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CRISPR/Cas systems for the detection of nucleic acid and non-nucleic acid targets

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ABSTRACT

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems are becoming powerful tools for disease biomarkers detection. Due to the specific recognition, *cis*-cleavage and nonspecific *trans*-cleavage capabilities, CRISPR/Cas systems have implemented the detection of nucleic acid targets (DNA and RNA) as well as non-nucleic acid targets (e.g., proteins, exosomes, cells, and small molecules). In this review, we first summarize the principles and characteristics of various CRISPR/Cas systems, including CRISPR/Cas9, Cas12, Cas13 and Cas14 systems. Then, various types of applications of CRISPR/Cas systems used in detecting nucleic and non-nucleic acid targets are introduced emphatically. Finally, the prospects and challenges of their applications in biosensing are discussed.

KEYWORDS

clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas), detection, nucleic acid targets, non-nucleic acid targets

1 Introduction

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system, an adaptable immune system of archaea and bacteria that includes the CRISPR and Cas proteins, has been applied as a powerful RNA-guided DNA/RNA targeting platform for genome editing [1–3], transcriptional interference [4, 5], epigenetic regulation[6], target detection [7] and so on. The CRISPR portion contains spacer and repeat sequences to anchor exogenous sequences, while the Cas proteins can bind exogenous gene fragments and cleave them [8, 9]. In the CRISPR/Cas system, pre-CRISPR RNA (crRNA) or single guide RNA (sgRNA) acts as a guide to direct Cas effector proteins to perform nuclease function at the specified location with high target-dependent cleavage activity, greatly improving the specificity of target recognition in this system [10, 11]. Diverse CRISPR/Cas systems have been found, with the main difference being that different Cas proteins have different ways of nucleic acid recognition and cleavage. With the deepening of research, the understanding of the diversity of CRISPR/Cas systems has produced several rounds of classification evolution. It is now well accepted to divide CRISPR/Cas into two major categories [12, 13]. The classical class I CRISPR/Cas system has multi-protein complexes, which are mainly operated by complexes composed of multiple Cas nucleases. In contrast, Class II system is characterized by an effector module consisting of a single multidomain protein, and their domain architectures clearly distinguish as type II, V and VI systems [14–17]. Inside, Cas9, Cas12 and Cas13 represent the effectors of the type II, V and VI systems, respectively. Cas14 is also an effector of a subtype of type V.

At present, a large body of research has focused on the application of CRISPR/Cas technology in gene editing. In contrast to its powerful capabilities for gene editing, the CRISPR/Cas systems have stimulated great interest in developing novel detection tools due to its specific or non-specific cleavage activity of Cas proteins. These systems mainly exploit attractive features of various Cas proteins, including sequence-specific recognition, specific nucleic acid endonuclease activity, and *trans* cleavage activity of Cas12, Cas13 and Cas14. Using the diversity of CRISPR/Cas systems to design different gene sequences enables the specific and low-cost detection of different targets. Therefore, understanding and applying CRISPR/Cas technology will facilitate further exploration of the boundaries of disease diagnosis. Since the type II CRISPR/Cas system has only one single effector protein, convenient modification enables its widespread use in the field of biosensing. With the enhancement of CRISPR/Cas technology, the applications of the system have been gradually expanding from nucleic acids to non-nucleic acids such as proteins, metal ions and others. Among them, the detection of nucleic acids, the most basic genetic material of life, has been used in precision medicine, public health, and other fields. How to achieve rapid, accurate, and sensitive detection is a goal that

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researchers have been exploring. Polymerase chain reaction (PCR) is the most widespread nucleic acid amplification method and is commonly employed in professional testing facilities [18], but the complexity of the technique, expensive instrumentation and the requirement for thermal cycling limit its application in non-laboratory scenarios. The development of gentle amplification techniques such as exponential strand substitution amplification (E-SDA) [19, 20], exponential rolling loop amplification (E-RCA) [21], and loop-mediated isothermal amplification (LAMP) [22] have further contributed to the improvement of nucleic acid detection, but interference still exists in terms of sensitivity and specificity. The specific recognition of nucleic acids by the Cas system has led to its incorporation into a variety of signal amplification methods for sensitive detection of trace substances, as well as facilitating timely detection in the field for follow-up studies. How to convert non-nucleic acid signals to nucleic acid signals is the cornerstone of the construction of non-nucleic acid substance detection systems. The conversion and capture of non-nucleic acid signals are efficiently achieved with the strategy of antibodies or aptamers that specifically recognize a substance.

The emergence of CRISPR/Cas technology is extremely likely to be a milestone in the field of biosensing. In this review, we first summarize the different domain architectures, characteristics, and capabilities of the second class of CRISPR/Cas systems to better understand the similarities and differences between each Cas system (Table 1). Subsequently, by classifying Cas assay targets into nucleic acid and non-nucleic acid classes, their classical applications in bioassays are highlighted. As shown in Fig. 1, these assays have been employed to the detection of single nucleotide polymorphism (SNP), pathogens, proteins and so forth. Especially, the rapid and sensitive detection of Severe Acute Respiratory Syndrome Coronavirus2 (SARS-CoV-2) in public health epidemic prevention deserves our attention and expectation. It is foreseeable that with further technological development, CRISPR/Cas-related biosensors may revolutionize the discovery and diagnosis of diseases. Finally, we discuss the current challenges and future perspectives of CRISPR/Cas-based sensing methods.

2 Working mechanism of various CRISPR/Cas systems

2.1 Cas9

As the hallmark protein of type II system, CRISPR/Cas9, found by Jennifer A. Doudna and Emmanuelle Charpentier first in 2012, has demonstrated that it can cleave double-stranded DNA (dsDNA) *in vitro* [23, 24]. After soon, Zhang employed the CRISPR/Cas9 system to implement genome editing in Escherichia coli [2] and James J. Collins coupled CRISPR/Cas9 with nucleic acid sequence-based amplification (NASBA) to discriminate between Zika strains with single-base resolution [25]. CRISPR/Cas9 system contains three parts: Cas9 protein, crRNA and trans-activated crRNA (tracrRNA) [26]. Among them, the Cas9 protein adopts a two-lobed structure and has several subdomains such as the RuvC and HNH nuclease domain which are involved in the cleavage of target DNA [27]. crRNA can bind tracrRNA and form guide RNA (gRNA) to execute recruitment and guide function, and there are currently numerous studies that integrate the two into one sgRNA [28]. The site where the Cas9 protein acts on exogenous DNA is called protospacer-adjacent motif (PAM, a short DNA sequence of the form 5'-NGG-3', where N represents any nucleotide), and only at the PAM position does Cas9 exert efficient specific recognition and cleavage [2, 3]. Generally, the Cas9 protein requires to be pre-incubated with sgRNA (or crRNA and tracrRNA) to form Cas9-sgRNA complex,

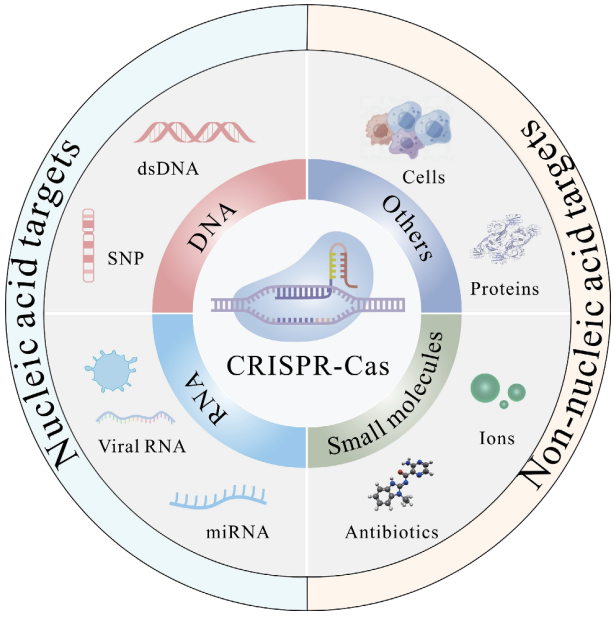

Figure 1 Scheme to illustrate the applications of CRISPR/Cas systems for the detection of nucleic acid and non-nucleic acid targets.

which is precisely positioned to the PAM target site of the original spacer sequence to achieve a precise cleavage function on dsDNA (Fig. 2(a)).

By specific binding to target nucleic acids and then activating nuclease activity, the CRISPR/Cas9 system enables the detection of pathogen nucleic acids, SNP genotyping and other areas, providing a powerful tool for more sensitive, efficient, and accurate detection. Noteworthy, by complementing the relevant PAM sequences, it can also be programmed to target single-stranded RNA (ssRNA) [29]. In parallel to the Cas9 protein which has the cleavage activity on dsDNA, an engineered nuclease-deficient Cas9 (dCas9), which lacks of DNA cleavage but retains DNA binding ability, has been exploited for specific sequence recognition and plays a special function in the field of nucleic acid detection [24, 30].

