Biosensors and Bioelectronics 215 (2022) 114559

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Novel non-nucleic acid targets detection strategies based on CRISPR/Cas toolboxes: A review

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ARTICLE INFO

Keywords:
CRISPR/Cas
Biotransduction elements
CRISPR-Dx
Non-nucleic acid targets
Challenges and perspectives

ABSTRACT

Various non-nucleic acid targets (ions, small molecules, polysaccharides, peptides/proteins/enzymes, cells, transcription factors) are important biomarkers. They play important roles in environmental protection, food safety and medical diagnosis. Therefore, it is necessary to detect non-nucleic acid targets from various samples before the situation deteriorates. Derived from prokaryotic immune systems, CRISPR/Cas tools have exhibited great promise in the field of biosensing, in addition to the well-known gene-editing function. However, most reported CRISPR/Cas-based biosensors are for nucleic acid detection and the application of non-nucleic acid targets is still in its infancy. To fully explore the potential of CRISPR/Cas-based biosensing systems, it is of great significance to summarize the strategies and prospects of CRISPR/Cas toolboxes in non-nucleic acid targets recognition. In this review, we introduced CRISPR/Cas systems and their characteristics in the field of detection. The progress of detecting six non-nucleic acid targets was outlined and reviewed based on CRISPR/Cas systems coupled with biotransduction elements, including aptamers, DNAzymes, riboswitches, enzymatic reactions, transcription factors, antigen-antibody interactions, allosteric probes, *in vitro* transcription processes, steric hindrance effectors, etc. The development challenges and prospects in this field were also put forward. As such, this comprehensive review would provide valuable information for the expansion of the powerful CRISPR/Cas toolboxes into multiple detection fields.

1. Introduction

The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas) are the important adaptive immune system in prokaryotes (Makarova et al., 2015). The CRISPR/Cas systems have been revolutionizing gene editing due to their high identification of target genes (Malinin et al., 2021). Accordingly, Emmanuelle Marie Charpentier and Jennifer Anne Doudna were awarded the 2020 Nobel Prize in Chemistry for their contributions to gene editing using the CRISPR/Cas systems.

Besides the gene-editing, many studies showed that the CRISPR/Cas systems can be developed for the field of detection due to their DNase/RNase properties (Kaminski et al., 2021). Specifically, the nucleic acid signals can be accurately identified and targeted by guide RNAs and Cas proteins. After recognizing ssDNA or dsDNA or ssRNA targets, the Cas12a/Cas13a/Cas14a proteins exhibited indiscriminate "*trans*-cleavage activity" to DNA or RNA sequence (Table 1) (Feng et al., 2021; Wu et al., 2021; Xie et al., 2021; Zhang et al., 2021d). This provides a theoretical basis for the application of biosensing. To this end, the CRISPR/Cas-based diagnostics (CRISPR-Dx) was first developed in 2016 (Pardee et al., 2016). In the last six years, many nucleic acid detection platforms based on CRISPR/Cas systems also have been successfully developed, including DETECTR (Chen et al., 2018; Harrington et al., 2018), SHERLOCK (Gootenberg et al., 2017), HOLMES (Li et al., 2018), CONAN (Shi et al., 2021), CCB-Detection (Zhou et al., 2020) and CDetection (Teng et al., 2019). Therefore, the CRISPR-Dx has become an important nucleic acid diagnostic technology related to agriculture and food safety, human health and environmental monitoring (Li et al., 2022c; Yin et al., 2021).

Notably, various non-nucleic acid targets are also biomarkers of environmental pollution, human disease and prognosis and food contamination. Traditional methods for detecting non-nucleic acid

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https://doi.org/10.1016/j.bios.2022.114559
Received 13 May 2022; Received in revised form 20 June 2022; Accepted 7 July 2022
Available online 13 July 2022
0956-5663/© 2022 Published by Elsevier B.V.

Table 1
Characteristics of different CRISPR/Cas systems.

Protein types	Cas12a	Cas13a	Cas14a
Targets (activators)	ssDNA, dsDNA	ssRNA	ssDNA, dsDNA
Guide RNAs	crRNA	crRNA	tracrRNA, crRNA
Sizes	~140 kDa	~140 kDa	40–70 kDa
Catalytic domains	RuvC	2 × HEPN	RuvC
Trans-cleavage substrates	ssDNA	ssDNA	ssRNA

Note: ssDNA represents single-stranded DNA; dsDNA represents double-stranded DNA; ssRNA represents single-stranded RNA.

targets include high-performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS), magnetic resonance imaging (MRI), enzyme-linked immunosorbent assay (ELISA), etc. However, these traditional methods are time-consuming, high cost and need expensive instruments and professional operators. In contrast, CRISPR/Cas biosensing systems provide the convenience of cheap, fast, accurate and point-of-care testing without professional equipment. Therefore, it is of great significance to promote the application of CRISPR-Dx for non-nucleic acid targets. However, unlike nucleic acid targets, non-nucleic acid target detection is still in the early stage. There are still many challenges in the detection of non-nucleic acid targets. As such, it is necessary to systematically summarize the reported CRISPR/Cas toolboxes for detecting non-nucleic acid targets to achieve new breakthroughs.

In this paper, we have reviewed the novel non-nucleic acid target detection strategies based on CRISPR/Cas toolboxes in recent years. Different non-nucleic acid targets are systematically classified into six categories. The detection characteristics and principles based on CRISPR/Cas toolboxes were summarized. The progress of detection of non-nucleic acid targets was further introduced by combining diverse biotransduction elements with CRISPR/Cas systems. Finally, the challenges and perspectives for detecting non-nucleic acid targets were discussed.

2. CRISPR/Cas-based biosensor for the detection of non-nucleic acid targets

2.1. Detection of ions

Metal ions are not only environmental pollutants but also have significant impacts on human health, such as kidney damage, infertility and hypertension (Mehta et al., 2022). Therefore, accurate detection of metal ions is essential. To this end, a CRISPR/Cas12a-DNAzyme-based biosensor was developed (Fig. 1A) (Li et al., 2020d). For Pb^{2+}-dependent DNAzyme, it can catalyze the nucleic acid substrate with a DNA activator, releasing the DNA activator in the presence of Pb^{2+}. Subsequently, the CRISPR/Cas12a system was activated. Based on the characteristics of CRISPR/Cas12a *trans*-cleaved ssDNA, the ssDNA fluorescent reporters modified with fluorophore and quencher at each end were cleaved, producing fluorescence signals. The intensity of fluorescence signals was proportional to the concentration of Pb^{2+} (LOD = 0.053 nM). Conversely, the DNAzyme showed no activity in the absence of Pb^{2+}. No fluorescence signals were produced. Similarly, a Na^{+}-dependent DNAzyme was also coupled to CRISPR/Cas12a for Na^{+} detection (Fig. 1B) (Li et al. 2020c, 2021b; Xiong et al., 2020a). In this platform, the fluorescence reporter and signal collector can be adjusted to enhance the performance of biosensor. For the former, an upconversion luminescent nanoparticle (UCNP) was developed to overcome background signal interference. For the latter, an emerging holographic optical tweezers technology was used to improve detection stability and throughput. The optimized biosensor showed quite satisfactory sensitivity and excellent specificity for Na^{+} (LOD = 4.3 nM).

Although CRISPR/Cas-based biosensors have made progress in the detection of cations, the detection of anions is relatively limited. To meet this need, the potential of F^{-}-responsive riboswitch was fully explored. Thus, a competitive binding mechanism between the F^{-}-responsive riboswitch and RNA polymerase was coupled with CRISPR/Cas13a to detect F^{-} (Fig. 1C) (Iwasaki and Batey, 2020). In this study, the recognition sites of RNA polymerase were occupied by the F^{-}-riboswitch, which prevented it from catalyzing the transcription process. When F^{-} was present, it was bound to the riboswitch, releasing RNA polymerase through competitive binding and allosteric effects. As a result, the transcription process was performed to obtain an RNA activator corresponding to the CRISPR/Cas13a system. The activated Cas13a protein can cleave RNA fluorescence reporters, producing fluorescence signals. Regrettably, the biosensing platform only demonstrated a limited detection range (1–100 μM). This is far from meeting the regulations of environmental protection organizations on the content of F^{-} in the environment. To overcome this deficiency, an improved biosensing platform was developed (Fig. 1D) (Ma et al., 2021a). In this platform, the DNA template was composed of promoter, spacer sequence, F^{-}-riboswitch and Cas13a targeting sequence. The F^{-}-responsive riboswitch directly triggered the transcription process, producing complete RNA products. The complete RNA can activate the detection system, generating fluorescence signals. In contrast, the transcriptional process was terminated and no fluorescence signals were generated. Notably, the platform greatly improved the detection range for F^{-} (0–800 μM) and displayed a low detection limit (LOD = 1.7 μM). This work further develops the application value of the CRISPR/Cas toolboxes in the field of anion detection. Meanwhile, this also accelerates the application of *in vitro* synthetic biology in the field of biosensing.

2.2. Detection of small molecules

Small molecule compounds are widely found in nature. They have significant impacts on human health, the environment, and food safety. As a new generation of molecular diagnostic tools, the CRISPR/Cas toolbox has realized the precise detection of various small molecules. To this end, this review systematically summarizes the research progress of CRISPR/Cas-based detection of small molecules in recent years (Table 2).

2.2.1. Detection of ATP

Adenosine triphosphate (ATP) is considered one of the most important life fuels (Li et al., 2019a). It is involved in almost all biochemical reactions and cellular metabolism (Nie et al., 2020). To this end, an aptamer-regulated CRISPR/Cas biosensing system was developed for the diagnosis of ATP (Fig. 2A) (Chen et al., 2022; Li et al. 2020c, 2021b; Xiong et al., 2020b). In this system, the DNA activator bound aptamer so that the DNA activator cannot recognize crRNA in the absence of ATP. Therefore, the *trans*-cleavage activity of Cas12a cannot be initiated. Conversely, ATP can bind the aptamer. Moreover, the binding force is greater than the complementary interaction force between the aptamer and the activator. As such, the DNA activator was released to activate Cas12a to cleave fluorescence reporters, producing fluorescence signals. In addition, steric hindrance effects and sandwich structures (aptamer-ATP-aptamer) have also been used in response to the CRISPR/Cas12a system (Li et al., 2021c; Niu et al., 2021; Peng et al., 2020). Through the aptamer and CRISPR/Cas12a coupling system, the generated fluorescence signals enable quantify ATP. Importantly, the lowest detection limit of 0.14 pM can be achieved. Moreover, effective detection of other targets is also expected to be realized by changing the aptamer. To broaden the application of the method, an electrochemical and an electrochemiluminescence (ECL) biosensor were also developed based on similar detection elements for ATP (Fig. 2A) (Qing et al., 2021; Xu et al., 2021). Through the platform, the methylene blue labeled probes were captured by an electrode modified with reduced graphene oxide (LOD = 0.46 pM) and the ferrocene labeled probes were captured by the cathode of the bipolar electrode (LOD = 0.48 nM). The difference is that the electrochemical intensity is a positive correlation and the ECL

