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Advances in biosensing: The CRISPR/Cas system as a new powerful tool for the detection of nucleic acids

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ABSTRACT

A main challenge in the development of biosensing devices for the identification and quantification of nucleic acids is to avoid the amplification of the genetic material from the sample by polymerase chain reaction (PCR), which is at present necessary to enhance sensitivity and selectivity of assays. PCR has undoubtedly revolutionized genetic analyses, but it requires careful purification procedures that are not easily implemented in point of care (POC) devices. In recent years, a new strategy for nucleic acid detection based on clustered regularly interspaced short palindromic repeats (CRISPR) and associated protein systems (Cas) seems to offer unprecedented possibilities. The coupling of the CRISPR/Cas system with recent isothermal amplification methods is fostering the development of innovative optical and electrochemical POC devices. In this review, the mechanisms of action of several new CRISRP/Cas systems are reported together with their use in biosensing of nucleic acids.

Contents

1. Introduction

The identification of an adaptive immune defense system present in Archaea and Bacteria is one of the most significant discoveries in recent years in the field of biology. CRISPR (clustered regularly interspaced short palindromic repeats) and CRISPR associated proteins (Cas), also referred as CRISPR/Cas systems, is a nucleic acid-based adaptive immune system which acts protecting these microorganisms from viral infection.

CRISPR/Cas systems rely on the bacterial ability to store a fragment of foreign phage genome in the CRISPR loci, as a memory of past encounters; these loci, together with surrounding repeats, are then used by Cas endonuclease proteins as guides to selectively recognize sequences in the foreign genomes and fight the invader.

Beside to the biological impact of these programmable enzyme systems, CRISPR/Cas systems have also attracted much interest in various biotechnology research fields where selectivity is at the heart of function, such as gene editing and development of innovative biosensing systems [1,2].

Therefore, due to their selectivity and programmability, CRISPR/Cas systems have been rapidly adapted as a recognition element in the development of biosensors and biosensing systems

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for the detection of nucleic acids, which are important targets in molecular diagnosis. Nucleic acids are widely analyzed to evaluate the presence of specific pathogenic microorganisms, antimicrobial resistance genes, gene mutations or as biomarkers (e.g. microRNA (miRNA)) for specific pathologies such as cancer and Duchenne muscular dystrophy [3,4].

In parallel, with the optimization of traditional laboratory molecular techniques mainly based on the polymerase chain reaction (PCR), the development of biosensors and new biosensing methods is becoming a hot topic for analytical applications [5]. In this review, we use the term biosensor when the biological element is bound to the transducer, whereas biosensing is used in the other cases [6,7].

The main purpose of these studies concerns the possibility of implementing traditional laboratory techniques with simple and portable point of care (POC) tests or devices for rapid diagnostic applications. These can provide rapid and selective information on-site to manage individual therapy or to quickly diagnose an infection, bypassing the often-time-consuming traditional methods and the need of specialized laboratories, particularly cumbersome for life-threatening conditions where a timely diagnosis can make a difference between life and death [8,9].

A timely and specific identification is of paramount importance for a prompt and adequate management of infectious diseases (e.g. Sepsis, Ebola, Zika, Dengue, Tuberculosis), both inside and outside hospital settings, but it is also crucial in unpredictable scenarios such as pandemic outbreaks. As we have seen in recent years, epidemics are increasingly affecting the world. Severe acute respiratory syndrome (SARS) and Middle East Respiratory Syndrome (MERS) viral infections are just some examples, which can easily turn into pandemics as happened with SARS-CoV-2 in January 2020 [10]. Therefore, it is increasingly necessary to invest in the development of rapid diagnostic tests to avoid the collapse of clinical laboratories and/or screen the largest possible number of potential infected subjects.

The major trend in research is thus the development of molecular POC devices following the so-called ASSURED criteria (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Deliver- able to end-users), whose performances should be comparable to traditional molecular techniques. However, such objective seems not to have been achieved yet [11].

Many efforts have been made to enhance the chances of future applicability of biosensors or biosensing systems. Further to improve reliability, researchers are also trying to enhance sensitivity by increasing the sensing surface with nanomaterials [12–18], and to reduce costs by fabricating sensors onto economic substrates (paper, flexible plastic) with technologies compatible with an industrial production (inject and screen printed technologies, micro and nano fabrication) [19–22].

In parallel with these efforts, the real challenge is the discovery of new strategies or new biological elements that in addition to sensitivity can also increase the selectivity and the specificity. The CRISPR/Cas era has just paved the road for a significant leap to be made in this direction [23,24].

2. The CRISPR/Cas system

The evolution of defense strategies in prokaryotes as an adaptation to their coexistence with phages has always been extensively studied. The understanding of such mechanisms provides relevant information for the improvement of biotechnological applications in the areas of phage therapy and industrial microbiology [25]. An outcome of such studies, i.e. the discovery of a unique adaptive immune system in prokaryotes, present in 90 % of Archaea and 50 % of Bacteria [26], has attracted the attention of scientists all over the world. This new system joins to the innate non selective immunity strategies already studied, such as: the prevention of adsorption, abortive infection, superinfection exclusion (Sie), and restriction-modification (R-M) [27]. Different from those, this new heritable system has a universal ability to adapt to the invasive genetic material from viruses and plasmids. It consists of memory CRISPR loci, where DNA fragment sequences derived from the invader are stored, and a Cas effector nuclease responsible for the recognition and degradation of invading element[28]. Further studies of this system have brought to light several Cas endonuclease enzymes with high selectivity and specific programmable activities, which emphasizes their potential application in numerous biotechnological approaches, such as gene editing and biosensing.

CRISPR/Cas systems work under the same principle but present a high structural and functional diversity. In fact, the large number of existing prokaryote viruses leads to a large variety of mechanisms against viral invasions, which means that both the CRISPR loci and the Cas proteins can rapidly evolve, generating many different systems. Thus, both the CRISPR array and the Cas enzymes differ from species to species, in length, composition and mode of action, respectively [29].

The CRISPR array is composed of a family of DNA repetitions (25–35 base pair, bp) interspaced by a spacer sequence (30–40 bp), which represents the recorded portion of DNA from the invader. The spacers keep the memory of invasions and are transmitted to the progeny during microbial cell division. The defense strategy can be summarized and divided into three different stages: adaptation, biogenesis of a CRISPR RNA (crRNA) and target interference (Fig. 1).

Each CRISPR array is associated with DNA modules (adaptation and effector) that encode the different Cas proteins, such as helicases and endonucleases. These are used in the adaptation of invading DNA and in contrasting phage infection.

