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CRISPR-Cas assisted diagnostics: A broad application biosensing approach

Annalisa Masi [a, 1], Amina Antonacci [a, 1], Maria Moccia [a], Valeria Frisulli [a], Mariarita De Felice [b], Mariarosaria De Falco [b], Viviana Scognamiglio [a, *]

[a] Institute of Crystallography, National Research Council, Department of Chemical Sciences and Materials Technologies, Via Salaria km 29.300, 00015, Monterotondo, Rome, Italy
[b] Institute of Bioscience and BioResources, National Research Council, Department of Biology, Agriculture and Food Science, Via P. Castellino 111, 80100, Naples, Italy

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ABSTRACT

In addition to its remarkable genome editing capability, the CRISPR-Cas system has proven to be very effective in many fields of application, including the biosensing of pathogenic infections, mutagenic defects, or early cancer diagnosis. Thanks to their many advantages in terms of simplicity, efficiency, and reduced time, several CRISPR-Cas systems have been described for the design of sensitive and selective analytical tools, paving the way for the development and further commercialization of next-generation diagnostics. However, CRISPR-Cas-based biosensors still need further research efforts to improve some drawbacks, such as the need for target amplification, low reproducibility, and lack of knowledge of exploited element robustness. This review aims to describe the latest trends in the design of CRISPR-Cas biosensing technologies to better highlight the insights of their advantages and to point out the limitations that still need to be overcome for their future market entry as medical diagnostics.

1. Introduction

In human history, numerous infectious diseases due to agents of different sources from bacteria to viruses, fungi, protozoa, helminths, and prions, continuously emerge threatening all countries worldwide [1]. The implementation of traditional laboratory techniques with forefront diagnostic tools for simple, sensitive, accurate, and rapid testing at the point of need is becoming a reality in recent years, where pathogenic contaminations are increasingly emerging at diverse levels, from pandemic outbreaks to food-borne infections and environmental pollution. The reason relies on the potential of such diagnostics to provide on-site information bypassing the cost and time-consuming conventional methods and the need for specialized laboratories. Undoubtedly, these skills derive from the perfect fusion of cutting-edge disciplines, capable of enhancing the analytical performance of such biosensing systems as sensitivity, accuracy, portability, and cost-effectiveness, such as nanotechnology, rational design, microfluidics, and material science. Parallel to these crosscutting technologies, the challenge is the discovery of new biotechnological strategies capable of further increasing sensitivity and specificity. In this context, the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas era is paving the way for a significant rise in this direction, helping not only to reshape our understandings of biomolecular systems but also to expand their applications to genome engineering, transcription regulation, and biosensing [2–4]. Indeed, CRISPR-Cas systems can be exploited for the design of biosensors to detect pathogenic nucleic acids, thus revealing the prospective for in-field diagnostic and genotyping applications [5,6].

In addition to infectious diseases, nucleic acids are also a crucial target for early stage cancer diagnosis where targeted treatments are the key targets to greatly improve cancer survival. Cancer is the second leading cause of death in the world. Referring to the data published in the Global Cancer Observatory in 2020, 19.3 million new cancer cases and about 10.0 million cancer deaths have been estimated worldwide. This data is also affected by the coronavirus disease 2019 (COVID- 19) pandemic; despite this, female breast cancer is still the most commonly diagnosed cancer, followed by lung, colorectal, prostate and stomach cancers [7,8]. A variety of

* Corresponding author.
E-mail address: viviana.scognamiglio@ic.cnr.it (V. Scognamiglio).
[1] Annalisa Masi and Amina Antonacci equally contributed.

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qualitative and analytical approaches have been established by several laboratories for the detection of cancer biomarkers, such as polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) detection [9,10]. However, each of these methodologies shows drawbacks that limit their diagnostic application [11,12]. Therefore, considerable efforts are being made to develop a new diagnostic technology that allows for early and targeted diagnosis without the need for complex and invasive instrumentation. In this context, the CRISPR-based genome and transcriptome engineering, and, in particular, CRISPR-Cas12a and CRISPR-Cas13a, appear to have the necessary characteristics, in terms of high detection sensitivity and specificity, as well as simple and fast operation, to be considered as robust detection tools for cancer therapy and diagnostics [13].

As stated by John Travis in his article "Making the cut" published in Science, the genome-editing CRISPR method is the Science's 2015 Breakthrough of the Year [14]. The reason for this recognition relies on the simplicity, efficiency, rapidity and cost effectiveness of the methodology over other genome editing technologies.

CRISPR-Cas system [15–18] has been discovered in the last decade as an adaptive immune defense system in Archaea and Bacteria that binds and cleaves foreign nucleic acids to protect from viral infections, giving rise to a valid, powerful, and programmable molecular scissors mechanism able to induce genetic cuts at specific target sites [19]. Since its discovery, CRISPR has been extensively used in "gene editing", namely a set of procedures for introducing desired changes at specific genomic *loci* and it proves a great promise for life sciences (e.g. for DNA or RNA recognition) [20].

Recently, the CRISPR-Cas-based biosensing system gives rise to a new era for precise diagnosis of infectious diseases, early-stage cancers, detrimental genetic conditions, and mutations of interest. In addition, CRISPR-Cas system is also a new hot spot technique to assist target monitoring in biosafety, food safety, and environmental monitoring. Moreover, CRISPR-Cas-based systems are highly specific, fast and convenient for detecting viruses and bacteria, nucleic acids and target proteins as well as tumor mutations [21–24].

For these reasons, CRISPR-Cas can be applied in many biosensing and bioimaging fields, since it possesses many advantages (e.g. easy use, low cost, and high specificity [25,26]. Moreover, it can be easily integrated with various nucleic acid-based signal amplification techniques to greatly promote sensitivity, selectivity, and specificity, finally providing an easy, rapid and efficient detection process [27].

1.1. Basic mechanisms of the CRISPR-Cas system

The first news regarding the CRISPR-Cas system dates back to the 1980s when anomalous repeated/inverted sequences were discovered. In 1987, Ishino and colleagues [28] described these sequences as atypical *loci,* in which palindromic sequences were interspersed with short repeated DNA sequences, but only in the 2000s the nature of these repetitions and their real function was really understood. Indeed, the origin of these sequences arises from phage or plasmid, inherited in turn from viruses that infected bacteria, acting as an adaptive immune system. In spite of these diverse origins, these systems are based on the same principle of action that includes three main stages: *i*) adaptation or acquisition of the spacer sequences, *ii*) biogenesis of the CRISPR RNAs (crRNAs) and *iii*) interference on the target DNA. Fig. 1 describes the main stages of CRISPR-Cas systems. Regarding the *adaptation, w*hen a bacterium survives a first virus attack, a distinct sequence of the invading MGE (movable genetic element), called protospacer, is incorporated into the CRISPR locus giving rise to a new element called spacer. Every single viral infection will bring the acquisition and introduction of new spacers at the end of the CRISPR leader palindromic sequence. After the insertion of each spacer, the palindromic region will be duplicated in order to allow the host organism to store the genetic material of the intruders and to give rise to the prokaryotic immune system [29]. Regarding the *biogenesis* of the crRNAs, the CRISPR sequence is transcribed into a long crRNA precursor (pre-crRNA) which is further processed into a mature crRNA containing the stored foreign sequence. The maturation of crRNAs is essential for the activation of the immunity system since it is necessary for the recognition and cleavage of viral DNA occurring during the following interference stage. Finally, regarding *interference and immunity* as the last stage of the process, this step leads to microorganisms' immunity, thanks to the mature crRNAs used as real guides to interfere with specific invading nucleic acids.

The basic mechanism of the CRISPR-Cas system interference operation is based on the crRNA which, bounding the Cas protein, can identify the corresponding protospacer and finally trigger the degradation of the cognate sequences of the invader genome.

Due to the advantages of its high specificity, gene-editing tool kits based on CRISPR-Cas system have been developed. The pioneers of this revolutionary precise genome-editing technology won the chemistry Nobel in 2020: Emmanuelle Charpentier and Jennifer Doudna.

Comparing the genomic context of CRISPR regions in many organisms, four conserved genes, regularly present in regions adjacent to CRISPR, were identified. These genes were designated as CRISPR-associated genes 1 to 4 (Cas1 - Cas4). Subsequently, many different types of Cas proteins will be recognized making a classification necessary (Table 1) [30]. Finally, the biological role of CRISPR-Cas systems as a prokaryotic immune system has been experimentally demonstrated by Barrangou R [31]. and van der Oost's groups [32].

