Challenges and perspectives of CRISPR-based technology for diagnostic applications

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ABSTRACT

The precision and versatility of CRISPR-based techniques, combined with the advantages of nucleic acid-based nanotechnology, hold great promise in transforming the landscape of molecular diagnostics. While significant progress has been made, current CRISPR-based platforms primarily focus on nucleic acid detection. To expand the applicability and fully leverage the advantages offered by CRISPR-based diagnostics, ongoing efforts explore molecular strategies to develop CRISPR sensors capable of detecting a diverse range of analytes beyond nucleic acids. In addition, challenges still persist in the adaptation of CRISPR platforms for point-of-care (POC) applications, involving concerns such as portability and automation, as well as the complexities associated with multiplexing. Here, we provide a detailed classification and comprehensive discussion of molecular strategies facilitating the conversion of non-nucleic acid target binding into CRISPR-powered outputs with an emphasis on their corresponding design principles. Furthermore, the second part of the review outlines current challenges and potential solutions for seamlessly integrating these strategies into user-friendly platforms and rapid tests specifically tailored for point-of-care (POC).

1. Introduction

CRISPR systems, found in microbial organisms, serve as adaptive immune systems where they detect and degrade foreign nucleic acids utilizing CRISPR-associated (Cas) enzymes [1,2]. This recognition of targets is facilitated by the complementary sequence matching between CRISPR RNA (crRNA) and the target molecules, allowing for programmable and engineered CRISPR systems that can target various DNA or RNA molecules. CRISPR systems are classified into two classes and six types based on evolutionary relationships [3] and hold immense potential also for the development of next-generation molecular diagnostics. The extensively employed CRISPR family in biosensing applications encompasses Cas9, Cas12, Cas13, and Cas14 [4]. Notably, the identification of collateral *trans*-cleavage nuclease activity associated with CRISPR type V (Cas12 and Cas14) and type VI (Cas13) systems has profoundly influenced the field of diagnostics [4–8]. These systems

present an optimal toolkit for molecular diagnostics, as they integrate target recognition, signal transduction, and amplification within a single detection system. Cas13 exhibits specificity for single-stranded RNA, while Cas12 and Cas14 recognize and cleave both double-stranded DNA and single-stranded DNA, constrained by a protospacer adjacent motif (PAM) sequence limitation for double-stranded DNA targets [9–11]. In the realm of diagnostic applications, the *trans*-cleavage activity of CRISPR systems is frequently harnessed by designing reporter molecules, such as single-stranded DNA or RNA, that are fluorescently quenched. Activation of the Cas effector by the specific target leads to the cleavage of these reporter molecules, resulting in the release of a fluorescent signal (Fig. 1). This innovative approach facilitates the sensitive and specific detection of target molecules in diagnostic assays [12]. In addition to the well-established Type V and VI systems, Type III CRISPR-Cas systems have been used in combination with allosterically activated proteins carrying cognate sensory domains (CARF-associated

enzyme) for nucleic acid detection [13,14]. The overreaching objective is to develop molecular detection platforms tailored for point-of-care (POC) applications, thereby ensuring accessible and timely disease diagnostics for individuals across diverse settings.

The predominant CRISPR-based sensing platforms designed for nucleic acid detection present numerous advantages. These include exceptional sensitivity, reaching detection limits in the attomolar range following isothermal pre-amplification steps, and notable specificity for single-base variations [15]. For a more in-depth exploration of these advantages and the broader landscape of CRISPR-based nucleic acid sensing, we encourage the reader to delve deeper into these reviews [16–18]. However, the lessons gleaned from recent pandemics, exemplified by COVID-19, underscore the importance of an effective diagnoses that encompass the detection of diverse biomarkers, ranging from proteins and small molecules to host biomarkers such as specific antibodies. Despite considerable strides, it is evident that CRISPR-based detection platforms have predominantly focused on nucleic acid targets. From a molecular standpoint, developing CRISPR-based sensors for non-nucleic acid targets poses a significant challenge. This is due to the need of employing a nucleic acid-mediated methodology to translate the target recognition event into the collateral cleavage activity of Cas effectors. Consequently, there is a pressing need for alternative molecular strategies to design switchable CRISPR sensors capable of detecting a broader range of targets other than nucleic acids [19]. Furthermore, an additional hurdle in translating CRISPR-based diagnostics into the market lies in achieving massive, rapid testing and/or screening, ideally extending to any class of biomarkers at the point-of-care (POC). This objective holds particular importance in ensuring the right to health, especially in emerging countries with limited economic and infrastructural resources. However, the translation of current CRISPR-based platforms for POC applications faces several challenges. These challenges encompass complex sample processing, time-consuming protocols, and reagent stability, among various other factors.

In light of the aforementioned considerations, our review is motivated by the dual objectives of investigating the molecular strategies employed to transduce the binding events of non-nucleic acid targets into CRISPR-powered signal outputs. By delving into the molecular intricacies that facilitate the conversion of non-nucleic acid target binding into CRISPR-powered signals, we aim to provide a comprehensive understanding of the innovative strategies driving this specific CRISPR application. While our primary focus is on elucidating these molecular strategies, we concurrently underscore the imperative of seamlessly integrating CRISPR-based assays into Point-of-Care (POC) devices. Our emphasis on the integration of CRISPR-based assays into POC devices underscores the practical applicability and accessibility of these technologies in real-world diagnostic scenarios.

2. Expanding CRISPR-based toolboxes for the detection of non-nucleic acid targets

CRISPR-based diagnostic methods have been adapted to detect a wide range of analytes, including proteins, small molecules, antibiotics, metal ions, and metabolites [19–21]. Here, our aim is to focus on the molecular strategies involved in the activation of Cas effectors, focusing on the challenges, limitations, and prospects for practical use. In many cases, the CRISPR system serves as a reporter or amplifier, while the actual sensing of the non-nucleic acid target molecule relies on a target-responsive molecular probe that undergoes a molecular reconfiguration upon target recognition. In this context, Table 1 provides an overview of various reported strategies for CRISPR-based non-nucleic acid target detection. Generally, these platforms utilize functional DNA/RNA probes, including aptamers, DNAzymes, riboswitches, or employ more intricate reaction networks as bio-transduction elements. In a more recent development, CRISPR readout has also been combined with antibody-based detection platforms. In the following section, we provide an highlight on some of these approaches.

The initial application of a biosensor utilizing Cas12a for ATP detection was introduced by Peng et al. [19]. In this system, a
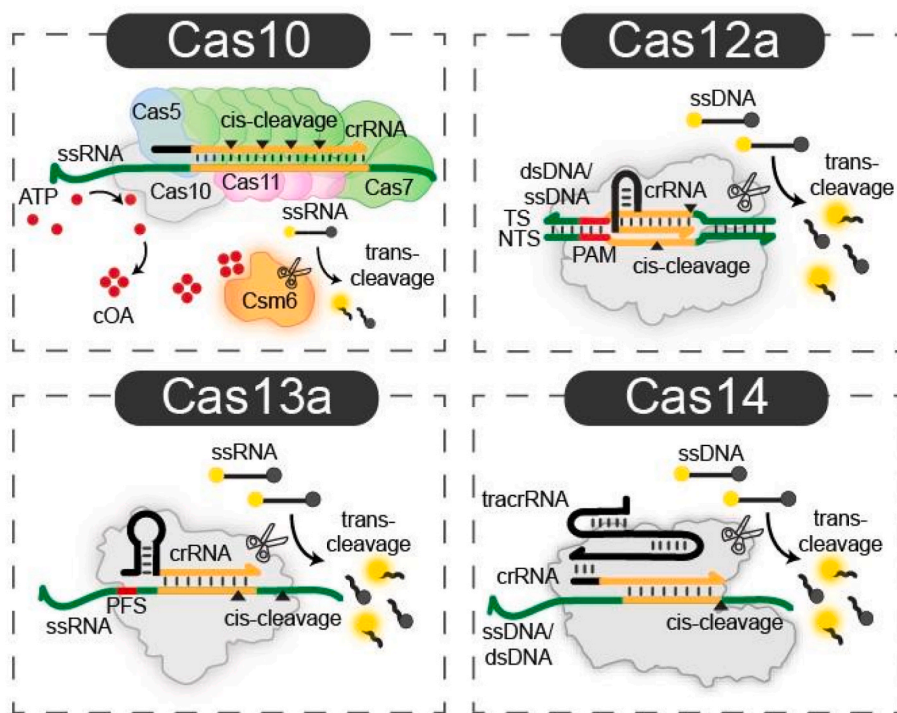
Fig. 1. The schematic provides an overview of key Cas enzymes widely employed in diagnostic applications. These enzymes play a central role in CRISPR-based diagnostic systems, exploiting their collateral *trans*-cleavage activity to generate a signal output. This signal output, reflecting the presence of target DNA or RNA molecules, is subsequently detected and quantified. The ability to generate a discernible signal enables sensitive and specific detection in various diagnostic applications, highlighting the versatility and applicability of CRISPR-based technologies in the field of molecular diagnostics.