2.2 Cas12

Cas12a (previously referred to as Cpf1), discovered by Zhang's team in 2015, is the first Cas12 nuclease to be utilized for genome editing [31]. Distinct from Cas9, Cas12a has only one RuvC catalytic structural domain that mediates recognition for target dsDNA with T-nucleotide-rich PAM (TTTN) by the guide of a single crRNA, and generates PAM distal dsDNA breaks with interspersed 5' and 3' ends, known as *cis*-cleavage [32, 33]. In 2018, Jennifer A. Doudna found that the combination of the Cas12a-crRNA complex and a guide-complementary single-stranded DNA (ssDNA) can unleash non-specific ssDNA trans-cleavage activity that may be ubiquitous among Cas12a proteins [34]. Once the *cis*-cleavage of target dsDNA is finished by Cas12a, the active site of RuvC is vacated for the access to any ssDNA [35, 36]. Other V-type CRISPR/Cas12 enzymes also possess this property of non-specific cleavage of single-stranded deoxyribonuclease (ssDNase) [37]. A DNA nuclease targeted CRISPR trans reporter gene (DETECTR) assay was developed by Jennifer A. Doudna to take advantage of the *trans*-cleavage activity of Cas12a on ssDNA to detect DNA. Overall, features such as specific recognition and *cis*-cleavage of dsDNA, non-specific trans-cleavage of ssDNA, and the guidance with only crRNA have attracted great excitement for nucleic acid detection applications with Cas12a (Fig. 2(b)). In addition, CRISPR/Cas12a demonstrated both *cis*- and *trans*-cleavage activity on ssDNA without PAM sequences [38].

As a unique V-B type system, CRISPR/Cas12b (also known as

C2c1) has been characterized as a dual RNA-guided DNA nucleic acid endonuclease system with features that distinguish it from Cas9 and Cas12a [39]. Unlike Cas12a, the binding of crRNA and tracrRNA to Cas12b is critical for the pairing between the guide and target strands. Notably, Cas12b is smaller in size than Cas9 and Cas12a, and is particularly attractive when working with viral vectors for cell delivery [40, 41]. Researchers have investigated the *trans*-cleavage characteristics of Cas12b and found that it is extremely sensitive to single-nucleotide mismatches [42].

2.3 Cas13

Cas13a (also named C2c2) from the Type VI system, identified by Eugene V. Koonin et al. in 2015, is a RNA-guided RNase that utilizes crRNA as a guide to recognize and cleave the target ssRNA [16]. Cas13a is characterized by two higher eukaryotics and prokaryotic nucleotide-binding (HEPN) structural domains (Fig. 2(c)). Abudayyeh et al. confirmed that Cas13a both specifically recognizes and shears targeted RNA fragments and indiscriminately cleaves collateral RNA sequences (similar to Cas12a) [43, 44]. Taking advantage of these properties, Zhang Feng established the first CRISPR/Cas13a-based nucleic acid detection system SHERLOCK (specific high sensitivity enzymatic reporter unlocking) to enable the detection of DNA and RNA targets with single-base resolution [7]. With a similar structure to Cas13a, Cas13b (formerly named as C2c6) is also from the type VI CRISPR/Cas system, which has a specific mechanism of action that can be repurposed in a programmable manner to target RNAs.

2.4 Cas14

Cas14 (also referred to as Cas12f) is a family of exceptionally compact RNA-guided nucleases (approximately 400–700 amino acids in size, half the size of currently known CRISPR/Cas in class II), which can specifically bind and cleave target ssDNA under the guidance of tracrRNA and crRNA (or sgRNA) without restrictive requirement of PAM site [45–47]. Similar to Cas12, Cas14a is also type V and is able to bind with target nucleic acids and subsequently activate their ssDNA trans-cleavage activity (Fig. 2(d)), which can be performed for molecular detection of target nucleic acids.

3 Detection of nucleic acid targets with CRISPR/Cas systems

The specific recognition of nucleic acids by the CRISPR/Cas system provides a unique advantage in the detection of nucleic acids, especially the specific recognition of target DNAs by Cas9 and Cas12, and target RNAs by Cas13. Signal conversion and amplification of trace raw concentrations in the target are key challenges in assay system. Nevertheless, the inferior concentration of the original target leads to the requirement of combining CRISPR/Cas system with nucleic acid amplification techniques for the purpose of detection. With the identification of the trans-cleavage activity of the Cas12a, Cas13a and Cas14 proteins, the amplification of the original signal can be achieved to a certain extent by utilizing the trans-cleavage activity activated after target binding. A series of amplification-free systems have been developed to enable highly sensitive and selective detection of nucleic acids by employing various Cas effector proteins as highly

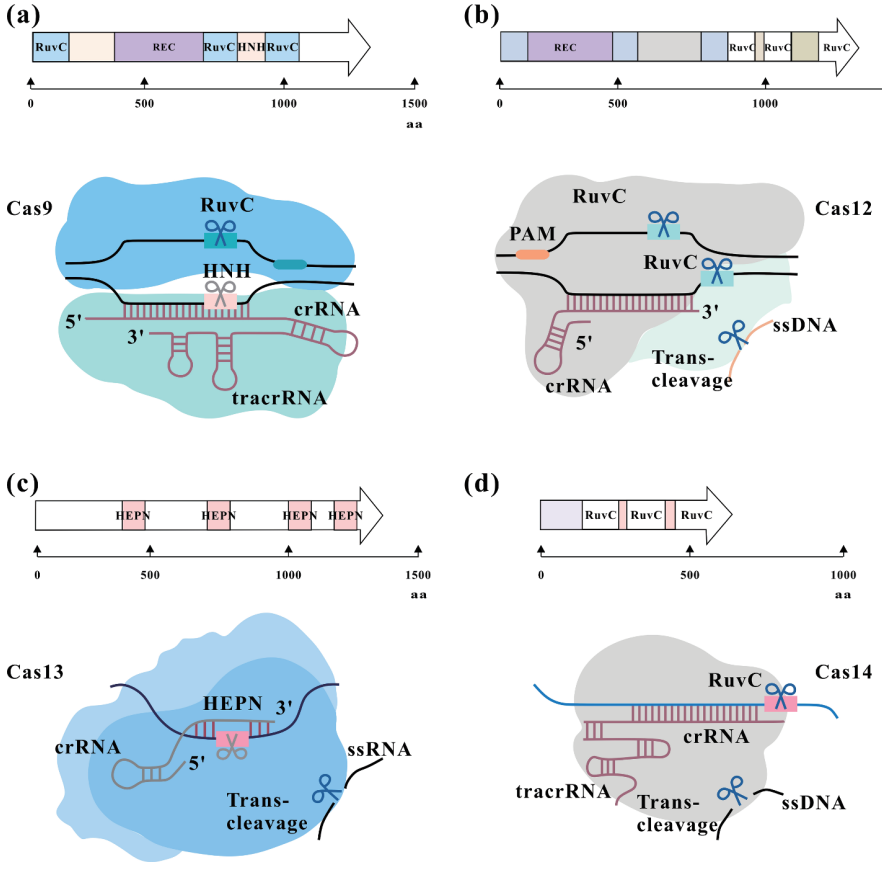

Figure 2 Schematic of CRISPR/Cas structure and cleavage activities. (a) Schematic of Cas9 protein mechanisms. crRNA can bind to tracrRNA and guide Cas9 to recognize and cleave specific dsDNA with PAM sequences. The HNH structural domain of Cas9 cleaves one strand of dsDNA complementary to crRNA, and the RuvC structural domain cleaves another strand of dsDNA. (b) Schematic of Cas12a protein mechanisms. Cas12a has only one RuvC catalytic structural domain. Under the guidance of crRNA, Cas12a can recognize the T-rich PAM sequence and specifically cleaves dsDNA while non-specifically cleaving surrounding ssDNA. (c) Schematic of Cas13a protein mechanisms. Cas13a contains two HEPN domains that can specifically recognize and cleave target ssRNA while non-specifically cleaving surrounding ssRNA under the guidance of crRNA. (d) Schematic of Cas14a protein mechanisms. Under the guidance of tracrRNA and crRNA, Cas14 can specifically bind and cleave target ssDNA without PAM, and non-specifically cleave ssDNA in the vicinity.