When the microorganism is invaded for the first time, the adaptation or “immunization” phase is activated. As a consequence, an adaptation module of CRISPR loci encodes a Cas enzyme, which recognizes and integrates a portion of the foreigner genetic material (known as protospacer), into a spacer in the CRISPR array. Therefore, the invasion is memorized. In the occurrence of a second challenge by the same phage, the adaptation phase is bypassed, and the biogenesis phase is activated. A crRNA guide is synthetized starting from the stored protospacer sequence, which is used by the Cas endonuclease enzyme to form a ribonucleoprotein (RNP) interference complex that recognizes and specifically cleaves the invader nucleic acid during the interference phase or “immunity” [28,30,31].

CRISPR/Cas systems are classified according to the use of specific Cas enzymes and the mechanism of interference. The most recent classification divides the different systems into 2 classes, 6 types and many subtypes [29]. The two main classes are divided according to the nature of the interference complex. The Class 1 uses a multi-subunit-crRNA-Cas protein whereas the Class 2 use single multidomain-crRNA-Cas proteins. The class 2 is only present in Bacteria and represents just the 5% of the known systems. Types I, III and IV in class 1, and types II, V and VI in class 2 are the most studied Cas enzymes (Fig. 2) [32].

The two classes share the same genes in the adaptation module encoding the Cas proteins (Cas1, Cas2), which are responsible for the specific cleavage in an adjacent protospacer motif (PAM), and the integration of the protospacer fragment into the CRISPR array [33]. Instead, they have different genes encoding Cas proteins that participate in the biogenesis and interference processes. The diversity between the CRISPR/Cas systems and the specific activities of the Cas effector proteins have been studied and exploited in the development of biosensing systems. In this review we aim to discuss the hallmark activity of Cas effector endonuclease enzymes

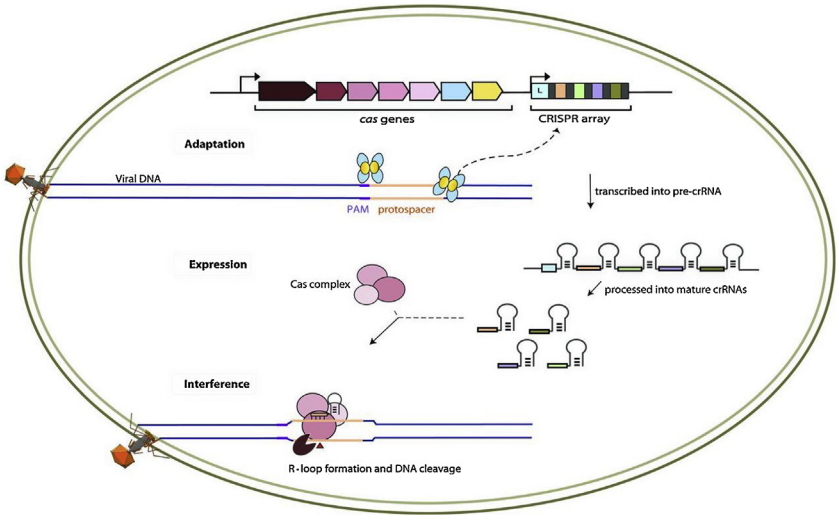

Fig. 1. CRISPR/Cas defense strategy (reprinted by [29] with permission of The American Association for the Advancement of Science).

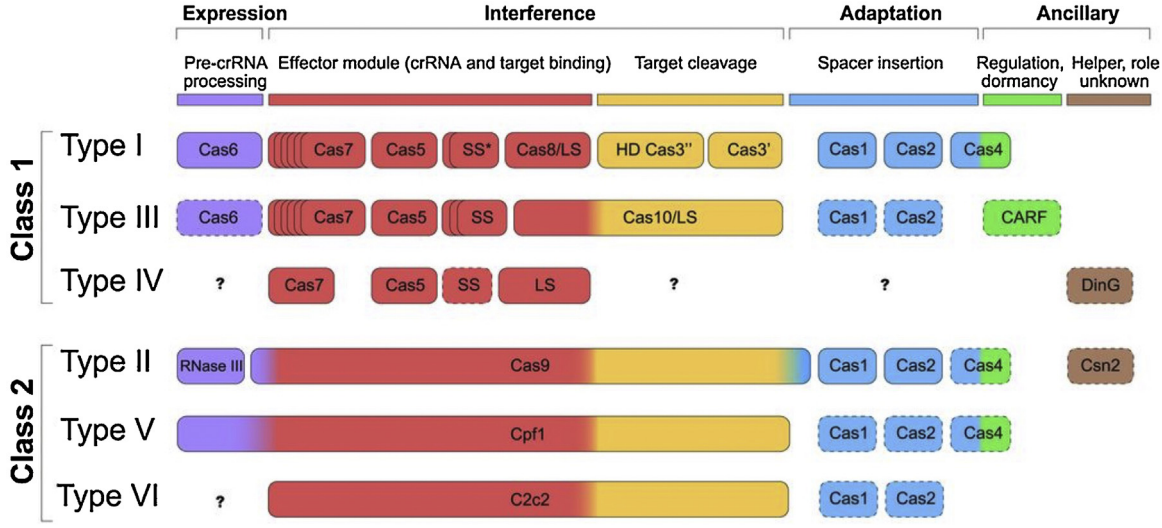

Fig. 2. Classification of Cas associated proteins (reprinted by [29] with permission of The American Association for the Advancement of Science).

belonging to class 2 (Cas9, Cas12, Cas13), which are most used in nucleic acid biosensing [34].

Class 2 encompasses three different enzyme types, II (Cas9), V (Cas12) and VI (Cas13) and different subtypes such as V-A (Cas12a), V-B (Cas12b), V-C (Cas12c), VI-A (Cas13a) and VI- B (Cas13b), which have evolved independent evolutionary ways. This led to an array of different enzymatic activities, but all of these are programmable endonucleases that share the hallmark use of a crRNA guide for nucleic acid target recognition. Endonucleases (Types II and V) can be programmed to recognize DNA, and (Type VI) to recognize RNA [35].

The first difference of Cas effectors can be found during the formation of the RNP complex between Cas protein and crRNA guide after the biogenesis process. The Cas9, Cas12b and Cas12c following tracrRNA dependent pathways (trans-activating crRNA) and non-Cas RNase proteins to process the crRNA. This mechanism has been largely studied in Cas9, while it remains to be elucidated for Cas12b and Cas12c [36,37]. During the formation of the tracrRNA:crRNA:Cas9 complex, the non-coding tracrRNA (75–110 bp) is responsible for the creation of a duplex RNA:pre-crRNA. Subsequently, a non-Cas RNase III ribonuclease cleaves (5′ repeat derived tag) the tracrRNA: pre-crRNA to make a ready-to-use tracRNA:crRNA for the Cas9 interference complex [38].