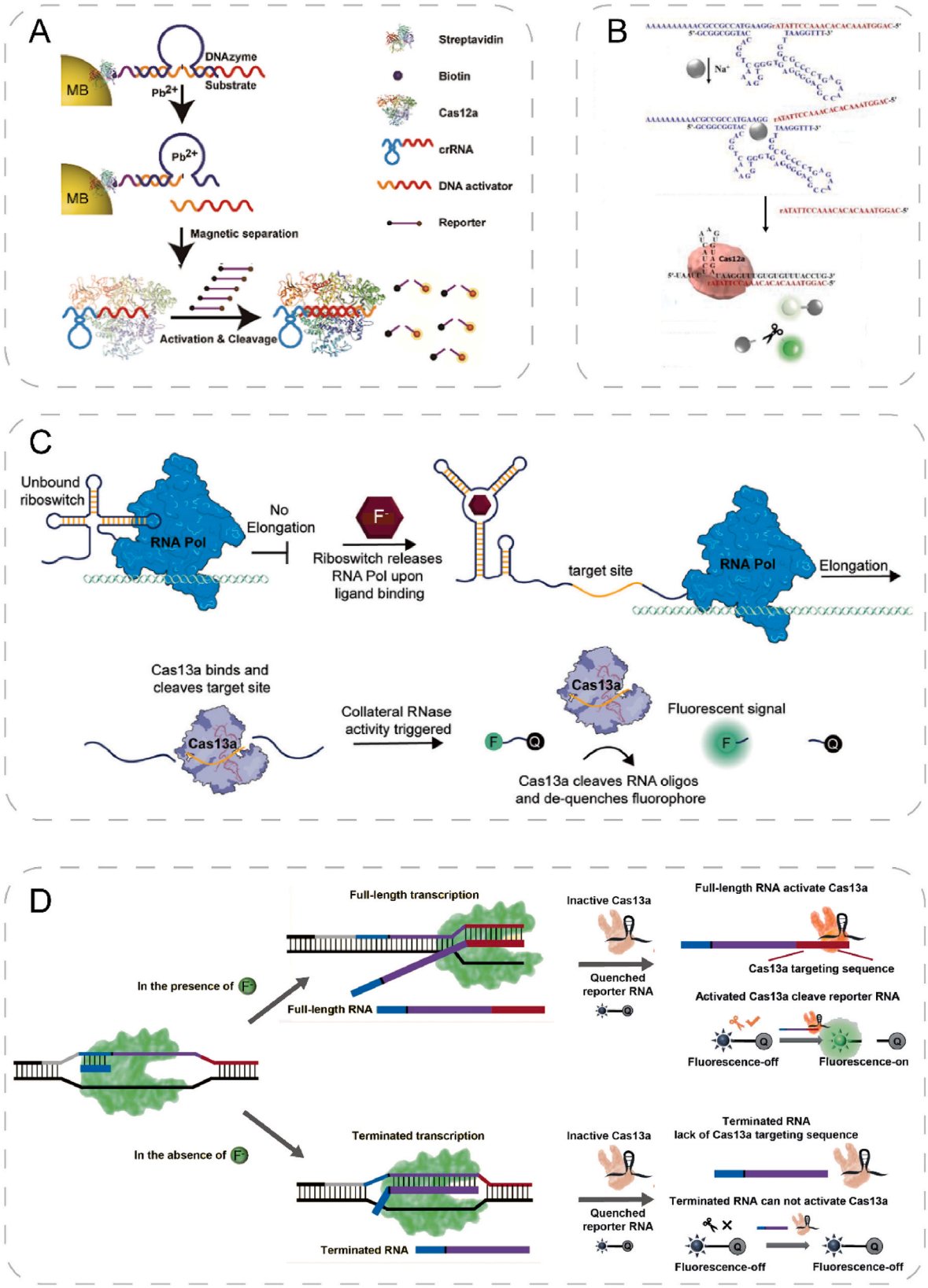


Fig. 1. Schematic illustration of the CRISPR/Cas biosensors to detect ions. (A) CRISPR/Cas12a biosensor regulated by Pb^{2+}-dependent DNAzyme to detect Pb^{2+}. (B) CRISPR/Cas12a biosensor regulated by Na^{+}-dependent DNAzyme to detect Na^{+}. (C) *In vitro* transcription-coupled CRISPR/Cas13a detection of F^{-} by fluorine ion riboswitch regulation. (D) Detection strategy for F^{-} via a fluriboswitch in tandem with CRISPR/Cas13a based on *in vitro* synthetic biology.

Table 2
Characteristics of various strategies for detecting small molecules.

Detection targets	Detection elements	Detection time	Detection linear ranges	Detection limits	Ref.
ATP	Aptamer, CRISPR/Cas12a	<15 min	0.78–25 μM	0.21 μM	Xiong et al. (2020b)
		80 min	0.1–2000 nM	87 pM	Li et al. (2021b)
		~15 min	30 nM-100 μM	18 nM	Li et al. (2020c)
		>135 min	2.5–800 μM	0.47 μM	Chen et al. (2022)
		>80 min	0–800 pM	0.14 pM	Li et al. (2021c)
		25 min	25–500 μM	104 nM	Niu et al. (2021)
		40 min	1–200 μM	400 nM	Peng et al. (2020)
	Aptamer, CRISPR/Cas12a, Rolling circle amplification, Reduced graphene oxide modified electrode	>110 min	10 pM-100 nM	0.46 pM	Qing et al. (2021)
	Aptamer, CRISPR/Cas12a, Bipolar electrode, Polydimethylsiloxane molds, $[Ru(bpy)_3]^{2+}$ emitter	130 min	1–500 nM	0.48 nM	Xu et al. (2021)
	ATP-dependent DNA ligase, CRISPR/Cas12a	>40 min	0.5–100 nM	50 pM	Zhao et al. (2021a)
NAD^+	NAD^+-dependent DNA ligase, CRISPR/Cas12a	>40 min	0.01–200 nM	10 pM	Zhao et al. (2021a)
Ampicillin	Aptamer, CRISPR/Cas14a, Metal isotope terbium, Plasma mass spectrometry	45 min	5–100 nM	2.06 nM	Hu et al. (2021b)
Kanamycin	Aptamer, CRISPR/Cas12a, Metal isotope thulium, Plasma mass spectrometry	30 min	8–120 pM	4.06 pM	Hu et al. (2021a)
Tobramycin	Aptamer-based allosteric probe, CRISPR/Cas12a, Klenow fragment DNA polymerase	75 min (dsDNA activators) 135 min (ssDNA activators)	10–300 pM 5–30 pM	3.719 pM 1.542 pM	Li et al. (2022a)
Tetracycline	Allosteric transcription factor, CRISPR/Cas12a, In vitro transcription	120 min	0–10 μM	2 μM	Mahas et al. (2022)
Uric acid	Allosteric transcription factor, CRISPR/Cas12a	35 min	25–500 nM	10 nM	Liang et al. (2019)
p-Hydroxybenzoic acid	Allosteric transcription factor, CRISPR/Cas12a	35 min	9–180 nM	1.8 nM	Liang et al. (2019)
Glucose	Glucose oxidase, Pistol-like DNAzyme, CRISPR/Cas12a	>90 min	0.1–10 μM	0.1 μM	Zhang et al. (2021a)
Melamine	Aptamer, CRISPR/Cas12a	20 min	0.1–25 μM	38 nM	Qiao et al. (2021)
Cocaine	Aptamer, CRISPR/Cas12a	>110 min	0.5–15 μM	0.34 μM	Zhao et al. (2021c)
	Aptamer, Terminal deoxynucleotidyl transferase, CRISPR/Cas12a, $[Fe(CN)_6]^{3-/4}$ modified gold electrode	>250 min	40–150 pM	15 pM	Abnous et al. (2022)
Deoxynivalenol	Aptamer, CRISPR/Cas12a, Gold nanoparticle-decorated $Ti_3C_2T_x$ MXene, ssDNA modified upconversion nanoparticles	>100 min	1–500 ng/mL	0.64 ng/mL	Lin et al. (2022)

intensity is a negative correlation with ATP levels.

Furthermore, some enzymatic reactions are also involved in the detection of small molecules. Recently, a biosensor driven by the NAD^+/ATP-dependent DNA ligase and CRISPR/Cas12a was developed (Fig. 2B) (Zhao et al., 2021a). In this system, a dsDNA with a gap cannot activate the CRISPR/Cas12a biosensing system. Therefore, the DNA ligation reaction mediated by NAD^+/ATP-dependent DNA ligase was used as a biotransduction element. When the DNA ligation reaction was performed, an intact dsDNA was obtained. The *trans*-cleavage activity of Cas12a was activated to generate the fluorescence signals. For this platform, NAD^+/ATP is the key to the DNA ligation reaction. Consequently, NAD^+ and ATP were successfully detected (LOD of NAD^+ = 10 pM; LOD of ATP = 50 pM). This indicates that small molecule-dependent enzymatic reactions will become important biotransduction elements for the future development of biological detection.

2.2.2. Detection of antibiotics

As one of the greatest discoveries of humans, antibiotics have been widely used for more than 80 years (Douafer et al., 2019). However, the abuse of antibiotics not only caused drug resistance but also caused the pollution of the water body and terrestrial environment. To this end, an antibiotic bioassay platform was designed based on ampicillin (AMP) aptamer, CRISPR/Cas14a and metal isotope-labeled probes (Fig. 2C) (Hu et al., 2021b). In this platform, the "lock-and-activate" detection system is the same as the aptamer-based mechanism described above. Uniquely, the ssDNA probes were modified with terbium (^{159}Tb) and biotin at each end. During this process, streptavidin-coated magnetic beads were used to capture uncleaved probes and biotin-modified moieties after cleaving. Ultimately, the remaining probes can be quantitatively detected by inductively coupled plasma mass spectrometry (ICPMS) due to metal isotope. As such, AMP was detected with high sensitivity and efficiency within 45 min (LOD = 2.06 nM). Similarly, the thulium(^{169}Tm)-tagged CRISPR-Cas12a bioassay platform was also established to quantitatively detect kanamycin within 30 min (LOD = 4.06 pM) (Fig. 2C) (Hu et al., 2021a). Although the above method achieved effective detection of antibiotics, both ^{159}Tb and ^{169}Tm are rare earth elements with very small reserves and are war preparation materials. This is not conducive to the large-scale promotion of this method.

Besides ampicillin and kanamycin, tobramycin is also an important antibiotic detection target. To ultrasensitively detect tobramycin, an aptamer-based allosteric probe was integrated into the CRISPR/Cas12a system (Li et al., 2022a). In the presence of tobramycin, it was recognized by the aptamer-based allosteric probe to release the primer template. Under the action of Klenow fragment DNA polymerase (Klenow), the primer template was amplified to form ssDNA or dsDNA activators.

