Trends in Biotechnology

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Review

CRISPR/Cas Systems towards Next-Generation Biosensing

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Beyond its remarkable genome editing ability, the CRISPR/Cas9 effector has also been utilized in biosensing applications. The recent discovery of the collateral RNA cleavage activity of the Cas13a effector has sparked even greater interest in developing novel biosensing technologies for nucleic acid detection and promised significant advances in CRISPR diagnostics. Now, along with the discovery of Cas12 collateral cleavage activities on single-stranded DNA (ssDNA), several CRISPR/Cas systems have been established for detecting various targets, including bacteria, viruses, cancer mutations, and others. Based on key Cas effectors, we provide a detailed classification of CRISPR/Cas biosensing systems and propose their future utility. As the field continues to mature, CRISPR/Cas systems have the potential to become promising candidates for next-generation diagnostic biosensing platforms.

Highlights

CRISPR/Cas biosensing systems transfer the sequence information of target nucleic acids to detectable signals such as fluorescence and colorimetric values.

CRISPR/Cas biosensing systems are versatile platforms for nucleic acid detection that can be used for pathogen detection and genotyping, cancer mutation detection, and single nucleotide polymorphism (SNP) identification.

The biosensing methods employing these Cas effectors rely on the collateral cleavage activities of Cas13 and Cas12.

CRISPR/Cas biosensing allows highly sensitive, specific, rapid, cost-efficient, and multiplex detection of target nucleic acids, and support point-of-care use without the need for technical expertise and complicated equipment.

CRISPR/Cas System Coming into Biosensing Applications

Molecular diagnostics is critical for life sciences, biosecurity, food safety, and environmental monitoring [1,2]. Detection of nucleic acids is a major molecular diagnostic practice that has been continuously growing in the past few decades. Besides various PCR-based nucleic acid detection methods, different isothermal amplification and nucleic acid hybridization methods have also been established [3,4]. However, most of the existing techniques have trade-offs in performance metrics such as sensitivity and specificity. They normally require sophisticated systems and tedious sample/reagent treatment, which relies on well-established laboratories with dedicated instruments or well-trained operators. The incidence of newly emerged or re-emerging infectious diseases, global antimicrobial resistance, and food and environmental contamination continues to increase, especially in underdeveloped countries or resource-limited regions. Therefore, there is an urgent need to develop novel nucleic acid detection technologies with high sensitivity and specificity, especially ones that can be used for rapid and versatile point-of-care (POC) diagnostic applications.

CRISPR/Cas systems (see Glossary) have taken center stage in biotechnology since the modified CRISPR/Cas9 system was applied for gene editing in mammalian genomes [5–8]. This system has thereafter been seen as a revolutionary gene editing toolbox and could be described as a 'magic wand' after being expanded to almost all genomic targets [9–11]. **Nuclease-deactivated Cas9 (dCas9)** and its fusion proteins have also been applied in gene regulation, epigenetic engineering, genome imaging, genetic screening, and other areas [12,13]. Furthermore, Cas9 has recently demonstrated its 'magic power' in biosensing applications. Combining Cas9 cleavage activity and nucleic acid amplification, approaches such as CRISPR/Cas9 triggered isothermal exponential amplification reaction (CAS-EXARP) and nucleic acid sequence-based amplification (NASBA)-CRISPR cleavage (NASBACC) can be used to genotype pathogens and differentiate single nucleotide polymorphisms (SNPs)

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[14,15]. Advanced studies on bacterial adaptive immune systems continue to push the boundary of understanding mechanisms of various CRISPR/Cas systems and discovering new *cas* genes with new enzymatic activities, perhaps thanks to the spotlight on the CRISPR/Cas9 toolbox. In 2016, a comprehensive study of the type VI CRISPR/Cas system demonstrated a 'collateral cleavage' activity (Box 1) of Cas13a after its target-specific cleavage [16]. This collateral activity was further harnessed to cut labelled RNA reporters for detection of target nucleic acids, which may come from bacteria, viruses, or eukaryotic cells [17]. Since then, a brand-new field has developed using CRISPR/Cas systems as novel biosensing platforms for nucleic acid detection, with inherent ultra-high specificity and sensitivity.

Figure 1 illustrates the timeline for development of CRISPR/Cas biosensing technology, which is now considered as an innovative approach towards next-generation diagnostics, simultaneously satisfying a variety of detection criteria. This technology has the potential to significantly affect the field of biosensors by offering a much more rapid and precise method for ultrasensitive nucleic acid detection. Even CRISPR/Cas-based deployable POC paper devices may be possible [18,19]. Due to the great potentials offered by CRISPR/Cas-based biosensing, several articles have been published as reviews, news, comments, or insights on **CRISPR diagnostics** since 2017 [20–27], which provided general information on the partially existing CRISPR/Cas systems without detailed classification and discussion. Herein, besides classifying current CRISPR biosensing systems and introducing their applications with underlying principles, we present a comprehensive comparison of their pros and cons, and propose future perspectives for CRISPR/Cas systems in biosensing applications.

Classification of CRISPR/Cas Biosensing Systems

In a CRISPR/Cas system, a pre-CRISPR RNA (crRNA) is transcribed from the CRISPR array and further processed to yield the mature crRNA, which serves as the guider to navigate the Cas effectors. The Cas effectors, which can be formed by either a single protein or a complex of proteins, are the enzymatic units possessing the target-dependent cleavage activity. Among the established CRISPR/Cas-based nucleic acid biosensing systems, the fundamental difference remains in implementing different Cas effectors (Cas9, Cas13, and Cas12), although the combination of different components is observed. The currently reported CRISPR/Cas biosensing systems can be classified into three groups based on the different Cas effectors. A comparison of major characteristics for these three classes is listed in Table 1 (Key Table). Box 1 introduces the mechanism of each system for a systematic understanding of their basic principles, which is critical for further modifying or improving the performance of CRISPR/Cas biosensing systems, or even developing new systems in the future. Three key parts (signal

Box 1. Distinct Principles of CRISPR/Cas-based Biosensing Systems

The Cas9 effector is famous for its ability to edit genomes by cleaving target dsDNA under the guidance of a single guide RNA (sgRNA). When combined with other technologies, CRISPR/Cas9 can be harnessed to create a biosensing system. Unlike Cas9, nuclease-deactivated Cas9 (dCas9) retains the ability to bind target dsDNA, which can be fused with various modules such as a split fluorescent protein or a split enzyme to develop a CRISPR biosensing system. Binding of dCas9 to target nucleic acids allows the re-integration of the split fluorescent protein or enzyme, generating signals for convenient detection.

