CRISPR-Cas system for biomedical diagnostic platforms

Zhen Wang | Wenguo Cui

Shanghai Institute of Traumatology and Orthopaedics, Shanghai Key Laboratory for Prevention and Treatment of Bone and Joint Diseases, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, People’s Republic of China

Correspondence
Shanghai Institute of Traumatology and Orthopaedics, Shanghai Key Laboratory for Prevention and Treatment of Bone and Joint Diseases, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, 197 Ruijin 2nd Road, Shanghai 200025, P. R. China.
Email: wgcui80@hotmail.com

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Abstract
Clustered short palindrome repeats with regular intervals, abbreviated as CRISPR, and functions as a self-defense system for prokaryotes, detecting particular pathogenic nucleic acid, interfering with the functions of exoteric DNA, and protecting them against foreign invaders. In recent years, CRISPR has attracted increasing interests in the in vitro diagnostic field because of its inherent allele specificity, which is one of the critical factors for the successful application of this technology in the development of high-precision treatment and diagnosis. Herein, this review article aims to provide an overview of CRISPR-CRISPR associated proteins (Cas) based biomedical diagnostics, including the biological mechanism, biomaterials, and applications. This paper first briefly introduces the development history and biological characteristics of the CRISPR-Cas system, and then summarizes the application status and development trend of the CRISPR-Cas system in the detection and identification of particular pathogens, specifically displaying a brilliant prospect in the most recent outbreak of novel coronavirus (formerly named 2019-nCoV). Moreover, its potential diagnostic power in oncogene mutations and single nucleotide variations detecting are assembled. Finally, we discuss challenges and future prospects of CRISPR-Cas system based diagnostic platforms in biomedicine, hoping to further inspire the development of biomedical diagnostics.

KEYWORDS
bacterium, CRISPR-Cas, in vitro diagnostics, novel coronavirus, virus

1 | INTRODUCTION

The nucleic acid test is an important technique to detect a specific nucleotide sequence and thus usually for identifying particular species, often pathogens isolated from the blood, urine, secretion, tissue, and so on.¹ In recent years, advances in nucleic acid-based diagnostics such as polymerase chain reaction (PCR) and sequence alignment as the representative have revolutionized the way clinical laboratories diagnosing human pathogens.² These new methods allow accurate and rapid diagnosis of a wide array of infectious diseases and facilitate the monitoring of responses to the treatment of infections, such as those caused by immunodeficiency virus and cytomegalovirus.³,⁴ However, in regions with underdeveloped medical resources, lacking professionals and relevant equipment severely limited the applications of these sophisticated tests.⁵,⁶ Thus, for years, calls have been
FIGURE 1  Brief summary of the development history of the CRISPR-Cas system

made to identify diseases rapidly and conveniently without the need for vocational training, auxiliary equipment, or power supplies. It is essential for optimizing clinical care, guiding disease prevention, and health care policy to limit the prevalence of the disease in the construction of basic medical engineering.7

The discovery of the CRISPR is being championed as a flexible and robust tool for genome editing.8 It contains two components, including a guide RNA (gRNA) and a CRISPR-associated endonuclease (Cas protein), which can perform gene reprogramming operations such as deletion or insertion on a DNA sequence. The gRNAs refer to an RNA sequence, which is necessary for Cas binding. It provides an about 20 nucleotides spacer for modifying the genomic target. Hence, a small change in the certain sequence of the gRNAs can lead to the variation of the genomic target. As the earliest known presence of a prokaryotic genome, these novel repeated DNA sequences were first reported in 1987 by Ishino in Escherichia coli and then Mojica considered them generally existing in bacteria in 2000.9,10 And later, the spacer sequences matching plasmids and phage genomes were realized, which indicated CRISPR-Cas would be an immune mechanism in 2005.11,12 The primary function of Cas proteins is to promote the adaptive immunity of bacteria via the processes of adaptation, production of CRISPR RNA (crRNA), and then interference. In the defense process, exogenous genetic substance is processed and selected to integrate into the CRISPR sequences to provide recognition material during repeated infections. The pre-crRNA is transcribed into long precursors and processed into mature crRNA forms to induce the Cas protein cutting the complementary sequences (interferences) of exogenous materials to digest them.13 As the functions and mechanisms of CRISPR systems are gradually revealed, new tools are emerging through transforming system components. It was first reported that Cas9-RNA mediated site-specific genome engineered in human cells in early 2013, being the beginning of new era of gene editing.14 Besides, in 2016, the Cas9 nuclease was used to deplete as low as 250 pg RNA nucleotide sequences.15 Beginning in 2017, CRISPR technology was designed for direct molecular diagnosis, down to a single molecule with high sensitivity.16 Subsequently, CRISPR-associated diagnosis testing exploded rapidly with a wide variety of applications for different pathogens identifying (Figure 1). CRISPR proteins match DNA or RNA sequences, and then real-time signals can be generated, making CRISPR useful for detecting any disease using nucleic acid biomarkers, which suggests a specific molecule testing by simply designing gRNAs. Developing a platform with CRISPR-Cas as the core mechanism enables fast, sensitive, specific, inexpensive, and reliable diagnostics. CRISPR-based diagnostic testing will be a revolution in the field of modern medicine, which is expected to be the front line of pathogen detection during epidemic outbreaks caused by bacteria, fungi, viruses, or parasites, while also having the potential to screen for cancer and genetic diseases. Several recent reviews have summarized the CRISPR systems, with most of them focusing on biological mechanisms and their applications. However, very few reviews have discussed the combination of CRISPR and biomaterials in one set.