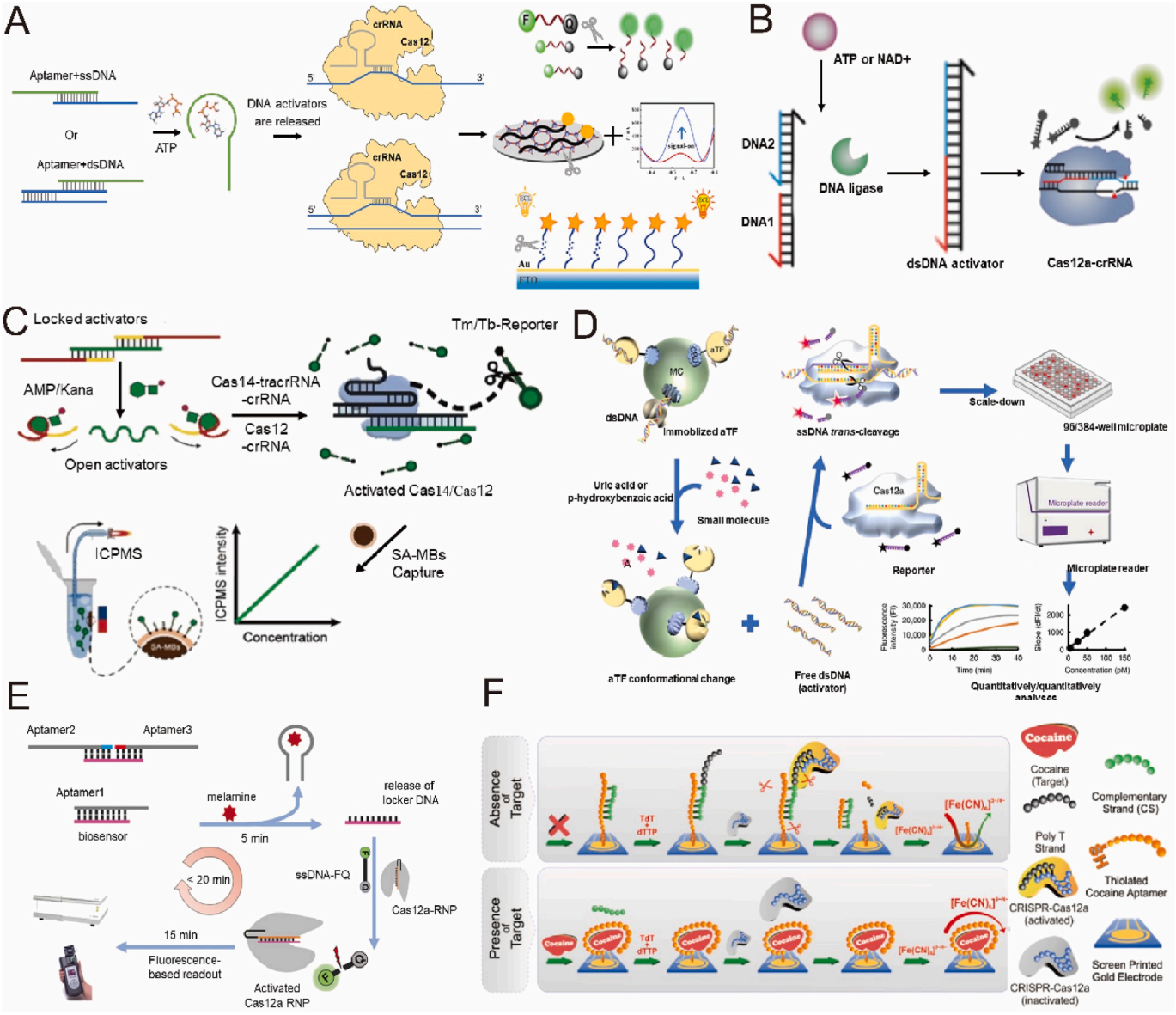


Fig. 2. Schematic diagram of detecting small molecules. (A) The principles of fluorescence/electrochemical/electrochemiluminescence biosensors based on aptamer and CRISPR/Cas12a for detecting ATP. (B) The biosensors based on ATP-dependent DNA ligase and CRISPR/Cas12a for detecting ATP. (C) The strategy of detecting ampicillin or kanamycin based on aptamers, CRISPR/Cas systems and terbium/thulium-labeled probes. (D) The CaT-SMelor platform for detection of uric acid and *p*-hydroxybenzoic acid. CBD represents the cellulose-binding domain; MC represents microcrystalline cellulose. (E) The strategy for detecting melamine using aptamers and the CRISPR/Cas12a system. (F) The electrochemical biosensor for detecting cocaine based on aptamer, terminal deoxynucleotidyl transferase (TdT), CRISPR/Cas12a and gold electrode.

As a result, the CRISPR/Cas12a system was triggered to produce fluorescence signals. Ultimately, the tobramycin was quantified by the intensity of the fluorescence signals (LOD = 3.719 pM). Conversely, this allosteric probe spontaneously formed an intramolecular hairpin and failed to activate the *trans*-cleavage activity of Cas12a. Although the biosensor of ssDNA activators exhibited a lower detection limit, the rapid accumulation of ssDNA resulted in rapid saturation of the signaling platform. This narrowed its detection range. Accordingly, the biosensor based on dsDNA activators is more advantageous.

Furthermore, allosteric transcription factor (aTF) was also introduced into CRISPR/Cas biosensing system to detect antibiotics. To do so, a biosensor was established based on CRISPR/Cas12a-aTF for detecting tetracycline (Mahas et al., 2022). In this biosensor, aTF was bound to the operator sequence, hindering the *in vitro* transcription process. In the presence of tetracycline, tetracycline was bounded to aTF, releasing of aTF from the operator sequence. Because of this, the *in vitro* transcription process was executed normally to obtain crRNA. Therefore, the CRISPR/Cas12a system was activated, generating fluorescence signals. Additionally, an adapted mobile phone program and a simple hand-held visualizer were used to further simplify the detection procedure. Notably, other aTF-based biosensors (ROSALIND) without CRISPR/Cas system have also been used to detect tetracycline (Jung et al., 2020). However, the comparison results show that the readout signal intensity is significantly enhanced after using the CRISPR/Cas12a system. Importantly, these antibiotic biosensing systems provide the strong guarantee for drug resistance and antibiotic pollution.

2.2.3. *Detection of metabolites*

Uric acid and *p*-hydroxybenzoic acid are important monitoring targets in both fermentation and physiological processes. To detect these targets quantitatively, a small molecule detection platform (CaT-SMelor) was developed by combining aTFs and the CRISPR-Cas12a system

(Fig. 2D) (Liang et al., 2019). For CaT-SMelor, the small molecule can bind to the corresponding aTF to release the dsDNA activators. Subsequently, the CRISPR/Cas12a system was activated to generate the fluorescence signals. Conversely, there are no detectable fluorescence signals in the absence of small molecule. Ultimately, this biosensor platform was successfully used to detect uric acid (LOD = 10 nM) and *p*-hydroxybenzoic acid (LOD = 1.8 nM) within 35 min. Similarly, the CRISPR/Cas13a-aTF/riboswitch coupled *in vitro* transcription platform (SPRINT) has also been constructed to detect cofactors and amino acid metabolites (Iwasaki and Batey, 2020). However, tedious multi-step operations and immobilization procedures are required for the above biosensors. This is inconvenient for point-of-care testing. Consequently, an integrated and portable detection kit will undoubtedly have more application value.

Glucose is also one of the most important compounds in life activities. The content of glucose is often used to diagnose various diseases, especially diabetes (Qu et al., 2016). Accordingly, a novel biosensor based on enzymatic reactions and the CRISPR/Cas12a system was developed for the first time to detect glucose levels (Zhang et al., 2021a). In the presence of glucose, glucose was catalyzed by glucose oxidase into gluconic acid and hydrogen peroxide (H_2O_2). Subsequently, the pistol-like DNAzyme was activated by H_2O_2 and spontaneously cleaved into part a, part b and part c. Among them, part b can trigger the CRISPR/Cas12a system to generate fluorescence signals. Finally, glucose levels were quantified from tears and saliva. Although the glucose concentration range (0.1–10 μM) detected is not very prominent, it provides a new idea for the non-invasive diagnosis of glucose, which has strong scalability and practicability.

2.2.4. Detection of melamine

Melamine is a triazine heterocyclic organic compound and is widely used in chemical fertilizer, leather, plastic and resin industries (Wang et al., 2020b). Angrily, melamine is also used by unscrupulous businessmen as a food additive to increase the protein content of foods (Daniel et al., 2017). Therefore, it is urgent to develop effective methods to detect melamine to ensure food safety. For this purpose, a biosensing platform based on aptamer and the CRISPR/Cas12a system was developed (Fig. 2E) (Qiao et al., 2021). In this platform, three combinations of locker DNA and aptamers were designed to form three "lock--and-activate" detection systems. Although only two were successful in the end, it did not affect the detection of melamine. With the same response mechanism as the previously described aptamer-CRISPR/Cas system, the melamine content was converted into fluorescence signals. Significantly, the detection platform showed a low detection limit (LOD = 38 nM), which was lower than the detection threshold of melamine in infant milk (1.0 mg/kg). Moreover, the detection process only takes 20 min and does not require complex sample processing.

2.2.5. Detection of poisons

Cocaine has become one of the most widely used illicit drugs in the world due to its strong stimulating effect on the central nervous system (Barreto et al., 2020). In this context, an aptamer coupled CRISPR/Cas12a biosensor was developed to detect cocaine (Zhao et al., 2021c). Consistent with the principle of other aptamer-CRISPR/Cas biosensing systems, the amount of cocaine was transformed into fluorescence signal intensity. However, the biosensor showed only moderate sensitivity to cocaine (LOD = 0.34 μM). Fortunately, a highly sensitive electrochemical biosensor was developed to detect cocaine very recently (Fig. 2F) (Abnous et al., 2022). In the electrochemical biosensor, cocaine aptamer and its complementary strand (CS) were modified on the screen-printed gold electrode surface. In the absence of cocaine, the CS was extended from the 3′-end by TdT to produce polymerized T sequences as activators. Subsequently, the *trans*-cleavage activity of Cas12a was activated. This resulted in non-specific cleavage of the existing ssDNA region, releasing the aptamer and CS from the electrode surface. Therefore, the oxidation marker $[Fe(CN)_6]^{3-/4-}$ can be easily moved to the electrode surface, producing electrochemical signals. On the contrary, the aptamer and CS were still retained on the electrode surface, hindering the generation of electrochemical signals. Importantly, the biosensor exhibited high sensitivity (LOD = 15 pM) and wide detection range (40 pM–150 nM). Additionally, another highly toxic substance deoxynivalenol was also detected through the coupling of aptamer-CRISPR/Cas12a with nanomaterials (Lin et al., 2022). In this platform, ssDNA modified UCNPs were used as luminescent labels and MXene (a two-dimensional metal carbide) modified with nano-gold was used as an enhanced quencher. Eventually, deoxynivalenol in the range of 1–500 ng/mL was successfully measured (LOD = 0.64 ng/mL). These biosensing devices are expected to greatly reduce the workload of safety supervision departments and improve their work efficiency.

2.3. Detection of polysaccharides

Lipopolysaccharide (LPS) is a component of the outer membrane of the cell wall of gram-negative bacteria (Posha et al., 2021). LPS can cause fever, diarrhea, shock, organ failure and even death (Niu et al., 2019). To do so, a biosensor was developed to detect LPS based on aptamer, DNAzyme and the CRISPR/Cas12a system (Wang et al., 2021b). During this process, the LPS aptamer (LPSA) first formed an LPSA/ASCII complex with the ASCII chain of DNAzyme. In the presence of LPS, it competitively bound LPSA leading to the release of ASII. The released ASII formed a partial duplex containing substrate chain with ASI through base complementary pairing and hydrazone reaction. Subsequently, DNAzyme hydrolyzed the substrate, producing DNA activators. The CRISPR/Cas12a system was activated to produce fluorescence signals. This hydrazone ligand-assisted biosensor provided a new model for LPS signal output (LOD = 7.31 fg/mL). However, conventional fluorescence reporters have relatively high background signals. To overcome the disadvantage, MXene was coupled with LPSA and CRISPR/Cas12a to detect LPS (Sheng et al., 2021). Due to its hydroxy-terminated surface and complete metal atomic layer, MXene can adsorb ssDNA via hydrogen bonds. MXene can also quench fluorescence. Because of the excellent quenching constant (353.59 g/L), this allows MXene to significantly reduce the background signals and improve the detection accuracy (LOD = 11 pg/mL).