Table 1 Structures and features of different CRISPR/Cas systems

Class 2	Cas9	Cas12a(Cpf1)	Cas13a(C2c2)	Cas14(Cas12f)
Subtypes	Type II [23]	Type V [32, 33]	Type VI [16]	Type V [45]
Nuclease domains	HNH, RuvC [26]	RuvC [32, 33]	HEPN [16]	RuvC [45]
Guide RNA	tracrRNA, crRNA [26]	crRNA [32, 33]	crRNA [16]	crRNA, tracrRNA [45]
Target (activator)	dsDNA [23] ssDNA [48], ssRNA [29]	dsDNA [31], ssDNA [38]	ssRNA [43, 44]	ssDNA, dsDNA [45]
Trans-cleavage substrates	—	ssDNA [36]	ssRNA [43, 44]	ssDNA [45]
PAM sequence	5′-NGG-3′[26]	TTTN [32, 33]	Non-G-PFS [43, 44]	—
Advantages and disadvantages for biosensing	Genotyping and SNP identification with high specificity [49]; PAM sequence restriction [26]; Additional signal amplification means [49, 50]	DNA target detection [32, 33]; signal amplification by *trans*-cleavage [36]; PAM sequence restriction for dsDNA detection [32, 33]; Lower specificity for ssDNA detection [38]	RNA target detection; signal amplification by *trans*-cleavage [7]; Multiple cleavage sites [43, 44]; PAM sequence restriction [43, 44]	Smaller size; No PAM sequence restrictions; Distinguish single base differences [45]; Longer sgRNA [45]

specific sequence recognition elements, in combination with various signal output methods such as electrochemical, colorimetric and fluorescent methods.

3.1 Detection of DNA

The ability of Cas9 and Cas12 to specifically target DNA for recognition and cleavage allows for a wide range of applications in DNA detection. In particular, the *trans*-cleavage activity of Cas12a allows the system to perform indiscriminate cleavage of nearby ssDNA, which results in the cleavage of signal probes and the production of detectable signals such as fluorescence and electrical signals. The CRISPR/Cas9 system exhibits high site-specific cleavage of dsDNA under the guidance of sgRNA. The combination of CRISPR technology and classical PCR can fully utilize the high specificity of CRISPR and the high sensitivity of PCR. Bae S and colleagues have developed a method called CRISPR-mediated, ultrasensitive detection of target DNA (CUT)-PCR, which can cleave wild-type sequences by using CRISPR/Cas endonucleases to reduce background DNA signals and enrich cancer-specific mutant DNA signals with PCR amplification [50]. Related experiments demonstrated that CUT-PCR has the capability to accurately detect sequences with oncogenic mutations. Zhang and colleagues combined the advantages of site-specific cleavage of CRISPR/Cas9 and the kinetic advantages of isothermal exponential amplification technology to build up a CRISPR/Cas9-triggered isothermal exponential amplification reaction (CAS-EXPAR) method, which has the function of rapid and site-specific nucleic acid detection. Since PAMmer (antisense ssDNA or ssRNA) can activate CRISPR/Cas9 to cleave target DNA, CAS-EXPAR is free from the need for exogenous primers (Fig. 3(a) reproduced from Ref. [51]). During the reaction, primers are generated and accumulated by cleavage of DNA sequences, which permits DNA detection within one hour. By converting single-base methylations into single-base mutations, CAS-EXPAR is able to achieve site-specific DNA methylation detection, which is essential for monitoring the diagnosis and prognosis of tumors. Furthermore, the strategy of combining CRISPR/Cas9 with loop amplification technology has achieved effective amplification of the target signal, enabling accurate detection at the single-base level. Taking benefits of the RCA, Li and coworkers engineered a CRISPR/Cas9-mediated proximity ligation assay (CasPLA) to examine single nucleotide variants (SNV) of mtDNA in single cell. Associated with two Cas9 probes, CasPLA sequentially targets mtDNA and determines the spatial localization of individual wild types by proximity ligation and *in situ* RCA, which is essential to observe the heterogeneity and complexity of diseases associated with mutations in mtDNA [49].

The cleavage properties of Cas12a for dsDNA and ssDNA provide a particular strength in signal amplification after target recognition. Once Cas12a forms a complex with crRNA and target DNA, the *trans*-cleavage activity of the system is effective in fragmenting any ssDNA in the system [35]. The trans-cleavage of Cas12a revealed a mentality for the detection of target DNA and the amplification of signal: by loading the non-specific reporter (e.g., ssDNA) into the Cas12a system, the reporter will be cleaved to release signals such as fluorescence while the target DNA is detected. Making the best of this feature, Jennifer A. Doudna exploited a system termed DETECTR, which includes Cas12a, crRNA and a fluorophore quencher (FQ)-labeled reporter (Fig. 3(b) reproduced from Ref. [36]). DETECTR was successfully applied to monitor human papilloma virus (HPV) by combining recombinase polymerase amplification (RPA) and Cas12a. Once Cas12a detects and cleaves the target DNA sequence, it will activate the *trans*-cleavage activity and cut the ssDNA tagged with a fluorophore-quencher pair, which causes the cleavage of FQ-labeled reporter. The presence of target DNA can be accurately quantitated by monitoring the fluorescence signal. The results demonstrated that the DETECTR system can accurately detect HPV types other than types 16 and 18 from clinical samples [36]. One-hour lost-cost multipurpose highly efficient system (HOLMES) was developed by the *trans*-cleavage function of Cas12a on fluorescent labeled ssDNA. The HOLMES differs from the DETECTR system in that the HOLMES amplifies the experimental template with the aid of PCR. In the presence of the target DNA, Cas12a-crRNA forms a ternary complex with the target DNA and cleaves ssDNA in *trans*, generating a fluorescent signal. SNPs are the most common heritable genetic variations in humans and have a strong association with the susceptibility and incidence of diseases. By introducing the relevant PAM sequence into the primers, the HOLMES platform is capable of detecting the existence of SNP site [52].

As research proceeded, Cas12b was found to have similar cleavage activity to Cas12a and detect the nucleic acid at single-base resolution. By utilizing the trans-cleavage activity of Cas12b, Wang and coworkers developed a one-pot one-step diagnostic system (HOMLES V2) based on LAMP and Cas12b, which will be subsequently described with more details in next section of RNA detection [53]. Li and colleagues found that a Cas12b system extracted from Alicyclobacillus acidophilus Cas12b (AaCas12b) is sensitive to dsDNA [41]. Hence, the AaCas12b-sgRNA-dsDNA-activator system was designed as a Cas12b-based DNA detection (CDetection) platform [54]. When the activator concentration