Both Cas12- and Cas13-based biosensing methods depend on the collateral effects of these Cas effectors. After the formation of a ternary complex of Cas12, crRNA (or sgRNA) and target nucleic acid, the collateral ssDNA reporter is *trans*-cleaved to small fragments. Similarly, the collateral cleavage activity of Cas13 against the ssRNA reporter was triggered by the guide RNA and target RNA. The ssDNA (or ssRNA) reporter can either be labelled with fluorescence and quencher or biotin-FAM, and dissociation of the reporter can be conveniently detected by either a fluorescence reader or colorimetric change in a paper lateral flow assay.

Glossary

CRISPR/Cas system: a type of unique genomic element originally discovered in bacteria and archaea, serving as an adaptive immune system to defend the invasion of phage or other foreign nucleic acids. The system comprises a short repeated DNA array called the clustered regularly interspaced short palindromic repeats (CRISPR) and a sort of CRISPR-associated proteins (Cas) expressed by *cas* genes.
CRISPR diagnostics: an approach to molecular diagnostics that employs Cas effectors. For example, Cas13-based SHERLOCK and Cas12-based HOLMES or DETECTR rapidly detect target nucleic acids with high sensitivity, specificity, and simplicity and low cost. CRISPR diagnostics has been successfully applied in pathogen detection and genotyping, single nucleotide polymorphisms (SNPs) discrimination, and cancer mutation detection.
Nuclease-deactivated Cas9 (dCas9): Cas9 with mutations in the cleavage domains (D10A in the RuvC domain and H840A in the HNH domain) that loses ability to cleave target dsDNA. However, under the guide of sgRNA, dCas9 still retains the ability to specifically bind to target dsDNA. dCas9 has now been widely used in transcriptional regulation, epigenetic studies, and genomic imaging and has also been demonstrated as an effective biosensing platform for pathogen detection and genotyping.
Protospacer-adjacent motif (PAM) sequence: a short DNA sequence immediately following the DNA sequence targeted by the CRISPR effectors; it is critical for some CRISPR effectors to bind and cleave target dsDNA sequences. For example, SpCas9 effector recognizes the PAM sequence of 5′-NGG-3′, and the PAM sequence of LbCas12a is 5′-TTTN-3′. For RNA-targeting and RNA-cleaving Cas effectors, the protospacer flanking site (PFS), instead of the PAM sequence, is necessary for target RNA binding and cleaving.

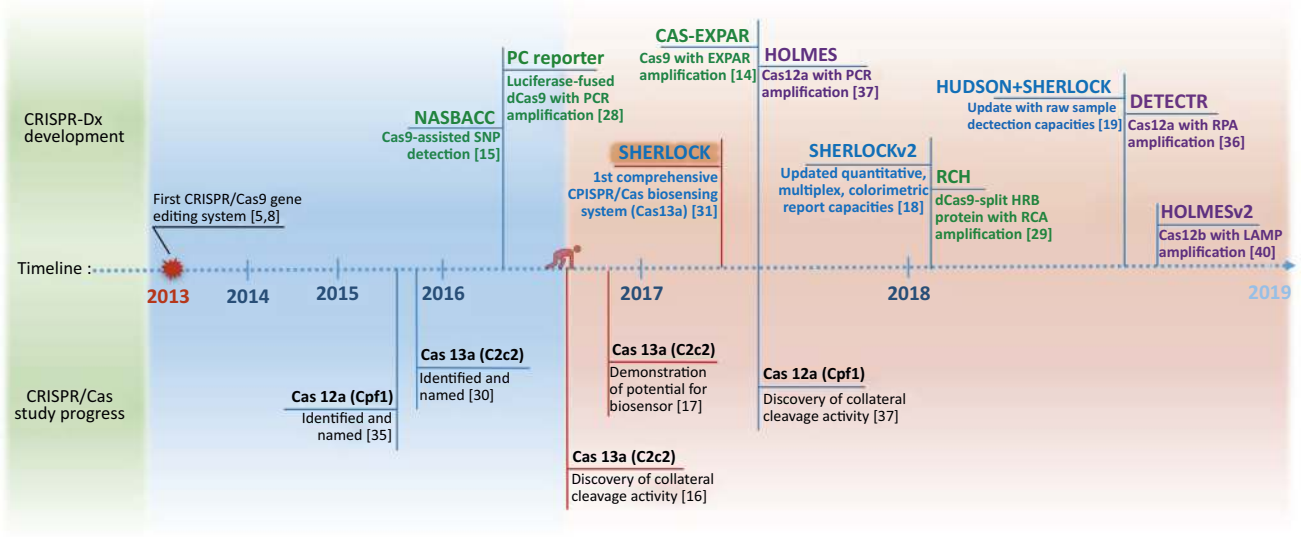

Figure 1. The Development of CRISPR/Cas Biosensing Technology. The timeline is based on the original publication date. Since the CRISPR/Cas9 system was first recognized as a novel biotech tool for gene editing, more Cas effectors (Cas12a, Cas13a, etc.) have been discovered. The attempts to harness the site-specific cutting or binding capability of (d)Cas9 have recently resulted in several *in vivo* biosensing systems. However, the era of CRISPR/Cas-based biosensing began with the discovery of Cas13a's collateral cleavage activity in 2016, and the first comprehensive and applicable system was not developed until the release of SHERLOCK system in 2017. Since then, different systems have been established within the past 2 years, reflecting a rapid development of this newly emerged biosensing technology. Green: Cas9-based system; blue: Cas13-based system, purple: Cas12-based system. (See [5,8,14–19,28–31,35–37,40]). dCas9, Nuclease-deactivated Cas9; HOLMES, one-hour low-cost multipurpose highly efficient system; HRP, horseradish peroxidase; HUDSON, heating unextracted diagnostic samples to obliterate nucleases; LAMP, loop-mediated isothermal amplification; NASBACC, nucleic acid sequence-based amplification-CRISPR cleavage; PC, paired dCas9; RCA, rolling circle amplification; RCH, RCA-CRISPR-split-HRP; RPA, recombinase polymerase amplification; sgRNA, single guide RNA; SHERLOCK, specific high sensitivity enzymatic reporter unlocking; SNP, single nucleotide polymorphism.

amplification, signal transducing, and signal reporting) are included for comparing different CRISPR/Cas sensing systems in this review.