CRISPR-Cas systems are largely distributed, indeed they have been found in about 85% of archaeal and 40% of bacterial completely sequenced genomes [53]. They have been classified into two wide classes, including in total six types, classified on the basis of phylogeny, cas gene composition and mechanisms of action [54,55]. Class I includes types I, III and IV, and 12 subtypes, is characterized by the involvement of several units of Cas proteins. Class II is found to exhibit one single, large effector protein. This feature makes the Class II system an ideal candidate for biotechnological applications, displaying the basis for genome engineering. For class II, the most widely used subtypes are type II, type V, type VI, characterized by proteins Cas 9, Cas 12 and Cas 13, respectively [55,56]. Fig. 2 describes the functional organization of CRISPR-Cas systems class I and II.

In type I system, Cas3, thanks to the presence of helicase and HD nuclease domains, has the function of cleaving the target DNA, which is first recognized by the Cascade multiprotein-crRNA complex (CRISPR associated complex for antiviral defense). In type II system, Cas9 is the specific protein required for interference. In type III system, Cas10 protein is assembled into a cascade-like interfering complex in order to find and destroy the target. System IV is featured by the uncharacterized protein Csf1, the latter is thought to be part of the cascade complex; however, these systems often exist alone as *cas* gene without any allied CRISPR array [54]. In type V system Cpf1, C2c1 or C2c3, depending on the subtype, can represent cas9 analogue nucleases [55]. Type VI system is characterized by having a single large protein, C2c2 which possesses two HEPN RNase domains (higher eukaryotic and prokaryotic nucleotide binding) [55].

2. Application fields of CRISPR-Cas biosensing

The first CRISPR-Cas-based sensing approach for the detection

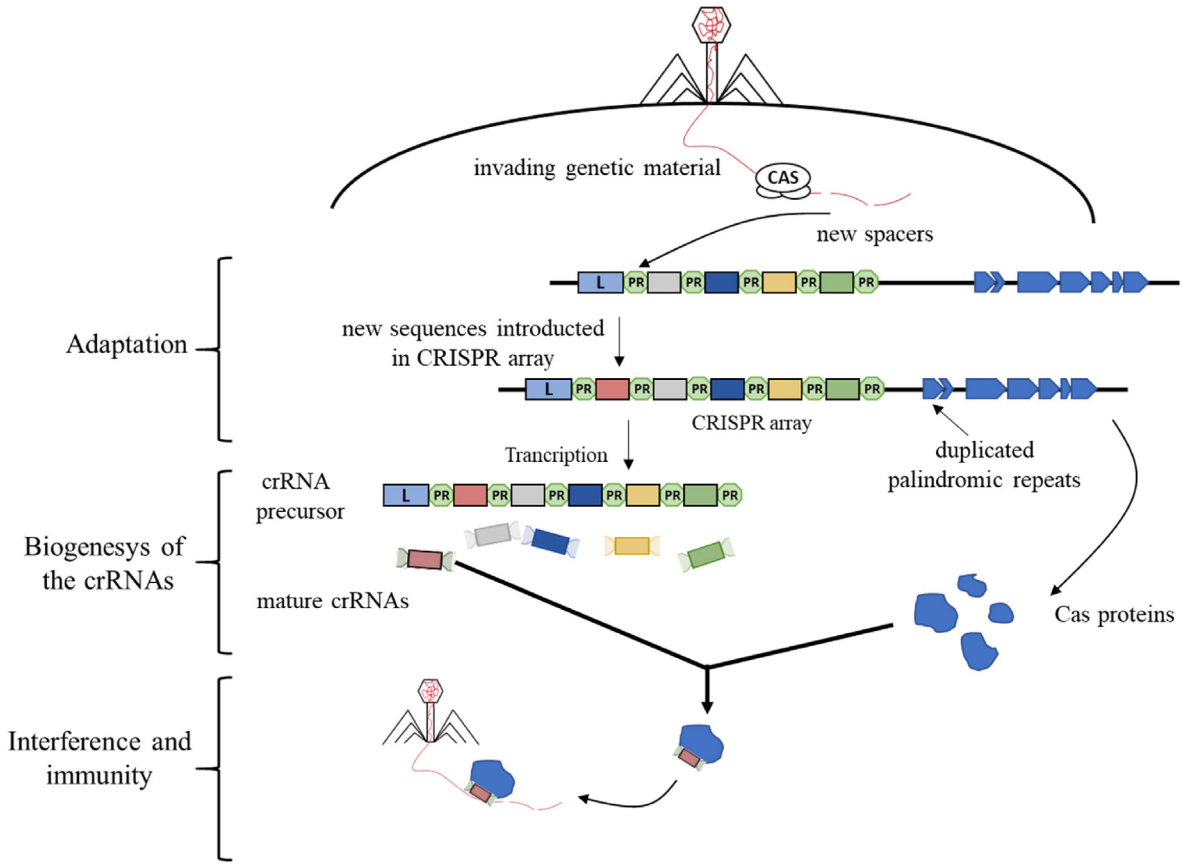

Fig. 1. CRISPR-Cas system main stages. The bacterial cell is infected by a bacteriophage and starts the adaptation step, the first stage of the CRISPR-Cas defense mechanism. A small fragment of the invader DNA is incorporated into the host CRISPR array giving rise to a genetic "memory" of the infection. These sequences are stored as spacers (colored squares) at the leader-proximal (L) end of the array and between two palindromic repeats (PR). The Cas1 and Cas2 proteins form a Cas1-Cas2 complex (CAS). CAS complex catalyzes the addition of a spacer from the phage genome (red) into the CRISPR array. The biogenesis of the crRNAs, the second stage of CRISPR-Cas defense, requires CRISPR array transcription and crRNA precursor maturation in order to generate a mature crRNA. Each mature crRNA includes a single spacer flanked by parts of the PR sequences (green). During the interference and immunity step, mature crRNAs bind to Cas effector proteins (light blue) to form crRNA-effector complexes that recognize the invading sequence and cleaves it causing its destruction.

Table 1
CRISPR-Cas systems families and biochemical functions.

Family	Biochemical features
Cas1	Metal-dependent DNase [33,34]
Cas2	RNase specific for U-reach sequences [35], dsDNA nuclease [36]
Cas3 (helicase and HD domain)	ssDNase (HD domain)/ATP-dependent helicase [37]
Cas3" (stand-alone HD nuclease)	Metal-dependent DNase specific for double-stranded oligonucleotides [38]
Cas4	PD-(DE)xK superfamily nuclease [39]; ssDNA exonuclease [40]
Cas5	Cascade complex subunit; interacts with large subunit and Cas7; binds the 5′-handle of crRNA [33] Cas5 replaces Cas6 in the subtype I–C system [41]
Cas6	Metal-independent endoribonuclease responsible for crRNAs maturation [42]
Cas7	Cascade complex subunit that binds crRNA [43]
Cas8abcef (large subunit)	Cascade complex subunit that recognizes PAM sequences [44]
Cas9	Cas9 generates crRNA and cleaves the target DNA [45,46] through RuvC and HNH nuclease domains [47]
Cas10 (large subunit)	Cascade (Cmr) complex subunit [48]
Small subunit	Small subunit of Cascade complex [49]
Cas12a	RNA-guided deoxyribonuclease and cleaves both dsDNA and ssDNA as target through RuvC [50]
Cas13a	Bind and cleave RNA through two HEPN domains [51]
Cas14a	Cleaves ssDNA using RuvC nuclease domain [52]

of pathogens is known as Specific High-Sensitivity Enzymatic Reporter Unlocking (SHERLOCK), based on CRISPR-Cas13a and recombinase polymerase amplification strategy for collateral cleavage and target isothermal amplification [56]. From now on, many bio-sensing platforms have been developed using the CRISPR-Cas system, with wide applications in different fields, from agro-environment to health.