Table 1
CRISPR/Cas-based detection of non-nucleic acid targets.

Recognition element	Target	Cas effector	Mechanism	Pre-amplification	Signal Transduction	LOD	Dynamic range	Sample type	Ref
Aptamer	ATP	Cas12a	Structure-switching	None	Fluorescence	400 nM	1–200 µM	5% fetal bovine serum (FBS)	[22]
Aptamer	ATP	Cas12a	Structure-switching	Hybridization chain reaction (HCR)	Fluorescence (smartphone)	1.0 nM	0.1–750 µM	5% fetal bovine serum	[23]
Bivalent aptamer	ATP	Cas12a	Structure-switching	None	Colorimetric (LFA)	0.85 µM	5–500 µM	10% human blood serum	[24]
	Kanamycin	Cas12 a				14.8 nM	25–800 nM	Pretreated milk	
Aptamer	Melamine	Cas12a	Structure-switching	None	Fluorescence	0.038 µM	0–25 µM	Milk samples	[25]
Aptamer	ATP	Cas14a	Structure-switching	None	Fluorescence	80 nM	100 nM to 2 µM	Diluted human blood serum	[26]
	Histamine					30 nM	40 nM to 1.5 µM	Buffer	
	Aflatoxin B1					16 nM	20 nM to 3 µM	Peanuts	
	Thrombin					36 nM	40 nM to 2 µM	Buffer	
	Cd^{2+}					4 nM	5 nM to 1.5 µM	3-fold diluted lake water	
	Acetampirid	Cas12a	Structure-switching	None	ECL	2.7 pM	0.1 nM to 0.1 mM	Homogenized lettuce	[27]
Aptamer	*Salmonella* Enteritidis cells	Cas13a	Allostery	T7 RNA polymerase transcription	Fluorescence	1 CFU	$1-10^5$ CFUs	Drinking water and 10-fold diluted milk	[28]
Allosteric transcription factor (aTF)	Uric acid p-Hydroxybenzoic acid	Cas12a	Allostery	None	Fluorescence	10 nM 1.8 nM	25–500 nM 9–180 nM	10-fold diluted blood	[29]
Allosteric transcription factor (aTF)	Tetracycline	Cas12a	Allostery	None	Fluorescence	2 µM	0–10 µM	Environmental water samples	[30]
DNAzyme	Pb^{2+}	Cas12a	Metal-dependent substrate cleavage	None	Fluorescence	0.48 nM	0.48 nM–48 nM	Drinking water	[31]
DNAzyme	Pb^{2+}	Cas III-A Csm6	Metal-dependent substrate cleavage	None	Fluorescence	70 pM	0.5–100 nM	Fresh eggs and processed biopsy from liver, kidney, colon and feces from mice.	
DNAzyme	Serum amyloid A-1 protein (SAA1) Coagulation factor V (FV)	Cas12a/ Cas13a	Metal-dependent substrate cleavage	None	Fluorescence	30 pg mL^{-1} for SAA1 200 pg mL^{-1} for FV	0.1–30 ng mL^{-1} 1–50 ng mL^{-1}	Pretreated human plasma later diluted 1:50	[32]
DNAzyme	Alkaline phosphatase (ALP)	Cas12a	Metal-dependent substrate cleavage	None	Fluorescence	0.04 U L^{-1}	0.1–10 U L^{-1}	150- fold diluted human serum samples and 20-fold mouse serum samples	[33]
DNAzyme	ATP *E. coli* *Klebsiella pneumoniae*	Cas12a	Metal-dependent substrate cleavage	None	Fluorescence	500 nM 10^2 CFU mL^{-1} 102 CFU mL^{-1}	–	10% human plasma and lysed cells from urine samples	[34]
DNAzyme	Pb^{2+}	Cas12a	Metal-dependent substrate cleavage	None	Fluorescence / Photonic crystal chip	86 fM 24 pM	0.1 pM to 1 µM 100 pM to 1 µM	Processed serum, air particles, and soil samples	[35]
Polyclonal antibody	interleukin-6 (IL-6) proteins	Cas12a	Proximity binding	Isothermal amplification (proximity primer extension)	Fluorescence	100 fM	1 pM to1 nM	Human blood serum	[36]
Antibody and protein	SARS-CoV-2 Ab	Cas12a	Proximity binding	Recombinase polymerase amplification (RPA)	Fluorescence / Optical (Lateral Flow)	10 aM Qualitative	10 aM to 1 pM –	Undiluted human blood serum and clinical samples from patients.	[37]
Peptide	Panitumumab Ab	Cas12a	Proximity binding	Catalytic hairpin assembly (CHA)	Fluorescence	0.62 pM	1 pM to1 nM	10 fold diluted human serum	[38]
Antibody	Chemokine ligand 9 protein	Cas12a	Sandwich immunoassay	None	Fluorescence	14 pg/mL	32–800 pg/mL	Buffer and urine samples	[39]
Antibody	Human IL-6, human VEGF	Cas13a	Sandwich immunoassay	T7 RNA polymerase transcription	Fluorescence	2.29 fM, 0.81 fM	8 to 5000 fM, 4 to 2500 fM	Buffer and in 20% and 100% serum samples	[40]

Table 1 (*continued*)

| Recognition element	Target	Cas effector	Mechanism	Pre-amplification	Signal Transduction	LOD	Dynamic range	Sample type	Ref
Antibody and aptamer	Carcino-embryonic antigen (CEA)	Cas12a	Sandwich immunoassay	None	Fluorescence	69.5 aM	0.6–20 μM	Buffer and serum samples	[41]
	Prostate specific-antigen (PSA)					175 aM	0.5–150 μM		
Antibody	Biotin	Cas12a	Competitive immunoassay	None	Fluorescence	13 pM	13 pM to 50 nM	20-fold diluted human serum	[42]
	Digoxin					0.125 pM	0.125–125 nM	20 fold diluted serum	
	Folic acid					50 pM	50 pM to 50 nM	Diluted serum	
Antibody	Biotin	Cas12a	Competitive immunoassay	None	Fluorescence	12.5 pM	12.5–32 pM	Buffer and 10-fold diluted human serum samples	[43]
	Digoxin					63 pM	63 pM to 16 nM		
	Folic acid					0.39 nM	0.39–50 nM		