2.4. Detection of peptides/proteins/enzymes

2.4.1. Detection of peptides

Microcystin-LR (MC-LR) is a cyclic peptide hepatotoxin that harms human health and the ecosystem (Zhang et al., 2021b). Considering its toxicity, a novel biosensor was established based on aptamer-CRISPR/Cas14a and 2D-PMOF (2D porphyrin metal-organic framework nanosheet) to detect MC-LR (LOD = 19 pg/mL) (Fig. 3A) (Wu et al., 2022). 2D-PMOF is considered another ideal fluorescence quencher to overcome high background signals besides MXene (Chen et al., 2021a). Using competitive binding between MC-LR and activator on aptamer, the unbound activator was used to trigger the *trans*--cleavage activity of Cas14a, cleaving fluorescence reporters on 2D-PMOF. Since ssDNA fluorescence reporters were pre-adsorbed on the 2D-PMOF surface, they were cleaved and then left 2D-PMOF, producing fluorescence signals. Significantly, the ssDNA fluorescence reporters can be strongly adsorbed on 2D-pMOF via a conjugated π-electron system, leading to fluorescence quenching without chemical reaction. As such, the magnificent performances of 2D-pMOF ensure the feasibility and practicability of the scheme for detecting MC-LR, including low fluorescence background, high signal output, excellent ssDNA absorption capacity, high fluorescence quenching efficiency and extremely short quenching time.

2.4.2. Detection of biomarker proteins

The traditional ELISA method is far from satisfying the requirement of detecting biomarker proteins, especially the ultra-low concentration

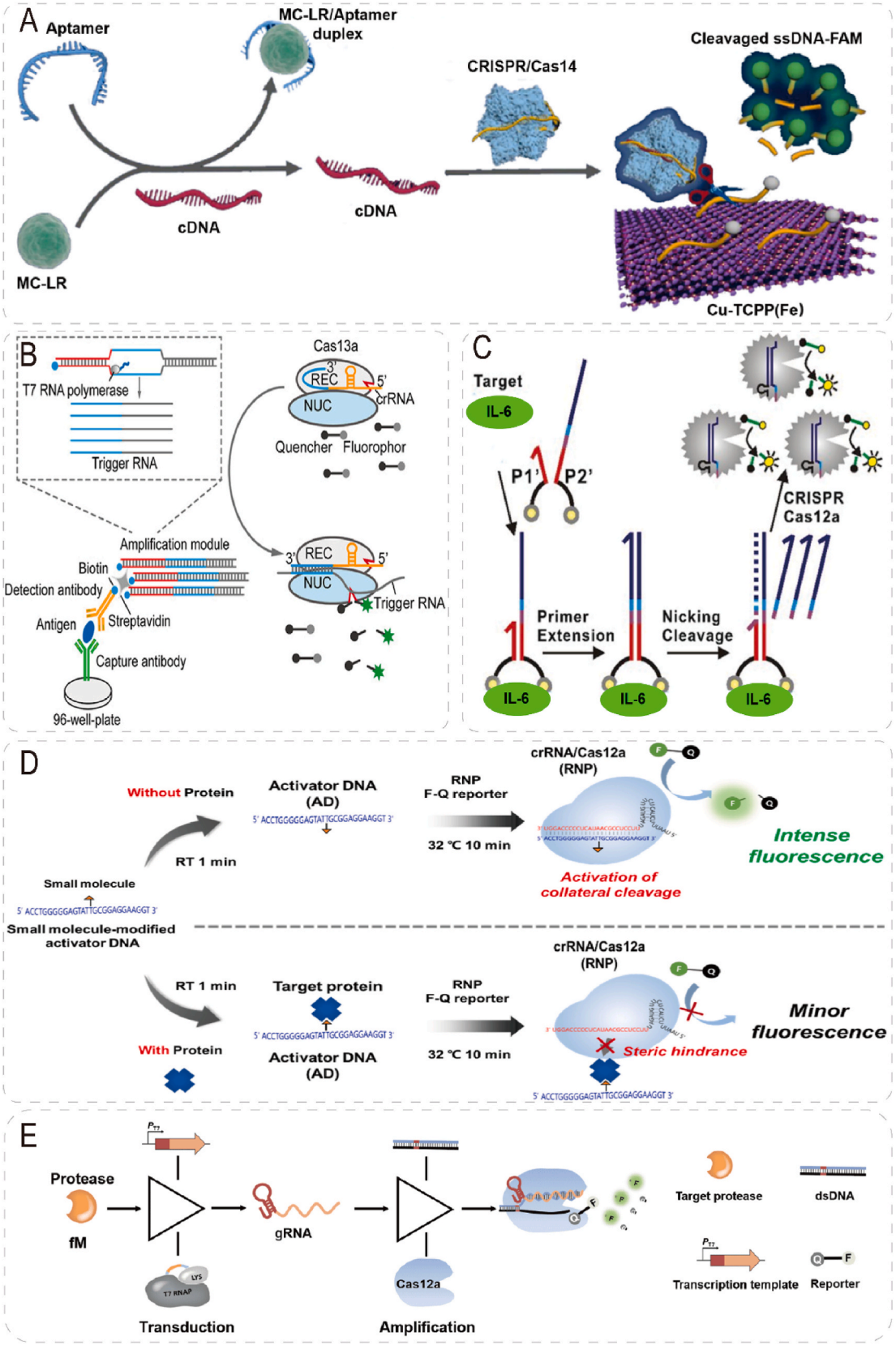


Fig. 3. (A) Schematic diagram of detection of MC-LR based on aptamer, 2D-PMOF (Cu-TCPP(Fe)) and the CRISPR/Cas14a system. (B) Strategy to detect interleukin-6 based on aptamer, *in vitro* transcription, antigen-antibody interaction and the CRISPR/Cas13a system. (C) Strategy to detect interleukin-6 based on isothermal proximity, antigen-antibody interaction and the CRISPR/Cas12a system. (D) Strategy to detect streptavidin and antidigoxigenin based on steric hindrance effect and the CRISPR/Cas12a system. (E) Schematic illustration of protease detection by *in vitro* transcription and the CRISPR/Cas12a system.

for early clinical disease diagnosis. To this end, various biosensor platforms were developed to improve the sensitivity and detection range of proteins (Table 3). Particularly, different aptamers were coupled with CRISPR/Cas systems to detect proteins (Wang et al., 2021a; Zhao et al. 2020a, 2020b, 2021c). In this method, the "lock-and-activate" detection system was still employed. The signals of proteins were transformed into fluorescence signals. To facilitate the separation of DNA activators and inactive parts with magnets, the complementary ssDNA was usually modified on magnetic beads through streptavidin-biotin interaction. In addition, an enzyme-free entropy driven catalysis (EDC) was used for intermediate signal amplification to enhance sensitivity. At the same time, double cycle amplification (DCA) based on replication and transcription was used to directly synthesize crRNA, which can increase the applicability and reduce the storage cost of biosensing systems. Notably, the essence of detecting extracellular vesicles (EVs) and exosomes is to detect the membrane-specific protein CD63. Therefore, aptamer coupled CRISPR/Cas biosensing platforms were successfully used to detect alpha-fetoprotein (LOD = 0.07 fM), thrombin (LOD = 0.4 nM), exosome (LOD = 3×10^3 particles/μL) and EV (LOD = 10^2 particles/μL). In short, this platform provides a new method for detecting proteins in disease diagnosis with the advantages of convenient operation and high sensitivity.

Moreover, various aptamer-CRISPR/Cas systems were also integrated into the antigen-antibody interaction process for the detection of biomarker proteins (Li et al., 2021c; Zhao et al., 2021b). In this biosensor, a new sandwich structure (antibody/aptamer-protein-aptamer) was formed on the plates/magnetic beads. The target protein was captured by the capture probe (antibody/aptamer). Then, the detection probe (aptamer-CRISPR/Cas12a) was added to identify the protein, generating fluorescence signals. Similarly, an antibody-protein-antibody sandwich structure was used to detect target proteins (Lee et al., 2021). To enhance detection sensitivity, a biotinylated DNA barcode activator with multiple CRISPR/Cas12a recognition sites was conjugated to the detection antibody. Therefore, the amount of protein is consistent with the fluorescence signal intensity. Through the biosensor, various proteins were detected, including carcinoembryonic antigen (LOD = 69.5 aM), prostate-specific antigen (LOD = 175 aM), platelet-derived growth factor-BB (LOD = 550 aM), and chemokine ligand 9 protein (LOD = 14 pg/mL) (Table 3). The ultra-sensitive detection platform can quantitatively analyze atto-molar level of proteins. Its sensitivity and detection range greatly exceed commercial ELISA kits. More importantly, the platform is direct quantification of proteins by catalytic and recognition elements, rather than the previously reported quantification by indirect transduction. This can significantly improve the work efficiency of the detection process.

Besides fluorescence biosensors, an electrochemical biosensor (E-CRISPR) was also established based on aptamer-CRISPR/Cas12a to detect transforming growth factor β1 (TGF-β1) protein (Table 3) (Dai et al., 2019). The difference is that the nucleic acid aptamer itself is the DNA activator instead of releasing it through allosteric effects. Moreover, the ssDNA reporters were modified with a methylene blue label and thiol at each end. As a result, the TGF-β1 protein was diagnosed quantitatively with electrochemical signals (LOD = 0.2 nM). Similarly, an electrochemical biosensor was also developed to detect human hepatoma cells HepG2 (LOD = 20 cells) (Table 3) (Li et al., 2021c). Of note, the essence of detecting HepG2 cells is to detect nucleolin proteins on the HepG2 cells. As such, this also provides a new idea for the study of protein-cell interactions.

Moreover, *in vitro* transcription was also integrated into CRISPR/Cas coupled immunoassay system to detect proteins (Fig. 3B) (Chen et al., 2020a). In this study, a sandwich complex was also constructed with a capture antibody-protein-detection antibody. After the target protein responds, the sandwich structure was formed and *in vitro* transcription was performed, obtaining an RNA activator. Subsequently, the *trans*--cleavage activity of Cas13a was triggered to produce fluorescence signals. Through this biosensor, human interleukin-6 (inflammatory factor, LOD = 2.29 fM) and human vascular endothelial growth factor (tumor marker, LOD = 0.81 fM) were detected, respectively. Furthermore, this method realizes the highly sensitive detection of low abundance proteins.

For the detection of interleukin-6, a biosensor was also built based on the isothermal proximity CRISPR/Cas12a assay (iPCCA) coupled antigen-antibody immunoadsorption (Fig. 3C) (Li et al., 2021g). In this platform, two polyclonal antibodies modified primers P1 and P2 were designed with a 6-nt complementary sequence and a 15-nt thymine spacing to ensure correct extension. The two modified primers can be bound to the same antigen interleukin-6. After the affinity interaction brought the two modified primers into proximity, a dsDNA activator was generated through the binding-induced primer extension reaction and nicking cleavage. Subsequently, the CRISPR/Cas12a system was triggered to generate fluorescence signals (LOD = 100 fM). Importantly, these platforms demonstrated greater sensitivity and a wider detection range than the traditional ELISA method. Furthermore, this also provides a wash-free way to detect proteins.