In this review, the paper will start with a brief introduction to the history and biology of the CRISPR-Cas system. Current and emerging applications of the CRISPR-Cas system to detect and identify particular pathogens will be summarized, along with their potential diagnostic power and recent advances in cancer diagnosis. Finally, the challenges and future prospects of the CRISPR-Cas system based diagnostic platforms in biomedicine are also assembled in order to look towards the future development of in vitro diagnostics.

2 | THE BIOLOGY OF THE CRISPR-Cas SYSTEM

The CRISPR-Cas system is characterized by providing immunity with hereditary adaptability to extraneous genetic elements of invaders for prokaryotes.17 The
CRISPR loci act as memory storage units in which spacer sequences of nucleic acids extracted from foreign genetic factors are isolated and stored. Then, the recall of sequences guides the Cas enzymes to specifically eliminate exogenous factors. And CRISPR-Cas plays an essential role in adaptation, crRNA maturation, and interference with important biodiversity (Figure 2). At present, in accordance with the classification of genes encoding Cas protein and the properties of interferential complex, CRISPR-Cas systems are divided into two classes and further divided into six categories. Some subtypes that each has unique Cas-coding genes. In class 1 systems (type IV, III, and I), interference is performed by multiple Cas protein complexes, while class 2 systems, including types VI, V, and II, use a single effector. Although subtle differences, these Cas enzymes have similar components: probes, gRNAs, buffers, and requirement of nucleotide activators. They have two different enzymatic domains. One is combined with a nucleotide activator, and another domain cleaves target nucleotide sequences when it is activated, playing the role of nuclease.

2.1 Adaptation

Adaptation, the step in the formation of previously infected memory, is also known as septal acquisition and is also the reason for the adaptability and heredity of CRISPR-Cas immunity. In the process of adaptation, the foreign nucleic acids are identified, protospacer sequences are processed and selected, and the integrations of spacer sequence into CRISPR array are performed (Figure 2). To avoid the damage and autoimmunity of the CRISPR array, the CRISPR-Cas system has to correctly differentiate self or extraneous DNA sequences. For example, in type I and II systems, a recognition of a protospacer adjacent motif (PAM) is helpful to distinguish endogenous and extraneous DNA. In the subtype of the I-E system, the Cas1-Cas2 protein complex identifies a PAM and then cuts exogenous DNA and adjusts the size of protospacer to integrate into a CRISPR array. The integration of newly acquired spacer often takes place right following the AT-rich leader sequence of the CRISPR array, leading to produce a sequential timeline of foreign genetic factors. An integrated host factor (IHF), a CRISPR-independent protein that serves as an integrase complex, sharply bends the DNA structure, enabling the Cas1-Cas2 to identify the leader sequence in the CRISPR array and unmistakably locate the spacer. In the subtype II-A system, in addition to Cas1-Cas2 and Cas9, Csn2, and transactivating crRNA (tracrRNA) are also required to obtain the spacer. Unlike described above, the identification of the leader sequence in type II-A is independent of the IHF, and requiring the Cas1-Cas2 complex directly identifying a leader anchoring site (LAS) to locate the spacer acquisition correctly.

2.2 crRNA maturation

Generation of mature crRNA begins with the transcription of a long precursor crRNA (pre-crRNA), which initiates from the leader sequence preceding the CRISPR array and
produces various spacer and repeat fragments. To recognize the extraneous target, the fragments are cut into single mature crRNAs that guide the recognition of Cas proteins (Figure 2).

### 2.2.1 Class 1 crRNA maturation

In type I and III systems of class 1, Cas6 proteins cut duplicated fragments in the pre-crRNA to produce mature crRNA (Figure 3A). In the subtype I-C subtype system, Cas5d functions instead of cas6, whereas possibly functions in subtype III-C and III-D systems. And Cas6, acting as a composition of the interference complex, is combined with crRNA in the vast majority of type I systems. The exceptions are subtypes of I-A and I-B, in which Cas6 turns into an uncombined form after a repeat sequence is cleaved. Cas6, a kind of dimeric protein, also becomes uncombined in the type III system. In subtype IV, however, the mechanism has not been clarified.
2.2.2 | Class 2 crRNA maturation

In class 2 systems, the same Cas proteins in an interference stage are employed to process crRNA but, in some circumstances, non-Cas proteins are used for processing (Figure 3B). In subtype II-A and B systems, Cas9 binds to mature crRNA and tracrRNA together and the host protein RNase III is recruited for cleaving the repeat section of pre-crRNA. In type II and subtype V-B systems, tracrRNA is necessary for crRNA maturation, whereas it is not a necessity in other class 2 systems. And both crRNA processing and interference are performed by Cas12 and Cas13 proteins in type V and VI systems, respectively.