single-stranded DNA (ssDNA) aptamer serves as target molecule for the crRNA/Cas12a complex (Fig. 2A). In the absence of ATP, the aptamer binds to the ribonucleoprotein complex, activating *trans*-cleavage activity. Consequently, the fluorescence resonance energy transfer (FRET)-labelled DNA reporter undergoes digestion, generating a detectable signal. The presence of the target molecule suppresses this signal, providing a mechanism for sensitive and specific ATP detection in the biosensor [22]. This biosensor exhibited a limit of detection (LOD) of 400 nM for ATP. Interestingly, Xiuping et al. [24] developed a bivalent aptamer-assisted CRISPR/Cas12a-lateral flow assay (BA-CASLFA). This is a turn-on colorimetric assay, and the test yields a positive output only when ATP is recognized by the ATP-binding aptamer. The BA-CASLFA method demonstrates excellent performance in terms of specificity and sensitivity (LOD = 0.85 μM) and high versatility since the same approach have been deployed to achieve sensitive detection of an aminoglycoside antibiotics (Kanamycin, LOD = 14.8 nM). It's worth noting that the method for ATP detection involves more than a single step to generate the signal [24]. Unlike commercially available ATP determination kit recombinant based on firefly luciferase and its substrate D-luciferin, which provide a straightforward and rapid one-step detection process, this particular CRISPR biosensor may involve multiple steps in the signal generation process. The complexity of the method suggests that, at its current stage of development, it may not be directly equivalent to the simplicity and immediacy offered by bioluminescent assays. Further refinement and optimization may be needed to enhance the biosensor's efficiency and bring it closer to the market. In 2020, Shen et al. [28] presented a CRISPR-Cas13a-based detection system for pathogens utilizing allosteric hairpin probes that demonstrated exceptional sensitivity with a limit of detection (LOD) of 1 colony-forming unit (CFU). This approach offers the advantage of direct pathogen identification without the need for DNA extraction, and it is also rapid, thus enabling quantification of *Salmonella Enteritidis* cells (from 1 to 10^5 CFU) in various sample types, such as milk, within a time frame of less than 3 h [28]. The methodology involves the utilization of a single-stranded DNA (ssDNA) allosteric probe comprising distinct domains: a switchable aptamer domain for specific recognition of *S. Enteritidis* and a primer binding site that facilitates DNA polymerase activity, leading to the generation of a double-stranded DNA (dsDNA) template for subsequent T7 RNA polymerase transcriptional activity. This transcriptional activity results in the release of single-stranded RNA (ssRNA) products, which subsequently undergo Cas13-based amplification. Despite the remarkable performance of this method, its practical application and translation into a point-of-care (POC) device remain challenging due to the substantial number of biological components required for the assay. The complexity involved in implementing and integrating these components poses hurdles for practical deployment.

As an alternative to aptamers, also DNAzymes have been widely used as bio-transducers for CRISPR activation. DNAzymes are probes with a specific catalytic activity that can be modulated by various effectors [6]. For instance, different metal ions serve as crucial cofactors for the catalytic function of DNAzymes. By taking advantage of this principle, researchers have integrated DNAzymes with CRISPR technology to fabricate assays specifically designed for the detection of metal ions. In one study, the DNAzyme substrate was designed to be cleaved in the presence of the Pb^{2+} ions [31]. As a result of the site-specific cleavage activity, a single-stranded DNA was generated, in order to trigger Cas12a signal amplification (Fig. 2B). A detection limit of 0.48 nM was achieved by this method. In another study, Yand et al. proposed a tandem, one-pot detection assay called cDNAzyme for sensitive lead (Pb^{2+}) detection [44]. An important aspect of this system is the activation of the GR-5 DNAzyme, which generates cleaved substrates that act as activators for CRISPR/Cas III-A Csm6. Specifically, the 5'-fragment of the cleaved substrate has a 2'3'-cyclic phosphate at the 3'-terminus, that activates the RNase activity of Csm6, thus resulting in the non-specific cleavage of collateral single-stranded RNA (ssRNA) reporters. Compared to the original GR-5 DNAzyme, the Csm6-DNAzyme tandem enhances the sensitivity for detecting Pb^{2+} (lead ions) by 6.1 times, while maintaining a detection limit of 70 pM, using a simple one-step, room temperature format. The method has demonstrated successful detection of lead in water and food samples (i.e. eggs), and it has been employed to investigate lead accumulation in mice. An interesting aspect of this approach is that it expands the capabilities of the Csm6 toolbox by eliminating the need for complex design and optimization of guide RNAs, which are typically required in CRISPR-powered amplification systems for non-nucleic acid detection. Metal-dependent DNAzymes have been also utilized as bio-transducer elements for the detection of other targets using CRISPR/Cas systems (eg., exosomal proteins [32], alkaline phosphatase [33], and ATP [34]). However, DNAzyme-regulated CRISPR systems have limitations in their applicability to diverse targets, as the specificity of DNAzymes depends on the design and selection process. Thus motivated, achieving high specificity, especially in complex biological samples containing complex mixture and interfering substances, can be challenging.

As a matter of fact, the fundamental principle underlying the aforementioned platforms is to establish artificial communication between nucleic acids (NA) biotransducers and non-nucleic acids (non-NAs) targets by engineering mechanisms and interfaces that enable probing, processing, and conversion of target binding into an amplified CRISPR-based signal. The functional nucleic acid module is modified to serve as a substrate for Cas protein binding only upon interaction with the target molecule. This is often accomplished by designing switchable DNA/RNA elements that can adopt different conformational states in the presence or absence of the target molecule [45]. However, this approach faces challenges in generalization, as the bio-transduction elements are often limited by sequence constraints of the functional unit. Additionally, these assays typically involve competitive binding, where the target
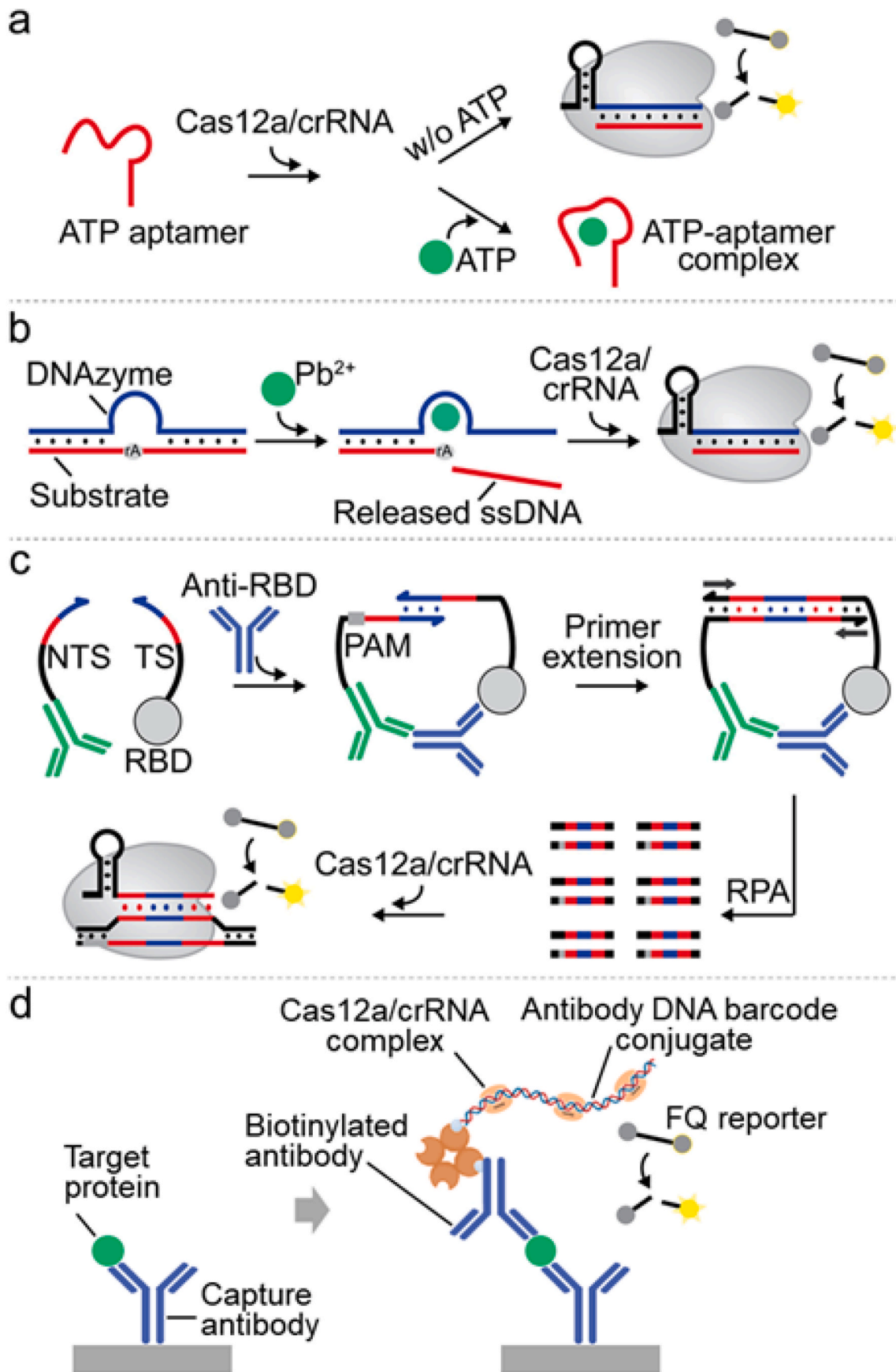
(caption on next page)