2.1. Agro-environmental applications of CRISPR-Cas biosensing

The idea of monitoring ecosystems through the genetic material released into the environment by local organisms is today a very

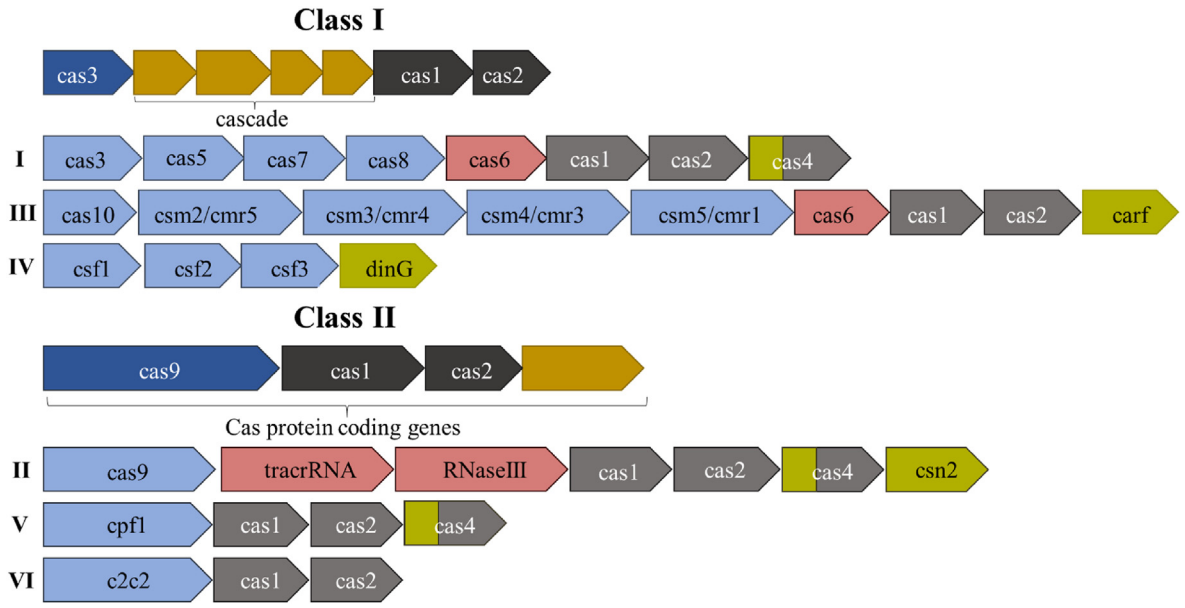

Fig. 2. Functional organization of CRISPR systems. The figure shows the two large classes of the CRISPR system and the specific representative operons for each type. The two colors for cas4 indicate the involvement of the gene product in two stages. Genes involved in interference, crRNA synthesis, adaptation and accessory role are represented in blue, red, dark grey and green, respectively.

active line of research [57]. In addition to environmental monitoring, food control within the production chain is gaining momentum in identifying pathogens that pose a risk to human health. To this end, CRISPR-Cas systems represent fundamental support for detecting the presence of nucleic acids of pathogenic bacteria and viruses in agro-environmental matrices.

The basic principle of this innovative monitoring technique is based on the capability of microorganisms, as bacteria and viruses, to release their genetic heritage into the surrounding environment (eDNA) in which they live. To date, the identification of eDNA is based on the use of PCR-based techniques, characterized by minimal invasive impact and very high specificity and sensitivity. Currently, the challenge in this sector is to develop reliable tools capable of guaranteeing logistical monitoring on site. In this context, Williams and colleagues [58] developed a biosensor device for rapid in field detection of *Salmo salar* collected in Irish river waters, coupling isothermal amplification to a CRISPR-Cas12a detection system. In particular, the authors reported a procedure characterized by the collection and filtration of water samples before DNA extraction. Subsequently, the target DNA was amplified by isothermal recombinase polymerase amplification (RPA) preceding CRISPR-Cas12a mediated fluorescence detection of the target using a ssDNA fluorophore-quencher (FQ) reporter. This technique allowed for the optical detection of the target with an attomolar sensitivity after incubation at 37 °C.

Concerning reliable solutions for multiple targets detection in clinical diagnostics and food safety, Wu and colleagues recently proposed a CRISPR-Cas12a based portable biosensor for the simultaneous detection of CaMV35S promoter (one of the most used elements in GM soybean) and Lectin gene (as internal control) from genetically modified soybean powders (Roundup Ready@) [59]. In particular, the biosensor, made of polymethylmethacrylate, was equipped with three channels and three detection chambers. A preloaded chamber was designed to allocate the CRISPR-Cas12a detection reagents and it was connected to the whole system by screw threads. Once the amplification occurred combining ordinary PCR, rapid PCR and Loop Mediated Isothermal Amplification (LAMP), the amplicons were directed by swinging towards the three detection chambers to ensure the simultaneous double detection. With a LED light centered at 490 nm wavelength, it was possible to observe the positive (green fluorescence) or negative (black) samples. The authors obtained as low as 0.1% transgenic ingredients in soybean powders in GM maize powders (MON810, GA21), GM soybean powders (DP305423), non-GM peanut and rice as targets.

Another interesting example in the context of the control of the food chain was recently proposed by Bu and colleagues [60]. In particular, an electrochemical biosensor for point-of-care (POC) testing of pathogenic bacteria *Escherichia coli* O157:H7 was developed. The concept was based on a cascade signal amplification of CRISPR-Cas12a and primer exchange reaction (PER). In particular, the presence of pathogenic bacteria triggers the unlocking of the hairpin of PER, previously established by the presence of specific aptamers able to block the fork of the PER. Hence, the presence of target bacteria determines the primers' extension in a long ssDNA, capable of forming a Cas12a-crRNA-target DNA ternary complex, which can subsequently activate the ssDNase activity of Cas12a. This latter finally determined the breaking of the loop region of the MB probe into short fragments, and thus a detectable decrease of the electrochemical signal was observed. This electrochemical biosensor was validated on real milk samples reaching a detection limit of 19 CFU mL^{-1}, which means a bacteria concentration from 10 to 10^6 CFU mL^{-1}.

Moreover, Li and colleagues introduced a novel electrochemical biosensor for *Listeria monocytogenes*, an important foodborne pathogen [61]. In detail, the *trans*-cleavage activity of CRISPR-Cas12a was blended with recombinase-assisted amplification (RAA) to develop a cost-effective, specific, and ultrasensitive method (0.68 aM of genomic DNA and 26 CFU/mL in pure cultures). The device operation (Fig. 3A) was based on a target that can induce the number change of the surface signaling probe, and consequently a variation in the electron transfer of the electrochemical tag. The exploitation of the Cas12a system provided a higher signal change between the presence and absence of the target.

An analogous system was described by Liu and colleagues, which is an electronic biosensor for the detection of pathogenic

