strategies, CRISPR/Cas-based methods can fulfill digital quantification of unamplified nucleic acids with excellent sensitivity. Nonetheless, these methods depend on bulky fluorescence readout instruments and complex preparation of microchambers and droplets which limit their widespread application.

CRISPR/Cas system can easily detect multiple sequences by introducing corresponding multiple crRNAs [83], but the detection of multiple targets in one reaction tube is difficult due to indiscriminate *trans*-cleavage activities of Cas effectors. Currently, several multiplexed CRISPR/Cas detection have been proposed. One of the most common methods for multiple detection is spatial-separation-based method, which designs multiple separated channels or chambers to respectively detect multiple targets from one sample. However, the complexity and cost of spatially-separated device, and the cross-contamination risks between each space are the challenges remaining to be solved. In addition to spatial separation, other methods are yet to be investigated in CRISPR-based amplification-free multiplex detection, including employing various fluorophore-, enzyme-, or bead-labeled reporters, and using different orthogonal Cas effectors [84].

5. Conclusion

CRISPR/Cas-based nucleic acid detection is well-known for its high specificity and programmability. The detection strategies are based on binding activity, *cis*-cleavage activity and *trans*-cleavage activity of Cas effectors. Combining with various signal transduction technologies, such as electrical, electrochemical, fluorescence, and colorimetric methods, CRISPR-based assays have been demonstrated to be capable to directly detect unamplified nucleic acids with fM level sensitivity. The current main amplification-free detection approaches based on CRISPR/Cas systems are presented in Table 2. Among these, electrochemical-based methods are cheap, portable, and allow simple samples processing, while the long detection time limits their application to a certain extent. Colorimetric detection assay based on metal nanoparticle technology and turbidity measurement could visually indicate the presence

Table 2
Summary of amplification-free detection methods based on CRISPR/Cas system.

Cas effectors	Cas activities	Targets and sample type	Readout mechanisms	Sensitivity	Time	Advantages	Disadvantages	Ref.
Cas9 or Cas12a	*Cis*-cleavage	PB-19 in spiked human serum	SWV	100 fM	60 min	A broad linear range	Time-consuming	[39]
dCas9	Binding	ctDNA in spiked human blood	EIS	0.65 nM	40 s	Rapid and cheap, single nucleotide specificity	Poor sensitivity	[40]
dCas9	Binding	MDR bacteria in mouse tissue	SERS	fM level	60 min	Without the need of gene purification step	May cause infections	[41]
dCas9	Binding	Cultured MRSA in LB medium	FISH	10 CFU/mL	30 min	Simple, rapid, without gene purification	Bulky instrument	[42]
dCas9	Binding	SNPs from HEK genomic material	gFET	1.7 fM	15 min	Can bind target in whole-genome samples	Expensive, qualitative	[46]
Cas12a	*Trans*-cleavage	HIV-1 DNA in human blood	Solid-state nanopores	10 nM	60 min	Requiring smaller quantity of ssDNA reporter	Poor sensitivity	[70]
Cas12a	*Trans*-cleavage	HPV-16 in spiked human blood	ECL	0.48 pM	70 min	Quantitative determination	Time-consuming	[69]
Cas12a	*Trans*-cleavage	HPV in spiked vaginal/anal swabs	DPV	30 pM	60 min	Being stable in raw samples	A narrow linear range	[66]
Cas12a	*Trans*-cleavage	DENV plasmids in water	SWV	100 fM	30 min	Rapid	A transformation step	[67]
Cas12a	*Trans*-cleavage	dsDNA target in water	Fluorescence	100 fM	46 min	Optimizing the reaction conditions		[72]
Cas12a	*Trans*-cleavage	Virus DNAs in clinical serum samples	Droplet fluorescence	~100 fM	60 min	Single-molecule digital DNA quantification	Bulky equipment	[74]
Cas12a/Cas13a	*Trans*-cleavage	ASFV in pig serum sample	Colorimetric	~500 fM	60 min	Simple, visual discrimination	A centrifugation step	[60]
Cas12a	*Trans*-cleavage	SNP in spiked serum sample	Colorimetric	10 pM	60 min	Without centrifugation and bulky equipment	Poor sensitivity	[77]
Cas13a	*Trans*-cleavage	miRNAs in human serum	Amperometric readout	2–18 pM	3.5 h	Multiplexing and quantitative detection	Possible cross-reactivity	[55]
cas13a	*Trans*-cleavage	miRNAs in spiked human serum	CHA	2.6 fM	2.5 h	Good sensitivity	Complex reporter probes	[56]
Cas13a	*Trans*-cleavage	RNA targets in water	Turbidity	nM level	60 min	Visual discrimination	Poor sensitivity	[58]
Cas13a	*Trans*-cleavage	Synthetic Ebola in water	Fluorescence	~100 fM	5 min	Automation, multiplexing and quantitative	Strong background signal	[18]
Cas13a	*Trans*-cleavage	SARS-CoV-2 in nasal swab	Fluorescence	~0.2 fM	30 min	Quantitative detection	Sequence limit	[62]
Cas13a	*Trans*-cleavage	Viable *Bacillus cereus*	Fluorescence	10 CFU	/	Quantitative detection	background fluorescence	[61]
Cas13	*Trans*-cleavage	SARS-CoV-2 in spiked samples	Fluorescence	~10 fM	5 min	Fast, quantitative detection	Bulky equipment	[64]
Cas13a	*Trans*-cleavage	SARS-CoV-2 in nasopharyngeal swab	Droplet fluorescence	~10 aM	60 min	Absolute digital quantification	Nucleic acid extraction	[63]
Cas13a + Cas14a	*Trans*-cleavage	miRNAs in spiked human serum	Fluorescence	1.33 fM	80 min	Good sensitivity	Time-consuming	[78]
cas10	Polymerase	SARS-CoV-2 in nasal swab	Colorimetric or fluorescence	10^6 copies/μL	within 30 min	Fast	Poor sensitivity	[79]

of the targets. Though the LOD of most colorimetric methods is not as good as that of fluorescence readout assays, the capacities of direct visualized discrimination and low cost make colorimetric assay an applicable tool for on-site detection. Fluorescence-based detection utilizes ssDNA/RNA reporters labeled with fluorophore to produce fluorescence signals. It is sensitive, rapid and stable. Moreover, digital quantification detection has been realized by introducing droplet microfluidics and microchamber technologies. CRISPR/Cas13a-based droplet microfluidics technology provides the sensitive detection with a LOD of ~10 aM. Nonetheless, the output result reading relies on costly and bulky optical equipment, which limits its widespread and on-site application.

In general, CRISPR/Cas system is a powerful tool for amplification-free detection of nucleic acids. With the discovery of new Cas effectors, it is promising to avoid off-target effects and to broaden the application for various target nucleic acids. The recognition accuracy can be further improved by engineering high-fidelity Cas mutants.

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