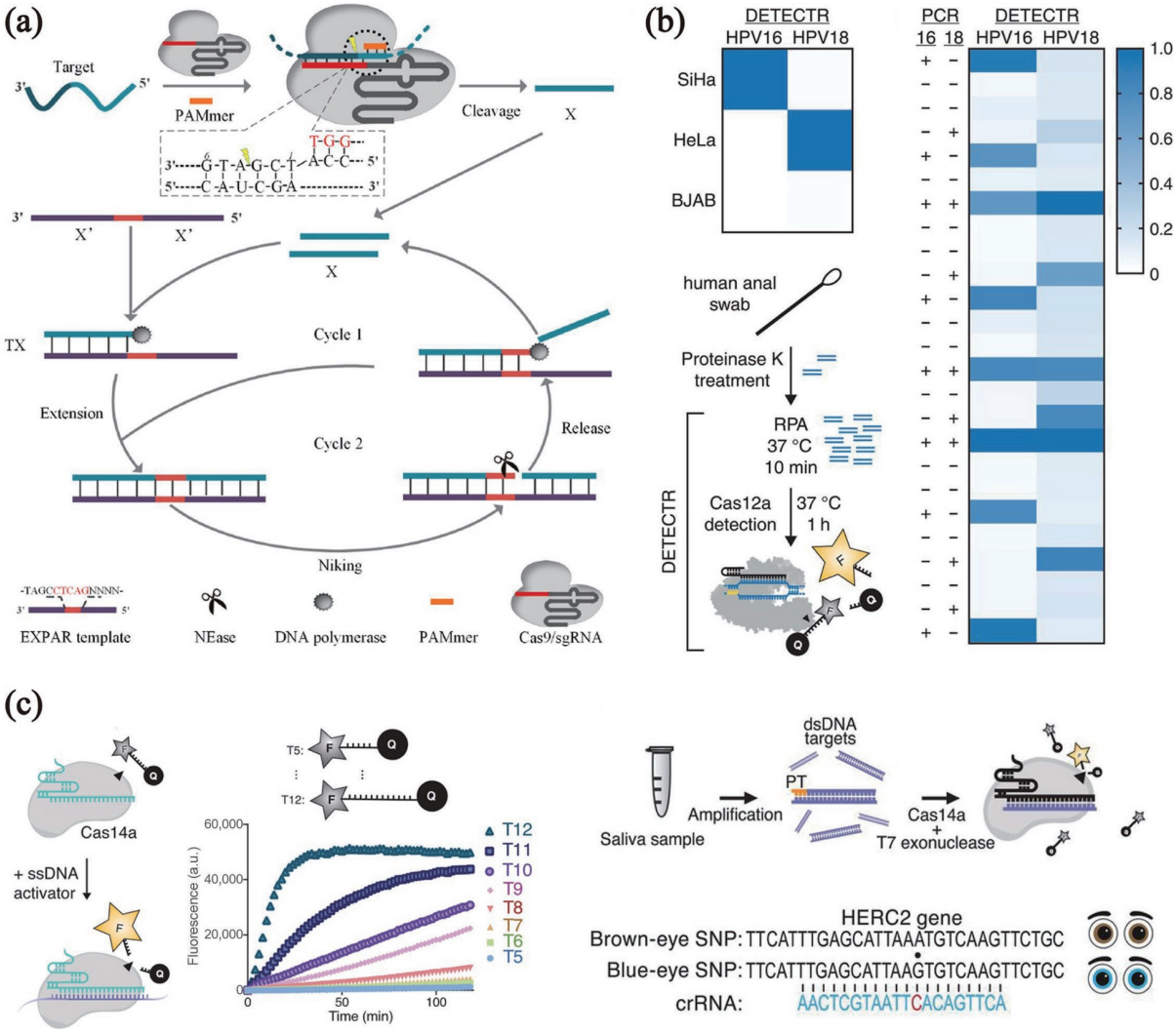

Figure 3 Application of CRISPR/Cas system in the detection of DNA. (a) Using CAS-EXPAR to detect site-specific DNA methylation. Reproduced with permission from Ref. [51], ©American Chemical Society 2017. (b) DETECTR constructed by combining Cas12a and RPA for the detection of HPV16 and HPV18. Reproduced with permission from Ref. [36], © Chen, J. S. et al., some rights reserved; exclusive licensee American Association for the Advancement of Science 2018. (c) Cas14a-DETECTR was developed to realize the high-fidelity SNP genome typing. Reproduced with permission from Ref. [45], © Harrington, L. B. et al., some rights reserved; exclusive licensee American Association for the Advancement of Science 2018.

exceeds 10 nM, CDetection can not only detect HPV but also distinguish HPV16 and HPV18. With the introduction of tuned guide RNA (tgRNA), CDetection can distinguish differences at the single-base level and determine ABO blood types with high accuracy.

Combining the principle of DETECTR with the non-specific cleavage activity of Cas14a, the Cas14a-DETECTR was developed, which can achieve high-fidelity SNP genome typing [45]. The results illustrated that Cas12a was incapable of distinguishing both ssDNA targets when detecting HERC2 genes associated with blue or brown eyes, while Cas14a performed enhancement in identifying blue eye SNPs. This can be attributed to the fact that Cas14a requires complementarity in the region for ssDNA substrate recognition, which allows Cas14a binding to its target with higher specificity than Cas12a. This supports the assertion that Cas14a is more appropriate for the high-fidelity detection of SNPs in saliva samples from blue and brown eyed individuals (Fig. 3(c) reproduced from Ref. [45]).

3.2 Detection of RNA

RNA is not only an essential genetic material in genetic coding, regulation and gene expression, but is also the genetic material of several high-risk human pathogens. RNA-targeting CRISPR/Cas systems include two categories, the first of which modifies DNA-targeted CRISPR systems to target and modify RNA [55]. For example, Cas9 targets both DNA and RNA, but it is inefficient in detecting RNA. The other category is CRISPR/Cas systems that specifically recognize ssRNA such as Cas13.

It has been found an abundance of Cas13 target sites in the genomes of human-associated viruses, suggesting the potential application of Cas13 for the detection and inhibition of a wide range of mammalian ssRNA viruses [56]. To improve the positive rate of Cas13 for disease diagnosis, Zhang Feng and coworkers proposed SHERLOCK in which the RPA was employed to amplify the target and single molecules of DNA and RNA were detected at the low concentrations (Fig. 4(a) reproduced from Ref. [7]). In 2018, Sabeti and coworkers modified SHERLOCK to a method named heating unextracted diagnostic samples to obliterate nucleases (HUDSON) (Fig. 4(b) reproduced from Ref. [57]). The HUDSON, which examines viral nucleic acids directly from bodily fluids, brings simplicity to the procedure, when in comparison with SHERLOCK in the detection of Zika and dengue viruses [57]. In 2019, Sabeti and Zhang have proposed Cas13-assisted restriction of viral expression and readout (CARVER) based on SHERLOCK, which adds the cleavage function of Cas13 for rapid diagnosis and treatment [58]. Lymphocytic choriomeningitis virus (LCMV), influenza A virus (IAV) and vesicular stomatitis virus (VSV) were employed as the examples to

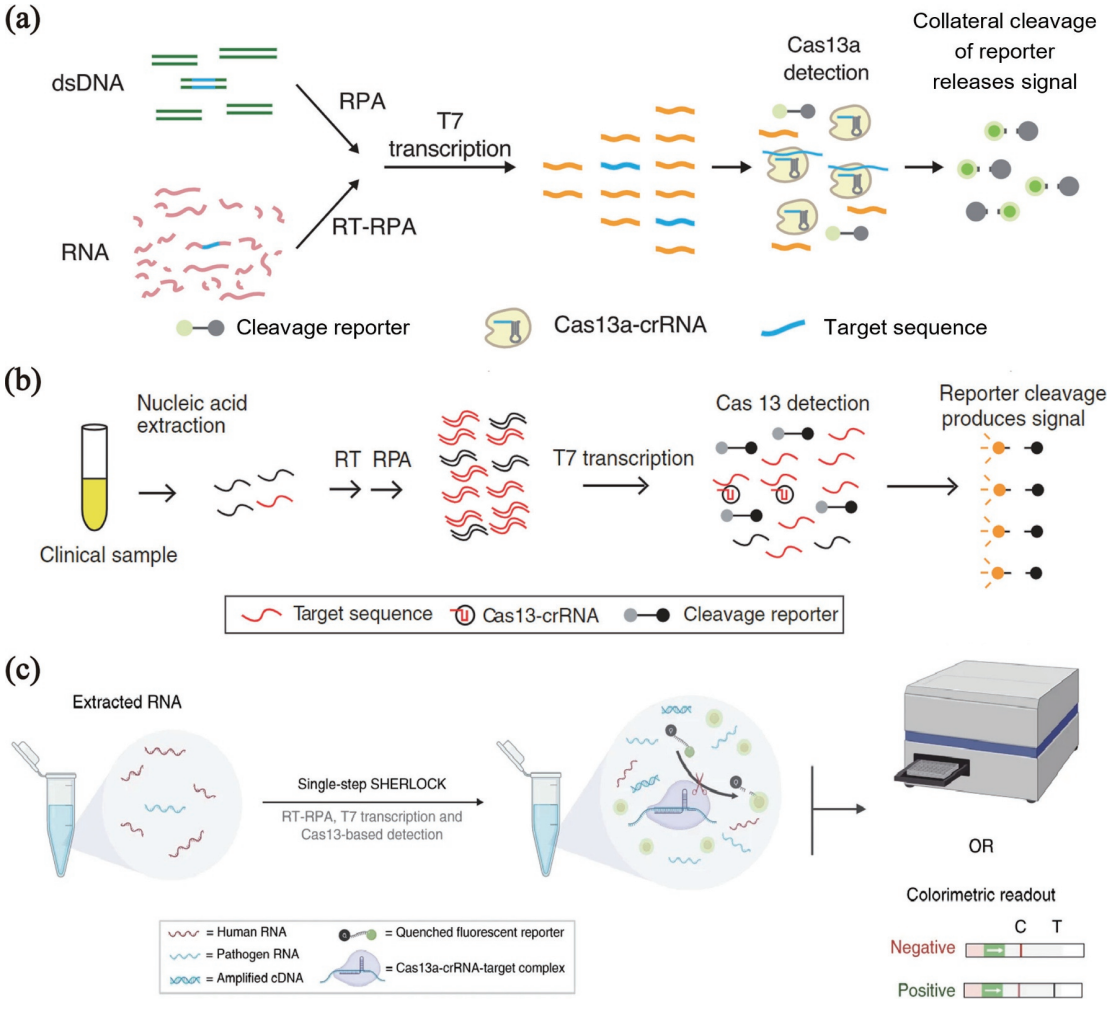

Figure 4 Application of CRISPR/Cas system in the detection of RNA. (a) A SHERLOCK system which combined Cas13a with RPA was established to detect single molecules of DNA and RNA. Reproduced with permission from Ref. [7], © American Association for the Advancement of Science 2017. (b) A HUDSON system which can detect viral nucleic acids directly from bodily fluids without the requirement of extraction step. Reproduced with permission from Ref. [57], © Myhrvold, C. et al., some rights reserved; exclusive licensee American Association for the Advancement of Science 2018. (c) SHINE, a sensitive and specific diagnostic tool that can detect SARS-CoV-2 RNA from unextracted samples. Reproduced with permission from Ref. [60], © Arizti-Sanz, J. et al. 2020.

demonstrate the high efficiency of CARVER. Integrating CRISPR with DNA polymerase, which performs 5' to 3' DNA polymerase activity, the Japanese encephalitis virus can be detected in less than one hour with great success.