2.3 | Interference

In the subsequent stage of interference, the mature crRNAs guide nuclease complex, the interference machinery to complementary sequences termed protospacers and thus cleave the nucleic acids of foreign invaders. Generally, class I CRISPR-Cas systems apply nuclease complexes containing several Cas proteins for interference, whereas in class 2 CRISPR-Cas systems it is accomplished by a single effector.

2.3.1 | Class 1 interference mechanism

The type I CRISPR-Cas system, the most widely spread one, uses a crRNA-bound multiprotein complex called a CRISPR-associated complex for antiviral defense (abbreviated as Cascade) for recognizing exogenous complementary targets, as well as the effector protein Cas3 for sequences degradation (Figure 3C). Although the cascade components are varying between different subtypes, the constant characteristics in almost all type I systems consist of PAM recognition pattern, Cas6 or Cas5 family mediated crRNA binding and processing, the seed sequence, R-loop structure to stabilize, cleavage of target by nuclease Cas3 and a helical backbone made of Cas7. The subtype I-E interference (usually employed as an illustration for type I interference) complex has been commendably characterized and understood, consisting of Cas5 and Cas6 bound to 5’ and 3’ repeats of the crRNA, respectively, the backbone of six centrally connected Cas7 proteins, “belly” formed by two Cas11 and the Cas8 tail. Identification of the PAM in the target double-strand DNA (dsDNA) is accomplished by the Cas8e tail of Cascade, which also initiates the local unwinding of DNA double helix and the subsequent binding of crRNA to the complementary strand of the protospacer. In the above procedure, the nontarget strand is bound by two Cas11e subunits, leading to the formation of the R-loop structure for stabilization, which is accompanied by substantial conformational changes of the small and large subunits and therefore allows the recruitment of the nuclease Cas3 for target cleavage.

The Type III complex of interference is also Cascade type, but in this system the combination of the interference complex and RNA of foreign DNA transcription determines the cleavage of extraneous DNA. The subtypes III-A and III-B interference complexes are called Csm and Crm, respectively, which are formed by Cas5 combining with the mature crRNA 5’ repeat end, a Cas7 backbone, and “belly” and “tail” subunits of Cas10 and Cas11. Some type III systems require to recognize RNA PAM (in the transcribed RNA) or PAM but not all, in which other particular implementations are applied. In case the interference complex binding, the single-stranded RNA (ssRNA) transcript is cleaved by Cas7 at regular intervals and target DNA is cleaved by Cas10. Recently, in the subtype III-A Csm complex, it is observed that the DNA cleavage of Cas10 triggers to produce secondary messengers (cyclic adenylyl), which initiate robust nonspecific RNA cleavage by an RNase, Csm6.

2.3.2 | Class 2 interference mechanism

In contrast to class 1 interference, class 2 systems in interference are carried out by an individual nuclease effector rather than a complex (Figure 3D). The type II system is characterized by a bilobate Cas9 endonuclease protein along with the necessity for dual RNA (tracrRNA and crRNA) to guide. tracrRNA bears the complementary sequence to the repeat regions of crRNA and facilitates the binding of Cas9. Once bound to mature dual RNA and with sufficient complementarity, PAM sequences on target DNA are recognized by Cas9, and the crRNA-tracrRNA complex is paired with the cDNA, finally leading to cleave Cas9 double strands of target DNA and generate a blunt break. Additionally, in the type V system, Cas12 is the characteristic protein, divided into subtypes V-A, V-B, and V-C corresponding to nuclease effectors of Cas12a, Cas12b, and Cas12c, respectively. The activation of Cas12b needs tracrRNA which is not required for Cas12a, while the active mechanism of Cas12c awaits further characterization. After the crRNA-Cas12 complex recognizing the PAM and enough base pairing, target dsDNA is cleaved in a staggered way, leaving double-stranded breaks with overhangs of 5- or 7-nucleotides. tracrRNA is not required for type VI systems, which use a Cas13 nuclease having characteristic higher eukaryotic and prokaryotic nucleotide (HEPN)-binding domains. ssRNA is the target of type VI systems, which is similar to type III systems. The complex of crRNA-Cas13 identifies a protospacer flanking site (PFS) on the 3’
FIGURE 4  Applications of CRISPR-Cas–based in vitro diagnostic platforms in different pathogenic factors detection: (A) virus, (B) bacterium, (C) fungus, (D) spirochete, (E) Chlamydia, (F) parasite, and (G) tumor

and 5’ ends of the protospacer for Cas13b interacting and a PFS adjacent to the complementary spacer of the ssRNA 3’ end for Cas13a. The combination of the target and Cas13 to the PFS initiates the cleavage of both nonspecific and target RNA within the two HEPN-binding domains of the protein. Similar to type III csm6 activity, Cas13 indiscriminately cleaves ssRNA.