The Cas12a and Type VI (Cas13) proteins follow a crRNA self-processing pathway. In fact, they are able to process the crRNA into their nuclease domains without the help of RNA or additional proteins.

Besides the mechanism of formation of the ribonuclease complex, Cas proteins also show different cleavage activities and targets.

The Cas9 uses tracrRNA:crRNA guide first to find a specific PAM sequence (5′ – NGG – 3′) in a non-target DNA strand, distant 10–12 nucleotides (nt) of the PAM, to make a duplex with the target strand. This PAM recognition process gives high specificity to the subsequent cleavage. The HNH and RuvC are two nuclease domains, (belonging to the nuclease NUC domain) responsible of the cleavage activity. The target and non-target DNA strands are bound by HNH and RuVC respectively, and their cleavage occurs in the presence of metallic ions, making a blunt break 3bp upstream of the PAM. After the cleavage, the dsDNA remains in the nuclease domain, so the enzyme will not be able to cut again (single turn-over) [36].

In the type V the most studied is the Cas12a (cpf1), which is able to self-process a pre-RNA by cutting, before stem-loop, to make

Table 1
Main characteristics of the Cas endonucleases used in the development of biosensing systems.

Name	Cas9	Cas12a	Cas13a
Type/Subtype	II-A	V-A	VI-A
tracrRNA	Required	Not required	Not required
Guide RNA biogenesis Pre-crRNA	Require RNAse III intervention	Self-processing	Self-processing
PAM	5′ – NGG – 3′	5′ – TTTV – 3′	PFS
Active site	Two domains (HNH and RuvC)	Single domain (RuvC)	Two domains HEPN
Target	dsDNA	dsDNA/ssDNA	ssRNA
Collateral activity	Not present	ssDNAse after target binding	ssDNAse after target binding

it a mature crRNA guide useful for the formation of an interference complex. Upon the crRNA:Cas12a formation, a conformational change occurs, similar to the fit-induced mechanism in Cas9, and this shows a dsDNA cleavage activity [39]. The crRNA: Cas12a recognizes a T-rich PAM (5′-TTTN-3′) in a non-target DNA strand, while RuvC domain Mg^{2+}, Ca^{2+} dependent, cleaves the target DNA strand, 8 nt distant from PAM, giving a 5′ sticky overhang. This interference mechanism is also present in Cas12b and Cas12c [40]. In addition to the Cas12a dsDNA activity (cis-activity), it has been discovered that LbCas12a shows a non-programmable ssDNA collateral activity (trans-activity). This no single turn-over activity, triggered by the cis-activity, has raised much interest in the development of biosensing systems [41,42].

The collateral trans-activity towards ssDNA is also present in the Type IV endonucleases, whose trans-activity is instead triggered by a ssRNA cis-activity, mediated by a self-processed mature crRNA. Two HEPN domains in the NUC lobe are activated after the crRNA:Cas13 complex formation and these are responsible for both the cis- and trans-activity [43]. The crRNA guides the crRNA:Cas13 complex to target the ssRNA sequence recognizing a PFS (protospacer flanking site) sequence. The PFS composition and position can vary among species and change according to the experimental conditions. In general, the Cas13a enzymes need a PFS at the 3′-protospacer strand. In contrast, Cas13b enzymes require a PFS in each protospacer strands. Further studies are necessary to better understand the mechanisms of these enzymes [43,44].

Table 1, summarizes the main characteristics of the Cas endonucleases used in the development of biosensing systems.

3. CRISPR/Cas biosensing for nucleic acid detection and molecular diagnostics

Nucleic acids are important targets for molecular diagnostic techniques detecting infectious diseases, antimicrobial resistance genes, mutations associated to the development of cancer or genetic diseases (e.g. Huntington, Cystic fibrosis and Duchenne muscular dystrophy), single nucleotide polymorphism (SNPs) and biomarkers (miRNA) [45,46]. In general, conventional molecular methods used in diagnostics can be classified according to the need of an amplification step. Fluorescence in Situ Hybridization (FISH) is a non-amplification cytogenic technique mainly used for the detection of gene mutations and the identification of microbial species. This method exploits synthetic fluorochrome-labeled oligonucleotide probes to hybridize a specific complementary target sequence, whose presence is assessed by fluorescence microscopy. In diagnostic applications, FISH is mostly used for the recognition of chromosomal mutations associated with a variety of diseases (Leukemia, multiple myeloma, Myelodysplastic syndromes and cancer diseases) or in the detection of a pathogenic microorganism rRNA. This technique however is time consuming, requires dedicated instrumentation and is limited to the availability of the labeled oligonucleotide probes. In addition, one of its major drawbacks is the high limit of detection (LOD) of fluorescent microscopes, therefore an amplification step is necessary [47]. Molecular techniques based on amplification are considered as a "gold standard" and are routinely used in clinical molecular laboratories. They are based on PCR, which has revolutionized molecular diagnostic approaches. The specific amplification of given nucleic acid sequences by PCR allows to easily overcome the LOD of the associated detection technique, usually based on fluorescence. This reaction requires specific primers, a DNA thermostable polymerase enzyme, nucleotides and a strictly cyclic temperature control (thermocyclers). The PCR is one step in the workflow of analysis and depending on the information sought, it bridges to different techniques such as: DNA microarray (single polymorphism), gel electrophoresis (identification and visualization of genetic material), real-time PCR (rapid and quantitative detection of pathogenic microorganisms) (Fig. 3) [48].

PCR- based approaches are routinely used in laboratory analyses; however, they require dedicated instrumentation, long sample/reagent treatments, and a well-equipped laboratory and trained personnel [49].

The amplification step is required for a rapid, specific and sensitive identification; unfortunately, it is not easily *implementable* in portable devices. In the last few years, several attempts have been made to develop a new and cheap tool for isothermal amplification such as: loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), rolling circle amplification (RCA), sequence-based amplification (NASBA), exponential amplification reaction(EXPAR), strand displacement amplification (SDS) [50–52]. These techniques demonstrated a reduced sensitivity and specificity than the traditional PCR and failed to fill the existent gap between isothermal based laboratory techniques or POC devices and PCR based laboratory diagnosis techniques.