Novel coronavirus pneumonia caused by SARS-CoV-2 is a persistent pandemic that has emerged a serious challenge to public health and the global economy. Exploitation of CRISPR-mediated molecular diagnostics offers a novel insight into the detection of SARS-CoV-2. Building on the SHERLOCK technology, Zhang Feng and coworkers developed a platform called STOP (SHERLOCK testing in one pot) [59]. The procedure eliminates the need for the purification of RNA from patient samples, and limits the chemical reaction steps to one single tube, effectively preventing possible contamination from additional sample transfer steps. The technique demonstrated 100% specificity and 97% sensitivity in 12 positive samples and 5 negative samples for novel coronavirus. Myhrvold and coworkers have developed a platform which is called streamlined highlighting of infections to navigate epidemics (SHINE). It combined the reverse transcriptase-RPA (RT-RPA) and the detection processes of Cas13a in one single step [60]. The system has enabled accelerated inactivation of SARS-CoV-2 RNA to diminish the risk of contamination while providing an excellent assurance of sample containment during the procedure (Fig. 4(c) reproduced from Ref. [60]). Additionally, Maxwell and coworkers proposed a Cas13-based, robust, equitable, and scalable testing (CREST) system for the efficient, sensitive, and cost-effective detection of SARS-CoV-2. A lateral flow test strip was constructed by coupling the PCR with CRISPR/Cas [61]. However, additional kits are still indispensable to extract RNA from CREST. To address this restriction, an alternative procedure called PEARL (precipitation enhanced analyte retrieval) was proposed, which uses a lysis solution to disrupt the viral envelope and cell membrane to separate pathogens from host nucleic acids. Coupling PEARL to CREST can avoid additional RNA extraction and simplify the testing process [62].

To detect SARS-CoV-2, isothermal amplification and simultaneous reverse transcription of RNA extracted from oropharyngeal swabs or nasopharyngeal swabs were performed with DETECTR. The feedback on the inspection can be ascertained by observing the fluorescence signal [63]. In 2020, Li and coworkers established CRISPR-assisted detection (CASdetec) to detect SARS-CoV-2 by combining CDetection, sample treatment protocols and recombinase-aided amplification (RAA). CASdetec executes reactions in a single tube, optimizing the workflow and reducing the risk of aerosol contamination [64].

3.3 CRISPR/Cas systems for detecting nucleic acid without target pre-amplification

Amplification-free detection of nucleic acids is a critical component of diagnosis, which offers multiple benefits, including the avoidance of potential aerosol contamination and shortened

detection time. Since the sensitivity and specificity is not intense sufficiently for practical application, current CRISPR systems are frequently integrated with amplification technologies to achieve target enrichment and signal amplification. Exploitation of amplification-free systems is a megatrend evolution for CRISPR/Cas in detection. The enhancement of *trans*-cleavage activity that generated by CRISPR/Cas is one of the strategies for the construction of amplification-free system. An amplification-free precision assay based on CRISPR/Cas13a with a simple detection device for neo-coronavirus was developed, enabling amplification-free detection of nasal swab samples within 30 min with a sensitivity of 100 copies/μL [68]. In this methodology, a number of crRNAs were concatenated to enhance the detection sensitivity of CRISPR diagnostics and enzyme kinetic parameters are utilized to quantify the viral load. Jennifer A. Doudna and coworkers have constructed a system incorporating Cas13a and Csm6, which is capable to detect target RNA directly at concentrations of up to 30 copies per microliter in less than 20 min. Assisted by the RNA endonuclease activity of the Csm6 protein, the amplification effect of fluorescence signaling molecules was achieved by the system [65]. The trans-cleavage activity of Cas13a/crRNA was triggered by the target RNA and the ssRNA probe was cleaved, activating Csm6 to shear the fluorescent and burst the molecule to enlarge the signal. In a further attempt to increase the sensitivity, the work has also combined multiple crRNA probes. With eight crRNA probes in one system, the detection sensitivity was boosted again from 1000 copies per microliter to 31 copies per microliter (Fig. 5(a) reproduced from Ref. [65]). Zhou et al. have proposed a strategy to raise the concentration of target nucleic acids by reducing the volume of CRISPR reaction to the minimum concentration required for Cas13a protein (Fig. 5(b) reproduced from Ref. [66]). A microdroplet approach was invented to improve the concentration to the pM level by drastically reducing the reaction

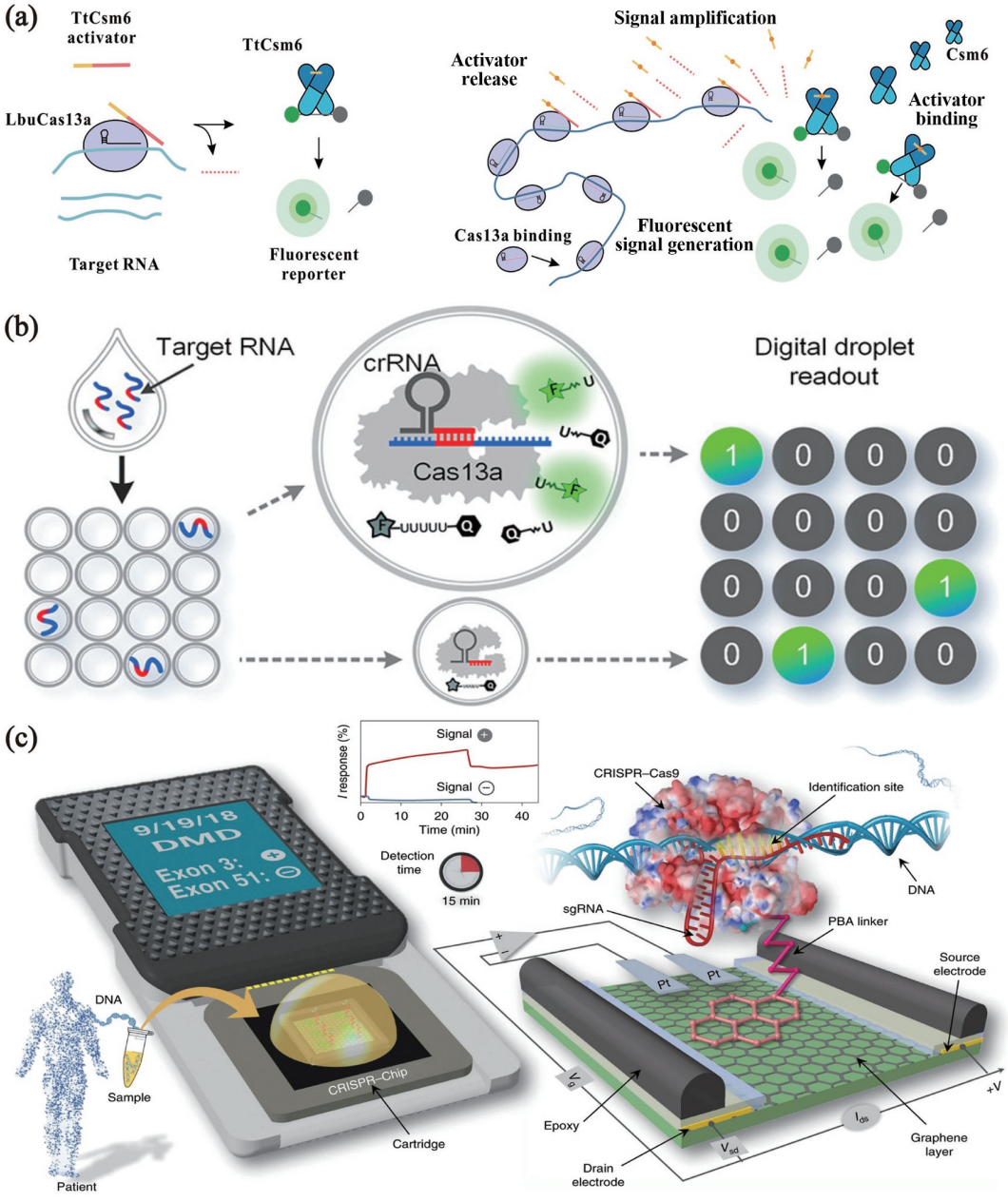

Figure 5 Application of CRISPR/Cas system in the detection of nucleic acid targets without target pre-amplification. (a) Coupled CRISPR-Cas13 protein with Csm6 to establish FIND-IT method for sensitive amplification-free direct detection. Reproduced with permission from Ref. [65], © Liu, T. Y. et al., under exclusive licence to Springer Nature America, Inc 2021. (b) Decrease the volume of the individual reaction system to increase the target concentration. Reproduced with permission from Ref. [66], © American Chemical Society 2020. (c) CRISPR-Chip: CRISPR-based graphene field-effect transistor technology for amplification-free direct detection of target DNA. Reproduced with permission from Ref.[67], © Hajian, R. et al., under exclusive licence to Springer Nature Limited 2019.

volume to achieve amplification-free detection at the single molecule level [66].