In addition to the typical antibody-analyte-antibody sandwich structure, a steric hindrance effect has also become an important biotransduction element for protein detection through antigen-antibody interaction. In the case of streptavidin-biotin, the steric hindrance

Table 3
Characteristics of various strategies for detecting proteins.

Detection targets	Detection elements	Detection time	Detection linear ranges	Detection limits	Ref.
α-fetoprotein	Aptamer, CRISPR/Cas12a	<20 min	0.24–977 fM	0.07 fM	Zhao et al. (2021c)
Thrombin	Aptamer, EDC reaction, CRISPR/Cas12a	>120 min	1–80 nM	0.4 nM	Wang et al. (2021a)
CD63 protein (Exosome)	Aptamer, CRISPR/Cas12a	120 min	3×10^3–6×10^7 particles/μL	3×10^3 particles/μL	Zhao et al. (2020b)
CD63 protein (EV)	Aptamer, DCA, CRISPR/Cas12a	90 min	10^2-10^6 particles/μL	10^2 particles/μL	Zhao et al. (2020a)
Carcinoembryonic antigen	Antibody-target-aptamer complex, CRISPR/Cas12a, AuNPs	>300 min	0.6–120 ng/mL	13.9 fg/mL	Zhao et al. (2021b)
Prostate-specific antigen	Antibody-target-aptamer complex, CRISPR/Cas12a, AuNPs	>300 min	0.5–150 ng/mL	5.6 fg/mL	Zhao et al. (2021b)
Platelet-derived growth factor-BB	Aptamer-target-aptamer complex, CRISPR/Cas12a	>110 min	1-400 fM	550 fM	Li et al. (2021c)
Transforming growth factor β1	Aptamer, CRISPR/Cas12a, Gold electrode	>90 min	1–50 nM	0.2 nM	Dai et al. (2019)
Nucleolin (HepG2 cells)	Aptamer, CRISPR/Cas12a, Gold electrode	80 min	20-10000 cells	20 cells	Li et al. (2021c)
Human vascular endothelial growth factor	Antibody-target-antibody complex, In *vitro* transcription, CRISPR/Cas13a	>16 h	160 fg/mL-0.1 ng/mL	32.27 fg/mL	Chen et al. (2020a)
Chemokine ligand 9 protein	Antibody-target-antibody complex, CRISPR/Cas12a, DNA barcode	1 h	32–800 pg/mL	14 pg/mL	Lee et al. (2021)

effect coupled CRISPR/Cas12a system was developed to detect streptavidin (Fig. 3D) (Kim et al., 2021). In this strategy, the ssDNA activator was modified with biotin. The streptavidin can bind biotin on the DNA activator, forming the steric hindrance effect. This prevented the DNA activator from being recognized by the CRISPR/Cas12a system. Accordingly, the activity of Cas12a protein was inhibited, resulting in fewer fluorescence signals. Conversely, more fluorescent signals were generated and quantified with streptavidin (LOD = 0.03 nM). Although the platform only showed the detection sensitivity of nano-molar level, it had great advantages in extremely short detection time (11 min). If the platform ignores the limited types between proteins and ligands, it has strong popularization. To further improve the detection sensitivity, hybridization chain reaction (HCR) or polymerase chain reaction (PCR) can be coupled to the biosensor (Li et al., 2021d; Xing et al., 2020). The HCR and PCR can amplify the intensity of nucleic acid signals, increasing the sensitivity of detection. Ultimately, diverse biomarker proteins were detected, including thioester-containing protein CD109 (LOD = 10^2 particles/mL), epidermal growth factor receptor (EGFR) (LOD = 10^2 particles/mL) and programmed death ligand 1 (PD-L1) (LOD = 10^2 particles/μL). Although it increases the complexity of the operation, it is a good choice for the detection of some ultra-low proteins.

2.4.3. Detection of interferon gamma

The cytokine interferon gamma (IFN-γ) is an important protein analyte in the immune system and various pathological activities (Januarie et al., 2022). Recently, a CRISPR/Cas12a-assisted fiber immunosensor (CAFI) was developed to detect IFN-γ (Deng et al., 2022). The glass fiber was modified by polyethylene glycol to improve the antifouling performance. The biotin-modified antibody was immobilized on the sensing interface of glass fiber by streptavidin. After the sensing interface was in contact with IFN-γ, the aptamer was added to form the antibody–IFN–γ-aptamer ternary complex, releasing the ssDNA activator from the aptamer. Finally, the CAFI system developed was used for in situ detection of IFN-γ in complex biological samples (LOD = 58.8 aM). Its detection limit is not only as low as atto-molar level but also its detection sensitivity is 1000-fold that of the traditional ELISA. Most importantly, it only requires a low detection sample of 100 μL volume, which makes it a practical tool for determining protein in real samples. Despite the advantages of the new technology, it would undoubtedly be more practical to optimize existing and mature commercial kits. As such, a fluorescence signal amplifier was incorporated into the commercial ELISA kit to detect IFN-γ (Li et al., 2021e). In the final step of the commercial ELISA kit assay, the anti-horseradish peroxidase (HRP) antibody-ssDNA complex was added in place of the traditional colorimetric substrate tetramethylbenzidine. Then, the complex was conjugated to the formed antibody–IFN–γ-antibody with an HRP label sandwich structure. Finally, the CRISPR/Cas12a system was activated to produce fluorescence signals. Compared with commercial ELISA kits, the detection range of the system was increased by two orders of magnitude and the detection limit was reduced by 260-fold (LOD = 1.2 pg/mL). Moreover, the method does not need to change the original reagent and operation scheme of the commercial ELISA kit. However, the detection limit and range of the above schemes are still limited. To this end, an ssDNA-anti–IFN–γ antibody hybrid conjugate was directly utilized to improve the performance of this protocol by the same team (Li et al., 2022b). By the similar principle, IFN-γ was detected by the CRISPR/Cas12a system coupled to the conjugate as a primary or secondary antibody. Ultimately, the sensitivity of the platform was reduced to 1 fg/mL (50 aM) and the detection range was extended to six orders of magnitude. Regrettably, the protocol still needs to be improved, especially the reaction time of 1 h and the additional elution step.

2.4.4. Detection of proteases

Proteases play crucial roles in various pathological and physiological processes (Bond, 2019). Inspired by synthetic biology regulatory sensing systems, a protease-activated RNA polymerase coupled CRISPR/Cas biosensor was developed for the detection of proteases using the inhibitory effect of T7 lysozyme (T7 LYS) on T7 RNA polymerase (T7 RNAP) (Fig. 3E) (Yang et al., 2021). In this strategy, T7 LYS and T7 RNAP were linked by a short peptide, which is a substrate of protease. The T7 RNAP was inactivated due to the proximity effect of the inhibitor. In the presence of a protease, the linked short peptide was hydrolyzed by the protease. Then, the T7 RNAP was activated. Therefore, RNA sequences were obtained through the T7 RNAP-mediated *in vitro* transcription process. The obtained RNA sequences were transformed into dsDNA activators by amplification *in vitro*. As a result, the CRISPR/Cas12a system was activated, producing the fluorescence signal output. Finally, the biosensor was used to detect matrix metalloproteinase-2 (MMP-2) (LOD = 5.4 fM) and thrombin (LOD = 47.8 fM). Moreover, the biosensor platform showed 5–6 orders of magnitude higher sensitivity than the standard peptide-based method.

2.4.5. Detection of enzyme activities

The activities of enzymes can accurately indicate the state of pathology and pharmacological action (Sinharay et al., 2018). As the functional biomarkers to measure diseases, enzyme activities-based diagnostics can achieve specific and sensitive disease detection (Soleimany and Bhatia, 2020). To do so, it is nececssary to develop biosensing systems to detect various enzyme activities. As we all know, 5′-phosphate and 3′-hydroxyl are the guarantee for the formation of phosphodiester bonds during the process of nucleic acid strand amplification (Srivastava et al., 2021). If the nucleic acid chain extension rule can be connected with enzymatic reactions, it will undoubtedly open up a new scheme for the detection of enzyme activities. Fortunately, TdT can use a DNA fragment with 3′-OH end to synthesize polymerized T sequence. The polymerized T sequence can activate the CRISPR/Cas12a biosensing system. As such, different enzymes were used to obtain a DNA fragment with 3′-OH end, which is crucial to detecting enzyme activities.

For example, (1) Uracil-DNA glycosylase (UDG) was used to specifically repair uracil damage, forming an apyrimidinic (AP) site (Fig. 4A) (Du et al., 2021). Subsequently, the AP site was cleaved by endonuclease IV (Endo.IV) to release a DNA fragment with 3′-OH end. (2) Nucleic acid primer with the AP site was cut by endonuclease (APE1) into a DNA fragment with 3′-OH end (Fig. 4B) (Chen et al., 2021b). (3) The DNA fragment with 3′-phosphate group was converted into a DNA fragment with 3′-OH end by T4 polynucleotide kinase (PNK) (Fig. 4B) (Chen et al., 2021b; Zhang et al., 2021c). (4) The hairpin substrate with 5′-GATC-3′ palindrome was methylated by DNA adenine methylation methyltransferase (Dam MTase) to 5′-G-mA-TC-3′ in the presence of methyl donor S-adenosine-L-methionine (Fig. 4C) (Chen et al., 2020b). The methylated product obtained was cleaved by restriction enzyme *Dpn*I into a DNA fragment with 3′-OH end. Subsequently, the TdT-CRISPR/Cas12a biosensing system was coupled to convert various enzyme activities into fluorescence signals.

In addition, telomerase catalyzed reaction was also utilized to extend DNA strands with repeated TTAGGG sequences. Accordingly, the DNA fragment with 3′-OH end or PAM-less conditional DNA (pcDNA) can be directly transformed by telomerase into dsDNA with PAM or PAM-less DNA (pDNA), respectively (Fig. 4D) (Chen et al. 2021b, 2022). For the detection of PNK, Klenow and DNA ligase were used to obtain complete dsDNA activators from DNA strands catalyzed by PNK (Wang et al., 2020a; Zhao et al., 2021a). The subsequent detection process is the same as the above. As such, coupling other enzymes with the CRISPR/Cas12a system can convert enzyme activities into detectable fluorescence signals. Lastly, different enzyme activities were detected, including T4 polynucleotide kinase (LOD = 3.3×10^{-6} U/mL), telomerase (LOD = 8 cells/mL), apurinic/apyrimidinic endonuclease APE1 (LOD = 2.52×10^{-4} U/mL), DNA adenine A methyltransferase (LOD = 1.26×10^{-3} U/mL) and uracil-DNA glycosylase (LOD = 5×10^{-6} U/mL). Based on fluorescence strategy, these platforms realize simple, visual and