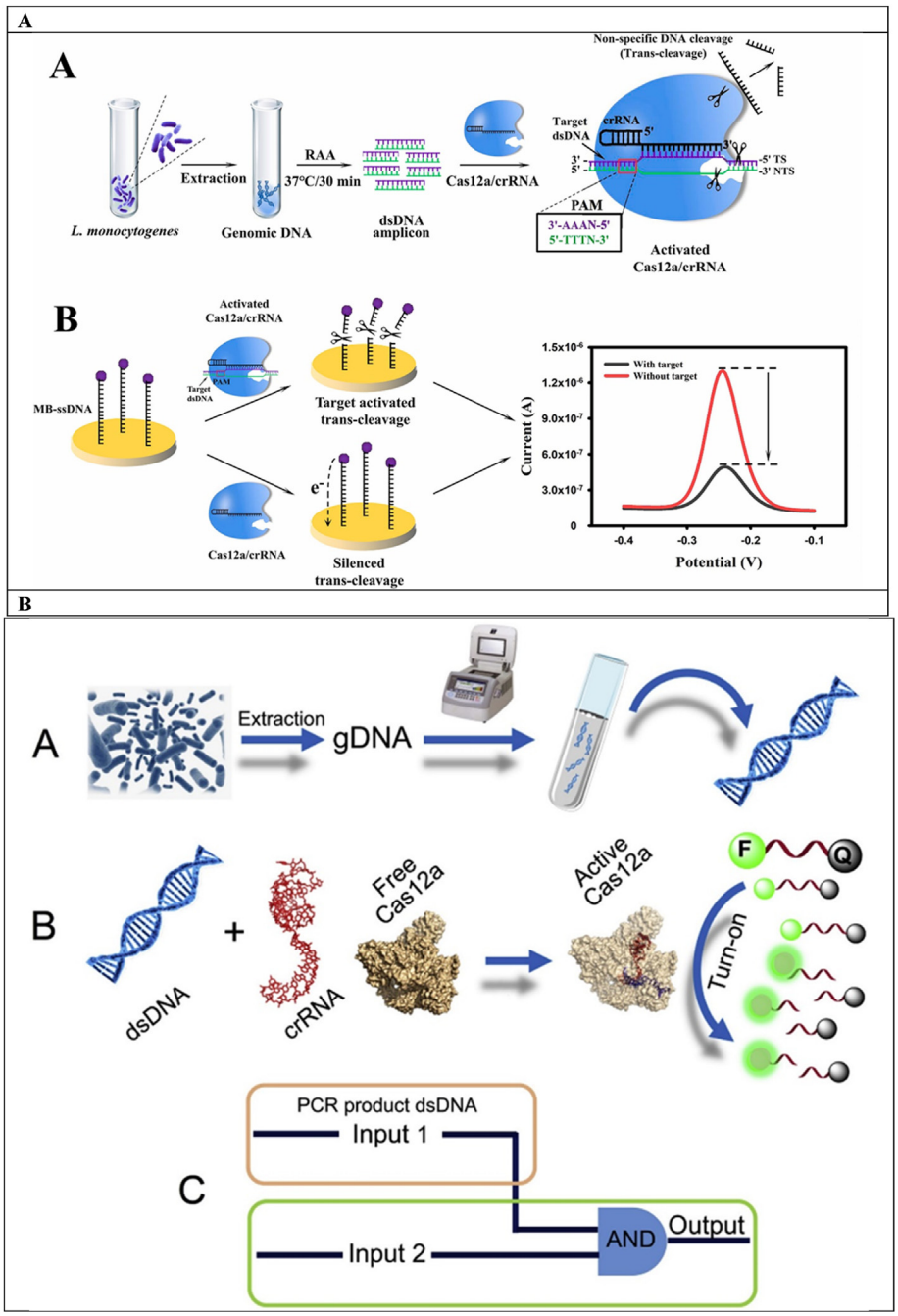

Fig. 3. A) Principle of the RAA-based E-CRISPR biosensor. *Reprinted with permission from Li, F., Ye, Q., Chen, M., Zhou, B., Zhang, J., Pang, R., Wu, Q. (2021). An ultrasensitive CRISPR-Cas12a based electrochemical biosensor for Listeria monocytogenes detection. Biosensors and Bioelectronics, 179, 113073. Copyright 2021 Elsevier.* B) The schematic illustration of CRISPR-Cas12a based logic gates with the function for detecting *S. aureus*. *Reprinted with permission from Peng, L., Zhou, J., Yin, L., Man, S., Ma, L. (2020). Integration of logic gates to CRISPR-Cas12a system for rapid and sensitive detection of pathogenic bacterial genes. Analytica Chimica Acta, 1125, 162–168. Copyright 2020 Elsevier.*

bacterium *Salmonella typhimurium* with a detection limit of 20 CFU/mL [62]. In particular, this analytical tool combined the Lba Cas12a (Cpf1) from the *Lachnospiraceae* bacterium with a hybridization chain reaction (HCR). In this case, the authors exploited dynabeads to immobilize the polymer double-stranded DNA of HCR by *S. typhimurium* aptamer.

Besides, Chen and colleagues recently presented an electrochemical biosensor for sensitive and specific detection of *E. coli* O157:H7 based on Cas12a and immuno-rolling circle amplification (immuno-RCA) [63]. In particular, the mechanism was based on the generation by immuno-RCA of a long single-stranded DNA with several alternating specific aptamers of *E. coli* O157:H7 and target repeated sequences, to allow for the complex formation between CRISPR-Cas12a and crRNA. When the bacteria target was present its quantification became possible because a non-specific trans cleavage occurred and the hairpin DNA on the Au electrode was cut determining a measurable reduction of the peak current. The authors reported a wide detection range from 10 to 10^7 CFU mL^{-1} and a LOD of 10 CFU mL^{-1}.

Moreover, in the context of an innovative CRISPR-Cas based

solution for pathogens detection in food contamination, Peng and colleagues proposed the construction of three 2-input elementary AND, OR, INHIBIT logic gates with CRISPR-Cas12a system [64]. In detail, the authors translated the intriguing advantages of programmability for the development of sensitive detection of *Staphylococcus aureus* with high sensitivity and specificity (LOD of 10^3 CFU/mL, and the dynamic range was 10^3–10^7 CFU/mL). The method was based on target amplification (*S. aureus* femA gene) using PCR. This step guarantees the synthesis of a dsDNA (input 1), which in combination with the cognate crRNA (input 2) can trigger the *trans*-cleavage of the reporter, cutting off a fluorophore and a quencher modified ssDNA. All these logic gates 'turn-on' fluorescent signals; in fact, only if all four elements (LbCas12a, crRNA, target and ssDNA reporter) were present, the enhanced fluorescence occurred (AND gate). On the contrary, in the gate OR only basal level of fluorescence was appreciable because the *trans*-cleavage on ssDNA reporter was not possible due to the absence of a correct input target. Meanwhile, the gate INHIBIT was constituted by LbCas12a, crRNA and ssDNA reporter, not recognizable crRNA as the not correct substrate for LbCas12a, and for this reason unable to generate any fluorescence signal (Fig. 3B). This study represented an example of a complete set of logic gates and their possible connection for a multi-level or cascade circuitry with others and more complex purposes.

Again, for the detection of *S. aureus*, considered one of the most important foodborne pathogens for its invasiveness, virulence, and antibiotic resistance, Zhou and colleagues developed a CRISPR-Cas12a based fluorescence-enhanced lateral flow biosensor with QD-modified streptavidin [65]. In detail, this POC platform proposes a combined system with a later flow assay and CRISPR-Cas, this latter to replace the traditional method for the amplicons production (i.e. LAMP or RPA), and hence to avoid false positives due to the primers and primer dimers. In fact, this unconventional method was characterized by the unique *trans*-cleavage activity induced by the target DNA. In particular, when a target DNA was present a was generated thanks to the recombinase-assisted amplification; this double strand triggers the *trans*-cleavage activity of Cas12a/crRNA. The concentration of the DNA target was easily identifiable by the T line (a weaker T line means a higher target concentration due to the biotin-probe trans cleavage). The strengths of this bacterial detection system for food and clinical diagnosis onsite testing requirements were certainly the simplicity, low cost, almost equipment-free, and a good detection limit of 75 aM achievable within 70 min.

Table 2 reports the main biosensors powered by CRISPR-Cas technology for the detection of nucleic acids in agro-environmental samples.

2.2. Biomedical applications of CRISPR-Cas biosensing

2.2.1. Cancer diagnosis

In the last years, various CRISPR-Cas-based systems have been developed for the detection of different tumor biomarkers. Due to its biochemical properties, CRISPR-Cas enables early detection and monitoring of cancer markers from liquid biopsy samples, such as circulating tumor DNA, tumor-derived proteins, and RNA [66]. Through liquid biopsy, cancer can be detected in the early stages and the clonal evolution and treatment response can be simply monitored without the complications of tissue biopsy allowing to apply personalized therapy. To this aim, several biosensing configurations have been described in the literature in the last few years exploiting CRISPR-Cas technology.

Among the various markers from the liquid biopsy, **circulating tumor DNA** (ctDNA) represents one of the promising for cancer diagnostics and prognosis, and it is obtained from body fluids such as blood, urine, saliva, and cerebrospinal fluid [67,68]. The great interest in this subject has led to several groups developing various systems for its detection [69].

In 2017, Gootenberg and colleagues [70] described the realization of a SHERLOCK-based platform, combining nucleic acid pre-amplification and subsequent CRISPR/Cas13a or 12a detection of pathogenic bacteria and viruses, genotype human DNA, and cancer-associated mutations in cell-free DNA. The advantages of this platform (Fig. 4) are high sensitivity and specificity, low component costs and speed of analysis.

This method finds application in several studies, as the one from Zhang and co-workers, which demonstrated that Sherlock using RPA and CRISPR-Cas13 can detect with high sensitivity and specificity two different cancer mutations, EGFR L858R and BRAF V600E, in mock cell-free DNA (cfDNA) samples with allelic fractions as low as 0.1% [71]. The detection of EGFR mutations in ctDNA is crucial to develop targeted treatment, and early prognosis of non-small cell lung cancer (NSCLC), the leading type of lung cancer, and represents a challenge for several research groups.

Using the same methodology, EGFR-L858R, EGFR-T790 M, and EGFR mutations in cfDNA were detected via fluorescent readouts in liquid biopsy in non-small-cell lung cancer (NSCLC), demonstrating high sensitivity. This version of the SHERLOCK platform proved to be improved in terms of signal sensitivity by increasing it by 3.5 fold and allowing a quantitative measurement starting from 2 aM [72]. In addition, this system was capable to provide analysis on a very low single-molecule volume of 250 μL and 540 μL.