Upgrading the sensitivity of the signal detector is also one of the approaches for the construction of amplification-free systems, as the *trans*-cleavage activity of Cas proteins is also diminished when the target nucleic acid concentration is in a low level. A CRISPR-Cas12a based electrochemical biosensor (E-CRISPR) system has been exploited to elevate the detection sensitivity by two magnitudes [69]. The *trans*-cleavage of Cas12a could be activated in the existence of the target nucleic acid and the methylene blue (MB) modified ssDNA coupled to the gold electrode could be cleaved, leading to the detachment of MB and the transformation in conductivity. Through the E-CRISPR system, the target nucleic acid could be monitored by the change of current value. In conjunction with aptamer, the transforming growth factor-β1 (TGF-β1) protein has been identified by E-CRISPR and a pathway for the detection of non-nucleic acid target by CRISPR/Cas was offered. Furthermore, a MB-modified stem-loop ssDNA was incubated on the gold electrode to construct a CRISPR/Cas system enhanced electrochemical system. Cas9 and Cas12a have been tested to demonstrate that E-DNA allows the detection of nucleic acid at the pM-level in an amplification-free situation [70]. Moreover, graphene and CRISPR/Cas9 were integrated to develop a CRISPR-based graphene field-effect transistor technology which is called CRISPR-Chip (Fig. 5(c) reproduced from Ref. [67]). This platform allows for amplification-free direct detection of the target DNA with a sensitivity of 1.7 fM and a time control of 15 min.

4 Detection of non-nucleic acid targets with CRISPR/Cas systems

Contrary to nucleic acid detection, the detection of non-nucleic acid targets by CRISPR/Cas is still in the initial stage. A number of non-nucleic acid detection systems have been developed based on the association of CRISPR/Cas with biological progenitors including aptamers, DNAzymes, and antigen-antibody reactions for the detection of proteins, small molecules, cells and so forth.

4.1 Detection of proteins

The challenge of protein detection is how to perform specific recognition and efficient signal transduction. The conversion and output of non-nucleic acid signals can be effectively accomplished with nucleic acid aptamers, antigen antibodies, etc. due to their ability to specifically bind to target proteins. Nucleic acid aptamers are short oligonucleotide sequences that can bind to the corresponding ligands with high affinity and specificity, which are frequently called "chemical antibody" [71]. Aptamers tend to exhibit predominantly unstructured in solution. Once the aptamers associate with their ligands, they fold into molecular architectures and the ligands become an intrinsic part of the nucleic acid structure. The specificity of the aptamer-ligand association usually relies on the precise stacking of flat moieties, specific hydrogen bonding, and molecular shape complementarity [72, 73]. Thus, aptamer can effectively bind to target protein, producing the nucleic acid activators recognized by the CRISPR/Cas system. Various aptamers with specific recognition of proteins have been developed, laying the foundation for protein detection.

Park and colleagues developed a method to identify the interactions of protein/small molecule by using the cleavage activity of CRISPR/Cas12a [74]. The relevant small molecules can be tagged to single-stranded activator DNA (AD) with the use of hydroxyl or carboxy. The presence of proteins interacting with small molecules resulted in the inability of small molecule-modified AD to bind to crRNA. Conversely, Cas12a is capable of being activated to cleave the fluorescently labeled reporters, and the fluorescence intensity was evaluated to ascertain the amount of protein.

An E-CRISPR system can convert biological signals into electrical signals for the amplification-free detection. The E-CRISPR system has also been incorporated with aptamer to detect the TGF-β1 protein, suggesting an application direction of CRISPR to detect non-targeted nucleic acids (Fig. 6(a) reproduced from Ref. [69]). A platform for protein detection that exploits the trans-cleavage activity of Cas14a to drive changes in the mechanical properties of DNA hydrogels was constructed. In the existence of the target, the aptamer-DNA complex was dissociated by competitive magnetic separation which resulted in the EXPAR and producing complementary DNA (cDNA) to activate Cas14a. The activated Cas14a protein is able to indiscriminately cleave hydrogel DNA and release platinum nanoparticle (PtNPs) decorated metal-organic framework (MOF) tetra (4-carboxyphenyl) porphyrin (TCPP) nanosheets (PtNPs/Cu-TCPP(Fe)) which has been pre-encapsulated, triggering a TMB reaction for quantitative detection of creatine kinase MB (CK-MB) (Fig. 6(b) reproduced from Ref. [75]).

Antigen-antibody reaction is one of the effective strategies for protein recognition, which can be combined with CRISPR-Cas systems to achieve more sensitive protein detection. The key point is that in this combined approach, the recognition of antigens and antibodies needs to be converted into DNA/RNA activators to initiate the cleavage activity of the Cas protein. A DNAzyme walker-triggered CRISPR-Cas12a/Cas13a system was designed for the detection of serum amyloid A-1 protein (SAA1) and coagulation factor V (FV) by combining antigen and antibody reactions. DNAzyme walkers were instrumented to convert SAA1 and FV into activators (P1 and P2) for initial signal amplification to activate the CRISPR Cas12a/Cas13a [77]. Carr and colleagues developed genomic locus proteomics (GLoPro) for the discovery of proteins associated with specific genomic loci on the basis of dCas9. GLoPro was demonstrated to be universally applicable and significant enrichment of multiple proteins such as mitogen-activated protein kinase (MAPK)14 by the use of myelocytomatosis (MYC) gene [78]. Long noncoding RNAs (lncRNAs) play a significant role in genetic regulation and cell differentiation. Yan and coworkers have established CRISPR-assisted RNA-protein interaction (CARPID) by the interaction of lncRNAs and RNA-binding proteins (RBPs). The CARPID system constructed by the design of CasRx (Cas13d from the intestinal bacterium Flavobacterium flavum) is capable of targeting specific lncRNA to recognize the relevant protein in the cell. This system has been applied to lncRNA X-inactive specific transcripts (XIST) and successfully identified XIST-associated proteins in living cells [79].

4.2 Detection of exosomes and cells

As thumbnail sketches of cells, exosomes are the promising biomarkers for non-invasive diagnosis and treatment of diseases. Exosomes and tumor cells often overexpress specific protein receptors on their surfaces, which are excellent markers for cell subpopulation classification and have been effectively employed for the detection of exosomes and cells. In the CRISRP/Cas detection strategy, aptamers are generally applied to bind with cell surface receptors, resulting in the signal transmission from cell to nucleic acid and the activation of CRISPR system.

An approach to exosome detection based on CD63 aptamer and CRISPR/Cas12a has been reported. CD63 aptamer could capture CD63-bearing exosomes and translate the exosome volume into nucleic acid detection, while CRISPR/Cas12a is responsible for highly specific amplification of nucleic acid signals.