3  |  CRISPR/Cas–BASED IN VITRO DIAGNOSTICS

There has been considerable progress in the CRISPR-Cas–based in vitro diagnostic platforms, with various types of pathogenic factors being detected and identified. This section reviews in detail the status of CRISPR-Cas in vitro diagnosis (Figure 4).

3.1  |  CRISPR-Cas–based diagnostic test for virus

Virus, the tiny vector of infection, can only have its biological function in the living cells. They primarily consist of the genetic material and a protein coat, along with an outside envelope of lipids in some cases via particles or something like that. It is demonstrated that viruses have the ability to infect all kinds of life forms. With
various shapes of these virus particles, they spread and transmit in many pathways. It requires vectors and host range that a virus can infect. For instance, after contacting the contaminated water or food, humans are likely to be infected by norovirus, a common virus which can appear in vomitus or excreta. Existing virus detection methods are generally nucleic acid amplification and detection toolkit like PCR that depend most on thermal cycling. It opens up opportunities that very few DNA samples can be amplified and then identified. Although primitive nucleic acid amplification and detection at one time are somehow sensitive and adjustable, the major difficulty of viral diagnosis mainly lies in the fact that they require a mass of sample manipulation and a large amount of money in machinery. They may especially fail to discriminate between associated viruses, which share a similar phenotype.

### 3.1.1 Human papillomavirus

Human papillomavirus infection is an infection caused by a DNA virus, the human papillomavirus (HPV). People can become infected with more than one type of HPV. In these cases, HPV16 and HPV18 account for appropriate 70%. A rapid and accurate test is reported by Chen et al to detect and distinguish HPV types 16 and 18 that are carcinoma-linked from clinical samples. It is discovered that vital nonspecific ssDNA transcleavage shows the moment when cleaving dsDNA in a sequence-specific manner by means of CRISPR-Cas12a proteins. The author extracted HPV dsDNA from anal swabs of patients and amplified through isothermal preamplification using recombinase polymerase amplification, an approach which can do without thermal denaturation and function under the low and identical temperature. They selected an ideal sequence close to TTTA PAM, which differed from each other by six base pairs. HPV16- or HPV18-containing plasmids were incubated with LbCas12a-crRNA, which targeted the HPV16 or HPV18 segment together with a ssDNA-FQ reporter showing the signal after cognate target advents as shown in Figures 5A, B and D. The activation of transcleavage of ssDNA is attributed to the fact that the Cas12a-crRNA complex combines and interferes with target HPV dsDNA. Upon cleavage, it generates a fluorescent signal with a combined fluorescent reporter. The above-mentioned method is termed as DNA endonuclease-targeted CRISPR trans reporter (DETECTR), which definitely provides a new platform where efficient and precise in vitro diagnosis for HPV subtypes is available (Figure 5C).

### 3.1.2 Zika virus

In addition, Zika virus (ZIKV), one of the emerging viruses in the world, is a member of the virus family Flaviviridae. Since its discovery in Uganda in 1947, ZIKV has continued to expand its geographic range and threatens to devastate the population. Myhrvold et al reported a specific and sensitive method for in vitro diagnosis of the dengue virus (DENV) and ZIKV, directly. To facilitate the SHERLOCK technique and expand its application in detecting viral nucleic acids conveniently from body fluids, they developed heating unextracted diagnostic samples to obliterate nucleases, which is also abbreviated as HUDSON. By means of heating and chemical reduction, the virus particles are dissolved and the high concentration of ribonuclease in body fluids is inactivated. After the samples extracted from plasma, urine, or whole blood, the results can be provided in no more than 2 h just with limited equipment or sample preparation. A novel approach is introduced to protect and release from degradation viral nucleic acids without the necessity for nucleic acid extraction. This way is a process of chemical and heat reduction which inactivates large quantities of ribonucleases (RNases) and resolves viral particles by disorganizing the viral envelope, after which nucleic acids are released into solution. This group relates it with a Cas13-based nucleic acid detection platform, which combines a Cas13 guided by RNA and recombinase polymerase amplification (RPA), resulting in the collateral cleavage of nucleic acids. Treated samples can be added to RPA reaction mixtures directly without a purification step, inhibiting subsequent detection or amplification. Trying to enhance sensitivity to some extent, a fluorescent signal that is augmented via enzymatic activity is produced by the cleaved RNA, which is integrated to the fluorescent reporter (Figures 5E and F). At the same time, Pardee et al reported a paper-based and cell-free sensor for in vitro diagnosis to detect the Zica virus. The author combined isothermal RNA amplification with the toehold switch sensor. RNAs containing a hairpin structure can be unfolded when they bind a trigger RNA, on the freeze-dried and paper-based platform. This is a module based on CRISPR-Cas9 that can tell the difference between Zika genotypes or with other unknown viruses like dengue virus. Only by virtue of a NGS (any nucleobase followed by two guanine nucleobases) sequence of PAM, it can exploit Cas9 to selectively cut DNA. It is demonstrated that several strain-specific PAM sequences can be employed for lineage discrimination. This new method utilized a reverse primer targeted to attach the trigger sequence of a synthetic toehold switch. After the complex of gRNAs targeting sites and appropriate PAM sequence gRNAs targeting sites are prepared, the double-stranded
DNA is synthesized with the help of Cas 9, causing a truncated RNA product unable to activate the sensor H toehold switch as shown in Figure 5G. Otherwise, the generated full-length RNA product can successfully activate sensor H. Triggering RNA is only amplified from DNA sequences which is not cleaved by Cas9 allowing for strain-specific identification using the toehold sensor H. The purple color proves that LacZ expression is activated from the toehold switch, while the yellow color shows the toehold switch remained inactive (Figures 5H, I and J).