Programmable Cas enzymes have proven to be powerful bio-recognition elements for the detection of nucleic acids, improving the isothermal amplification based molecular diagnostic laboratory techniques in terms of sensitivity, specificity, cost and time of analysis. More importantly, they have been successfully integrated in POC devices with performances comparable to routine techniques.

3.1. Type II cas effector

Cas9 is the most popular Type II enzyme attracting a wide attention for its gene editing applications [53,54], but in recent years it proved to be a good bio-recognition element. Table 2 summarizes the latest publications regarding the implementation of existing molecular laboratory techniques as well as the development of portable biosensing systems and biosensors.

In these applications, the wild type (Cas9) and gene deactivated Cas9 (dCas9) enzymes were mostly used. Cas9, as shown in section 2, is able to recognize and cut the DNA target in a single-turnover activity. Nevertheless, the deactivated enzyme dCas9 works as a recognition element, specifically binding to the target DNA sequence without cutting it [55].

In 2017, K. Guck and coworkers implemented dCas9 by putting it into the FISH technique workflow, for the detection of methicillin-resistant *Staphylococcus aureus* (MRSA). Although FISH has been successfully used in several applications, it has several disadvantages such as a time-consuming protocol, strict control of

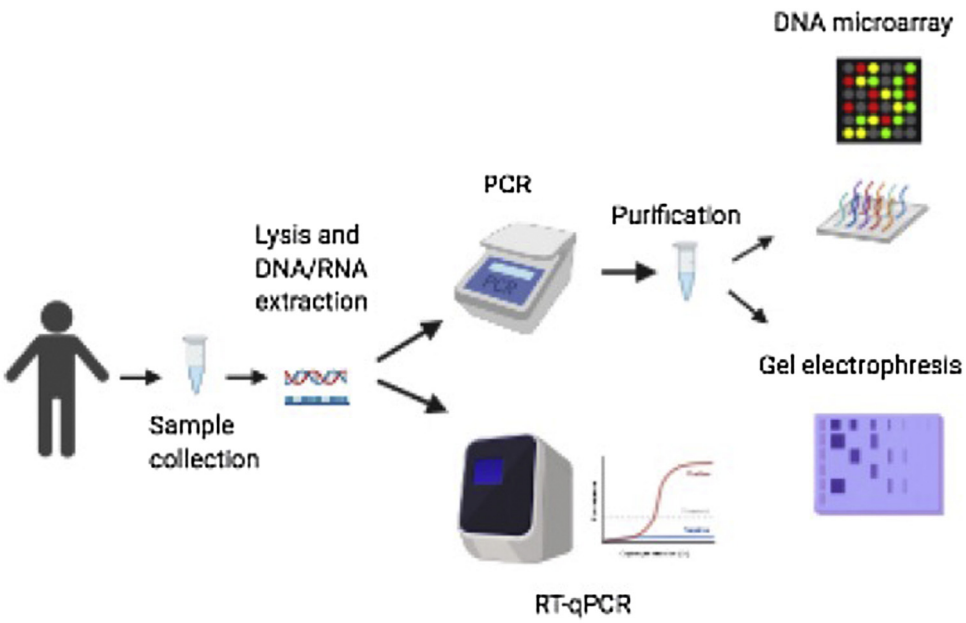

Fig. 3. PCR based molecular diagnostic workflow.

temperature and it is limited to the availability of the labeled oligonucleotide probes.

In the described system, the labeled oligonucleotide probes were replaced by a flexible and highly specific dCas9/sgRNA complex combined with SYBR green 1 dye as staining label, and fluorescence was registered using a laboratory microplate fluorescent reader. The system successfully detected MRSA and was able to discriminate it from a methicillin-susceptible *S. aureus* (MSSA), with a higher sensitivity than the classic FISH method [56].

Amplification based molecular laboratory techniques have also been improved, and new strategies based on isothermal amplification methods coupled with Cas9/sgRNA complex element have been used to replace an expensive conventional PCR protocol.

The rapid kinetics of isothermal exponential amplification reaction (EXPAR) was combined with the specific cutting activity of Cas9/sgRNA for the detection of nucleic acids (CAS-EXPAR), and the fluorescence was recorded by real-time fluorescent laboratory instrument mediated by SYBR green I dye.

The use of Cas9 enzyme has overcome the biggest drawback of EXPAR. In fact, this amplification method requires an exogenous oligonucleotide primer for long ssDNA or RNA amplification. These primers often lead to target-independent amplification, so a false positive signal is given, making EXPAR useless for detection applications. The Cas9/sgRNA complex was used to recognize and cut the ssDNA target, and its products were used as internal primers for the amplification. This strategy opened the way for the use of a rapid and efficient EXPAR amplification, until now mainly applied only for mRNA, long ssDNA or RNA detection [57].

Also, Sun and coworkers have used primers derived from the Cas9/sgRNA enzyme activity for the isothermal amplification step. They used a modified Cas9(H840A)/sgRNA complex, which is able to cut only the non-target strand, working like a nicking endonuclease. But if compared to the classical nicking enzyme, Cas9(H840A) is programmable and more selective. Therefore, two Cas9/sgRNA1 and Cas9/sgRNA2 complexes were used to recognize the dsDNA target in two different nucleic acid sites. The ssDNA product was processed by a strand displacement amplification (SDA), then becoming the primer to amplify a long DNA fragment by the rolling circle amplification (RCA). Combining the Cas9(H840A)/sgRNA activity and two different isothermal amplification steps with a metal-organic framework (MOFs) including a short fluorescent ssDNA fragment Ui066, it has been possible to obtain a new molecular detection method for *E. coli* 0157:H7, with a three order of magnitude lower LOD than a traditional RT-PCR [58].

The Cas9 and dCas9 can be easily and successfully adapted in applications that do not required a data laboratory acquisition system. In fact, they have been used in development of portable and easy able to end user biosensing system and biosensor.

The Cas9 enzyme has been implemented by Pardee and colleagues in their “toehold switch sensor” for Zika virus detection. This system is based on an engineered oligonucleotide sensor which, upon target recognition, triggers the translation of the LacZ enzyme converting the yellow substrate (chlorophenol red-b-D-galactopyranoside) on a paper disc into a purple product (chlorophenol red).The Zika target was amplified by a nucleic acid sequence-based amplification (NASBA), and the Cas9 was combined with the oligonucleotide sensor for the target detection. This biosensing system was then named NASBACC (NASBA-CRISPR). Thanks to use of Cas9, the NASBACC was able to detect and distinguish two different Zika virus genotypes with a single-base resolution [59].