(d)Cas9 Effector-based CRISPR/Cas Biosensing Systems

Cas9 cleavage can be combined with nucleic acid amplification to detect specific nucleic acid sequences, which can be used for genotyping pathogens and discriminating SNPs. For example, Pardee and colleagues achieved the remarkable result of successfully integrating NASBA with a Cas9 effector for detecting and genotyping Zika virus [15]. Based on their paper-based isothermal toehold switch biosensing system, this CRISPR/Cas9 approach was able to enhance the specificity to single-base resolution. Specifically, a trigger sequence of the toehold sensor was added to the amplification products after NASBA amplification. Then, a single guide RNA (sgRNA) was designed, with a strain-specific **protospacer-adjacent motif (PAM) sequence** covering an SNP that exists only in the genome of American Zika but not African Zika. With the presence of the sgRNA, Cas9 was only able to cleave the amplification products from American Zika strain; thus American Zika failed to initiate the toehold activation because the trigger sequence was lost after Cas9 cleavage. The method was employed for discrimination of distinct Zika genotypes and described as NASBACC [15] (Figure 2A).

Recently, Huang and colleagues developed a CAS-EXPAR that is capable of detecting a DNA target with attomolar (aM) sensitivity and single-base specificity within 1 h [14]. This system has been optimized for amplification efficiency, sensitivity, and specificity, and then successfully verified for its capability of detecting DNA methylation and *Listeria monocytogenes hly* mRNA. By designing a

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Key Table

Table 1. Major Characteristics for Classified CRISPR/Cas-based Biosensing Systems[a]

Classification	System name	Effector[b]	Signal Amplification	Sensitivity[c]	Specificity	Quantitative	Multiplex	Readout[d]	Time	Sample type	Target type	Sensing environment	Refs
Cas9-based class	RCH	Sp-dCas9	RCA	fM	1 nt	Y[e]	N	C (TMB)	<4 h	Pretreated/ raw	RNA	*in vitro*	[29]
	NASBACC	SpCas9	NASBA	fM	1 nt	N	N	C (chlorophenol red-β-D-galactopyranoside)	~3 h	Pretreated	RNA	*in vitro*	[15]
	PC reporter	Sp-dCas9	PCR	One copy	NA	N	N	B (Luciferase)	10 min after PCR	Pretreated	DNA	*in vitro*	[28]
	CAS-EXPAR	SpCas9	EXPAR	aM	1 nt	N	N	F (SYBR Green I)	<1 h	Pretreated	DNA/ RNA	*in vitro*	[14]
Cas13-based class	SHERLOCK	LwCas13a	RPA	aM	1 nt	N	N	F (FAM)	2–5 h	Pretreated	DNA/ RNA	*in vitro*	[31]
	SHERLOCKv2[f]	CcaCas13b PsmCas13b LwaCas13a	RPA	zM	1 nt	Y	Y	F (FAM, TEX, Cy5, HEX); C (Gold-NP, anti-FAM Abs)	0.5–3 h	Pretreated	DNA/ RNA	*in vitro*	[18]
	HUDSON + SHERLOCK	LwCas13a	RPA	aM	1 nt	N	N	F (FAM); C (Gold-NP, anti-FAM Abs)	<2 h	Raw	DNA/ RNA	*in vitro*	[19]
Cas12-based class	HOLMES	LbCas12a	PCR; RT-PCR	aM	1 nt	N	N	F (HEX)	~1 h	Pretreated/ raw	DNA/ RNA	*in vitro*	[37,39]
	DETECTR	LbCas12a	RPA	aM	6 nt[g]	N	N	F (FAM)	~2 h	Pretreated	DNA	*in vitro*	[36]
	HOLMESv2	AacCas12b	LAMP; RT-LAMP; Asymmetric PCR	aM	1 nt	Y	N	F (HEX, FAM)	~1 h	Pretreated/ raw	DNA/ RNA	*in vitro*	[40]

[a]All features were claimed by the original publications; zM, 10^{-21} M or zeptomole/l; aM, 10^{-18} M or attomole/l; fM, 10^{-15} M or femtomole/l; N, no; NA, not applicable; nt, nucleotide; Y, yes.
[b]Sp-dCas9 = *Streptococcus pyogenes* dCas9; SpCas9 = *S. pyogenes* Cas9; LwaCas13a = *Leptotrichia wadei* Cas13a; CcaCas13b = *Capnocytophaga canimorsus* Cc5 Cas13b.
[c]Only the highest sensitivity was shown in the table. The sensitivity for each system before amplification was not provided thoughtfully. Before RPA, the level of sensitivity reported in SHERLOCK system was ~50 pM; HOLMES and HOLMESv2 systems reported a level of ~100 pM sensitivity before nucleic acid amplification. PsmCas13b = *Prevotella* sp. MA2016 Cas13b; LbCas12a = *Lachnospiraceae bacterium* ND2006 Cas12a; AacCas12b = *Alicyclobacillus acidoterrestris* Cas12b.
[d]B, Bioluminescent; C, colorimetric; F, fluorescent.
[e]Scale level quantitative results achieved.
[f]For maximum four-multiplex purpose in SHERLOCKv2, AsCas12a effector has also been applied as one component of the SHERLOCKv2 system.
[g]Differentiation of HPV on the basis of 6-nt difference was reported in DETECTR and no better resolution was reported later.