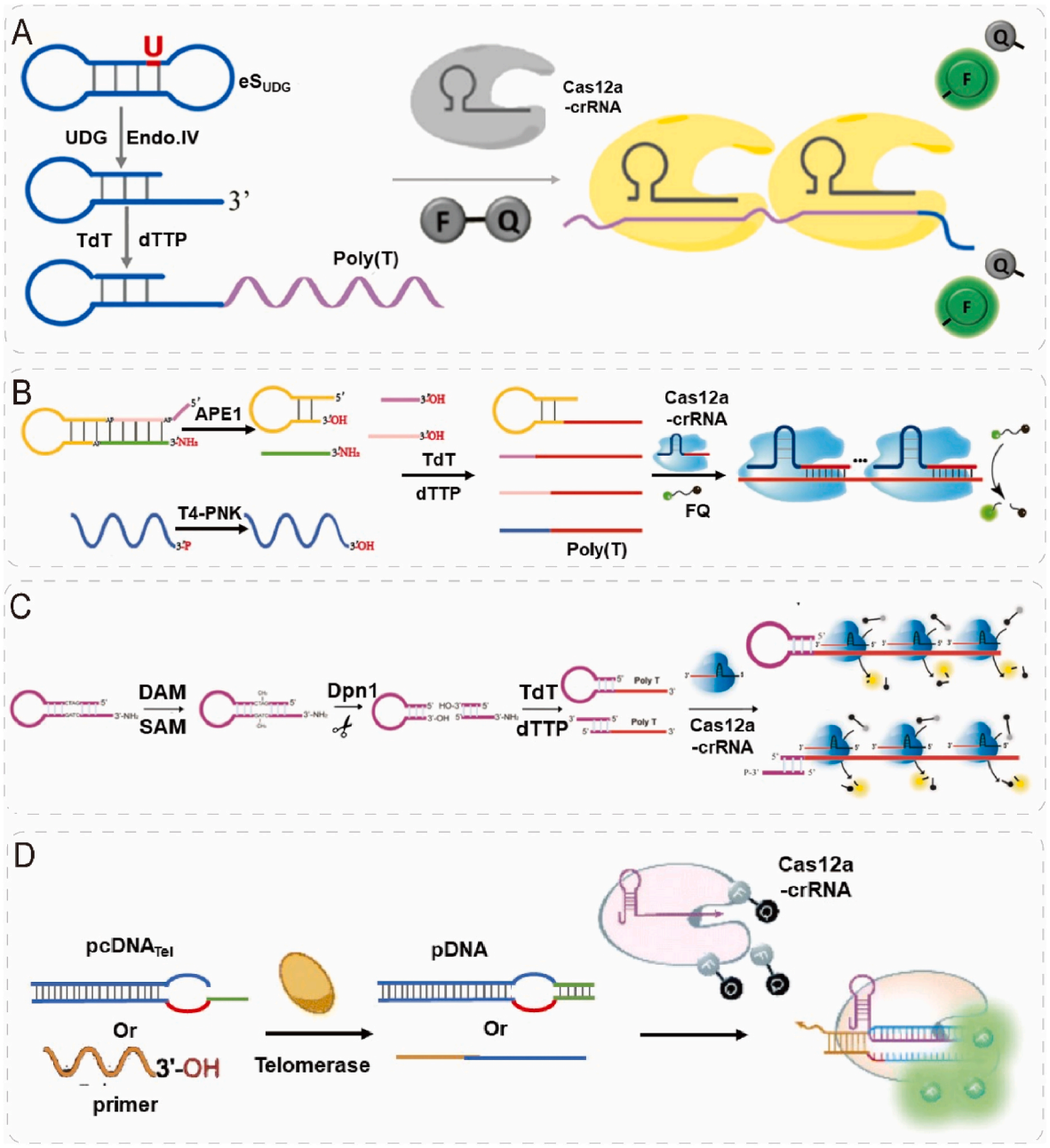


Fig. 4. Schematic diagram of detecting enzyme activities. (A) Detection activity of uracil-DNA glycosylase (UDG) based on terminal deoxynucleotidyl transferase (TdT) and the CRISPR/Cas12a system. (B) The strategy of endonuclease (APE1) or polynucleotide kinase (PNK) activity detection based on TdT and the CRISPR/Cas12a system. (C) The strategy of DNA adenine methylation methyltransferase (Dam MTase) activity detection based on TdT and the CRISPR/Cas12a system. (D) The strategy of telomerase activity detection based on the CRISPR/Cas12a system.

ultra-sensitive detection of various enzyme activities. If the detection range can be further expanded, it will have the potential to be suitable for the construction of a high-throughput screening platform for enzymes in synthetic biology.

2.5. Detection of cells

Pathogenic microbial cells and tumor cells are the main hazards to the global public health system (Lian et al., 2022). However, it remains a huge challenge for the accurate detection of these pathological cells. To do so, an allosteric probe-initiated detection system was developed based on aptamer, nucleic acid amplification and the CRISPR/Cas13a system (Fig. 5A) (Shen et al., 2020). Normally, the allosteric probe formed an inactive hairpin structure. In the presence of target cells, the inactive allosteric probe was activated through the allosteric effect of the aptamer. Under the action of external primer, DNA polymerase and T7 RNA polymerase, an ssRNA activator was generated. Subsequently, the CRISPR/Cas13a biosensing system was activated to generate fluorescence signals. Finally, the *Salmonella* Enteritidis cells were quantitatively detected (LOD = 1 CFU/mL). Additionally, an antibody-coupled CRISPR/Cas12a biosensor was developed to detect *Cryptosporidium parvum* cells (Fig. 5B) (Li et al., 2021f). In this system, the *C. parvum* cells were captured by antibodies immobilized on 96-well plates. Then, an anti-*Cryptosporidium* antibody-ssDNA conjugate (Abs-ssDNA) was prepared and used to recognize the cells captured. As a result, an antibody-cells-Abs-ssDNA sandwich structure was formed. Thus, the CRISPR/Cas12a biosensing system was triggered to generate fluorescence signals. The linear range of 6.25–1600 cells/mL was implemented

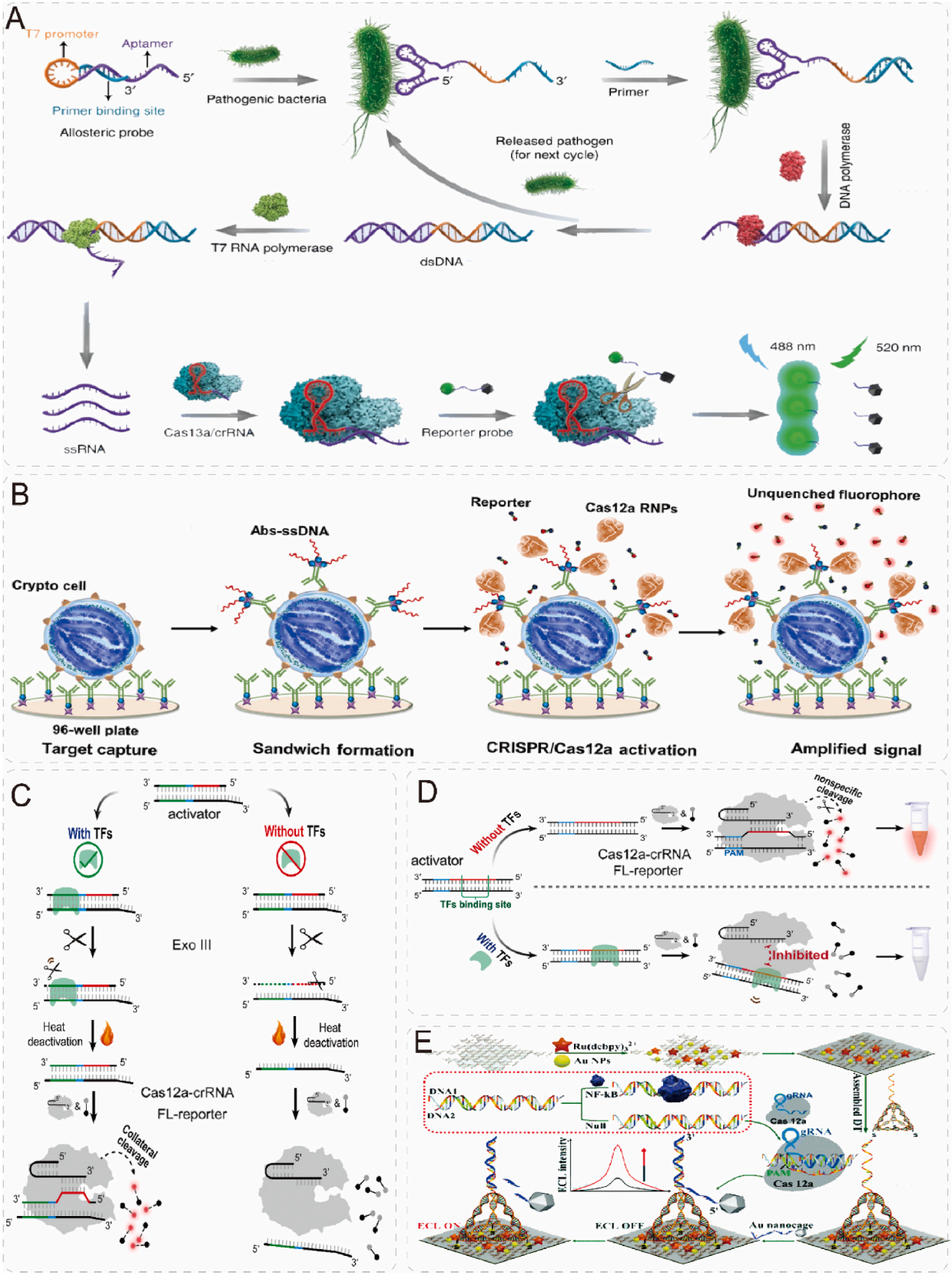


Fig. 5. Schematic diagram of the detection of cells and transcription factors. (A) The detection strategy for pathogenic microbial cells based on an allosteric aptamer probe, *in vitro* transcription and the CRISPR/Cas13a system. (B) The strategy to detect *Cryptosporidium parvum* cells through antigen-antibody interactions and the CRISPR/Cas12a system. (C) The biosensing mechanism to detect transcription factors based on nuclease protection and the CRISPR/Cas12a system. (D) The biosensing mechanism to detect transcription factors based on the steric hindrance effect and the CRISPR/Cas12a system. (E) The electrochemiluminescent biosensor for the detection of transcription factors based on tetrahedral DNA scaffolds and the CRISPR/Cas12a system.

to detect *C. parvum* cells (LOD = 1 oocyst/mL). Moreover, the system can reveal how cells regulate various physiological functions and stress responses during biological processes.

2.6. Detection of transcription factors

In addition to being a biotransduction element, transcription factor (TF) is also closely related to many physiological functions (Li et al., 2019b). Therefore, a series of TF sensors were developed (Li et al., 2020b). However, these sensors are not widely used due to their limited selectivity, low sensitivity and complex operating conditions. To this end, a nuclease protection assay coupled with CRISPR/Cas12a was first integrated for the detection of nuclear factor-kappa B (NF-κB) p50, which is a common TF in mammals (Fig. 5C) (Li et al., 2021a). Specifically, a dsDNA activator was connected to the binding domain of NF-kB p50. The binding domain was recognized by NF-κB p50 to form a stable TF-DNA complex. This complex used the steric hindrance effect to protect the dsDNA activator from degradation by exonuclease. Afterward, the TF and exonuclease were dissolved by thermal inactivation. As a result, the CRISPR/Cas12a system was triggered to generate fluorescence signals (LOD = 0.2 pM). This method was capable of detecting NF-κB p50 in the range from 0.5 pM to 1.6 nM. Furthermore, this method can be further extended to the screening of TF inhibitors, which is helpful to the research of TF related drugs. Meanwhile, a similar biosensor was also developed to detect NF-κB p50 and other TFs (Fig. 5D) (Li et al., 2020a). Uniquely, no exonuclease was required. Consequently, the steric hindrance effect of TF was only used to inhibit the activity of Cas12a but not that of exonuclease. Likewise, the TF signals were converted into fluorescence signals. Finally, this biosensor was successfully used to detect NF-κB p50 (LOD = 2.7 pM). Through the modular redesign of activators, five TFs were also detected, including p53 (LOD = 3.6 pM), c-Myc (LOD = 2.3 pM), MITF (LOD = 4.2 pM), AP-1 (LOD = 4.5 pM) and Nanog (LOD = 6.0 pM). Significantly, the detection time, sample volume and detection cost of this biosensor are only 1/9, 1/10 and 1/14 of that of commercial ELISA kits, respectively. Considering the recoverability and reproducibility of reagents, an ECL biosensor was also developed to detect NF-κB p50 using the similar principle as above (Fig. 5E) (Fan et al., 2021). In this biosensor, a complex was used as the emitter of ECL through the combination of Ru $(bpy)_3^{2+}$ modified graphene oxide and Au nanocage (quencher). This emitter not only showed high emitting efficiency but also was very stable in solution. The emitter of ECL was immobilized on DNA tetrahedral scaffolds (DTs), which can improve the hybridization efficiency of nucleic acid sequences. Accordingly, the ECL signals can be used to measure the amount of NF-κB p50. Meaningfully, this method reduced the detection limit of TFs to femto-molar level (LOD = 10.9 fM) for the first time. Moreover, this facilitates the study of the mechanism of other elements interacting with nucleic acids.