Another easy to read and portable biosensing system called CASFLA (Cas mediated lateral flow assay) was developed [60] In this case, Cas9 was integrated with a lateral flow assay (FLA) to overcome the typical disadvantages of the nucleic acid-based FLA test, like PCR amplification and tagged amplicon hybridization steps. The authors proposed a highly specific universal FLA platform based on a two-step target recognition by using RCA amplification and Cas9 activity. The target was amplified using a biotinylated primer and the amplicons were reidentified by specific Cas9/sgRNA complex. Then, the Cas9/sgRNA/biotin-target complex was immobilized on a paper substrate containing the streptavidin probes. Positive results could be directly observed by naked eyes, thanks to an engineered universal gold nanoparticle- DNA (AuNP-DNA) probe, which hybridize the sgRNA hairpin loop in the Cas9/sgRNA/biotin-target complex.

Having in mind that the dCas9 shows a recognition activity, R. Hajisan and coworkers immobilized it on transducer to develop a nucleic acids biosensor.

Table 2
Molecular laboratory techniques, portable biosensing systems and biosensors exploiting Type II Cas effectors.

Name	Cas enzyme	Target	Amplification	Transduction method	LOD	Linear range	Detection time	Sample	Year	Ref
DNA-FISH	dCas9	*S.aureus* mecA	/	Fluorescence	10 CFU/mL	$10\text{-}10^7$ CFU/mL	1 h	Genetic DNA or Cell lysate	2017	[56]
CAS-EXPAR	Cas9	ssDNA	EXPAR	Real-Time Fluorescence	0.82 amol		1h	Buffer contains Target	2018	[57]
CRISP-Cas9-SDA-RCA	Cas9(H840A)	*E. coli* 0157:H7	SDA + RCA	Fluorescence mediate by Metal Organic Framework	$4.0 * 10^1$ CFU/ml	$1.3*10^2\text{-}6.5*10^4$CFU/ml	2 h	Spike spring water	2020	[58]
NASBA-CRISP (NASBACC)	Cas9	ika Virus	ASBA	olorimetric	fM		h		2016	[59]
CASLFA	Cas9	ASFV	RPA or PCR	Colorimetric (Lateral flow assay)	150 Copies		40 min	Swine serum	2020	[60]
CRISPR-CHIP	dCas9	DMAS	Amplification free	Field-effector transisotr	1.7 fM		15min	buffer	2019	[61]

They designed a dCa9-graphene-field effect transistor (CRISPR–CHIP), obtaining a surprisingly powerful amplification-free and reagentless biosensor.

This biosensor combined the programmable specificity of dCas9/sgRNA with the high sensitivity of a liquid-gate graphene field effect transistor (gFET). The dCas9 enzyme was functionalized with 1-pyrenebu-tanoic acid (PBA) and connected to the graphene surface by non-covalent interactions (π - π aromatic stacking). The interaction between the Cas9/sgRNA and the gene target led to a change in the graphene channel current. The CRISPR–CHIP was tested for the detection of two distinct mutations occurring in the Duchenne muscular dystrophy (DMAS), but it is suitable for testing with any nucleic acid target gene. This biosensor could open a new way for the CRISPR/Cas9 technology for the development of rapid and specific diagnostic tests [61].

3.2. Type V and VI cas effector

This paragraph explains how Cas effectors belonging to types V (Cas12a) and VI (Cas13a) can be used to develop biosensing systems (

Table 3). Since their discovery, these enzymes immediately attracted attention due to their differences from Cas9.

As reported in section 2, these Cas effectors have a collateral activity consisting in an indiscriminate cleavage of single strand oligonucleotide sequences upon recognition of a specific target (DNA sequence for type V, or RNA in the case of Type VI). If the first enzyme activity is a single turnover, the triggered activity shows a multi-turnover mechanism, which is very useful in the design of biosensing systems. Indeed, the Cas enzyme can recognize its nucleic acid target and then its triggered activity can be used to report this event in an amplified way. Several publications reported the use of these enzymes coupled with different read-out strategies such as fluorescent, colorimetric and electrochemical methods.

In 2017, Zhang and collaborators first discovered the Cas13a collateral activity and demonstrated its possible use for biosensing thanks to a Highly-sensitive Enzymatic Reporter UnLOKing (SHERLOK) system. In this system, the RNA or DNA gene target was amplified and transcripted by RT-RPA + T7 (or RPA-T7) and then recognized by a specific Cas13a/crRNA complex. This triggered the collateral activity and the cleavage of a quenched RNA reporter leading to an increased fluorescence, which was registered in real-time (Fig. 4a).

The sensitivity of the system was similar to digital-droplet polymerase chain reaction (ddPCR) and quantitative PCR (qPCR). SHERLOK was able to distinguish the Zika from the Dengue virus genome in spiked samples of human serum and urine. The system was also tested for the identification of bacterial species and cancer associated mutations, opening a new CRISPR-based diagnostic approach [62].

In the same way, Doudna and coworkers in 2018 discovered the collateral activity of Cas12a and introduced it into the biosensing research, developing a DNA Endonuclease Target CRISPR Trans Report (DETECTR) biosensing system. The Cas12a/crRNA complex was able to detect the DNA target amplified by RPA and the collateral activity was used to cleave a fluorescent-quencher ssDNA reporter (Fig. 4b).

DETECTOR was tested against dsDNA targets of two human papilloma virus types (HPV16 and HPV18), whose presence is associated to cervical cancer, but authors claimed it was capable to detect any dsDNA target [63].

The Cas12a collateral activity was also independently discovered in the same year in China, where Wang's group demonstrated the possible use of this enzyme as bio-recognition element. They combined the Cas12a/crRNA complex and its collateral activity with PCR and a fluorescent-quencher ssDNA reporter, design-