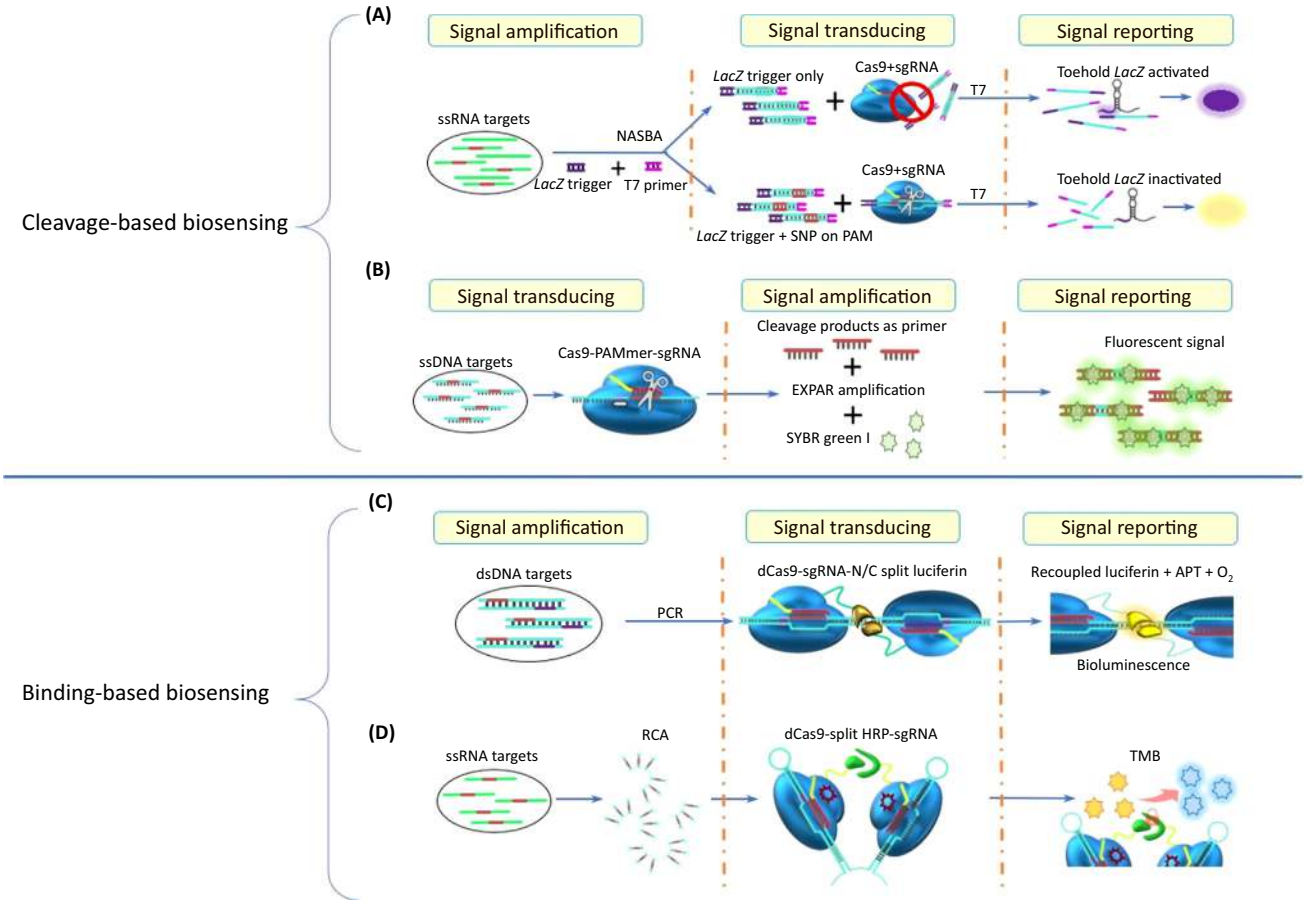

Figure 2. Schematics for Cas9-based Biosensing Systems. Cas9-based biosensing systems can be classified into two groups: harnessing the specific target cleavage activity of Cas9 effector or combining the target-specific binding activity of dCas9 effector with split signal transducing proteins. (A) NASBACC system. The RNA targets are reverse transcribed by the NASBA amplification system, generating a *lacZ* coding sequence that can be activated by a toehold switch. Cas9 then works as an assistant sensor to discriminate base variations in the target sequences. Specifically, strain-specific base differences are deliberately designed to be located within the PAM region, leading to distinct cleavage behavior by Cas9 to remove *LacZ* sequence. Therefore, only target sequences with PAM can be cleaved, and other sequences will retain integrity and can be activated to initiate the *LacZ* colorimetric reaction, generating signals. (B) CAS-EXPAR system. Primers are first generated by cleavage of the target ssDNA by Cas9, employing a specifically designed PAM sequence that is complementary to the target ssDNA and is called 'PAMmer'. Then, primers can hybridize with the EXPAR templates and the amplification performs cyclically, generating dsDNA for signal reporting by a real-time fluoresces monitoring method. (C) PC reporter. After PCR amplification, two closely located sequences can bind to two dCas9 effectors fused with the N and C terminal halves of the luciferase, which can lead to the recoupling of integrity luciferase enzyme that can generate the bioluminescence signal with APT and O_2 presented. (D) RCH system. Deactivated Cas9 effectors fused with split HRP protein have been engineered for activating the colorimetric change of TMB after recognizing and binding to the target. dCas9, Nuclease-deactivated Cas9; dsDNA, double-stranded DNA; HRP, horseradish peroxidase; NASBACC, nucleic acid sequence-based amplification-CRISPR cleavage; PAM, protospacer-adjacent motif; PC, paired dCas9; RCA, rolling circle amplification; RCH, RCA-CRISPR-split-HRP; ssDNA, single-stranded DNA; TMB, tetramethylbenzidine.

separate antisense PAM-presenting oligonucleotide (PAMmer) to activate CRISPR/Cas9 site-specific cleavage targeting single-stranded DNA (ssDNA), the downstream EXPAR system was able to harness these cleavage products as its own primers and initiate the isothermal amplification, generating a large amount of target amplified double-stranded DNA (dsDNA) fragments that could be bound by SYBR Green I for fluorescence signal readout [14] (Figure 2B).

In addition to harnessing the enzymatic cleavage activity of Cas9 effector, the enzymatically deactivated Cas9 effector (dCas9) with sequence-specific binding capability has also been

employed to develop several new CRISPR/Cas-based nucleic acid biosensing systems, which are similar to conventional *in situ* hybridization methods using transcription activator-like effectors (TALE) or zinc finger proteins [21]. One example is the paired dCas9 (PC) reporter system to detect *Mycobacterium tuberculosis* DNA [28]. The N and C terminal halves of the firefly luciferase enzyme were separately fused with two dCas9, which were then guided by a pair of sgRNAs complementary to the upstream and downstream segments of a target DNA sequence, respectively. If the target DNA existed, it could be amplified by PCR. The products of PCR could then be bound by a pair of dCas9 effectors in proximity, generating a reconstituted luciferase with luminescent signals for convenient detection (Figure 2C). Another example is the rolling circle amplification (RCA)-CRISPR-split-HRP (RCH) system for miRNA detection [29]. RCA is first recruited for isothermal miRNA target amplification, and then two engineered dCas9 effectors fused with half of the split horseradish peroxidase (HRP) protein, are applied to bind to the amplified target sequences. dCas9 effectors binding to nearby target sequences lead to the reconstitution of split HRP, which can generate a colorimetric signal readout by adding the chromogenic substrate tetramethylbenzidine (TMB) at the last step [29] (Figure 2D).

Cas13 Effector-based CRISPR/Cas Biosensing Systems

Within the CRISPR/Cas family, Cas13a (previously called C2c2) is an RNA-guided RNase belonging to the type VI CRISPR/Cas system [30]. Cas13a, containing two higher eukaryotes and prokaryotes nucleotide-binding (HEPN) domains to fulfil its crRNA guided single-stranded RNA (ssRNA) cleavage activity, was discovered to possess the target RNA triggered 'collateral cleavage' activity in 2016 [16]. After further exploring this activity, Gootenberg and coworkers developed the first comprehensive and applicable CRISPR/Cas13a-based nucleic acid detection system with a selected robust Cas13a from *Leptotrichia wadei* (LwCas13a) [31]. The method, called SHERLOCK (specific high sensitivity enzymatic reporter unlocking), is capable of achieving aM sensitivity for detecting both DNA and RNA targets with single-base resolution. This CRISPR/Cas13a biosensing system has been successfully applied for detecting Zika and dengue viruses, bacterial isolates, antibiotic resistant genes, human DNA genotypes, and cancer mutations [31]. Later, another Cas effector, Cas13b, which is an RNA-guided RNase from CRISPR type VI-B, was also found to possess collateral RNA cleavage activity and was used in SHERLOCKv2 [18].