Fig. 2. Schematic illustration of different CRISPR-based detection strategies for non-nucleic acid targets. (a) Aptamer-mediated CRISPR/Cas12a-based detection of ATP. ATP-binding aptamer acts as Cas12a activator by triggering *trans*-cleavage activity of Cas12a only in the absence of ATP. Adapted with permission from Ref. [22] (Copyright © 2020, Elsevier). (b) DNAzyme-regulated CRISPR-Cas12a systems for Pb^{2+} detection. In the presence of the Pb^{2+} ion, the DNAzyme substrate is cleaved and the ssDNA activator of Cas12a is released. Adapted with permission from Ref. [31] (Copyright © 2022, Elsevier). (c) CRISPR-based detection of anti-SARS-CoV-2 antibodies. Proximity binding of two recognition elements to the anti-Sars CoV-2 antibody leads to hybridization of DNA probes and primer extension that triggers the *trans*-cleavage activity of Cas12a. Adapted with permission from Ref. [37] (Copyright © 2022, Springer Nature). (d) The Immuno-CRISPR assay for the sensitive detection of the CXCL9 protein operates by forming a sandwich structure in the presence of the target protein. This structure involves the capture antibody, the CXCL9 protein, and the antibody-DNA barcode conjugate. The formation of this sandwich structure activates the *trans*-cleavage activity of Cas12a. Adapted with permission from Ref. [39] (Copyright © 2021, American Chemical Society).

molecule and CRISPR RNA (crRNA) compete for binding to the same functional nucleic acid. Consequently, these assays often require multiple steps of analysis, which restricts their practical application for field tests or point-of-care (POC) diagnostics. Another issue faced by many of these assays is the presence of background signals obtained in the absence of the target, that leads to potential issues with accuracy and specificity. The difficulty lies in engineering on-off switching mechanisms at the molecular level, especially when using RNA-based receptors that can intrinsically populate multiple conformational states. In most of these cases, the stability of these multiple conformational states can be influenced by the presence of the crRNA since the probe-crRNA hybridization can push the equilibrium toward a signaling state [46]. As a result, even in the absence of the target molecule, a small fraction of the switchable probes can populate the crRNA-binding conformational state, generating a significant background signal due to the high sensitivity of CRISPR-based detection (low pM). Addressing these challenges and improving the specificity of CRISPR biosensing for non-nucleic acid targets will require advancements in the design and engineering of molecular switches and probes [47,48], as well as a better understanding of the factors influencing conformational states and their stability [49,50]. These considerations are crucial for the development of highly accurate and specific CRISPR-based detection assays.

In recent advancements, proximity-based CRISPR assays have emerged as a promising strategy for detecting targets with multiple binding sites. These assays utilize proximity effects to achieve target recognition and activation of Cas activity, rather than relying on direct binding of the crRNA [36,51]. One example of this approach is an isothermal proximity CRISPR Cas12a assay for the highly sensitive and specific detection of proteins, as reported by Li et al. [36] The methodology involves the design of two DNA proximity probes, each possessing short complementary domains to discourage self-hybridization in the absence of the target. Upon the binding of the two probes to the same target, a three-way junction straucture is formed, thus initiating a primer extension reaction facilitated by a polymerase. This reaction generates a double-stranded DNA barcode, subsequently triggering the Cas12a/crRNA complex. Through the modification of the two probes with affinity ligands (e.g., anti-IL6 Ab), the authors successfully conducted quantitative profiling of IL-6 expression during allergen-mediated mast cell activation. Furthermore, the assay demonstrated the capability to detect IL-6 proteins at concentrations as low as 100 fM, highlighting the exceptional sensitivity and specificity of this isothermal proximity CRISPR Cas12a assay for protein detection applications. This modular and versatile approach has been also used in a follow-up work for the ultrasensitive CRISPR-based detection of SARS-CoV-2 antibodies in clinical samples (Fig. 2C) [37]. In this strategy, two DNA scaffolds are used, each conjugated to either anti-human IgG or IgM antibodies, or the receptor binding domain (RBD) of the SARS-CoV-2 spike protein. When the anti-RBD antibody binds to the RBD, it induces proximity-dependent DNA hybridization between the two DNA probes. This, in turn, leads to DNA extension and the generation of a double-stranded DNA barcode that can be targeted by CRISPR-Cas12a. By employing recombinase polymerase amplification (RPA), this approach achieves a remarkably low detection limit of 10 aM. The detection module can be switched between fluorescence and lateral flow readout, making it suitable for point-of-care applications. The ability to detect SARS-CoV-2 antibodies with high sensitivity and

flexibility demonstrates the potential of proximity-based CRISPR assays in clinical diagnostics. More recently, Zhang et al. took advantage of proximity-induced DNA hybridization for the detection of panitumumab, a cancer therapeutic monoclonal antibody, in human blood serum [38]. In this sensing platform, peptides conjugated by two DNA proximity probes specifically recognize and bind to panitumumab. This triggers the catalytic hairpin assembly (CHA) cycles which results in generating dsDNA output. Thanks to the dual signal amplification integrated by CHA amplification and CRISPR/Cas12a, a LOD of 0.16 pM was achieved for panitumumab.

Within the domain of immunoassays, traditional enzyme-linked immunosorbent assay (ELISA) has been integrated with CRISPR/Cas systems for the detection of diverse targets. This innovative integration includes combining of various sandwich design strategies employed in ELISA, such as antibody-target-antibody [39,40,52–55], antibody-target-aptamer [41,56,57], and aptamer-target-aptamer [56] with the *trans*-cleavage activity of Cas enzymes. These assays ingeniously capitalize on the efficient signal generation of the CRISPR/Cas system while leveraging the specific recognition capabilities of ELISA. The indiscriminate cleavage activity of the Cas enzymes, in particular, provides greater sensitivity and a wider detection range than the traditional ELISA. On the other hand, like traditional ELISA, CRISPR-based immunoassays are a general and versatile method that can be extended to detect various biologically and environmentally relevant targets without the need for redesigning crRNA. In an example, Lee et al. presented an immuno-CRISPR assay utilizing the conventional antibody-protein-antibody sandwich architecture for detecting urinary chemokine ligand 9 (CXCL9) by replacing enzyme labels with DNA barcode strands (Fig. 2D) [39]. In this method, biotinylated DNA barcodes containing multiple Cas12a recognition sites were assembled onto streptavidin and linked to the biotinylated anti-CXCL9 antibody. When the target protein (CXCL9) is present, a sandwich structure forms, involving the capture antibody, CXCL9 protein, and the antibody-DNA barcode conjugate. This structure triggers the *trans*-cleavage activity of Cas12a. The inclusion of an increased number of Cas12a recognition sites on DNA barcodes allowed the authors to achieve an impressive detection limit of 14 pg mL^{-1}, which is seven times lower than that of the conventional ELISA method. The immuno-CRISPR assay was applied to assess the presence of CXCL9 in urine samples from 11 kidney transplant recipients. The results demonstrated a remarkable 100% accuracy in clinical CXCL9 determination. Despite the high sensitivity and versatility of CRISPR-based immunoassays, there is a recognized need to integrate automation into these assays. This effort aims to enhance the time efficiency of the process, enabling the rapid screening of numerous samples simultaneously while maintaining simplicity in the analysis.