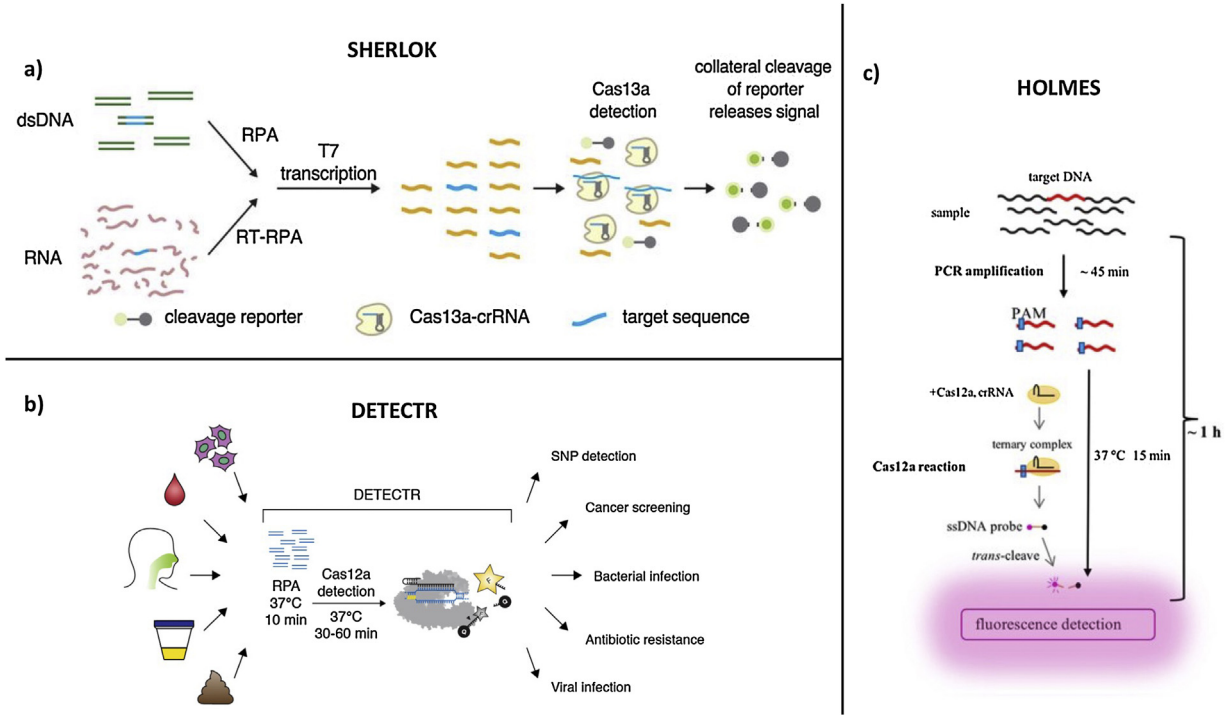

Fig. 4. Biosensing strategy of: a) SHERLOK (reprinted by [62] with permission of The American Association for the Advancement of Science); b) DETECTR (reprinted by [63] with permission of The American Association for the Advancement of Science); c) HOLMES (freely repinted by [64]).

ing a one−HOur Low-cost Multipurpose highly Effector System (HOLMES) (Fig. 4c). HOLMES was tested in genotype detection and proved able to distinguish the homozygous and heterozygous genotypic mutations in human 293 T cells. The authors also demonstrated that the combination of reverse transcriptase PCR (RT-PCR) and the Cas12a/crRNA complex could detect the RNA target of Japanese encephalitis virus (JEV), extending the potential applications of Cas12a as bio-recognition element [64].

These publications showed the potential of Cas13a and Cas12a effectors in biosensing application. Although little time has passed since their first description, further improvements have paved the way towards their implementation in POC devices.

SHERLOCK developers further improved the system, first by heating unextracted diagnostic sample to obliterate nucleases (HUDSON), and then by a multiplex quantitative detection platform SHERLOKv2. HUDSON is a new procedure designed for the direct use of biological samples without extraction of genomic RNA reducing the number of steps to measurement. The combination of HUDSON and SHERLOK was tested to detect the Zika and Dengue virus in saliva and urine samples, and results were compared with RT-PCR [65]. In the case of SHERLOKV2, a multiplex quantitative POC detection device was designed using four Cas/crRNA complexes detecting their targets in a same solution; in this case, the signal was produced by four specific ssDNA fluorescent reporters and read out after a lateral flow assay [66].

The HOLMES approach was also improved by the inventors, and a CRISPR-Cas12a-based method named “Cas12aVDet” and the HOLMESV2 systems popped out. The Cas12aVDET combined the Cas12a collateral activity with RPA in a single reaction unit, diminishing the analysis time from 2 h to 30 min. The use of an ssDNA colorimetric reporter allowed to assess results by naked eyes [67]. HOLMESV2 introduced a new Cas12b enzyme, which was more thermostable than Cas12a. The Cas12b/crRNA complex was combined to LAMP amplification and the possible use this system was demonstrated for the detection of single nucleotide polymorphism SNPs, virus RNA and mRNA from human cell and DNA methylation [68].

Based on previous bio-recognition principles, further steps were taken towards the development of POC devices. Cas13a was integrated in a microfluidic sample treatment device and combined with a portable fluorometric detector for the rapid on-site detection of Ebola virus [69]. Cas12a was also used in a portable fluorimeter to detect a dsDNA target relevant to the African Swine Fever Virus (ASFV) [70], and combined to a colorimetric lateral flow assay to detect a *Pseudomonas aeruginosa* gene target [71].

The big impact of this powerful, selective, flexible and low-cost bio-recognition system in biosensing is also understood by observing the development of rapid and portable molecular tests against the SARS-CoV-2. In fact, the DETECTR system was rapidly adapted to produce a lateral flow assay supporting the management of COVID-19 pandemic. This test is able to detect SARS-CoV-2 target gene from nasopharyngeal swab samples in about 40 min, in contrast to the time-consuming and non-portable traditional techniques [72].

The publications reported so far in this review show the use of Cas effectors as bio-recognition system coupled with a fluorimetric or colorimetric techniques, but these enzymes can also be used to develop electrochemical biosensing systems. In contrast to traditional electrochemical biosensors detecting nucleic acids (E-DNA) [73], based on the hybridization of an amplified target and its complementary strand immobilized on the electrode surface, these new biosensing systems exploit the Cas effector to recognize a specific target in solution and the collateral activity to cleave nucleic acids on the electrode surface.