Figure 3 illustrates a schematic of Cas13-based biosensing systems. After the captured targets are cleaved by Cas13-crRNA complex, the collateral cleavage of the complex is activated, and site-independent ssRNA will be cut hundreds to thousands of times, suggesting its inherent signal amplification potential [17,32]. This promiscuous cleavage activity was then used for signal reporting by applying either ssRNA quenched florescent or colorimetric reporters [18,19,31]. For the SHERLOCK system, recombinase polymerase amplification (RPA) was applied first to boost the sensitivity from pM to aM concentration [31]. This SHERLOCK system has been successfully used to detect both DNA and RNA by coupling RPA or reverse transcription-RPA (RT-RPA) and then by T7 transcription [31] (Figure 3A). For SHERLOCKv2, the updated version of SHERLOCK, a 3.5-fold increased sensitivity was achieved by combining Cas13a with Csm6, an auxiliary type III CRISPR effector nuclease [33,34] capable of coupling its reporter signal with Cas13a for signal boosting (Figure 3B). Diluted isothermal amplification primers were used for nonsaturated reaction, leading to relatively quantitative results (Figure 3C). A portable paper lateral flow assay (LFA) strip was created by applying an FAM-biotin reporter with anti-FAM antibody and gold nanoparticle (Figure 3D). In addition, four-channel multiplex detection has also been achieved by screening three orthogonal Cas13 effectors with different preferences in the dinucleotide cleavage motifs (LwaCas13a to AU, PsmCas13b to GA, CcaCas13b to UC), along with applying an AsCas12a effector for cutting its

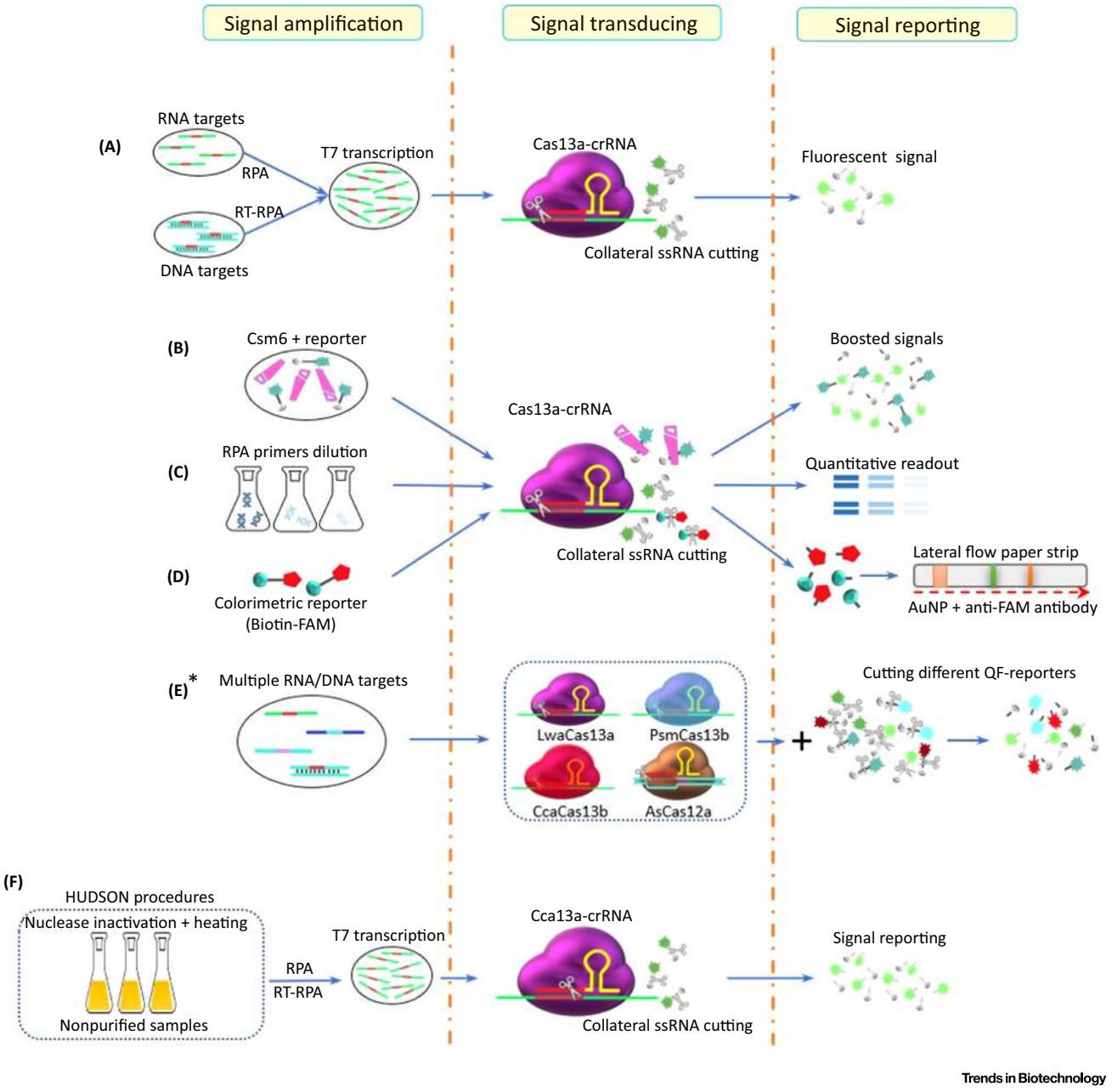

Figure 3. Schematics for Cas13-based Biosensing Systems

For a Figure360 author presentation of Figure 3, see the figure legend at https://doi.org/10.1016/j.tibtech.2018.12.005

(A) The first SHERLOCK system. (B) SHERLOCK system with Csm6 sensitivity boosting. (C) Quantitative SHERLOCK system. (D) SHERLOCK system combining colorimetric reporter for visualized readout. (E) Four-channel multiplex detection of SHERLOCK system. (F) SHERLOCK system combing with HUDSON for eliminating the nucleic acid extraction and purification procedures. *In four-channel multiplex target detection. A Cas12a effector was used. crRNA, CRISPR RNA; HUDSON, heating unextracted diagnostic samples to obliterate nucleases; RPA, recombinase polymerase amplification; RT, reverse transcription; SHERLOCK, specific high sensitivity enzymatic reporter unlocking; ssRNA, single-stranded RNA.

own specially designed ssDNA reporters [18] (Figure 3E). Later on, the heating unextracted diagnostic samples to obliterate nucleases (HUDSON) protocol, introducing heat and chemical treatment procedures for viral particle lysis and ribonuclease deactivation, was added to the SHERLOCK sample treatment step for detecting viral nucleic acid directly from clinical samples without DNA/RNA extraction and purification [19] (Figure 3F).