### 3.1.3 Severe acute respiratory syndrome coronavirus 2

COVID-19, an outbreak of serious acute respiratory syndrome infected by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), out broke in November 2019 and has since spread rapidly affecting 199 countries and territories around the world and two international conveyances. It can be diagnosed using RT-PCR
usually in hospitals, but the ever-increasing numbers of patients or inadequate access to reagents has hindered the virus detection.\textsuperscript{51,52} Feng Zhang et al described a protocol for using the SHERLOCK method for the quick diagnosis of COVID-19 (https://www.broadinstitute.org/files/publications/special/COVID-19%20detection%20(updated).pdf). Exploiting synthetic COVID-19 virus RNA segments purified from patient samples, they are able to distinguish COVID-19 target sequences in a range between 20 and 200 amol/L validated by 10-100 copies per microliter. Without any elaborate instrumentation, they can be displayed by using a dipstick in less than an hour. The detection can work in three steps including: (1) utilizing the RPA kit to amplify the samples after incubation for 25 min isothermally; (2) utilizing CRISPR-Cas13 to detect the amplified RNA of the virus in advance after incubation for 30 min; (3) utilizing a paper dipstick, which is available to display the result after incubation for 2 min. Beginning from nucleic acid extraction, which is commonly employed for qRT-PCR tests, it is supposed to be completed in 1 h. In a gesture to prove the advent of COVID-19 RNA in the samples, this group chose two targets in the COVID-19 genome, including Orf1ab (open reading frame) gene and the S gene. LwaCas13a CRISPR gRNAs and RPA amplification primers were devised for given detection. In a gesture to maximize the specificity of this method, they picked guide sequences, which minimized off-targets to related human respiratory virus genes. Utilizing sequential dilutions of synthetic COVID-19 Orf1ab gene and S gene RNA segments, they can detect the advent of synthetic COVID-19 RNA sequence in a range between 10 and 100 copies per microliter (Figure 6A). Broughton et al also introduced an accurate and quick CRISPR-Cas12 based lateral flow assay.\textsuperscript{53} First, they designed 72 primers which target the E (envelope) and N (nucleoprotein) genes of COVID-19.\textsuperscript{54} Then they devised Cas12 gRNAs to find coronaviruses in the E 80 gene and specifically in the N gene. They managed to detect PCR-positive samples from COVID-19 patients. Conventional RNA extraction or sample matrix can be used as an input to this CRISPR-Cas12 based lateral flow assay, which is visualized by a fluorescent reader or lateral flow strip after reacting at 37°C for 10 min (Figures 6B and C).

He et al presented a sophisticated technology for efficient detection of African swine fever virus (ASFV), a highly severe pathogen that results in almost a hundred percent death rate in domestic pigs.\textsuperscript{55} Since ASFV possesses a double-stranded DNA genome, CRISPR-Cas12a together with a crRNA is employed to detect the target DNA by cutting ssDNA probes after hybridizing the ASFV target. On ASFV DNA combing, the complex of CRISPR-Cas12a, crRNA, and ASFV DNA is supposed to be activated. In order to report the target DNA, this group employed the ssDNA probe, which links a quencher and a fluorophore. LbCas12a first binds with crRNA. Later in the solution LbCas12a-crRNA complex combines with A-DNA and then triggers the random cut of ssDNA. The fluorescence-sensing unit can detect the fluorophore on the ssDNA probe released in the assay (Figures 7A-C). They demonstrated that they were able to differ closely matched target samples with a detection limit of \(\sim\)1 pmol/L and matched the efficiency of high-throughput of commercialized PCR systems.