In 2019, Liu and coworkers first used the Cas12a collateral activity in a labeled electrochemical biosensing system termed electrochemical CRISPR (E-CRISPR). They immobilized a non-specific short ssDNA tagged with a methylene blue redox mediator (MB-ssDNA) on a gold electrode surface, and then used the Cas12a/crRNA complex to recognize the dsDNA target in solution and trigger the collateral activity, leading to cleavage of the MB-

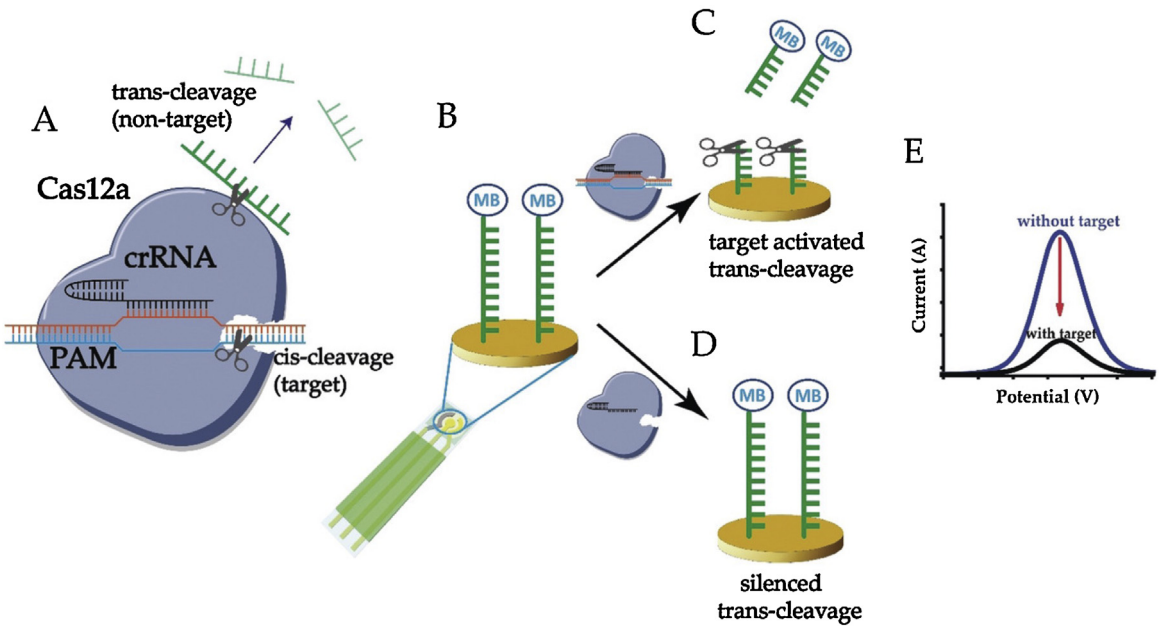

Fig. 5. E-CRISPR detection strategy (reprinted by [74] with permission of Angewandte Chemie International Edition).

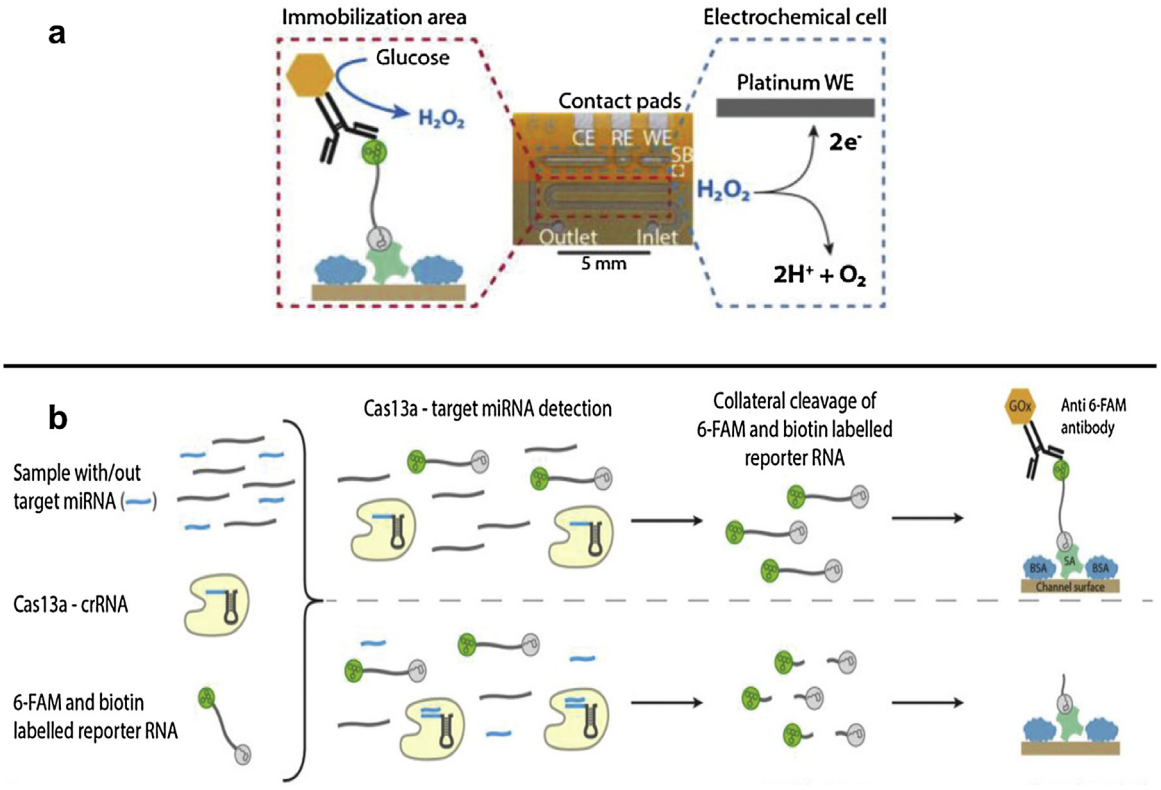

Fig. 6. a) Schematic representation of microfluidic electrochemical biosensors. b) Mechanism of detection mediate by Cas13a. (freely repinted by [77]).

ssDNA on electrode surface. The turn-off of the electrochemical signal was measured by square wave voltammetry (SWV) thanks to the residual MB redox mediator on the electrode surface after recognition (Fig. 5) [74].

In contrast with the E-DNA biosensors, this E-CRISPR electrochemical platform could detect any dsDNA and become a universal platform. Zhang and collaborators demonstrated that hairpin MB-hpDNA reporters on a gold electrode surface achieve lower LODs than linear MB-ssDNA reporters, as they are more accessible to the interaction with the Cas12a/crRNA complex, thus enhancing the efficiency of cleavage from the collateral activity [75].

The Liu's group recently proposed an E-CRISPR based on MB-hpDNA not using the collateral activity to cleave the reporters, but based on the combination of the of MB-hpDNA conformation change and the selective cleavage of Cas12a/crRNA complex. The interaction between ssDNA target in solution and the MB-hpDNA on the electrode surface led to a conformational change and the dsDNA formed was recognized and cleaved by Cas12a/crRNa activ-

Table 3
Molecular laboratory techniques, portable biosensing systems and biosensors exploiting Type V and VI Cas effectors.