Cas12 Effector-based CRISPR/Cas Biosensing Systems

In addition to Cas13, the class II type V-A Cas12a (previously called Cpf1) effector was also found to possess collateral cleavage activity (or *trans*-cleavage activity). However, different from Cas13 effectors, Cas12a effectors target DNA and *trans*-cleave collateral ssDNA [35–38]. The formation of a ternary complex of Cas12/gRNA/target DNA is required for the collateral cleavage activity, cleaving any collateral ssDNA by the RuvC pocket [38]. The first report of the Cas12a *trans*-cleavage activity, as well as its usage in nucleic acid detection (one-hour low-cost multipurpose highly efficient system or HOLMES), was patented in China in 2017 [37]. Later, the same group published two peer-reviewed papers describing the details of these findings [38,39]. During the same period, another American research group independently discovered the Cas12a-mediated *trans*-cleavage activity and invented a similar Cas12a-based nucleic acid sensing method, named as DNA endonuclease-targeted CRISPR *trans* reporter (DETECTR) [36].

HOLMES can detect DNA/RNA viruses and distinguish virus genotypes and human SNPs from either cell lines or clinical samples within 1 h with an aM sensitivity [38]. Similarly, DETECTR has been used to detect viruses and discriminate human papillomavirus (HPV) genotypes in either virus-infected human cell lines or clinical patient samples [36].

The class 2 type V-B CRISPR/Cas12b has also demonstrated the same DNA *trans*-cleavage activity, which was reported in the above-mentioned patent [37] and also a publication by Chen and coworkers [36]. Recently, Li and colleagues employed Cas12b to create an improved version of HOLMES (called HOLMESv2) [40]. Having a wide range of reaction temperature for *trans*-cleavage, Cas12b can be combined with various isothermal amplification methods, such as loop-mediated isothermal amplification (LAMP). HOLMESv2 is a one-pot nucleic acid detection system that supports quantitative determination of target DNA. In HOLMESv2, Bst 3.0 DNA polymerase containing 5′ to 3′ DNA polymerase activity with either DNA or RNA templates was applied to directly amplify RNA targets for a simplified RNA detection protocol [40]. It is the simplest CRISPR biosensing system for RNA detection so far.

Similar to Cas13-based biosensing systems, three Cas12 biosensing methods mentioned previously require target nucleic acid amplification to increase the detection sensitivity to aM concentrations. In addition to published examples of PCR, LAMP, and RPA, Cas12- and Cas13-based systems can be combined with other nucleic acid amplification methods [14,15,28,29], depending on the specific application. Cas12a *trans*-cleaves collateral ssDNA molecules at a rate of approximately 250 per second, and this process has greatly boosted the signal intensity for a faster outcome [36,37,39,40] (Figure 4). Similar to SHERLOCK, the ssDNA reporters in HOLMES or DETECTR can either be labelled with paired fluorescence/quencher for fluorescence detection [36,37,39,40] or combined with colorimetric strategies for naked-eye readout in LFA.

Pros and Cons of Different CRISPR/Cas Biosensing Systems

Most of these introduced CRISPR/Cas biosensing systems have advantages of simplicity to develop/re-develop, ultra-high resolution to single-base variation, at least fM or mostly aM concentration sensitivity, and no need for dedicated instruments. Additionally, these nucleic

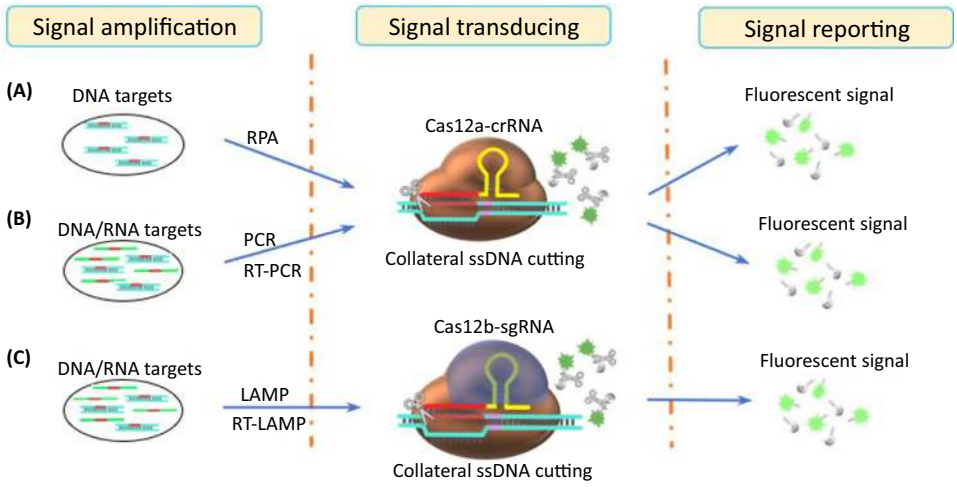

Figure 4. Schematics for Cas12-based Biosensing Systems. The Cas12a effector can both bind and cut ssDNA and dsDNA site-specifically, but the collateral cleavage remains only for ssDNA, indicating that an ssDNA reporter is needed. (A) DETECTR system. (B) HOLMES system. (C) HOLMESv2 system. All three systems have a similar schematic but varying amplification strategies and choice of Cas effectors. Fluorescent reporters can be changed according to individual needs. crRNA, CRISPR RNA; DETECTR, DNA endonuclease-targeted CRISPR *trans* reporter; HOLMES, one-hour low-cost multipurpose highly efficient system; LAMP, loop-mediated isothermal amplification; RPA, recombinase polymerase amplification; RT, reverse transcription; sgRNA, single guide RNA; ssDNA, single-stranded DNA.

acid biosensing systems have the potential to satisfy various needs of POC and field deployment diagnostics, as they can be modified to work on a variety of *in vitro* mediums efficiently and robustly. Their tolerance to different untreated samples and extremely low cost (<$0.1/reaction for reagents) make them extremely suitable for large-scale screening tasks in resource-limited settings, first-line laboratories, or clinical environments [14,17–19,29,31,36,39,40]. Table 2 illustrates the features of each type of CRISPR/Cas-based biosensing system matched with different criteria of biosensing performance. Nevertheless, there are still limitations for each CRISPR/Cas biosensing system.