In adherence to a similar design principle, Zhu et al. introduced competitive CRISPR/Cas12a-powered immunoassays tailored for the detection of small molecules [42,43]. In a more recent development, an active DNA linked to the target small molecule has a dual role: it acts both as a competitor for antibody binding and as activator of CRISPR-Cas12a [43]. In the absence of target small molecules, DNA probes are captured by the antibody on the microplate, initiating Cas12a-catalyzed cleavage of DNA reporters. Conversely, in the presence of small molecule targets, the DNA probes are displaced from the antibody. This leads to a reduction in DNA probes on the microplate and a decrease in activated Cas12a, ultimately resulting in a diminished

fluorescence signal. This method was successfully applied for the detection of biotin, digoxin, and folic acid in serum samples at the picomolar level with a flexible detection range adjusted by varying the concentrations of immobilized antibodies and DNA probes. For a more in-depth exploration of the various assays designed for non-nucleic acids, we recommend that the reader refers to additional comprehensive review articles [19,51].

Despite the notable achievements in CRISPR biosensing, its applicability to non-nucleic acid target detection using antibodies as recognition elements is currently constrained. This limitation arises due to the inherent challenges associated with antibody-based assays, which include multiple reaction steps linked to target binding and Cas processing. These complexities have direct implications for the time required for analysis and the overall assay complexity. As a result, while CRISPR-based biosensing has demonstrated remarkable success in various applications, overcoming the challenges associated with non-nucleic acid target detection using antibodies remains a significant hurdle that warrants further exploration and refinement in assay design and optimization.

3. CRISPR-Cas assay optimization for applications at the POC and in the field

According to the World Health Organization (WHO), a POC sensing platform should follow the REASSURED (Real-time connectivity, Ease of specimens, Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, Delivered) indications. There are many critical key points to be addressed for using CRISPR assays for POC applications. First, it is necessary to develop bio-assays operating with a minimal number of steps involved in sample processing, ideally providing a single-step sample-to-answer analysis. The need of multiple reagents and steps of analysis has an obvious effect on the time of analysis, and also on the overall cost of the CRISPR assay. Thus, the best advancement toward POC diagnostic platforms focuses at the molecular level on engineering DNA/RNA reporter to boost the sensitivity and to reduce the time of analysis. Other efforts of the scientific community are concentrated on i) optimal sample treatment - with minimal analytical steps - to allow the use of unprocessed patient samples; ii) the minimization of the steps of analysis (amplification and signal generation); iii) the introduction, at the device level of automated processing (paper-based platforms, microfluidics, etc.) with visual readout (see LFA section) or smartphone application enabling signal quantification to creating a user-friendly diagnostic kit; iv) the implementation of multiplexed detection of analytes to reach ease-of-use and cost-effective manufacturing; v) the improvement of CRISPR reagent stability compatible with downstream liquid handling and analysis (i.e. lyophilization, additives, etc.). Most of these issues does not belong only to CRISPR-based platforms but are common to many different molecular assays for POC applications.

In recognition of the acknowledged limitations of CRISPR-based point-of-care (POC) platforms for non-nucleic acid target detection, this discussion is dedicated to illuminate these challenges and highlight recent strategies that exhibit promise in mitigating the above mentioned issues. Acknowledging the existing scarcity of documented POC applications specifically tailored for non-nucleic acid molecule detection, we offer insights into examples from the literature that demonstrate technology with potential relevance in this domain, despite their reliance on standard nucleic acids. By exploring these strategies and examples, we aim to foster a comprehensive understanding of the current landscape, encourage further research into innovative solutions, and pave the way for the development of robust CRISPR-based POC platforms capable of sensitively and specifically detecting non-nucleic acid targets.

3.1. Toward automated/amplification-free CRISPR assays

In the context of non-nucleic acid detection using CRISPR-based assays, our focus is on pre-amplification-free CRISPR assays. In principle, the exclusion of a pre-amplification step holds significant technological advantages for the production of an integrated device. This departure from pre-amplification is particularly noteworthy given that one of the primary challenges in CRISPR-based detection of nucleic acids often needs pre-amplification to obtain sufficient sensitivity. Indeed, the inclusion of a pre-amplification step typically results in an increased number of procedural steps and a concomitant rise in assay complexity.

By opting for pre-amplification-free CRISPR assays in the realm of non-nucleic acid detection, we aim to streamline the assay process, reduce complexity, and enhance the overall efficiency of the detection methodology. This strategic choice represents a step forward in optimizing CRISPR-based detection for non-nucleic acid targets, aligning with the goal of developing more user-friendly and accessible diagnostic platforms. In this respect, an easy and effective approach is to use a high responsive device, as the graphene field-effect transistor (FET) reported by Hajian et al. [58] to identify genomic DNA mutations from HEK293T cells. The sensor detected the target sequence in 15 min with a sensitivity of 1.7 fM without the need for amplification. Despite the remarkable result, the prohibitive manufacturing cost for the development of single-use devices is still an issue. Another example of amplification-free assays is the work of Ma et al. [59] in which the SARS-CoV-2 RNA is extracted and reverse transcribed. Upon the recognition of the target the Cas12a cleaves the ssDNA reporter, tasked as a linker between two SERS nanoprobes consisting of Au NPs functionalized with ssDNA partial complementary to the reporter. In absence of the target the nanoprobes tends to aggregate and precipitate causing a low SERS signal, while the activation of the Cas12a due to the presence of the target cleaves the reporter and the nanoprobes remains dispersed in solution, causing a high SERS signal. With this method they achieved a LOD of 1.9 copies mL^{-1} within 45 min of assay. A further interesting approach to have a one-pot assay was proposed by Hu et al. [60]. The advantage of this assay lies in the CRISPR activity block using a protective nucleic acid to temporarily silence the crRNA, which can be activated only when illuminated by a UV source. Hence, the UV-light cleaves the photo-sensitive groups present in the protective nucleic acid, activating the cleavage reaction. They managed to integrate this one-pot assay into a device that requires only an optical system for detection, protective nucleic acid cleavage, and temperature control for amplification and enhance the Cas activity. They succeeded in obtaining a CRISPR-based device that quantify SARS-CoV-2 viral RNA within 30 min including amplification, crRNA reactivation, and CRISPR detection.

Test-tubes, microfluidic systems and paper-based platforms represent the main routes for the incorporation of the above mentioned highly sensitive reactions and transducers, in a fast, accurate, and cost-effective CRISPR point-of-care device. Test-tube platforms offer a simpler approach but might be more appropriate for centralized laboratory diagnostic tool with the potential for high-throughput screening and the need of a trained used for almost all the steps performed. Gootenberg's group [61] provided a clear example of the multiplexing capability of CRISPR systems by coupling Cas13 and Cas12a with different reporters, reaching four-channel multiplexing with orthogonal assays. The multiplexing capability, particularly with orthogonal assays, is a critical aspect for the future development of compact devices which detect non-nucleic targets without the requirement for additional steps.

Microfluidic systems allow for precise control of fluids at a small scale, enabling the integration of multiple steps of the CRISPR assay. Chandrasekaran [62] showcased a single-use microfluidic cartridge with an automated assay and readout which detect SARS-CoV-2 in saliva within 60 min. The assay requires only two steps: first, the sampling and nucleic acid extraction, and a second step where the amplification process and the cleavage are automated. The advantage of using CRISPR and microfluidics can be extended to proteins and small molecules detection, simplifying more the assay due to the absence of an extraction step to analyze the proteins, which are already present in the body fluids. Morever, microfluidics usually needs for instrumentation around

the chip to perform the analysis in a controlled and reproducible way. Pumps or centrifugal force are used to generate a controlled flow, necessitating relatively bulky instrumentation. Moreover, CRISPR assays requires an optimal temperature over 25 °C, thus requiring the integration of a heating system and temperature control.

Paper-based platforms provide a portable and low-cost option for point-of-care testing, especially in resource-limited settings. Yin et al. [63] proposed a fluorescence-based assay on a microfluidic paper-based analytical device (μPAD) to detect multiple SARS-CoV-2 genes. This device provides a 15 min amplification step guaranteed by the sucrose valve solubilization time, achieving a LOD of 102 copies in a nasal swab within 1 h assay. The advantage of employing such sensing platforms lies in the control over fluidics and their cost-effectiveness. The materials used for fabrication are typically inexpensive, including options like paper, certain sugars for time-programmable valves, and a 3D-printed holder. These devices align closely with the Point-of-Care (POC) concept, and their main development challenges include the need for proper stabilization of dried chemicals on paper and the optimization of fluidics for increased reproducibility and sensitivity. Overall, these platforms show significant promise in enhancing access to CRISPR-based diagnostics and advancing personalized medicine. Importantly, these devices can be easily adapted to detect non-nucleic acid targets, combining well with most aptamer/DNAzyme-based assays previously reported.