Qin et al developed a quick and thoroughly microfluidic Ebola virus detection via CRISPR-Cas13a, which uses its collateral RNA degradation.\textsuperscript{56} Nonspecific cleavage products of Cas13a are at once detected by a custom-integrated fluorometer that has an advantage of small size and convenience for in vitro diagnosis in field when the microfluidic hybridization and mixing begin automatically. They combined a self-acting and multiplexing CRISPR microfluidic chip with a custom especially designed benchtop fluorometer for quick and low volume (\(\sim\)10 μL) detection of Ebola (Figures 7D-G). At the same time, they mounted the microfluidic chip on the fluorometer for detection in situ and succeeded in detecting the advent of whole Ebola RNA only with a detection limit of \(\sim\)20 pfu/mL (5.45 × 10\(^7\) copies/mL). English et al reported that Cas12a-based hydrogels in the form of a paper fluidic device could enable diagnostic readouts.\textsuperscript{57} As shown in Figure 8, the intermediary layer includes PA-DNA gel precursors so that together with ssDNA cross-linker, it is supposed to form a hydrogel in the paper channels, which is related to the extent of degradation of the ssDNA gel cross-linker. In the presence of nonspecific dsDNA trigger during preincubation, ssDNA cross-linkers are not cut, which allows for a hydrogel assembly in the microchannel. Consequently, when Ebola-specific gRNA was preincubated with Cas12a for about 4 h, buffer flow through the μPAD caused short-circuiting of the radio-frequency identification (RFID) tag antenna compared with an unmodified reference RFID tag. In real time, the RFID-μPAD signals in Ebola-positive samples can be detected as a transformation in the signal strength.

### 3.2 CRISPR-Cas–based diagnostic test for bacterium

Bacteria, by far the smallest living cells, are classified as prokaryotes. They are single-celled creatures, and their internal composition is so uncomplicated that is short of a nucleus and consists of DNA either linear mass called the nucleoid or in circular fragments called plasmid.\textsuperscript{58} Bacteria are classified as pathogens if they form a parasitic
FIGURE 6  (A) Result for detecting of SARS-CoV-2 using CRISPR-Cas–based diagnostics. (https://www.broadinstitute.org/files/publications/special/COVID-19%20detection%20(updated).pdf)  (B) The minimum equipment required to run the protocol.  (C)Schematic of the workflow of SARS-CoV-2 DETECTR. Reprinted with permission from Cold Spring Harbor Laboratory, bioRxiv.
association with other living organisms. Pathogenic bacteria can lead to the diffusion and infection of diseases just as Staphylococcal pneumonia, tuberculosis, typhoid fever, syphilis, cholera, and foodborne illness. Representative identification of bacteria is accomplished by culturing in mediums for up to 48 h. The growth is identified by vision or genome. Afterward, the cultured organism is analyzed and observed to help recognize strains. However, this method is time- and labor-consuming. Due to the inherent limitations, there have been calls for rapid, reliable, easy-to-use, and inexpensive diagnostic tools. In one of the earlier studies, Gootenberg et al. using a CRISPR-Cas13a based molecular detection platform, successfully identified \( E. \) coli and \( P. \) aeruginosa, which both are Gram-negative, rod-shaped bacteria, and pathogenic varieties causing diseases in human. Based on CRISPR-Cas13a, this molecular diagnosis platform amplifies DNA (or RNA with reverse transcriptase) by recombinase polymerase amplification, an isothermal amplification of nucleic acid. Isothermal amplification, using a single temperature, does not need specialized instrumentation. The amplified nucleotide binds to Cas13a nuclease, which is a kind of guiding RNA matched with the target sequence, and a short nucleotide sequence combined with a fluorescence reporter and a quenching agent. If the sequence of interest appears in the amplified nucleotides pool, Cas13a’s nonspecific RNase is activated, and the RNA reporter is cleaved to activate the fluorophore. Hence, as a visible indicator, the signal of fluorescence reflects whether the sequence of interest exists in the nucleotides pool or not (Figure 9A). Consequently, the author named this in vitro nucleic acid detection platform SHERLOCK. In order to identify \( E. \) coli and \( P. \) aeruginosa, the V3 region of 16S rRNA gene is used as the target. In this region, the conservative flanking regions allow the application of RPA primers across bacterial species, and the various internal region allows species differentiation. In a group of five crRNAs that may target different strains and gDNA isolated from \( P. \) aeruginosa and \( E. \) coli (Figure 9B). SHERLOCK also genotyped the strain correctly and acted out low cross-reactivity (Figure 9C). Another method was obtained by Ai et al. developing a rapid assay basing on CRISPR for identifying tuberculosis. Tuberculosis, with a higher risk of death than HIV globally, is the main cause of death in infections. In order to estimate the CRISPR-MTB test (Figure 9D) for diagnosing MTB in various clinical samples, a polymerase amplification reaction was combined with CRISPR-Cas12a for target identification and 179 patients were conducted of a retrospective cohort study. First, they observed the outcome in the subcohort of pulmonary tuberculosis cases. CRISPR-MTB picked up 46 out of 51 pulmonary TB cases in this group, indicating that it
FIGURE 8  (A) Schematic diagram of the stackable μPAD composed of CRISPR based hydrogels and electrical readout. (B) RT-RPA μPAD hydrogel detection of ssRNA of Ebola virus. (C) Image of CRISPR detection and electronic readout. (D) Schematic illustration of the paper-fluidic device and RFID tag. (E) Representative signaling track of positive and negative results in blind experiments in RFID mPAD devices. Reprinted with permission from The American Association for the Advancement of Science. 57 μPAD, microfluidic paper-based analytical devices.
FIGURE 9  (A) Schematic of SHERLOCK. (B) Schematic illustration of applying SHERLOCK to discriminate specific bacterial strains. (C) Schematic illustration of applying SHERLOCK to achieve sensitive and specific identification of E. coli or P. aeruginosa. Reprinted with permission from The American Association for the Advancement of Science. (D) Schematic illustration of CRISPR-MTB. Reprinted with permission from Taylor & Francis Group. (E) Schematic of CRISPR-Cas–based particular gene sequences detection. (F) The workflow of the CRISPR-Cas–based particular nucleic acid detection by colorimetry. (G-H) Representative results for orthogonal identification of each bacterium. Reprinted with permission from American Chemical Society. SHERLOCK, Specific High-Sensitivity Enzymatic Reporter UnLOCKing; RPA, recombinase polymerase amplification; CRISPR-MTB, CRISPR-Mycobacterium tuberculosis