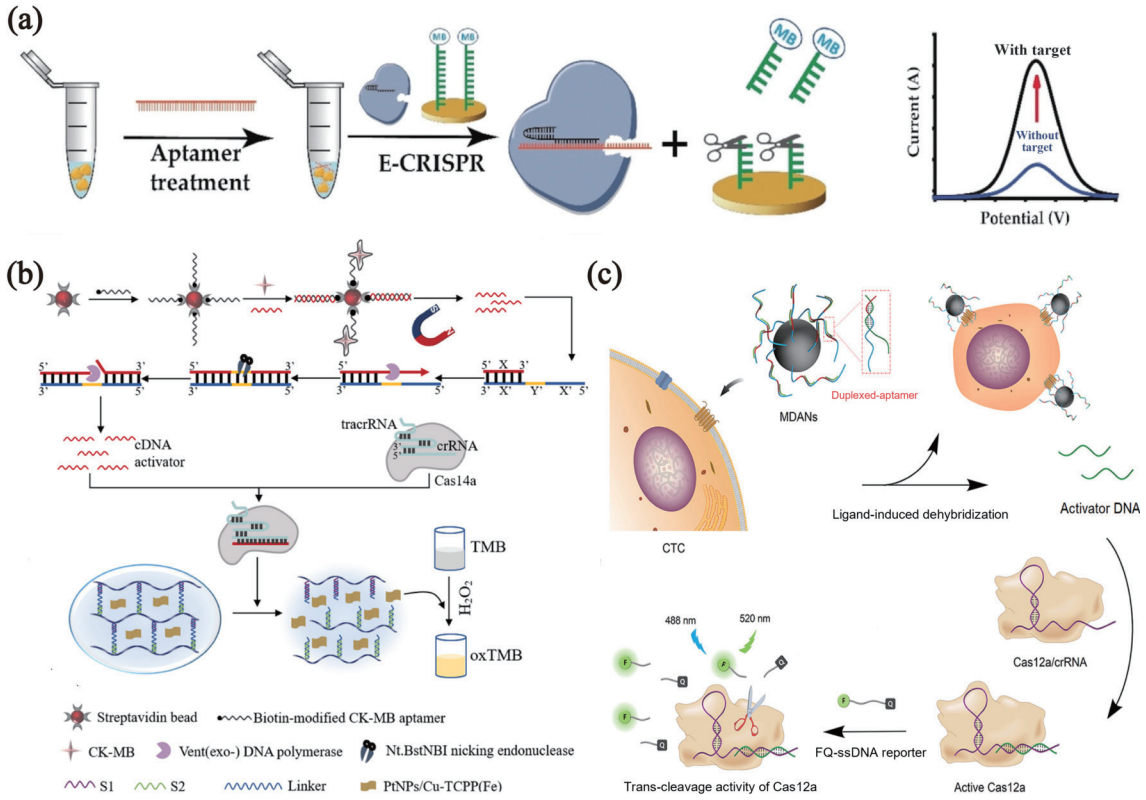

Figure 6 Application of CRISPR/Cas system in the detection of proteins and cells. (a) Electrochemical principle combined with CRISPR/Cas12a to detect TGF-β1. Reproduced with permission from Ref.[69], © Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim 2019. (b) DNA hydrogel based on EXPAR and CRISPR/Cas14a for ultrasensitive detection of creatine kinase MB. Reproduced with permission from Ref. [75], © Elsevier B.V. 2022. (c) MDANs-Cas12a can directly detect CTCs in human blood samples. Reproduced with permission from Ref. [76], © American Chemical Society 2021.

The combination of the CD63 aptamer and exosomes could trigger a conformational change in the aptamer and the release of blocker, which would be recognized by CRISPR/Cas12a. The intensity of the fluorescent signal is positively correlated with the concentration of the exosomes which could be attributed to the cleavage and release of fluorescent signals by CRISPR/Cas12a [80]. It provides a highly sensitive and specific method to detect exosomes with more than four magnitudes and offers a pathway for exosome-based disease diagnosis.

Circulating tumor cells (CTCs) are seeded from the primary tumor to peripheral blood during tumor development and progression, causing distant metastasis of the tumor [81]. Yang and coworkers have established a multivalent duplexed-aptamer networks detection of CTCs and the investigation of cancer metastasis. Multivalent double-stranded aptamer networks Cas12a (MDANs-Cas12a), which could accomplish the detection of CTCs and the investigation of cancer metastasis. MDANs, modified on the surface of magnetic beads, was designed to bind with protein receptors on the CTCs surface and undergo conformational reorganization. The presence of CTCs could force the chain to dissociate to activate Cas12a, which then contributes to the splitting and release of the fluorescent signal (Fig. 6(c) reproduced from Ref. [76]). The conversion of CTCs capture events to ssDNA probes for detection probably provides a strategy for CRISPR/Cas in cellular assays.

4.3 Detection of small molecules

Small molecules exist widely in nature and have great influence on human health, natural environment and so on. A series of aptamers that can specifically bind to small molecules have been developed, providing strategic support for the detection of small molecules by CRISRP/Cas. Adenosine 5'-triphosphate (ATP) is an essential indicator of the metabolic activity in living cells and is involved in the growth and metastasis of many diseases such as tumors. Functional DNAs (fDNAs), aptamers and DNAzymes were combined with CRISPR/Cas12a to allow the detection of small molecules [82]. Lu et al. designed an ATP aptamer pre-hybridized with a DNA activator to prevent the activation of the Cas12a protein. In the presence of ATP, it was captured by ATP aptamer, which releases the DNA activator. And then the released DNA activator activated the trans-cleavage activity of Cas12a, resulting in the cleavage of the ssDNA substrate labeled with a fluorophore and quencher at each end (Fig. 7(a) reproduced from Ref. [82]). In addition, Mirkin and colleagues introduced horseradish peroxidase (HRP) cascade amplification reaction into the CRISPR/Cas detection system while employing the aptamer to selectively bind ATP. The HRP released into the solution can be monitored by colorimetric, fluorescent, or luminescent methods, effectively improving the limit of detection (LOD) by ~ 30-fold compared to traditional fluorophore/quenched reporters in CRISPR/Cas detection systems. Such a cascade amplification method for HRP also provides a way of general thought at the development of CRISPR/Cas systems [83].

In addition, a type of DNA sequences has been discovered with the function of enzymes when binding to specific metal ions [84]. This type of DNA sequence is called DNAzyme and is another efficient method for signal conversion of small molecules. DNAzyme substrates can be designed to produce ssDNA that is complementary to crRNA and activate the trans-cleavage activity of CRISPR/Cas12a. The development of various metal-dependent DNA enzymes such as Mg^{2+}, Mn^{2+}, Pb^{2+}, etc. provides a strategy for CRISPR/Cas-based detection. For the selectively recognition of metal ions, Lu and coworkers employed the DNAzyme as a fDNA to couple with CRISPR/Cas12a for the detection of Na^{+}. A DNAzyme that is specific to Na^{+} and contains a DNA activator sequence at the 5' end was designed as a substrate strand (NaA43S') with an enzyme strand of NaA43E' [82]. When Na^{+}

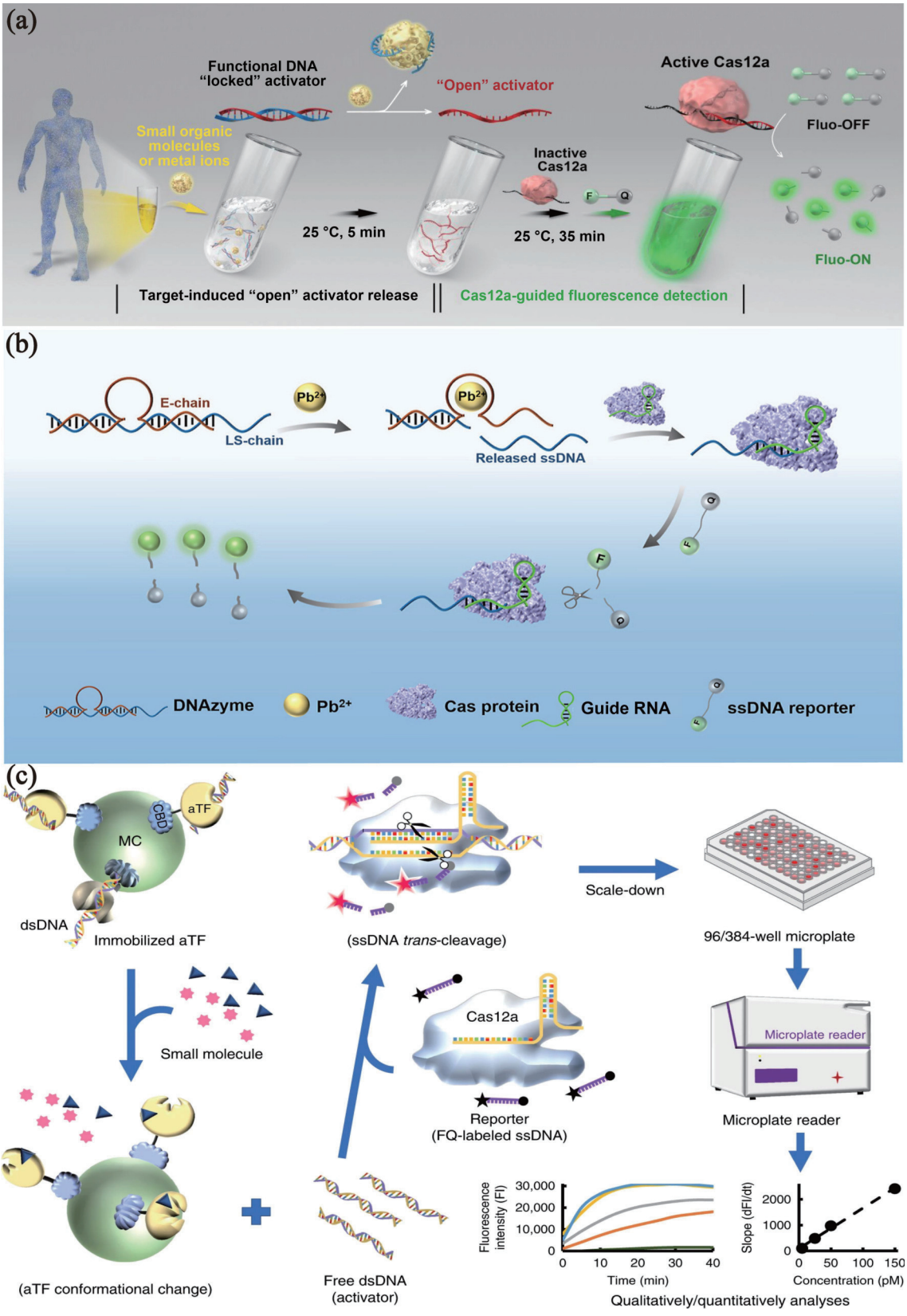

Figure 7 Application of CRISPR/Cas system in the detection of small molecules. (a) Quantitative and rapid detection of ATP by CRISPR diagnostic technology coupled with aptamer. Reproduced with permission from Ref. [82], © American Chemical Society 2019. (b) CRISPR/Cas technology allows the detection limit of Pb^{2+} below 0.48 nM. Reproduced with permission from Ref. [85], © Elsevier B.V. All rights reserved 2021. (c) Small molecule detection platform mediated by CaT-SMelor. Reproduced with permission from Ref. [88], © Liang, M. et al. 2019.