3. Challenges, perspectives and conclusions

The emergence of the CRISPR/Cas systems has not only revolutionized gene-editing technology but is also reshaping analysis and detection (Ma et al., 2021b, 2022; Yin et al., 2021; Zhuang et al., 2022). The CRISPR/Cas systems have rapidly evolved and been repurposed into powerful molecular diagnostic tools. Moreover, CRISPR/Cas-based diagnostics has been rated as one of the top seven technologies to focus on in 2022 by Nature journal (Eisenstein, 2022). Through specific target recognition and *trans*-cleavage activity, CRISPR/Cas-based biosensors showed better accuracy, adaptability, portability, timeliness and effectiveness than traditional methods. Therefore, the CRISPR/Cas biosensing systems are poised to meet diverse requirements and realize fit-for-purpose detection in various fields such as disease diagnosis, food safety inspection and environmental analysis.

The CRISPR/Cas-based biosensing utilizes the guided and programmable enzymatic activities of Cas effectors for sensitive, specific, and rapid nucleic acid detection, providing researchers with a powerful new biomolecular sensing tool for diagnosing diseases and understanding cellular events (Dai et al., 2020; Soleimany and Bhatia, 2020). The CRISPR/Cas-based biosensing is truly promising but not impeccable. It is mainly limited to the detection of nucleic acid targets. For non-nucleic acid targets, the development of these systems is still in its infancy. In principle, non-nucleic acid targets must be converted into recognizable nucleic acid signals by biotransduction elements that can be taken up by CRISPR/Cas. These biotransduction elements mainly include aptamers, DNAzymes, riboswitches, enzymatic reactions, transcription factors, antigen-antibody interactions, allosteric probes, *in vitro* transcription processes, steric hindrance effectors, etc. Subsequently, the obtained nucleic acid singles are used to activate CRISPR/Cas systems. In the activated systems, the Cas12a/Cas13a/Cas14a proteins can cleave the labeled reporters, producing detectable output signals.

Nevertheless, the CRISPR/Cas biosensing systems still have a few pending shortcomings for non-nucleic acid target detection. (1) It is technically difficult to detect multiple non-nucleic acid targets simultaneously. Simultaneous detection of multiple non-nucleic acid targets is ideal and often imperative for decision making for a multi-factors deterministic system. Multiplexed CRISPR/Cas biosensing can be pushed through different approaches such as assorted labeling (nucleic acids, enzymes, or nano-materials), spatial or temporal separation and local separation such as sensor arrays, chips, channels, micro-droplets, etc (Bruch et al., 2019). And indeed there is just little work concerning this and further research is expected. (2) The matrix effect is also troublesome for non-nucleic acid target detection. It is not only restricted to CRISPR/Cas-based detection, it is for almost all sorts of detection. To overcome this, on one hand, a pretreatment (extraction, concentration, enrichment) is desirable; on the other hand, the robustness and compatibility of CRISPR/Cas-based detection systems could be optimized and improved. (3) The standardization of CRISPR/Cas-based detection is not established officially and there are no approved products either. Nevertheless, the authors believe this situation could be changed along with the technical matureness of this detection technology. (4) The Cas proteins are instrumental in this technology and it has problems such as non-specific *trans*-cleavage. This leads to high background signals, decreased signal-to-noise ratio, or even false positives. (5) The emergence of new technologies would propel the development of CRISPR/Cas-based detection and it is intriguing to see what will happen if combining CRISPR/Cas with them to enhance the applicability. For example, artificial intelligence (AI) could be highly useful in rebuilding or predicting the dynamics of enzymes at work, which helps the researchers to figure out how the enzymes work at the real-time level by knowing the conformational changes of enzymes and key amino acids residues involved. This information would guide the researchers to locate and engineer such amino acid residues in purposes. Even, man-made proteins through AI-assisted *de novo* protein design that performs the activity of Cas effectors could be fulfilled one day in the near future. Also, machine learning and bioinformatics algorithms stemming from large-scale experimental data sets can develop more effective design tools to better predict guide RNA and target activator performance and improve the specificity and activity of Cas proteins. This strategy can also be used for rational design and directed evolution of Cas proteins to overcome nonspecific cleavage effects. Meanwhile, finding novel Cas proteins is also an option. The recently discovered Cas12i, Cas12j and Casϕ proteins, their functions have not been fully developed. (6) A major drawback of most CRISPR/Cas-based methods for detecting non-nucleic acid targets is that it remains a challenge to detect targets below the atto-molar range. This can be solved by a few methods, for example, the addition of the pretreatment step to concentration, the discovery of novel recognition modules that can respond to ultralow analytes (for example better nucleic acid aptamer with enhanced association capability with small molecules). (7) Integrated detection of small equipment is lacking. The current work is somehow limited by the separate steps in operation and the future work could

consider merging pretreatment, signals transduction and Cas *trans*-cleavage into one pot fashion to make the detection more simplified and streamlined. Also, bench-top mini-sized devices can be developed by integration, miniaturization and automation, achieving a sample-in-result-out detection to facilitate field use.

To sum up, this review has pinpointed the current development status, existing challenges, as well as the futuristic development directions for non-nucleic acid target detection using CRISPR/Cas toolboxes. For the CRISPR/Cas detection system, some reviews have summarized the related research progress (Dai et al., 2020). However, the topics of these reviews are not specific to non-nucleic acid targets. These reviews mainly introduced the function and detection utility of the CRISPR/Cas system, the diversity of nucleic acid target detection, and the combination with different amplification techniques and signal outputs. For the detection of non-nucleic acid targets, only served a small (certainly not major) or supplement parts within these review articles. Since the invention of this technology, there has been no systematic and comprehensive summary of CRISPR/Cas-based detection of non-nucleic acid targets. Furthermore, there is no critical review of the strengths, current challenges and future directions in this area. To the best of our knowledge, this is the first review article on the detection of non-nucleic acid targets based on the CRISPR/Cas toolboxes. Therefore, our review article would be timely and meaningful to supplement the above vacancies and deficiencies. In conclusion, we have tried to provide the readers with a concise profile of this emerging and fast-evolving technology and highlighted its potential applications in a range of scenarios about non-nucleic acid target detection. It will definitely have a significant economic and social impact on disease prevention, diagnosis and treatment, environmental monitoring and protection and food safety in the future.

Author contribution

Xinkuan Cheng: Conceptualization, investigation, writing original draft, review & editing. Yaru Li: Figure drawing, review & editing. Jun Kou: Figure drawing, review & editing. Dan Liao: Figure drawing. Wenlu Zhang: Revision. Lijuan Yin: Review & editing. Shuli Man: Conceptualization, writing-review & editing. Long Ma: Design, conceptualization, project administration, supervision, writing original draft, review & editing, funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

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

Acknowledgements

This work was financially supported by National Natural Science Foundation of China (No. 32072309, 21672161, 81503086), Tianjin Municipal Science and Technology Committee (19JCYBJC27800), State Key Laboratory of Food Nutrition and Safety, Tianjin University of Science & Technology (19PTSYJC00060), Scientific Research Project of Tianjin Education Commission (2019KJ239) and Tianjin graduate scientific research innovation project (2021YJSB208).