Sequence Limitation

CRISPR effectors such as Cas9 and Cas12, guided by CRISPR RNA, are able to recognize and cleave at any desired position, but they require PAM sequences adjacent to the target dsDNA [e.g., NGG for *Streptococcus pyogenes* Cas9 (SpCas9) and TTTN for *Lachnospiraceae bacterium* ND2006 (LbCas12)]. For most cases, such as pathogen detection, it is easy to find candidates for target sequences with suitable PAM sequences; however, there could be fewer choices when performing SNP-based discrimination and other short sequence detection, where the requirement of specific PAMs for each Cas effector may be hard to satisfy. For example, the Cas9-based NASBACC method requires a mutation in the PAM sequence to precisely distinguish the pathogen genotypes [15], which may limit its wider applications. To solve these problems, Li and coworkers invented a method to introduce the PAM sequence into the amplified products of PCR or LAMP by using PAM-containing primers in both HOLMES and HOLMESv2 [39,40], enabling HOLMES and HOLMESv2 to detect nucleic acids in a PAM-independent way. Moreover, the length of guide sequences, as well as the position of the mutation site within the guide sequences, can remarkably affect the signal-to-noise ratio in Cas12-based biosensing for discrimination of single-base differences [39,40]. Similarly, the **protospacer flanking site (PFS)** also affects the efficacy of Cas13a-mediated target

Table 2. Comparison of Different CRISPR/Cas Biosensing Systems in Fulfilling Biosensing Purposes

Category			
Criteria	Cas9	Cas13	Cas12
Sensitive	√[b]	√	√
Specific	√	√	√
Quantitative	√	√	√
Rapid	√	√	√
Cost efficient	√	√	√
Multiplex		√	
Visualized (naked eye)	√	√	
Sample tolerant	√	√	√
Real-time[a]			
In vivo (live cells)[a]			
Single-cell[a]			

[a]Currently, no CRISPR/Cas-based biosensing system can meet criteria of real-time, *in vivo*, or single-cell detection.
[b]Items selected are based on the best capability in each category.

cleavage [16], where the first base following the protospacer should be a non-G base. The Cas13-based detection methods can easily overcome the PFS limitation through shifting the protospacer sequence to avoid the first G base [31].

Quantitative Analysis

Quantitative analysis of target nucleic acids is essential, such as analyzing the RNA expression level for precise disease diagnostics. The RCH method for miRNA detection uses a colorimetric readout, and the detected value represents the expression level of the target miRNA [29]. SHERLOCKv2 has already demonstrated quantitative potential in biosensing, and when it is coupled with LFA, the band intensity can be used to roughly quantify the target nucleic acids [18]. For Cas12-based biosensing systems, HOLMES and DETECTR are not quantitative systems, but HOLMESv2 is able to quantify the nucleic acid target when combined with a real-time PCR reaction [40].

Multiple Analyte Detection

Up to now, only SHERLOCKv2 has shown capability for multiple detection [18]. However, this method is restricted to four targets at present, and more orthogonal Cas effectors with different cutting preferences are therefore needed to expand the number of detection targets in a single reaction system or other alternative strategies need to be explored.

Sample Pretreatment

So far, all published CRISPR biosensing methods require pretreatment of samples to facilitate subsequent amplification and detection. Among these pretreatments, heating could be the simplest and cheapest procedure; this was used in the HUDSON method prior to the SHERLOCK detection and has demonstrated effectiveness on serum, saliva, and urine samples [19]. A similar procedure has been applied to HOLMES for direct detection of saliva or cell samples before PCR amplification [39].

In the future, with the demanding applications of CRISPR/Cas-based biosensing systems for various diagnostic purposes, the sample sources could be expanded with distinct characteristics, such as containing different inhibited factors. Therefore, a suitable sample pretreatment

strategy may become essential for wider application, especially for POC diagnostics. In addition, more accompanying devices or instruments could be developed to facilitate the sample pretreatment in CRISPR/Cas-based biosensing systems.

Type of Nucleic Acid Targets

Cas9 and Cas12 are DNA-targeting enzymes, while Cas13 recognizes RNA targets. Unmodified Cas12 effectors are capable of cutting both dsDNA and ssDNA, while Cas9 can be activated by dsDNA or ssDNA/RNA with additional ssDNA PAMmer. However, the types of targets can be converted during nucleic acid amplification (e.g., DNA to RNA by transcription or RNA to DNA by RT), enabling these biosensing systems to detect both DNA and RNA targets.

Assay Time

The entire process of CRISPR/Cas biosensing systems may include sample pretreatment, nucleic acid amplification, CRISPR/Cas-mediated binding/cleavage, and signal detection. The whole time required for each method is shown in Table 1. For the sample pretreatment step, the time consumption may depend on the types of tested samples, and could be as short as 10 min in the HUDSON method for treating serum, saliva and, urine samples. The speed of nucleic acid amplification is strongly related to the method selected, where the ultra-fast LAMP and RPA methods need only 15–60 min. Cas-mediated reaction is the key step in CRISPR/Cas biosensing, and the assay time is 15 min in HOLMES, 30 min in HOLMESv2, 1 h in DETECTR, and 30 min to 3 h in SHERLOCK, mostly depending on the Cas effectors selected.

POC Capability

Most current CRISPR/Cas biosensing systems (e.g., SHERLOCK, SHERLOCKv2, HOLMES, HOLMESv2, DETECTR, RCH, and CAS-EXPAR) rely on fluorescent signals, which could be detected by portable fluorescence readers in the future. Moreover, signals in SHERLOCKv2 and NASBACC can be detected by paper-based colorimetric changes when they are integrated with LFA, which endows these methods with greater capability for POC diagnostics for different applications. With a similar strategy used by Cas13a biosensing systems, Cas12 biosensing methods also have the same POC testing potentials.

Nevertheless, when applying a CRISPR/Cas biosensing system for particular studies or purposes, the pros and cons of these systems need to be carefully considered and evaluated (Table 2).

Exploration of CRISPR/Cas for Additional Biosensing Purposes

The mechanism of dCas9-based genome imaging is similar to that of dCas9-based biosensing, but with a different purpose. When dCas9 is fused with a fluorescent protein, the fusion protein (e.g., dCas9-EGFP) can specifically bind to a target sequence in living cells, allowing dynamical observation of the specific loci [41]. To achieve high signal output, the fusion protein is often targeted to highly repetitive sequences such as telomeres and centrosomes, recruiting multiple copies of the fluorescent proteins. Alternatively, an array of sgRNA can be designed for targeting nonrepetitive sequences [41]. Later, by using dCas9-SunTag or an sgRNA scaffold with multiple protein binding sites, more fluorescent proteins can be recruited to a single locus to enhance the fluorescent signals [42,43]. Additionally, orthogonal dCas9s or sgRNA scaffolds can be employed to achieve multisite genome imaging [44,45]. Recently, a new CRISPR-mediated system called CRISPR-Sirius was developed for live cell imaging using RNA scaffolds with a significantly improved signal amplification efficiency (~60-fold) [46].