has a high sensitivity for pulmonary TB detection. They then made an assessment of the diagnostic outcome in the entire cohort of both pulmonary and extrapulmonary tuberculosis, and it was reported that the CRISPR-MTB picked up 91 out of 116, suggesting that it created a sensitivity of 79% in active TB cases. Moreover, they examined the specificity of this diagnostic tool which demonstrated a specificity of 98%. In addition, it is worth noticing that this diagnostic tool evaluated in this study requires only 500 μL of samples and an average time of 1.5 h. Another technique was developed by Yuan et al, which is a new diagnostic platform based on distance-associated optical characters of the AuNPs-DNA probes (Figure 9E). In this technique, universal linker ssDNA or ssRNA working as the transcleavage substrates for the Cas12a or Cas13a was applied, respectively. In addition, they then designed a pair
of AuNPs-DNA probes to hybridize to linker ssDNA or ssRNA. Lacking of a target cannot activate transcleavage, so in the reaction, the linker ssRNA or ssDNA keeps unaffected. The cross-linking induced by hybridization forms aggregation state by AuNPs-DNA probe. When the target DNA and RNA is identified by Cas12a/crRNA and Cas13a/crRNA, respectively, transcleavage is activated and the linkers ssDNA and ssRNA are degraded. The AuNPs-DNA probes pair loses their linkers which are for hybridizing and therefore becomes dispersed. And visually detecting is achieved by assessing the distance-associated optical characters of the cross-linked and dispersed AuNPs-DNA probes. After that, they proceeded to design a workflow (Figure 9F). And it was reported that the CRISPR-Cas13a based colorimetric assay provides a method for detecting bacteria, which can identify six bacteria which are pathogenic (Figure 9G). Besides, CRISPR/Cas12a-based colorimetric assay can identify seven pathogenic bacteria, including six pathogenic bacteria mentioned above and Neisseria encephalitis (Figure 9H), suggesting this platform holding potential promise in bacterial identification.