Name	Cas enzyme	Target	Organism	Amplification	Transduction method	LOD	Range	Detection time	Sample	Year	Ref
SHERLOK	Cas13a	DNA; RNA	Zika,Dengue,*E.coli,K. pneumo-niae*,Human SNPs	RPA	Fluorescence	2 aM	/	2−3h	Human Urine/Saliva Bacteria culture	2017	[62]
DETECTR	Cas12a	DNA	HPV16,HPV18	RPA	Fluorescence	~aM	/	40−70min	Culture infected human cell	2018	[63]
HOLMES	Cas12a	RNA; DNA	JEV,Pseudorabies virus (PRV)	PCR	Fluorescence	10 aM	/	1h	Culture infected cell	2018	[64]
HUDSON + SHERLOK	Cas13a	RNA	Zika,Dengue	RPA	Fluorescence	2 aM	/	< 2 h	Human Urine/Saliva	2018	[65]
SHERLOKV2	Cas13a Cas12a Cas13b	DNA/RNA	Zika,Dengue,*E. coli,K. pneumoniae* Human SNPs	RT-RPA + T7 or RPA + T7	Fluorescence Later flow Assay	2 aM	/	< 2 h	Human Urine/Saliva Bacteria	2018	[66]
Cas12VDET	Cas12a	DNA	Mycoplasma	RPA	Colorimetric naked eyes	10 aM	/	30 min	Culture cell	2019	[67]
HOMESV2	Cas12b	DNA/RNA	JEV,Pseudorabies virus (PRV),Human SNPs, DNA methylation	LAMP	Fluorescence	~10 aM	/	1 h	Culture infected cell	2019	[68]
Microfluidic Ebola virus detection	Cas13a	RNA	Ebola	No	Microfluidic chip; Portable fluorimeter	20 pfu/mL	Linear 20−2000pfu/mL	5 min	Virus Stock	2019	[69]
Cas12a fluorescent based point of care system	Cas12a	DNA	ASFV	No	Portable fluorimeter	1 pM	Linear1−100pM	2 h	Synthetic DNA gene B646L	2020	[70]
CIA	Cas12a	DNA	*P, aeuroginosa*	LAMP	Colorimetric Lateral flow assay	1 cfu/mL	/	50min	Recombinant plasmid E.coli	2020	[71]
RT/LAMP Cas12a DETECTR	Cas12a	RNA	SARS-CoV-2	RT-LAMP	Lateral flow assay	10 copies/ul		40	Nasopharyngeal swab	2020	[72]
E-CRISPR	Cas12a	DNA	HPV16	No	SWV	50pM	Dynamic~(pM-uM)	/	Amplified ssDNA Spike sample	2020	[74]
CRISPR/Cas12a-Mediated Interfacial Cleaving of Hairpin DNA Reporter for Electrochemical Nucleic Acid Sensing	Cas12a	DNA	HPV16, HPV18	No	DPV	30pM	Dynamic 50pM-100nM	60	Amplified ssDNA Spike sample	2020	[75]
Surpassing the detection limit and accuracy of the electrochemical DNA sensor through the application of CRISPR Cas systems	Cas12a	DNA	Parvovirus B19	No	SWV	10fM	Dynamic fM-uM	>60	Amplified ssDNA Spike sample	2020	[76]
CRISPR/Cas13a-Powered Electrochemical Microfluidic Biosensor for Nucleic Acid Amplification-Free miRNA Diagnostics	Cas13a	RNA	miR-19B	No	Amperometric	10pM	Dynamic 0,1−1000pM	<4h	Serum from children	2019	[77]

ity, causing a current signal change. This new strategy was more accurate and had a lower LOD than the E-CRISPR [76].

Cas13a has also been used for electrochemical biosensing. Bruch and collaborators designed a microfluidic device including an electrochemical biosensor exploiting CRISPR/Cas13a for the detection of miRNA without amplification. This microfluidic chip included a channel with an immobilization area, whose surface was functionalized with streptavidin and bovine serum albumin as blocking agent, and an electrochemical cell containing working, reference and counter electrodes (Fig. 6a). The Cas13a/crRNa complex was incubated with a sample containing the miRNA target and a 6-fluorescein amidate (6FAM)-biotin-ssRNA label. After the recognition step, the solution was added with anti-fluorescein antibodies coupled to Glucose Oxidase (GOx) and pushed through the channel. The biotin-6Fam-ssRNA reporters interacted with the streptavidin, and the uncut reporters bound the antibodies (Fig. 6b). The collateral activity of Cas13a/crRNa complex was registered by amperometric technique adding a glucose enzymatic substrate. So, the signal was proportional to the reduction H_2O_2, produced by GOx enzymatic catalytic activity, and inversely proportional to the miRNA concentration in the sample [77] (Table 3).

4. Conclusion and perspectives

The discovery of the CRISPR/Cas immune system and the characterization of Cas enzymes is revolutionizing gene editing and molecular diagnostics, leading to an impressive series of fast evolving applications. Cas effectors are classified into two main classes and six different types based on their characteristics. Cas9, Cas12a, Cas13a, which belong to the second class and to I, V, VI types, respectively, have become effective biorecognition elements used in many biosensing systems due to their high selectivity and specificity towards nucleic acids. These key features may allow to overcome some critical limitations in the isothermal amplification used in the molecular tests by POC devices and make their performances comparable to those of PCR based techniques. Although Cas9 has been mainly used in gene editing applications, its modification in a deactivated form (dCas9) is particularly interesting for bio-recognition due to specificity, so that this “antibody like” mechanism originated to a new generation of CRISPR based diagnostic devices (CRISPR−CHIPs). The multi-turnover collateral activity of Cas12a and Cas13a has been exploited to amplify a target recognition event, with systems such as SHERLOK, DETECTR and HOLMES pioneering the field of novel nucleic acid detection systems. Since their first publications, the number of related works is exponentially growing together with the increasing awareness of the scientific community concerning the Cas endonuclease potential.

The CRISPR technology potential beyond the scientific community has been demonstrated in the actual Covid-19 pandemic scenario, where the need for the development of new molecular, portable and rapid tests, has accelerated the entry of CRISPR-based devices in the POC market. Indeed, the SHERLOK and DETECTR systems described in section 3.2, have been rapidly adapted and modified to detect the SARS-CoV-2 coping with the diagnostic market requirements and these tests are now being commercialized by SHERLOK Biosciences and Mammoth Bioscience respectively.

We believe that the combination of the Cas endonuclease ability in target detection and the latest transducer technology could irreversibly revolutionize the new POC molecular diagnostics market. Furthermore, the discovery of new Cas effectors in the near future may add further tiles to the multifaceted puzzle of bio-recognition elements.

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.

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