References

Abnous, K., Abdolabadi, A.K., Ramezani, M., Alibolandi, M., Nameghi, M.A., et al., 2022. Talanta 241, 123276.
Barreto, D.N., Ribeiro, M., Sudo, J.T.C., Richter, E.M., Munoz, R.A.A., et al., 2020. Talanta 217, 120987.
Bond, J.S., 2019. J. Biol. Chem. 294, 1643–1651.
Bruch, R., Urban, G.A., Dincer, C., 2019. Trends Biotechnol. 37, 791–792.
Chen, J., Zhu, Y., Kaskel, S., 2021a. Angew. Chem. Int. Ed. 60, 5010–5035.
Chen, J.S., Ma, E., Harrington, L.B., Da Costa, M., Tian, X., et al., 2018. Science 360, 436–439.
Chen, Q., Tian, T., Xiong, E., Wang, P., Zhou, X., 2020a. Anal. Chem. 92, 573–577.
Chen, S., Wang, R., Peng, S., Xie, S., Lei, C., et al., 2022. Chem. Sci. 13, 2011–2020.
Chen, X., Cao, G., Wang, X., Ji, Z., Xu, F., et al., 2020b. Biosens. Bioelectron. 163, 112271.
Chen, X., Cao, G., Zhang, J., Deng, Y., Luo, X., et al., 2021b. Sens. Actuators B Chem. 333, 129553.
Dai, Y., Somoza, R.A., Wang, L., Welter, J.F., Li, Y., et al., 2019. Angew. Chem. Int. Ed. 58, 17399–17405.
Dai, Y., Wu, Y., Liu, G., Gooding, J.J., 2020. Angew. Chem. Int. Ed. 59, 20754–20766.
Daniel, S.C.G.K., Nirupa Julius, L.A., Gorthi, S.S., 2017. Sens. Actuator. B Chem. 238, 641–650.
Deng, F., Li, Y., Qiao, L., Goldys, E., 2022. Anal. Chim. Acta 1192, 339351.
Douafer, H., Andrieu, V., Phanstiel, O.t., Brunel, J.M., 2019. J. Med. Chem. 62, 8665–8681.
Du, Y.C., Wang, S.Y., Wang, Y.X., Ma, J.Y., Wang, D.X., et al., 2021. Biosens. Bioelectron. 171, 112734.
Eisenstein, M., 2022. Nature 601, 658–661.
Fan, Z., Ding, Y., Yao, B., Wang, J., Zhang, K., 2021. Chem. Commun. 57, 8015–8018.
Feng, W., Newbigging, A.M., Tao, J., Cao, Y., Peng, H., et al., 2021. Chem. Sci. 12, 4683–4698.
Gootenberg, J.S., Abudayyeh, O.O., Lee, J.W., Essletzbichler, P., Dy, A.J., et al., 2017. Science 356, 438–442.
Harrington, L.B., Burstein, D., Chen, J.S., Paez-Espino, D., Ma, E., et al., 2018. Science 362, 839–842.
Hu, J., Song, H., Zhou, J., Liu, R., Lv, Y., 2021a. Anal. Chem. 93, 14214–14222.
Hu, J., Zhou, J., Liu, R., Lv, Y., 2021b. Chem. Commun. 57, 10423–10426.
Iwasaki, R.S., Batey, R.T., 2020. Nucleic Acids Res. 48, e101.
Januarie, K.C., Uhuo, O.V., Iwuoha, E., Feleni, U., 2022. Anal. Bioanal. Chem. 414, 907–921.
Jung, J.K., Alam, K.K., Verosloff, M.S., Capdevila, D.A., Desmau, M., et al., 2020. Nat. Biotechnol. 38, 1451–1459.
Kaminski, M.M., Abudayyeh, O.O., Gootenberg, J.S., Zhang, F., Collins, J.J., 2021. Nat. Biomed. Eng. 5, 643–656.
Kim, H., Lee, S., Yoon, J., Song, J., Park, H.G., 2021. Biosens. Bioelectron. 194, 113587.
Lee, I., Kwon, S.J., Sorci, M., Heeger, P.S., Dordick, J.S., 2021. Anal. Chem. 93, 16528–16534.
Li, B., Shao, Z., Chen, Y., 2021a. Anal. Chim. Acta 1165, 338478.
Li, B., Xia, A., Zhang, S., Suo, T., Ma, Y., et al., 2020a. Biosens. Bioelectron. 173, 112619.
Li, B., Xie, S., Xia, A., Suo, T., Huang, H., et al., 2020b. Trends Anal. Chem. 132, 116039.
Li, C.Y., Zheng, B., Li, J.T., Gao, J.L., Liu, Y.H., et al., 2021b. ACS Nano 15, 8142–8154.
Li, C.Y., Zheng, B., Liu, Y.H., Gao, J.L., Zheng, M.Q., et al., 2020c. Biosens. Bioelectron. 169, 112650.
Li, H., Guo, Z., Xie, W., Sun, W., Ji, S., et al., 2019a. Anal. Biochem. 578, 60–65.
Li, H., Li, M., Yang, Y., Wang, F., Wang, F., et al., 2021c. Anal. Chem. 93, 3209–3216.
Li, H., Xing, S., Xu, J., He, Y., Lai, Y., et al., 2021d. Talanta 221, 121670.
Li, J., Yang, S., Zuo, C., Dai, L., Guo, Y., et al., 2020d. ACS Sens. 5, 970–977.
Li, S.Y., Cheng, Q.X., Wang, J.M., Li, X.Y., Zhang, Z.L., et al., 2018. Cell Discov. 4, 20.
Li, X., Yang, J., Yuan, R., Xiang, Y., 2019b. Biosens. Bioelectron. 142, 111574.
Li, Y., Deng, F., Goldys, E.M., 2021e. Sens. Actuator. B Chem. 347, 130533.
Li, D., Ling, S., Wu, H., Yang, Z., Lv, B., 2022a. Sens. Actuator. B Chem. 355, 131329.
Li, Y., Deng, F., Goldys, E.M., 2022b. Talanta 246, 123469.
Li, Y.R., Man, S.L., Ye, S.Y., Liu, G.Z., Ma, L., 2022c. CRISPR-Cas-based detection for food safety problems: current status, challenges, and opportunities. Compr. Rev. Food Sci. Food Saf. https://doi.org/10.1111/1541-4337.13000.
Li, Y., Deng, F., Hall, T., Vesey, G., Goldys, E.M., 2021f. Water Res. 203, 117553.
Li, Y., Mansour, H., Watson, C.J.F., Tang, Y., MacNeil, A.J., et al., 2021g. Chem. Sci. 12, 2133–2137.
Lian, M., Shao, S., Liu, M., Shi, Y., Zhang, H., et al., 2022. Talanta 238, 123071.
Liang, M., Li, Z., Wang, W., Liu, J., Liu, L., et al., 2019. Nat. Commun. 10, 3672.
Lin, X., Li, C., Meng, X., Yu, W., Duan, N., et al., 2022. J. Hazard Mater. 433, 128750.
Ma, Y., Mou, Q., Yan, P., Yang, Z., Xiong, Y., et al., 2021a. Chem. Sci. 12, 11740–11747.
Ma, L., Peng, L., Yin, L., Liu, G., Man, S., 2021b. CRISPR-Cas12a-powered dual-mode biosensor for ultrasensitive and cross-validating detection of pathogenic bacteria. ACS Sens. 6, 2920–2927.
Ma, L., Yin, L., Li, X., Chen, S., Peng, L., Liu, G., Ye, S., Zhang, W., Man, S., 2022. A smartphone-based visual biosensor for CRISPR-Cas powered SARS-CoV-2 diagnostics. Biosens. Bioelectron. 195, 113646.
Mahas, A., Wang, Q., Marsic, T., Mahfouz, M.M., 2022. Anal. Chem. 94, 4617–4626.
Makarova, K.S., Wolf, Y.I., Alkhnbashi, O.S., Costa, F., Shah, S.A., et al., 2015. Nat. Rev. Microbiol. 13, 722–736.
Malinin, N.L., Lee, G., Lazzarotto, C.R., Li, Y., Zheng, Z., et al., 2021. Nat. Protoc. 16, 5592–5615.
Mehta, P.K., Jeon, J., Ryu, K., Park, S.H., Lee, K.H., 2022. J. Hazard Mater. 427, 128161.
Nie, Y., Liu, Y., Zhang, Q., Zhang, F., Ma, Q., et al., 2020. Anal. Chim. Acta 1127, 190–197.
Niu, C., Wang, C., Li, F., Zheng, X., Xing, X., et al., 2021. Biosens. Bioelectron. 183, 113196.
Niu, J., Hu, X., Ouyang, W., Chen, Y., Liu, S., et al., 2019. Anal. Chem. 91, 2360–2367.
Pardee, K., Green, A.A., Takahashi, M.K., Braff, D., Lambert, G., et al., 2016. Cell 165, 1255–1266.

Peng, L., Zhou, J., Liu, G., Yin, L., Ren, S., et al., 2020. Sens. Actuators B Chem. 320, 128164.
Posha, B., Kuttoth, H., Sandhyarani, N., 2021. Langmuir 37, 257–265.
Qiao, B., Xu, J., Yin, W., Xin, W., Ma, L., et al., 2021. Biosens. Bioelectron. 183, 113233.
Qing, M., Chen, S.L., Sun, Z., Fan, Y., Luo, H.Q., et al., 2021. Anal. Chem. 93, 7499–7507.
Qu, F., Guo, X., Liu, D., Chen, G., You, J., 2016. Sens. Actuators B Chem. 233, 320–327.
Shen, J., Zhou, X., Shan, Y., Yue, H., Huang, R., et al., 2020. Nat. Commun. 11, 267.
Sheng, A., Wang, P., Yang, J., Tang, L., Chen, F., et al., 2021. Anal. Chem. 93, 4676–4681.
Shi, K., Xie, S., Tian, R., Wang, S., Lu, Q., et al., 2021. Sci. Adv. 7, eabc7802.
Sinharay, S., Randtke, E.A., Howison, C.M., Ignatenko, N.A., Pagel, M.D., 2018. Mol. Imag. Biol. 20, 240–248.
Soleimany, A.P., Bhatia, S.N., 2020. Trends Mol. Med. 26, 450–468.
Srivastava, P.N., Narwal, S.K., Mishra, S., 2021. DNA Repair 101, 103078.
Teng, F., Guo, L., Cui, T., Wang, X.G., Xu, K., et al., 2019. Genome Biol. 20, 132.
Wang, C., Han, C., Du, X., Guo, W., 2021a. Anal. Chem. 93, 12881–12888.
Wang, D.X., Wang, J., Du, Y.C., Ma, J.Y., Wang, S.Y., et al., 2020a. Biosens. Bioelectron. 168, 112556.
Wang, M., Zhao, W., Lei, S., Zou, W., Hu, W., et al., 2020b. Talanta 208, 120359.
Wang, P., Liu, Y., Yu, Y., Zhang, Y., Peng, J., et al., 2021b. Anal. Chim. Acta 1174, 338747.
Wu, H., Chen, X., Zhang, M., Wang, X., Chen, Y., et al., 2021. Trends Anal. Chem. 135, 116150.
Wu, P., Ye, X., Wang, D., Gong, F., Wei, X., et al., 2022. J. Hazard Mater. 424, 127690.
Xie, S., Ji, Z., Suo, T., Li, B., Zhang, X., 2021. Anal. Chim. Acta 1185, 338848.
Xing, S., Lu, Z., Huang, Q., Li, H., Wang, Y., et al., 2020. Theranostics 10, 10262–10273.
Xiong, Y., Zhang, J., Yang, Z., Mou, Q., Ma, Y., et al., 2020a. J. Am. Chem. Soc. 142, 207–213.
Xiong, Y., Zhang, J., Yang, Z., Mou, Q., Ma, Y., et al., 2020b. J. Am. Chem. Soc. 142, 207–213.
Xu, Z.H., Zhao, Z.Y., Wang, H., Wang, S.M., Chen, H.Y., et al., 2021. Anal. Chim. Acta 1188, 339180.
Yang, M., Shi, K., Liu, F., Kang, W., Lei, C., et al., 2021. Sci. China Chem. 64, 330–336.
Yin, L., Duan, N., Chen, S., Yao, Y., Liu, J., Ma, L., 2021. Ultrasensitive pathogenic bacteria detection by a smartphone-read G-quadruplex-based CRISPR-Cas12a bioassay. Sens. Actuators B Chem. 347.
Yin, L.J., Man, S.L., Ye, S.Y., Liu, G.Z., Ma, L., 2021. CRISPR-Cas based virus detection: recent advances and perspectives. Biosens. Bioelectron. 193, 111354.
Zhang, C., Yao, H., Ma, Q., Yu, B., 2021a. Analyst 146, 6576–6581.
Zhang, S., Du, X., Liu, H., Losiewic, M.D., Chen, X., et al., 2021b. Environ. Res. 192, 110254.
Zhang, X., Zheng, C., Ding, L., Wu, Y., Xu, H., et al., 2021c. Sens. Actuator. B Chem. 330, 129317.
Zhang, Y., Wu, Y., Wu, Y., Chang, Y., Liu, M., 2021d. Trends Anal. Chem. 137, 116210.
Zhao, J., Tan, Z., Wang, L., Lei, C., Nie, Z., 2021a. Chem. Commun. 57, 7051–7054.
Zhao, Q., Pan, Y., Luan, X., Gao, Y., Zhao, X., et al., 2021b. Biosens. Bioelectron. 190, 113450.
Zhao, X., Li, S., Liu, G., Wang, Z., Yang, Z., et al., 2021c. Sci. Bull. 66, 69–77.
Zhao, X., Zeng, L., Mei, Q., Luo, Y., 2020a. ACS Sens. 5, 2239–2246.
Zhao, X., Zhang, W., Qiu, X., Mei, Q., Luo, Y., et al., 2020b. Anal. Bioanal. Chem. 412, 601–609.
Zhou, J., Yin, L., Dong, Y., Peng, L., Liu, G., et al., 2020. Anal. Chim. Acta 1127, 225–233.
Zhuang, J., Zhao, Z., Lian, K., Yin, L., Wang, J., Man, S., Liu, G., Ma, L., 2022. SERS-based CRISPR/Cas assay on microfluidic paper analytical devices for supersensitive detection of pathogenic bacteria in foods. Biosens. Bioelectron. 207, 114167.