3.2. Toward multiplexed analysis using CRISPR-powered detection systems

The ability to analyze multiple analytes from a single sample is desired and often essential for the diagnosis or monitoring of specific diseases. However, at its current stage, one notable limitation of CRISPR biosensing technology is the restricted multiplexing capability. As clearly reported by Dincer and colleagues [64], the collateral cleavage capability of Cas effectors is an exceptional feature but presents a significant obstacle to achieving high-level multiplexing. Therefore, it is often necessary to employ different strategies to achieve a greater degree of multiplexing. Utilizing different labels in conjunction with spatial separation methods such as channel networks, droplets, electrode arrays, etc., proves to be a viable approach. However, when using fluorescent readout, the selection of labels is typically limited due to potential cross-interference among them. Also, the compartmentalization of the reaction chambers causes more difficult design and manufacturing process. In this regards, Roh et al. [65] proposed an alternative approach to achieve multiplexing in the same microfluidic channel with one reporter. The approach involves compartmentalizing the CRISPR/Cas reaction within spatially-encoded hydrogel microparticles (Fig. 3A). Each microparticle possesses a unique face code and exhibits fluorescence upon the presence of target DNA. They incorporated the capture of the hydrogel microparticles into a microfluidic device and measured three human papillomavirus (HPV) DNAs by fluorescence with high sensitivity (LOD = 145 aM). Of note, a machine-learning algorithm automatically recognizes the captured particles, further streamlining the process. Despite the main advantages, the assay requires 1 h at 37 °C and a preamplification step to be performed out of the device. However, the primary challenge in developing a successful multiplexed CRISPR/Cas system lies in incorporating a large number of multiplexed detection sites without adding complexity to the system. In the frame of non-nucleic acid detection, a significant step towards simplifying the system is the elimination of nucleic acid amplification which may help overcome this barrier.

3.3. Minimized automated devices for CRISPR-based analysis

Minimized automated devices are specifically developed to streamline and simplify the process of CRISPR-based analysis, making it more accessible and efficient. Thus, in addition to measuring multiple analytes, the minimization and automation of assay steps are also prominent areas of interest for CRISPR-based devices. These devices often integrate various components such as microfluidics, sample handling, reaction chambers, and detection modules into a compact and portable format. They aim to automate the steps involved in CRISPR-based analysis, reducing the reliance on manual manipulation, and minimizing the risk of human error due to the execution process of end-users who may not be familiar with the assay principle. However, there are challenges associated with minimizing and automating the assay steps. Additional instrumentation may be required for process automation, which adds complexity to the setup. Moreover, reducing the assay complexity to a minimal setup may lead to a potential loss of sensitivity. This trade-off between simplification and sensitivity needs to be carefully addressed in the design and optimization of these devices.

To solve this problem coupling a miniaturized and established measuring system, such as the glucose meter, and the CRISPR assay can be helpful to build up a cartridge around the assay. Jia et al. [66] proposed a strategy to measure the alpha-fetoprotein (AFP) in serum, by using a 3-steps assay for the detection of a non-nucleic acid. The assay (Fig. 3B) is harnessing the sensitivity of an aptamer for the protein target, and successively the CRISPR-Cas12a to release the invertase enzyme bound to a reporter immobilized on magnetic beads. Then the released enzyme in solution it is separated as supernatant and sucrose is added to this solution to generate glucose than has been measured using a glucose meter. They obtained noticeable results by measuring the AFP in spiked human serum in 60 min with a LOD of 10 ng mL^{-1}, but unfortunately, they still run most of the assay in a tube, without a full integration of the assay in the device. The main disadvantage of such sensing method lies in the use of multiple enzymes which leads to two main disadvantages. The use of two different buffers for the optimal activity of Cas12a and invertase, and the necessity of the user to be present during the measurement to perform several the several steps required by the assay. These limitations hinders the assay application in a protein sensing POC platform, as it reduces the ease of use.

Zhang et al. [68] proposed an alternative approach by employing CRISPR-Cas12a in a paper-based microfluidic sensor for the detection of Pb^{2+} in contaminated water (Fig. 3C). They harnessed the G-quadruplex formation resulting from the interaction between the single-stranded DNA (ssDNA) as a substrate for Cas12a and Pb^{2+} to inhibit *cis*-cleavage activity. The researchers utilized a ssDNA reporter immobilized on Au NPs. In the presence of the cation, the reporter is not collaterally cleaved and is captured by DNA nanoflowers in the detection zone through complementary base pairing. The color intensity, increased due to the Au NPs, is proportional to the concentration of Pb^{2+} and is measured by a smartphone, enabling quantification. The results demonstrated a linear range from 0.1 to 10 μM and a limit of detection (LOD) of 18.3 nM. The sensor exhibited low interference from other ions and maintained good stability over time, up to 11 days, with recovery rates ranging from 96% to 103%. This work showcases the promise of CRISPR-Cas12a's versatility, demonstrating its application not only in measuring nucleic acids or proteins but also in detecting ions within a low-cost and highly sensitive device.

An alternative strategy to streamline the assay and reduce the number of steps is to automate the addition of chemicals. Numerous studies have concentrated on optimizing multistep assays, with some introducing a sliding mechanism to automate the addition of reactants [69]. For a more user-accessible device, microfluidic paper-based analytical devices (μPADs) are a viable option [70], as they incorporate dried chemicals into the substrate of the μPADs and employ time-control microfluidic valves to increase the reaction time. Yin et al. [63] proposed a fluorescence-based assay on a μPAD to detect multiple SARS-CoV-2 genes. This device provides a 15 min amplification step guaranteed by the sucrose valve solubilization time, achieving a LOD of 10^2 copies in a nasal swab within 1 h assay. The assay still involves challenges such as DNA sample extraction and a long assay time.