presents, NaA43S' can be cleaved by NaA43E', and the DNA activator sequence is dissociated, which can activate the *trans*-cleavage activity of Cas12a and cause the release of fluorescent signals. The DNAzyme designed by Wu et al. consists of an enzyme strand (E strand) and a substrate strand (S strand). The S-strand would be cleaved in the presence of Pb^{2+} by the E-strand, and the released ssDNA could be recognized by the gRNA of the Cas protein (Fig. 7(b) reproduced from Ref. [85]). Furthermore, a split aptamer-regulated CRISPR/Cas12a and a gap-enhanced Raman tag-based lateral flow biosensor were constructed. The

splitting aptamer of 17-β-estradiol was able to bind competitively to regulate the trans-cleavage ability of CRISPR/Cas12a, which established a dual mode visible C for 17-β-estradiol. Coupling CRISPR/Cas12a with surface-enhanced Raman spectroscopy (SERS) lateral flow test strips provides a new perspective on the detection of non-nucleic acid targets [86].

Moreover, bacterial allosteric transcription factor (aTF) which can sense and respond to various small molecules via conformational changes is another effective strategy for signal conversion of small molecule substances to nucleic acids [87]. aTFs are proteins that can conjugate small molecules and specific dsDNA motifs. The affinity of aTF for dsDNA can be varied with the binding to various small molecules. Zhang and coworkers developed a small-molecule detection platform called CRISPR/Cas12a and aTF-mediated small-molecule detector (CaT-SMelor) [88]. The outcome demonstrated the reduced affinity of aTF with dsDNA motifs when it binds to other small molecules. Subsequently, dsDNA was released to trigger the trans-cleavage activity of CRISPR/Cas12a. By measuring the variation in the fluorescence signal, the target small molecules could be measured exactly (Fig. 7(c) reproduced from Ref. [88]).

Antibiotics are most widely used drugs, and overuse leads to residues in food to some extent. As such, detection of antibiotics in food is essential for food safety [89]. The transformation of antibiotics information into chemical signals can be effectively achieved by employing the biological recognition of aptamers, specific antibodies, and enzymes. A metal-labeled CRISPR/Cas12a system was constructed to achieve ultra-sensitive and highly selective assessment of antibiotics in fish samples. Featuring kanamycin as a modal analyte, the system has provided detection limits which are in the low range of 4.06 pM in a 30 min assay process [90]. Lv and his team proposed an elemental probe-based

Table 2 Application of CRISPR/Cas system for the detection of nucleic acid and non-nucleic acid targets

Application	Target	Cas	Technology	Target	References
Nucleic acid targets	DNA	Cas9	CAS-EXPAR	Methylated DNA	[51]
			CUT-PCR	ctDNA	[50]
			CasPLA	mtDNA SNV	[49]
		Cas12a	DETECTR	HPV	[36]
			HOLMES	SNP	[52]
			E-CRISPR	HPV-16, PB-19	[69]
		Cas12b	CDetection	HPV, ABO, BRCA1	[54]
		Cas14a	Cas14a-DETECTR	HERC2	[45]
		Cas12b	HOLMESv2	Japanese encephalitis virus	[53]
			CASdetec		[64]
		Cas12a	DETECTR		[63]
		Cas13	STOP	SARS-CoV-2	[59]
			SHINE		[60]
			PEARL		[62]
			CREST		[61]
			FIND-IT		[65]
			SHERLOCK	Zika, dengue viruses	[7]
			HUDSON	Zika viruses	[57]
			CARVER	LCMV, IAV, VSV	[58]
	Protein	Cas9	GLoPro	MAPK14	[78]
		Cas12a	Protein/small molecule interactions	Streptavidin	[74]
			E-CRISPR	TGF-β1	[69]
		Cas13a/12a	DNA enzyme walker	SAA1, FV	[77]
		Cas14a	EXPAR-PtNPs/Cu-TCPP (Fe)	Creatine kinase MB	[75]
		CasRx	CARPID	RBPs	[79]
	Others	Cas12a	CD63 aptamer	Exosomes	[80]
			MDANs-Cas12a	CTCs	[76]
			CaT-SMelor	aTF	[88]
Non-nucleic acid targets	Small molecules	Cas12a	fDNAs (two ATP aptamers)	ATP	[82]
			HRP-amplification		[83]
			Lateral flow biosensor	17β-estradiol	[86]
			DNAzyme (fDNA)	Na^+	[82]
			Metal-labeled CRISPR/Cas12a	Kanamycin	[90]
		Cas14a	Enzyme strand	Pb^{2+}	[85]
			An elemental probe-based CRISPR/Cas14	AMP	[91]

CRISPR/Cas14 detection platform that enables the sensitive detection of ampicillin (AMP) [91]. A locking strand containing two AMP aptamer sequences was designed to hybridize together with the activator strand. The aptamer would bind the AMP molecule once the AMP was recognized and result in the release of the activation strand to bind with CRISPR/Cas14, activating the *trans*-cleavage activity and cleaving the probe consisting of the Tb element and ssDNA. After cleavage, the ssDNA would be captured by streptavidin-coated magnetic beads (SA-MBs) and removed. Consequently, the concentration of Tb can be quantified by Tb ICP-MS (inductively coupled plasma mass spectrometry) intensity, while the target AMP concentration can be quantified.

5 Challenges and prospects

The last five years have witnessed the burgeoning of sensing platforms based on CRISPR/Cas systems for the detection of nucleic acid and non-nucleic acid targets (Table 2). Owing to the modular and programmable features, CRISPR/Cas systems can be combined with amplification technologies to realize the precise and sensitive detection of samples at ultra-low concentrations. Moreover, by coupling the CRISPR/Cas systems with other devices, such as paper-based lateral flow assays (LFAs), field-effect transistor (FET) and microfluidics devices, point-of-care testing can be implemented with high sensitivity and specificity. At present, combined with specific aptamers, the CRISPR/Cas systems have been successfully employed for non-nucleic acid detection, such as CTCs, metal ions. CRISPR/Cas systems have achieved gene editing within the cells, and would also be a powerful tool for intracellular detection.

Although CRISPR/Cas systems have highlighted a variety of advantages in biosensing, there are still several challenges that need to be overcome. First, since the procedure of target pre-amplification readily occurs errors, it is extremely critical to develop the detection method based on CRISPR/Cas with the expected sensitivity but without pre-amplification. Recently, strategies of combined multiple crRNAs have been designed to improve sensitivity and specificity, allowing directly quantification of viral loads. In addition, the approach of extending the 3'- or 5'-ends of the crRNA can improve collateral cleavage activity and recognition specificity of Lachnospiraceae bacterium Cas12a (LbCas12a). Second, Cas proteins exist certain background activity, which is different for different batches, leading to differences in the detection results of the samples. Third, crRNA and ssDNA/ssRNA signal reporters remain at risk of degradation in clinical complex samples. Adding RNase inhibitors and redesigning the reporter may avoid unwanted degradation.

In addition, for the detection of non-nucleic acid targets, it requires steps to convert them into nucleic acid signals beforehand. Aptamers and DNAzymes are commonly used ssDNA or ssRNA oligonucleotides that can bind specifically to non-nucleic acid targets, which were applied as activators for Cas proteins. However, the activity of Cas proteins obtained by single-stranded activators is lower than that obtained by double-stranded activators with PAM sequences. Designing a complementary stand of single-stranded activator to hybridize with is an alternative method. Besides, by rationally optimizing the sequence of single-stranded activators, it may be possible to improve the cleavage activity of Cas proteins. After understanding and addressing these challenges, we believe that CRISPR/Cas systems may become significant tools for disease diagnosis and facilitate the development of biosensing.

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