Despite these advantages, SHERLOCK has several limitations, such as poor accessibility, ease of sample preparation, and time-consuming, therefore its field of application is somewhat restricted.

For these reasons, considerable efforts are being made to

Table 2
Main biosensors powered by CRISPR-Cas method for agro-environmental applications.

Type of material	Target	Preamplification method	Sample used	Readout method	LOD	Ref
DNA	*Salmo salar*	Isothermal recombinase polymerase amplification	River waters	Fluorescence	10^{-7} nM	[58]
CaMV35S promoter	Soybean	Combined PCR, rapid PCR and LAMP	Soybean powders	Fluorescence	0.1% transgenic ingredients	[59]
DNA	*E. coli* O157:H7	CRISPR-Cas12a and primer exchange reaction	Milk	Electrochemical	19 CFU mL^{-1}	[60]
DNA	*L. monocytogenes*	Recombinase-assisted amplification	*F. velutipes* samples	Electrochemical	0.68 aM	[61]
DNA	*S. typhimurium*	Lba Cas12a (Cpf1) from *Lachnospiraceae* combined with hybridization chain reaction	Milk	Electrochemical	20 CFU/mL	[62]
DNA	*E. coli* O157:H7	Cas12a and immuno-RCA	–	Electrochemical	10 CFU mL^{-1}	[63]
DNA	*S. aureus*	PCR	Milk	Fluorescence	10^3 CFU/mL	[64]
DNA	*S. aureus*	LAMP or RPA	Milk	Fluorescence	75 aM	[65]

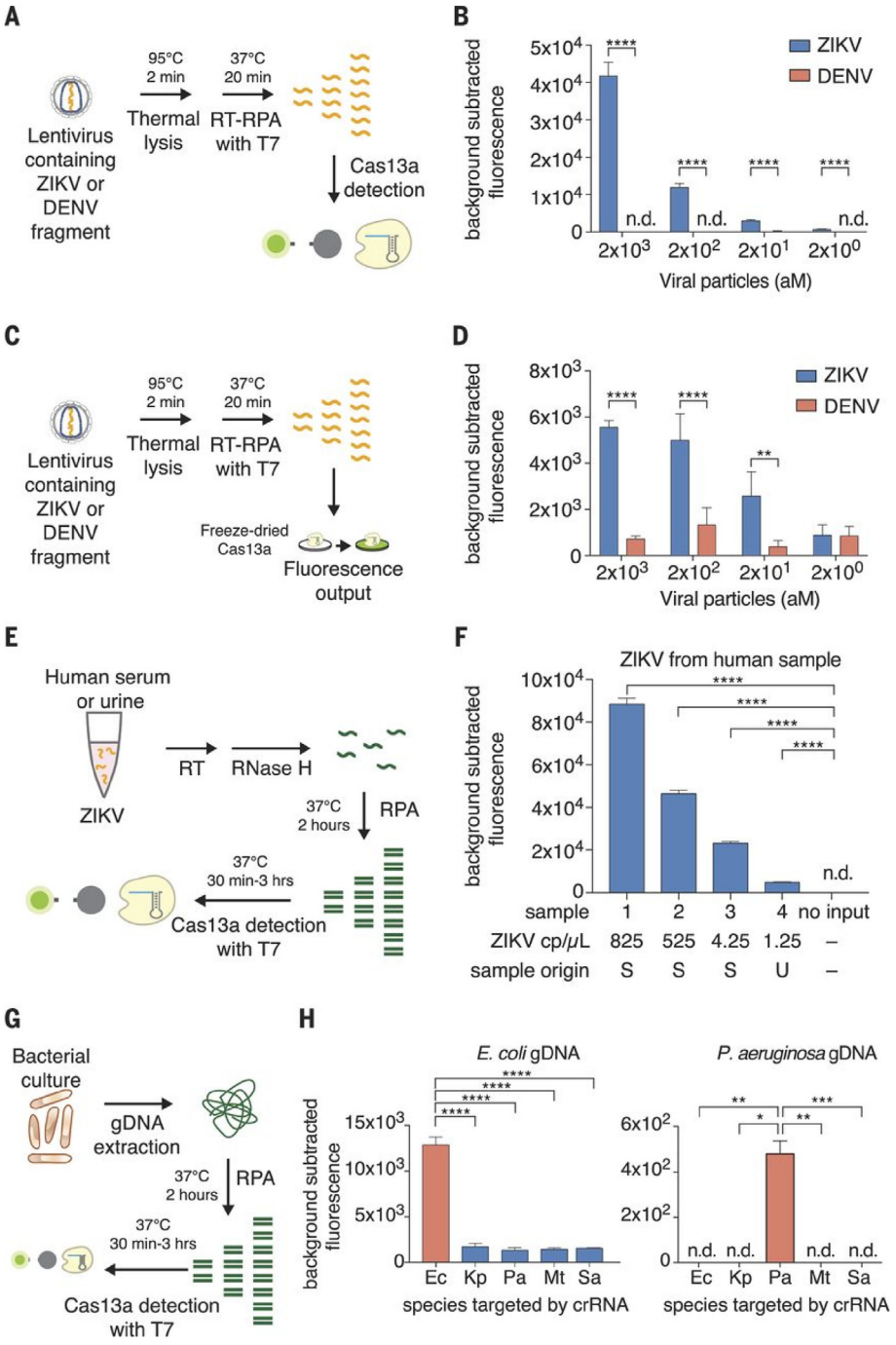

Fig. 4. Cas13a detection can be used to sense viral and bacterial pathogens. A) Schematic of ZIKV RNA detection by SHERLOCK. B) SHERLOCK is capable of highly sensitive detection of the ZIKV lentiviral particles. C) Schematic of ZIKV RNA detection with freeze-dried Cas13a on paper. D) Paper-based SHERLOCK is capable of highly sensitive detection of ZIKV lentiviral particles. E) Schematic of SHERLOCK detection of ZIKV RNA isolated from human clinical samples. F) SHERLOCK is capable of highly sensitive detection of human ZIKV-positive serum (S) or urine (U) samples. Approximate concentrations of ZIKV RNA shown were determined by qPCR. G) Schematic of how SHERLOCK is used to distinguish bacterial strains with a universal 16S rRNA gene V3 RPA primer set. (H) SHERLOCK achieves sensitive and specific detection of E. coli or P. aeruginosa gDNA. Ec, E. coli; Kp, K. pneumoniae; Pa, P. aeruginosa; Mt, Mycobacterium tuberculosis; Sa, Staphylococcus aureus. (B, D, F, and H) n = 4 technical replicates, two-tailed Student's *t*-test; *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001; n.d., not detected; bars represent mean ± SEM. *Reprinted with permission from Gootenberg, J. S., Abudayyeh, O. O., Lee, J. W., Essletzbichler, P., Dy, A. J., Joung, J., & Zhang, F. (2017). Nucleic acid detection with CRISPR-Cas13a/C2c2. Science, 356(6336), 438–442. Copyright 2017 Science.*

overcome these limitations. In 2020, Jang et al. [73] developed a new CRISPR-Cas 12a system to recognize circulating EGFR mutations (L858R and T790 M) in plasma. In this study, CRISPR systems and digital PCR (ddPCR) were compared, highlighting the rapid assay, low-cost and high detectability (100% sensitivity and 100% specificity) of CRISPR-Cas12a for the L858R mutation, comparable with ddPCR and a higher sensitivity (33% sensitivity and 100% specificity) for the detection of T790 M not detectable by ddPCR. Indeed, this system showed a LOD of 10×10^{-3} ng for L858R and of 10×10^{-4} ng for T790 M, respectively, while ddPCR showed a LOD of 10×10^{-1} only for L858R. On the other hand, analysis on the crude cell-culture preparation provided a detection limit of

10×10^{-2} ng for both EGFR mutations. However, the process was less labor-intensive and time-consuming being able to evaluate EGFR mutations directly in liquid fluids without DNA isolation.

Recently, an electrochemical biosensor, based on the CRISPR-Cas12a system and MB/Fe3O4@ COF/PdAu nanocomposites, was developed for the first time for the detection of EGFR L858R [74]. The system combines the recognition characteristics of CRISPR-Cas12a with the catalytic and signal amplification activity of the nanocomposite showing high specificity, stability and selectivity. This system was extremely innovative very precise, and reliable, showing a linear range from 10^{-17} M to 10^{-10} M and a limit of detection of 3.3 aM using chronoamperometry measurement, very competitive with respect to similar biosensors reported in the literature. The ctDNA sampled from the blood serum of 25 NSCLC patients was detected with a satisfactory concordance rate between the present biosensor and ddPCR (92%, 23/25 samples).