In general, while these user-friendly devices offer the advantage of
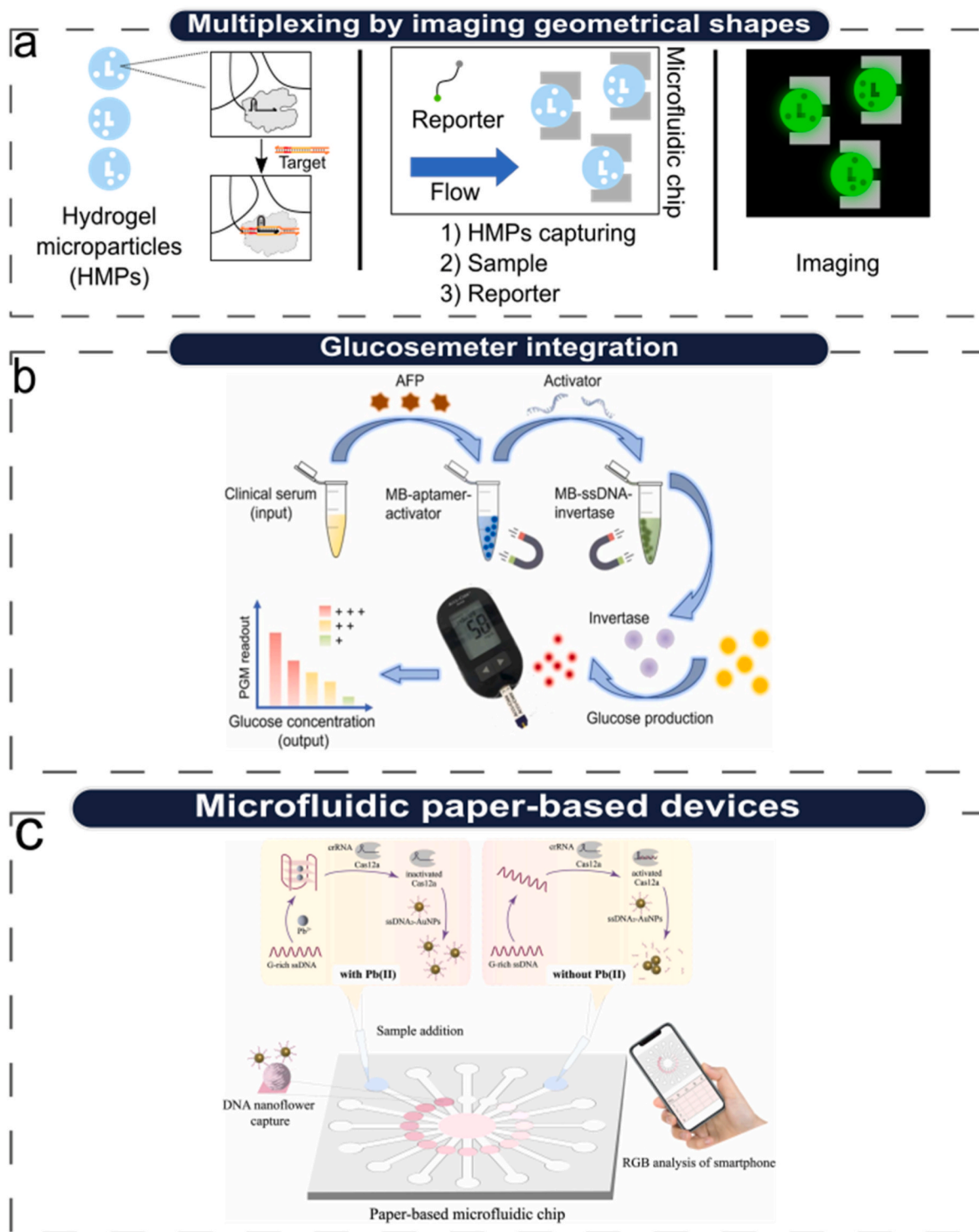
Fig. 3. (a) In the CRISPR-Cas assay multiplexed by imaging, multiple target detection is achieved by crosslinking Cas proteins in hydrogel microparticles (HMPs) with different pin-hole shapes. Each unique HMP shape is crosslinked to a specific CRISPR-RNA (crRNA):Cas complex, allowing for the recognition of different DNA targets (a, left). The sample, containing the target DNAs, and subsequently the fluorescent reporter, are flowed into the microfluidic chip (a, center), resulting in a geometric fluorescent signal that is dependent on the DNA sequence (a, right). Adapted with permission from Ref. [65] (Copyright © 2023, Wiley). (b) The versatility of an enzymatic coupling assay is demonstrated through its integration into a Point-of-Care (POC) device using various approaches. On the left, a multiple amplification approach is employed for detecting the non-nucleic acid target alpha-fetoprotein. This approach is characterized by a lower degree of process automation. Adapted with permission from Ref. [66] (Copyright © 2020, Royal Society of Chemistry). On the right, the integration involves a microfluidic system that enables automatic amplifications and facilitates the delivery of the sample to the sensor. Adapted with permission from Ref. [67] (Copyright © 2022, Nature). (c) Exploitation of the G-quadruplex formation to quantify the amount of Pb^{2+} in contaminated water with a paper-based microfluidics. Adapted with permission from Ref. [68] (Copyright © 2024, Elsevier).

requiring minimal equipment, they come with drawbacks such as poor reproducibility of results due to lower microfluidic control compared to chip-based devices with pumps, and the necessity for specific paper substrate treatment to ensure a longer shelf life. To overcome the limitations of microfluidic paper-based analytical devices (μPADs), an alternative approach that strikes a balance between repeatability, sensitivity, and execution complexity is represented by lateral flow assays (LFs).

3.4. State of art and perspectives of CRISPR-based lateral flow assays

The fast-growing field of CRISPR-Cas based diagnostics had been significantly limited by the requirements of trained personnel to perform the assays and specific equipment to read the fluorescent outputs in which the majority of assays are based [15], to mitigate these drawbacks lateral flow assays (LF) tests stand as ideal candidates. Gootenberg and co-workers [61] were the first to report the adaptation of CRISPR-Cas13a detection assay into LF reading approach. The authors elegantly applied the commercial lateral flow strips "Hybridetect – Universal Lateral Flow Assay Kit" supplied by "milenia biotec GmbH" using a short dual-labelled oligo having terminal biotin and Fluorescein (FITC) (Fig. 4). The LF strips used in this approach contain sample pads that consist of anti-FITC antibodies modified with gold nanoparticles (Ab-AuNP). When the sample is applied, it solubilizes the Ab-AuNPs which then migrate by capillarity towards the detection pad. If the reporter remains intact, it acts as a bridge between the Ab-AuNP and the streptavidin immobilized in the detection line. The accumulation of gold nanoparticles generates a pink colored line. However, if the reporter is cleaved, the biotin and FITC are separated, allowing the Ab-AuNP to flow past the streptavidin line and reach a second line containing antibodies specific for the IgG type of the anti-FITC. The presence of the analyte triggers the cleavage of the reporter, resulting in fainter control lines and more intense detection lines. This initial work served as inspiration for the development of various biosensors that combine CRISPR-Cas detection with LF reading. One notable publication in this field was published by Broughton and co-workers [71], where they report the detection of SARS-CoV-2, capable of detecting just 10 copies of viral RNA (genes E ot N) per μL in a 45 min assay. Other examples include the quantification of specific pathogenic bacteria [72,73], viruses [74] or parasites [75]. Although the wide use of Millenia Biotech's Hybridetect strips, other groups have adapted different commercial LF strips following distinct strategies. Tang and co-workers [76] adapted commercial pregnancy tests using a ssDNA reporter labelled with humanchorionic gonadotropin (hCG). Other authors have designed and produced their own LF strips: Mukama and co-workers [77] developed an assay capable of detecting DNA from P.aeruginosa, while Su and co-workers engineered their own LF strips to multiplex the assay for the detection of different nucleotide sequences [78].