Other explorations with CRISPR/Cas for biosensing purposes have been reported, such as cellular recoding and barcoding [47,48]. For example, taking advantage of the Cas9 effector's

gene editing ability, a rewritable in-cell recording system named CAMERA (CRISPR-mediated analog multi-event recording apparatus) was developed for sensing and recording single or multiple signal changes within live cells, including signal duration, strength, and order, among others. [49]. Schmidt and colleagues employed the CRISPR spacer acquisition ability to acquire RNA, therefore facilitating the storage of transcriptional information [50]. In addition, Kalhor and colleagues recently used CRISPR/Cas9 and homing guide RNAs to develop a platform for barcoding and lineage tracing in a mammalian model system [51]. Although these systems were not originally developed for a POC or field-deployable scenario, they are remarkably novel platforms for intracellular studies.

Concluding Remarks and Future Perspectives

Based on current understanding, the entirety of the discovered CRISPR/Cas family has been categorized into two classes with six subtypes [52,53]. Cas effectors from class 2 are commonly composed of only one protein to fulfil both the recognition and target cleavage functions. The simplicity and high efficiency have promised wide applications of Class 2 Cas effectors, including type II (i.e., Cas 9), type V (i.e., Cas12a and Cas12b), and type VI (i.e., Cas13a and Cas13b) effectors, not only in genome editing but also in biosensing systems [52,54,55]. Although some Cas9-based biosensing methods have been developed, it was not until the discovery of the promiscuous RNA cleavage effect of Cas13a in 2016 that much public attention had been paid to CRISPR/Cas biosensing applications. Recently, several nucleic acid detection methods (such as SHERLOCK, HOLMES, DETECTR, and HUDSON) have been rapidly developed with various Cas effectors, and these code names are just the beginning of this novel biosensing technology in the diagnostic arena. The great potential of CRISPR/Cas biosensing technologies is continually inspiring new research activities in developing next-generation nucleic acid detection platforms.

The current systems have successfully demonstrated the potential to develop highly sensitive, ultra-resolution specific, and time-efficient detection methods, combined with simple visual signal readouts and quantitative and multiplex capabilities. All of these extraordinary biosensing qualities are highly desirable not only for researchers and clinicians but also for individual end-users. The gradually unleashed power of CRISPR/Cas-based biosensing will help to satisfy various diagnosis or detection goals that have been evading modern analytical science for decades. The biggest challenge that the CRISPR/Cas biosensing system has solved is the low sensitivity problem that current POC analytical devices face. Additionally, the collateral cleavage activity has endowed CRISPR/Cas biosensing systems with self-signal amplification to further enhance their highly sensitive and ultra-fast biosensing capability while maintaining isothermal performance, easy retargeting, and simplified components. CRISPR/Cas biosensing systems are well suited for developing POC testing devices with equal or superior performance to conventional diagnostic practices, such as PCR or hybridization, but with affordable cost, which is desirable for resource-limited regions. These advantages have highlighted great potentials of CRISPR/Cas biosensing systems in automatic diagnostics, precise medication, and medical AI/big data analysis. As new CRISPR/Cas components or even new systems have been continuously discovered, and new Cas effector activities have been studied, it is reasonable to assume that more functions can be achieved, such as better multiplexing (i.e., more than four individual analytes), better quantitative capabilities, or less time consumption (see Outstanding Questions). Most recently, a new CRISPR protein, Cas14, was characterized with a collateral cleavage effect that is triggered by target ssDNA, and it could also potentially be employed for nucleic acid detection [56].

It is not an overstatement to think of CRISPR/Cas biosensing as an innovative technology with a significant impact on detection and diagnostic capabilities in many fields, especially for POC

Outstanding Questions

Besides the Cas proteins investigated so far, are there novel proteins (e.g., Cas proteins or others) with better nucleic acid detection performance?

Are there any new orthogonal proteins that can be combined with the Cas proteins used in the SHERLOCKv2 system to achieve multiplex detection of more than four targets?

Will it be possible to use the CRISPR biosensing technology for real-time detection of pathogen or other targets inside a single living cell?

Will it be possible to make a simple and cheap CRISPR/Cas biosensing device through integrating functional modules of sample treatment, nucleic acid amplification, Cas reaction, and signal detection?

Besides the applications investigated so far, are there any new fields suitable for CRISPR/Cas-based biosensing technology?

Can the CRISPR biosensing methods be used for absolute quantification of target nucleic acids? If yes, then how it can be done?

scenarios such as public healthcare and disease prevention and management. It is reasonable to assume it will be widely applied to broadly diverse fields in the near future. However, the CRISPR/Cas biosensing systems may still have several drawbacks to overcome before they can be widely translated into a practicable diagnostic tool for the above-mentioned scenarios. One drawback could be the relatively lower sensitivity of Cas protein detection alone. Although most CRISPR/Cas biosensing methods can directly detect nucleic acid targets, they would still benefit from combination with proper amplification methods. The coupled amplification procedures can not only greatly improve detection sensitivity but also convert target types during amplification (e.g. DNA to RNA by transcription and RNA to DNA by RT), which would enable each CRISPR/Cas system to detect both target DNA and RNA. In addition, introducing PAM sequences during amplification allows CRISPR/Cas biosensing systems to work in a PAM-independent way. Therefore, all current CRISPR/Cas biosensing systems rely on target nucleic acid amplification to improve sensitivity, which may create inconvenience and make the system less robust. Additionally, CRISPR biosensing methods can only be used to detect known DNA sequences, which could limit their application in some scenarios. Furthermore, particularly for achieving practical diagnostics, having strategies to immobilize CRISPR/Cas systems to various interfaces is one of the major issues for complex biomedical engineering designs.

Nevertheless, even as we advocate for the outstanding performance that the existing systems exhibit, it still remains to be seen whether they truly represent revolutionary next-generation diagnostics until they are applied efficiently in a variety of complex fields or conditions for detection of more diversified targets with superior specificity and sensitivity, and eventually are transferred from the laboratory bench to practical or clinical use.

Acknowledgments

This work was financially supported by funding from the Australia Research Council Future Fellowship (FT160100039), the ARC Centre of Excellence for Nanoscale BioPhotonics CE140100003, and the National Natural Science Foundation of China (Grant 21575045). Yi Li would like to thank the University of New South Wales for providing PhD Scholarship.

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