Among various ctDNA, BRAF V600E represents a specific biomarker correlated to papillary thyroid carcinoma. Using CRISPR-Cas12a, Yi and co-workers [75] detected the BRAF V600E gene. The platform features a fluorescence detection strategy combined with a 3D DNA walker with CRISPR-Cas12a. The system was highly specific and fast, reaching a detection limit of 0.37 fM, thanks to the presence of the specific endonuclease (Nb.BbvCI). Furthermore, the fluorescence signal was enhanced reaching a detection limit of BRAF V600E of 0.37 fM without DNA polymerase. Analysis time was also significantly reduced to 70 min overall, making it a potential candidate for early clinical diagnosis.

Mutation in the PIK3CA gene was also identified as having a crucial role in various human cancers, including colon, breast, brain, ovarian, liver, and lung cancers. Therefore, PIK3CA mutation appears to be a promising predictive biomarker; its importance in the early development of breast cancer and in treatment selection is the subject of study and research. In this context, Uygun et al. [76] designed an innovative system that combines CRISPR-Cas9 technology with graphene oxide screen-printed electrodes (GPHOXE) to detect the PIK3CA exon 9 mutation by electrochemical impedance spectroscopy (EIS) analysis. Thanks to the modified GPHOXE, there was an increase in conductivity and the high selectivity of the system opens the way to future and promising applications. This biosensor was able to detect PIK3CA within a good linearity between 0 and 20 nM and a detection limit of 0.65 nM. Real blood sample analyses were also performed with 96.53% recovery.

In a recent work, Chen et al. [77] developed a novel CRISPR-Cas9 cleavage-triggered filament displacement reaction (ESDR) system based on the 3D electrochemical biosensor GR/AuPtPd nanoflower to detect mutated EGFR-ctDNA. Thanks to the advantages of high specificity and sensitivity derived from the combination of Cas9/sgRNA, ESDR and 3D GR/AuPtPd, this platform represents a powerful tool for molecular diagnosis. Using differential pulse voltammetry (DPV) measurements, current responses with a linear dependency were observed in the range of target concentrations from 0.01 pM to 500 pM, with a limit of detection of 0.13 pM. Over similar biosensors present in literature, this system showed several advantages, as widely underlined by the authors. Indeed, the designed 3D GR/AuPtPd nanoflower biosensor confirmed to be an efficient, sensitive, and rapid electrochemical sensing platform, thanks to the three-dimensional flower-like structure, which provides a large specific area and high electrochemical activity.

Besides the circulating tumor DNA, proteins are also exploited as cancer biomarkers. **Proteins** are large and complex molecules that cover and play numerous critical roles in the survival of the organism. They are responsible for most of the processes that occur in each cell, but also necessary to guarantee the structure and good functioning of tissues and organs. Since their malfunction is related to various pathologies, numerous detection systems have been developed in recent years to guarantee predictive diagnosis and targeted therapy [78,79]. Prostate cancer is among the most frequent and lethal cancers in males around the world. Early diagnosis improves treatment effectiveness and the risk of mortality. Prostate specific antigen (PSA) has been established as a reference biomarker for this tumor. For this reason, numerous studies have been carried out to increase the sensitivity and specificity in the recognition of this glycoprotein. In a recent study, Wang and co-workers [80] realized an enzyme-free hybridization chain reaction (HCR)-powered CRISPR-Cas12a-based biosensor for the colorimetric detection of PSA. The system was designed to induce dispersion of gold nanoparticles (AuNPs) with color change in presence of PSA, showing that the absorbance considerably increased with PSA concentration from 0.2 ng mL^{-1} to 4.0 ng mL^{-1} with a limit of detection of 0.1 ng mL^{-1} (3δ/slope). These data demonstrate the proposed biosensor was able to detect PSA in a highly sensitive manner with a visual mode. Also, this was the first time a platform has combined both technologies, as the isothermal and enzyme-free amplifying power of HCR, ensuring high sensitivity and selectivity.

DNA-AuNPs nanotechnology was also used to activate the *trans*-cleavage activity of CRISPR-Cas12a for PSA recognition. To this aim, Zhao et al. [81] described a Nano-CLISA platform (nano-immunosorbent assay based on Cas12a/crRNA) for the detection of PSA biomarker, exhibiting a linear response in the range of 0.6–120 ng/mL and a limit of detection of 5.6 fg/mL (175 aM), which was about 1000-fold lower than the commercial human PSA ELISA kit (0.01 ng/mL). This demonstrated that the system allowed for highly sensitive analysis.

Recently Taghdisi et al. [82] developed a fluorescent aptasensor for PSA recognition using CRISPR-Cas 12a and the addition of PicoGreen (PG) as a fluorochrome. This system, exploiting the formation of cruciform DNA nanostructure in the presence of PSA, was proved to be a highly sensitive (LOD = 4 pg/mL within a linear range of 25 pg/mL - 350 ng/mL) and selective tool, which could also be applied for the recognition of other protein biomarkers. Several competitive targets were also analyzed as IgG, HSA, AFP, and CEA, with any change in the response of the aptasensor, highlighting a good capability in distinguishing PSA from other interferents. However, the disadvantage of this system was that PSA determination required multiple steps that increase the detection time.

Table 3 reports the main biosensors powered by CRISPR-Cas technology for the detection of biomarkers in bioclinical samples.

2.2.2. Virus infection diseases diagnosis

As we have seen in recent years, epidemics are increasingly affecting the world. We are currently facing the 2019 Coronavirus (COVID-2019) epidemic, but in the past, we have been affected by other pandemics such as severe acute respiratory syndrome (SARS), viral infections, and infectious diseases (e.g. Sepsi, Ebola, Zika, Dengue, Tuberculosis).

It is increasingly evident that their rapid and timely identification is of fundamental importance for their containment. Therefore, considerable efforts have been made to develop rapid and specific diagnostic tests for the recognition of the greatest possible number of viral diseases. Being nucleic acids important targets for molecular diagnostics [83–85], the development of CRISPR-Cas platforms is favoring the development of smart optical and electrochemical diagnostic devices [86]. In addition, the combination with different kinds of isothermal amplification methods has been further improved the sensitivity of nucleic acids detection, as exponential amplification reaction (EXPAR), RCA, hybridization chain reaction (HCR), and strand displacement amplification (SDA), among others [87,88]. In their study, Gong and coworkers [89] reported a strand displacement amplification assisted CRISPR-Cas12a (SDACC)

Table 3
Main biosensors powered by CRISPR-Cas method for biomedical applications.

Type of material	Target	Preamplification method	Sample used	Readout method	LOD	Ref
ctDNA	EGFR-L858R, BRAFV600E	RPA + T7 transcription + CRISP/Cas13	Mock serum	Fluorescence	–	[71]
ctDNA	EGFR-L858R, EGFR-T790 M, and EGFR exon 19 deletion	RPA + T7 transcription + CRISP/Cas13	Patient serum	Fluorescence, Lateral-flow	2 aM	[72]
ctDNA	EGFR-L858R EGFR-T790 M	CRISP/Cas12a	Plasma samples	Fluorescence	10×10^{-3} ng 10×10^{-4} ng	[73]
ctDNA	EGFR-L858R	CRISPR-CAS12a + MB/Fe3O4@ COF/PdAu	Plasma samples	Electrochemical biosensor	3.3 aM	[74]
ctDNA	BRAF V600E	CRISPR-CAS12a + 3D DNA walker Specific endonuclease (Nb.BbvCI)	Plasma samples	Fluorescence detection	0.37 fM	[75]
ctDNA	PIK3CA(Exon 9)	GPHOXE + CRISPR-dCas9	Blood samples	Electrochemical impedance spectroscopy	0.65 nM	[76]
ctDNA	EGFR	CRISPR-Cas93DGR/AuPtPd	Patient serum	Electrochemical biosensor	0.13 pM	[77]
Protein	PSA	CRISPR-Cas12a + HCR + AuNPs	Patient serum	colorimetric detection	0.1 ng	[78]
Protein	PSA	Cas12a/crRNA + AuNPs	Patient serum	colorimetric detection	5.6 fg/ml	[81]
Protein	PSA	CRISPR-Cas 12a + PicoGreen (PG)	Patient serum	Fluorescence detection	4 pg/mL	[82]

method for the colorimetric analysis of hepatitis B virus (HBV). This system was based on a ssDNA amplicon hybridized with template DNA to activate the *trans*-cleavage activity of CRISPR-Cas12a, which lead to a nonspecific cleavage of ssDNA on GOx-ssDNA-modified magnetic beads. The GOx released from the magnetic beads worked to catalyze the substrate solution and produce a color change observed by the naked eyes. This strategy was able to recognize a single-base mismatch and furnish for HBV DNA detection with a detection limit of 41.8 fM.