Due to the mechanism of detection, nucleic acids are the most intuitive and straightforward analytes for CRISPR-Cas-based lateral flow (LF) assays. As a result, it is not surprising to see that the literature on these biosensors is dominated by biosensors designed for nucleic acid detection. However, significant efforts are being dedicated to developing CRISPR-Cas-based LF assays for non-nucleic acid analyte detection as well. These assays typically rely on some type of inactivation or blocking action exerted by the analyte on the CRISPR-Cas activating
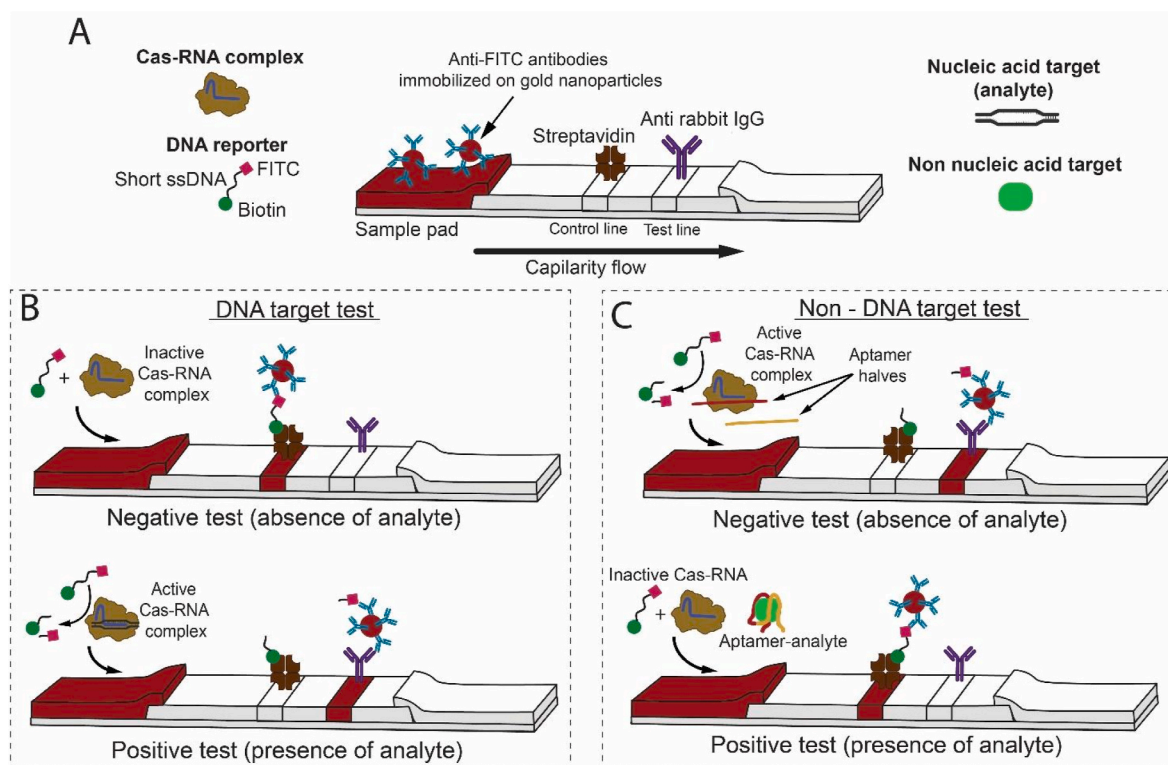
Fig. 4. (a) Typical lateral flow (LF) strips used in CRISPR-Cas assays (i.e., HybriDetect from Milenia biotech) are composed by a sample pad that works also as conjugate pad containing rabbit anti-FITC antibodies labelled with Au NPs. the detection pad is deposited in the second line and an absorbent pad at the end of the strip collects the excess of liquid. In the case of nucleic acid analytes (b), the absence of the analyte leads to an inactive CRISPR-Cas system. In this scenario, the intact DNA reporter serves as a bridge between streptavidin and anti-FITC AuNPs, resulting in the generation of a red line. However, when the target is present, it triggers collateral activity, causing the cleavage of the DNA sequence within the reporter. This cleavage separates the biotin from the FITC, preventing the anti-FITC AuNPs from binding to streptavidin. Instead, they bind to the anti-rabbit line, resulting in a distinct signal. For non-nucleic acid analytes (C), the absence of the analyte enables the aptamer's free sequences to trigger nuclease activity, leading to the cleavage of the reporter. This cleavage generates a signal in the anti-rabbit line. However, in the presence of the analyte, the analyte molecules bind to both halves of the aptamer, preventing its activation of Cas. In this scenario, the intact reporters facilitate the formation of color in the streptavidin line.

DNA strand. The presence of the target analyte leads to a decrease in the signal output, hindering the activation of collateral nuclease activity on the reporter molecule. As an example Li and co-workers [79] developed a CRISPR-Cas-based LF for the detection of 17β-estradiol. They achieved a limit of detection of 180 fM using a split aptamer approach. In the absence of the analyte, the aptamer is available to bind Cas12a and activate its cleavage activity resulting in a colored line in the test region. However, in the presence of the analyte, the two halves of the aptamer bind to it, rendering them unavailable for Cas12a binding. This prevents the activation of the cleavage activity, resulting in a colored line in the control region.

Although CRISPR-based lateral flow (LF) assays show promise for point-of-care (POC) applications in the field, there is value in incorporating orthogonal two-channel readouts into the LF format. This can effectively help avoid false-negative or false-positive results, which are commonly associated with LF systems that detect a single target. Another crucial aspect is the elimination of pre-amplification steps or the use of isothermal amplification at a controlled temperature (such as 37 °C), which offers significant advantages for POC applications. In this regard, techniques like RPA (Recombinase Polymerase Amplification) or RT-RPA (Reverse Transcriptase Recombinase Polymerase Amplification) appear more promising compared to other techniques that operate at higher temperatures, such as LAMP (Loop-mediated Isothermal Amplification). Additionally, considering the requirements for sample extraction during practical detections, there is a need to explore facile sample preparation procedures that are suitable for field-deployable POC testing. In our opinion, by integrating multiplexed target detection with a single-strip colorimetric readout, the CRISPR-Cas mediated multiplexed lateral flow assay may offer in the next future a promising alternative for routine POC analysis.

4. Conclusions

CRISPR-based technology holds the potential to establish a novel benchmark in molecular diagnostics, potentially revolutionizing disease detection and making it more accessible to a broader population. However, substantial challenges persist in adapting these innovations for point-of-care (POC) analysis and non-laboratory settings. Key hurdles include achieving portability, attaining high sensitivity without intricate amplification (for nucleic acid detection) or pre-enrichment (for proteins, antibodies, etc.), navigating complexities associated with multiplexing, and expanding practical applications beyond nucleic acids. Despite the inherent simplicity, affordability, and accessibility of CRISPR technologies, their translation to POC settings requires careful consideration of factors such as the speed of analysis and portability of the device. Similar to many other molecular POC assays, the CRISPR-based design for home or field use should integrate straightforward sample-preparation protocols with robust detection methods to deliver reliable results in variable or challenging contexts. These challenges encompass prolonged reagent storage, limited user training, and harsh environmental conditions. Also sensitivity and matrix effects pose significant challenges, particularly concerning non-nucleic acid targets. Although standard sample pre-treatment steps (lysis, inactivation of nucleases, and purification) are not required for non-nucleic acid detection, for protein and antibody detection, the treatment of samples with tailored non-denaturing detergents may become crucial [79]. These detergents should be capable of degrading cell membranes without denaturing proteins, preserving the integrity of the biological components in the sample. Optimizing detergent concentrations - and conditions (temperature, pH, ionic salt, etc.) could eventually lead to the development of equipment-free, one-step solutions, further enhancing the accessibility and usability of CRISPR-based diagnostics.

Achieving high sensitivity without pre-amplification steps demands innovative solutions also at technological levels. Various sample-to-answer solutions still have to be fully explored to encapsulate CRISPR reagents in a compact device and automate the processing of these reagents with the specific target. Examples may include tube-based devices, paper-based platforms, and microfluidics modules [61–63]. These technologies are already designed to create user-friendly diagnostic kits but still require tailored optimization for specific CRISPR POC applications. These approaches, however, have potential to streamline the diagnostic workflow, reducing complexities associated with sample processing and CRISPR detection.

In addition, from a molecular standpoint, it is mandatory to enhance the reliability and efficiency of CRISPR-based analysis. These improvements can be realized through the identification or engineering of novel Cas enzymes performing with lower-temperature requirements (e.g. thermostable Cas enzymes), higher sensitivity, or faster kinetics, thus enabling rapid and simple amplification-free detection with single-molecule sensitivity. Combining the collateral cleavage activity of Cas12/Cas13 enzymes with a downstream secondary enzyme activity (i. e. NanoLuc, glucose oxidase, etc.) may represent an alternative approach for enhancing detection sensitivity without the need of complex pre-enrichment steps. However, it does require the integration of additional enzymes that must switch on only in response to CRISPR activity, and under the same experimental conditions. Also multiplexing, especially in methods based on the collateral cleavage, introduces complexities related to accuracy and specificity. Addressing these challenges is imperative for the seamless integration of CRISPR-based diagnostics into diverse diagnostic scenarios. Extending applications beyond nucleic acids demands for engineering bio-transducer probes with on-off switching activity at the molecular level to minimize a-specific activation and signal background. Achieving this requires expertise in addressing challenges, advancing design tools, and gaining a better understanding of factors influencing probes conformational states and their stability. The challenges discussed in this article are anticipated to be resolved in the near future, paving the way for disruptive technologies in the rapidly expanding molecular diagnostics market.

CRediT authorship contribution statement

Stefano Del Giovane: Investigation. **Neda Bagheri:** Investigation. **Andrea Celeste Di Pede:** Writing – original draft. **Alejandro Chamorro:** Writing – original draft. **Simona Ranallo:** Writing – review & editing. **Davide Migliorelli:** Writing – original draft, Writing – review & editing. **Loïc Burr:** Writing – original draft, Writing – review & editing. **Samantha Paoletti:** Writing – original draft, Writing – review & editing. **Hatice Altug:** Conceptualization, Writing – original draft, Writing – review & editing. **Alessandro Porchetta:** Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Alessandro Porchetta reports financial support was provided by Italian Association for Cancer Research.

Data availability

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

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