In a recent work, Min and co-workers [90] developed a highly sensitive electrochemical biosensor using the CRISPR system with methylene blue (MB)-conjugated Au nanoparticles (MB-AuNPs) for the detection of Dengue virus (DENV). In detail, the target nucleic acid was activated on the CRISPR-Cpf1 complex for non-specific ssDNA *trans*-cleavage activity. The MB-AuNPs immobilized on the working electrode by SH-ssDNA-biotin were degraded by the activated CRISPR-Cpf1 complex and the electrochemical signal of the MB-AuNPs was recorded. Enhanced electrochemical signals were provided to measure a low concentration of dengue-4 viral RNA (DENV-4 RNA) with a detection limit of 100 fM and within 30 min, demonstrating high sensitivity and rapidity.

Zhao et al. [91] fabricated an "on-off" signal-switchable electrochemiluminescence (ECL) biosensor for the detection of two kinds of virus genes, human immunodeficiency virus (HIV) and human papilloma virus (HPV-16) DNAs, by the integration of spherical nucleic acid (SNA) with CRISPR-Cas12a. This system was based on two separate ECL detection: i) an "on" ECL signal obtained after incubating the biosensor with HIV DNA and ECL luminophores of carbon dots (CDs)-labeled SNA; and ii) an "off" ECL signal obtained after incubating the biosensor with assistant DNA, CDs-labeled SNA and Cas12a/crRNA/HPV-16 dsDNA complex. Using this strategy, HIV and HPV-16 DNAs awerere successfully quantitatively detected with detection limits of 30 fM and 0.32 pM.

Li et al. [92] introduced a new type of G-triplex (G3)-based CRISPR-Cas12a system, used to analyze patient samples potentially affected by HPV infection. The authors demonstrated, using different techniques such as fluorescence spectroscopy, circular dichroism (CD) spectroscopy, gel electrophoresis and nuclear magnetic resonance (NMR), that Cas12a *trans*-cleaves G3 structures within 10 min. The presence of the G3 reporter improves analytical sensitivity up to 20 times compared to the ssDNA-reporter based Cas12a system, and allows detection of unamplified and amplified HPV16 plasmids at 50 pM and 0.1 aM (one copy/reaction), respectively.

Reliable detection of African Swine fever virus (ASFV) and SARS-CoV-2 was also demonstrated by Tian et al. [93], which designed dual-gene amplified products from the multiplex RPA for the analysis of Cas12a and Cas13a in a single tube. This system exhibited 100% sensitivity with test results observed by the naked eye with a sensitivity of 8 copies/μL, as well as high specificity on 2 swab specimens for SARS-CoV-2 and 35 ASFV suspected swine blood samples.

In the last 2 years, such CRISPR-Cas biosensors widely demonstrated their high potential for the design of very sensitive, specific, and fast analytical tools for the monitoring of SARS-CoV-2 as well as for early treatment and control of the disease spread [94].

In this sense, a portable CRISPR-Cas12a assay was developed by Huang and colleagues [95] for the rapid and sensitive detection of SARS-CoV-2, combining the reverse transcription recombinase aided amplification (TRAA) under mild conditions of the virus genes, the CRISPR-Cas12a system for target recognition, a glucose-producing response for signal transduction, and a personal glucose meter (PGM) for final results readout. In comparison with qRT-PCR, the proposed PGMs-CRISPR system exhibited several advantages like lower material cost, shorter detection time, milder experimental conditions, and no requirement for a professional instrument. The analytical limit of detection was as low as 10 copies/μl.

To detect SARS-CoV-2, Li et al. [96] developed a new one-pot CRISPR-mediated test, which combined in one step the DNA or RNA amplification with CRISPR-Cas12b-mediated detection reducing the risk of contamination and the complexity of operation. This COVID-19 CRISPR-top assay was applied in the detection of ORF1ab (opening reading frame 1a/b) and NP (nucleoprotein) genes targets of SARS-CoV-2, working at 59 °C with a response interpreted by visual fluorescence or lateral flow readouts within 60-min. This system showed a detection limit of 10 copies (for each detection target). Furthermore, the diagnostic results obtained for COVID-19 CRISPR-top were similar to those obtained using RT-PCR, indicating its potential application in the clinic, field laboratories and primary care facilities.

Zhang and coworkers [97] presented the first CRISPR-Cas12a-based pH-induced regenerative biosensor for SARS-CoV-2 diagnosis. This system was based on an exonuclease III cleavage reaction-based isothermal amplification of nucleic acids with

CRISPR-Cas12a-mediated pH-induced regenerative Electrochemiluminescence (ECL) biosensor. It showed a detection limit of 43.70 aM, good stability, and reproducibility. Moreover, its long-term use was guaranteed by the regeneration at pH 10.

Huang et al. [98] developed a CRISPR-based assay that integrates a custom CRISPR-Cas12a/gRNA complex with a fluorescent probe to detect target amplicons produced by standard RT-PCR or RPA, enabling sensitive and robust detection with an analysis response within 50 min and a detection limit of 2 copies per sample. The performance of this assay was comparable to the CDC-approved quantitative RT-PCR (RT-qPCR) test performed in a state laboratory and superior to the clinical laboratory, reducing the required equipment.

Ma et al. [99] developed a CRISPR-Cas12a powered visual biosensor with a smartphone reading for ultra-sensitive and selective detection of SARS-CoV-2 (Fig. 5A). The mechanism was inspired by the "pull-down" method, already applied in previous works [100]. However, some improvements were made, as i) an additional centrifugation step, for clearer discrimination for "pull-down" samples; ii) a smartphone with the Color Picker app, which uses the built-in camera for image capture was greatly improved simplicity, detection and reduced portability. The advanced

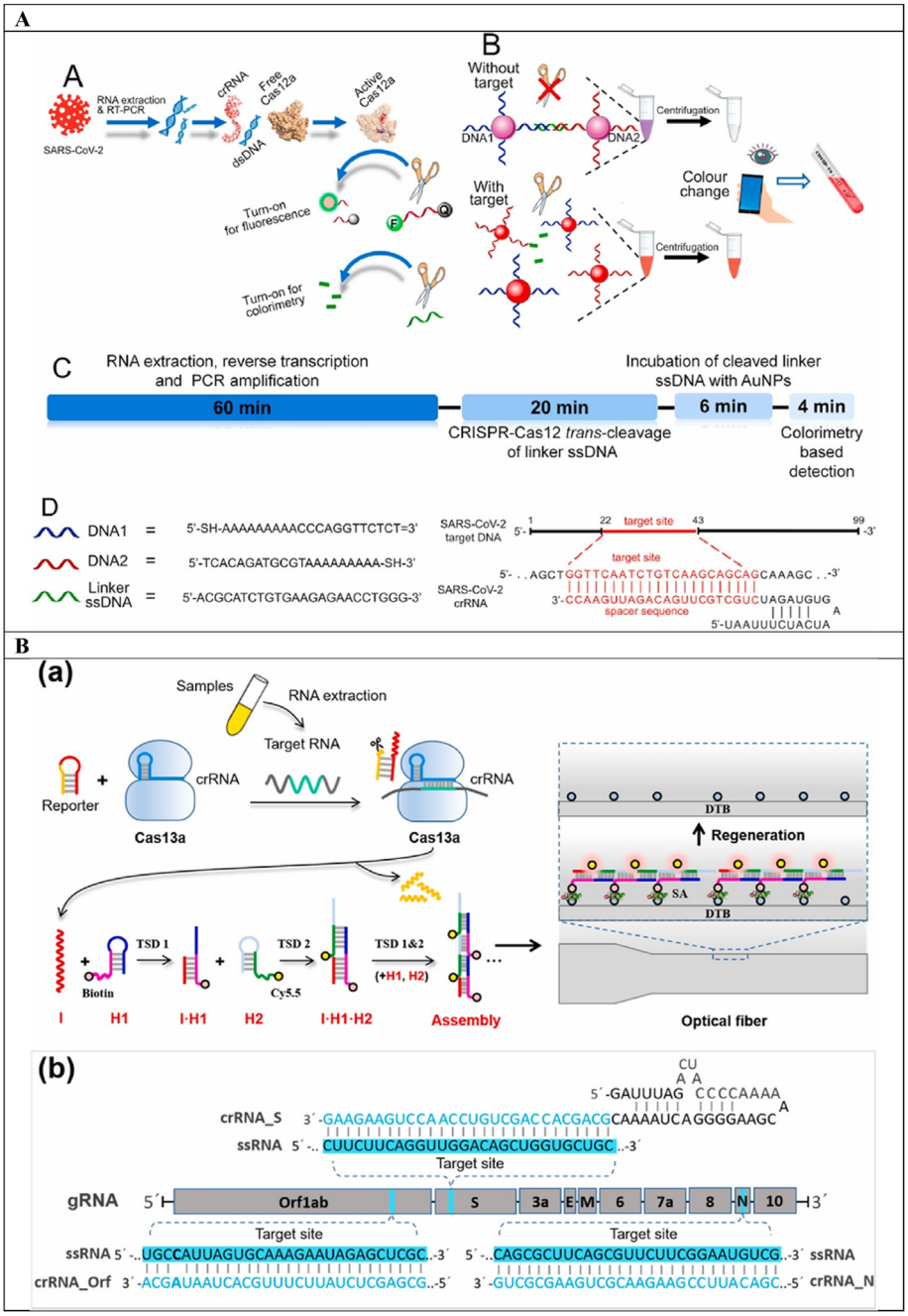

Fig. 5. A) The *trans*-cleavage of CRISPR-Cas12a can be utilized to devise fluorescent and colorimetric biosensors for SARS-CoV-2 detection. *Reprinted with permission from Ma, L., Yin, L., Li, X., Chen, S., Peng, L., Liu, G., & Man, S. (2022). A smartphone-based visual biosensor for CRISPR-Cas powered SARS-CoV-2 diagnostics. Biosensors and Bioelectronics, 195, 113646. Copyright 2022 Elsevier.* B) SARS-CoV-2 detection with enzyme-free amplification coupled CRISPR-Cas13a. *Reprinted with permission from Yang, Y., Liu, J., & Zhou, X. (2021). A CRISPR-based and post-amplification coupled SARS-CoV-2 detection with a portable evanescent wave biosensor. Biosensors and Bioelectronics, 190, 113418. Copyright 2021 Elsevier.*

Table 4
Main biosensors powered by CRISPR-Cas method to detect viruses in bioclinical samples.

Type of material	Target	Preamplification method	Sample used	Readout method	LOD	Ref
DNA	Hepatitis B virus	Strand displacement amplification	–	Colorimentric	41.8 fM	[89]
RNA	Dengue virus	Any amplification	–	Electrochemical	100 fM	[90]
DNA	Human immunodeficiency virus and human papilloma virus	Any amplification	Human serum	Electrochemi-luminescence	30 fM and 0.32 pM	[91]
DNA	Human papilloma virus	PCR amplified and unamplified	Human anal swabs	Fluorescent- and lateral flow	50 pM and 0.1 aM	[92]
RNA	SARS-CoV-2	Multiplex recombinase polymerase amplification	Swab specimens	Smartphone-based fluorescence imaging	100%	[93]
RNA	SARS-CoV-2	Reverse transcription recombinase aided amplification	Nasopharyngeal or oropharyngeal swabs	Fluorescence	10 copies/μl	[95]
RNA	SARS-CoV-2	Loop-mediated isothermal amplification	Respiratory swab samples	Colorimetric	10 copies per sample	[96]
RNA	SARS-CoV-2	Exonuclease III cleavage reaction-based isothermal amplification	Pharyngeal swabs samples	Electrochemi-luminescence	43.70 aM	[97]
RNA	SARS-CoV-2	RT-PCR or isothermal amplification of recombinase polymerase	Swab samples	Fluorescence	2 copies per sample	[98]
RNA	SARS-CoV-2	PCR	Throat swab samples	Colorimetric	1 copy/μL	[99]
RNA	SARS-CoV-2	entropy-driven amplification reaction	Serum	Electrochemi-luminescence	32.80 aM	[101]
RNA	SARS-CoV-2	stand-alone universal enzyme-free hybridization chain reaction	Oropharyngeal swabs samples	Evanescent wave	25 pM	[102]

platform featured a detection limit for SARS-CoV-2 pseudovirus of 1 copy/μL.1 copy, 100% agreement (both positive and negative) with qPCR results, and a sample-to-result time of approximately 90 min. However, the system showed also some limitations that should be addressed, such as: i) the PCR method for gene amplification still required thermal cyclers, which was one step away from POC detection. ii) the analysis procedure involved several steps that should be simplified.

Zhang et al. [101] presented an electrochemiluminescent biosensor for detecting the SARS-CoV-2 RdRp gene using a DNA triple helix structure to inhibit CRISPR-Cas12a activity. The authors exploited a segment of the SARS-CoV-2 RdRp gene by means of an entropy-driven reaction coupled to double-stranded DNA driving CRISPR-Cas12a to form triple-stranded DNA. The latter inhibited the double-stranded DNA binding interaction with CRISPR-Cas12a. The inhibited CRISPR-Cas12a was therefore unable to cut the modified nucleic acid on the electrode surface, resulting in the inability of the modified ferrocene (Fc) on the other end of the nucleic acid to diverge from the electrode surface, causing decreases in electrochemiluminescence in modified GOAu-Ru on the electrode surface in a concentration-dependent manner. This system was able to detect the SARS-CoV-2 RdRp gene with a detection limit of 32.80 aM.

The CRISPR-Cas test developed by Yang and colleagues [102] complemented the Cas13a system with a stand-alone universal enzyme-free hybridization chain reaction (HCR) (Fig. 5B). The detection of HCR groups was enabled through adsorption on optic fiber modified with dexbiotin, followed by the emission of fluorescence induced by an evanescent field. The platform showed a rapid response (<1 h) with an attomolar sensitivity towards three specific genes (S genes, N genes and Orf1ab genes) of SARS-CoV-2, demonstrating great potential for the POC diagnosis of COVID- 19.

Table 4 reports the main biosensors powered by CRISPR-Cas technology for the detection of viruses in bioclinical samples.

3. Conclusions and future perspectives

CRISPR-Cas-assisted biosensing has evolved to become a cutting-edge analytical approach capable of providing sensitive, specific and accurate analyses, being crucial claims for high-tech diagnosis in medicine. Indeed, such systems can provide information on the presence of a pathogenic infection as well as on the mutagenic defect or on the early diagnosis of cancer.

However, CRISPR-Cas-based biosensors still suffer from some limitations, including the need for target amplification, low reproducibility, and lack of knowledge about the robustness of the exploited elements and user accessibility. Furthermore, CRISPR-Cas-based diagnostics is critically constrained by poor sensitivity to clinical needs and, for this reason, such systems are often combined with target pre-amplification. Finally, mixing of reagents, potential off-target detection, and heavy reliance on lavels for signal generation impede the multiplexing capability of CRISPR-Cas, hampering POC applications that integrate sample preparation, detection, data collection, and analysis of results.

Therefore, despite the great research efforts made in recent years, CRISPR-Cas biosensing systems have not yet passed the clinical stage. Further studies should focus on.

i) the use of nanomaterials, known to improve analytical performance in terms of stability, sensitivity and repeatability;
ii) the structural design of the CRISPR-Cas molecular system;
iii) the enzymatic coupling;
iv) the combination with isothermal amplification methods;
v) protein engineering, e.g. for Cas12 and Cas13 to improve their *trans*-cleavage properties, thus simplifying biosensor design and lowering the limit of detection;
vi) the integration of diverse readouts and machine learning-assisted high-throughput multiplex sensing;
vii) the use of particularly robust macromolecules selected from bacteria and archaebacteria or suitably synthesized through a rational design.

This further research predicts that the future of CRISPR-Cas based biosensing will be a POC diagnostic platform, with amplification-free, ultrasensitive, highly specific, multiplexed, and large-scale screening capabilities. This vision makes CRISPR-Cas diagnostics a viable alternative to lab-setup PCR-based approaches, with expanded testing capability over current analytical facilities, as well as low-cost, time-saving, simple, and portable.

Declaration of competing interest

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

Data availability

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

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