CRISPR can not only detect pathogenic bacteria but also identify those resistant to antibiotics. Methicillin-resistant Staphylococcus aureus (MRSA), one of the most significant pathogens which have multiple resistance, resists to most common antibiotics and leads to wide-ranging problems from mild infections to severe diseases. In the past, there have been many studies to identify MRSA. One of the most famous is English’s research, which involved a group of stimuli-responsive hydrogels to respond to the programmable nuclease Cas12a. They creatively combined the CRISPR-Cas toolbox with DNA hydrogels. Specifically, CRISPR-Cas12a technique applied an effector Cas12a nuclease to target DNA complementing to the gRNA spacer sequence. And correct base-pairing leads to the activation of RuvC nuclease of Cas12a and cleaves any indiscriminate ssDNA with multiple-turnover general single-stranded deoxyribonuclease (ssDNase) activity or target dsDNA. In addition, this system is deeply modularized. Cas enzymes regulate the hydrogel’s properties with integrated DNA components that consist of an actuator and a switcher. The actuator involves ssDNA cross-linkers which actively tune the characters of hydrogels. The switcher includes a Cas12a-gRNA complex and a target dsDNA strand with switching states monitored by unmistakable activities of hybridization between target dsDNAs and gRNAs. By developing a Cas12a-gRNA and target dsDNA matching lock-key pair, they achieved a smart, universal, multifunctional, and sensitive hydrogel platform to diagnose fragments of the mecA gene in charge of MRSA. Similarly, Guk et al developed a CRISPR-mediated DNA-FISH method for detecting MRSA. Guk and colleagues used a CRISPR-associated 9/single-guide-RNAs (dCas9/sgRNAs) complex as a targeting substance to recognize MRSA by specifically identifying the mecA gene by the sgRNAs. Since DNA of most methicillin-susceptible S. aureus (MSSA) does not include the mecA gene, so MSSA is not influenced by the dCas9/sgRNAs. Then, the MRSA and MSSA are separated by Ni-NTA magnetic nanobeads, which are attached to dCas9. At last, SYBR Green I (SG I), a fluorescent probe, stains dsDNA and the intensity of fluorescence can show the MRSA concentration. Using this method, they spotted the dCas9/sgRNAs (#1539) onto a Ni-NTA glass surface. Then, they applied the DNA from MRSA and MSSA to the dCas9/sgRNAs (#1539) spotted glass slide. Finally, SG I was used to stain. The detection spot was specifically targeted by the MRSA, yet the fluorescent signals of MSSA were practically at background levels (Figures 10A-C). And they found that the method based on dCas9/sgRNAs-SGI can be used as an effective microarray platform to create the potential application (Figure 10D).

Consequently, a DNA-FISH method based on CRISPR-Cas systems for detecting MRSA therefore was developed.

Another significant application for CRISPR-based diagnosis is the genetic detections that cause antibiotic resistance in pathogenic bacteria. Muller and his colleagues combined CRISPR-Cas9 with an optical DNA map to detect antibiotic resistance genes in bacteria. Wild-type Cas9 was applied to identify resistance genes in bacterial plasmids (Figure 10E). Cas9 with a crRNA targeting a specific resistance gene was applied to cleave plasmids into their linear structures. They next stained DNA with YOYO-1 (δ1,1′-(4,4,8,8-tetramethyl-4,8-diazaundecamethylene)bis[4-[(3-methylbenzo-1,3-oxazol-2-yl)methylidene]-l,4-dihydroquinolinium] tetraiodide) and metropsin and visualized the position of double-strand breaks on the barcode by stretching the linearized plasmid in the nanochannels. If most breaks occur at the same location along the barcode, the targeted sequence appears on the plasmid. Using this technique, they distinguished plasmids that produced different extended-spectrum β-lactamases, containing cefotaxime 14 (CTX-M-14) and cefotaxime 15 (CTX-M-15). It also generated carbapenemases, involving Klebsiella pneumoniae carbapenemase (KPC) and New Delhi metallo-β-lactamase-1 (NDM-1). They have also integrated some significant parameters, which were traditionally applied for plasmid detection, including gene identification and size determination, in one single assay that furthermore provided a fingerprint of the plasmid that was used for further plasmid identification and tracking (Figure 10F). Additionally, the same effect can be achieved by using other CRISPR-based diagnostic techniques. For example, Gootenberg et al are able to use the above SHERLOCK technique to distinguish clinically isolated K. pneumoniae via two resistance genes including KPC and NDM-1.
Fungi are a group of eukaryotic organisms that possess relatively rigid cell walls and conduct as an important infectious disease. Their way of living may be saprophytic or parasitic, and fungi take in soluble nutrients by diffusing through their cell surfaces. Approximately, 300 fungi are known to be pathogenic to humans. For clinical laboratories to diagnose, the growth of fungi occurs on enriched bacteriologic media. However, numerous fungal cultures need days to weeks of incubation for first growth and the proliferation of bacteria present in the specimen is more rapid. The initial appeal of CRISPR is that it makes diagnosis fast, easy, and cheap. Based on this approach, iGEM (International Genetically Engineered Machine) team from University of Oslo developed a rapid CRISPR-dCas9–based detection kit for vulvovaginal Candida albicans.
infections, one of the most common women vulvovaginal yeast infections (The International Genetically Engineered Machine Competition: UiOslo_Norway, http://2018.igem.org/Team:UiOslo_Norway, accessed March 2020). In this work, upon a suspected infection, a vaginal sample will be treated with glucanase to selectively lyse yeast cells walls in selectivity, exposing the DNA of fungi. Afterward, modified dCas9 enzymes fused with split β-lactamase are added. Using distinguishingly designed gRNAs, the dCas9 complexes bind adjacently and specifically on C. albicans DNA sequences. This can activate the β-lactamase to cleave its substrate nitrocefin, producing a colored product suggesting that the C. albicans DNA is present (Figure 11A). In addition, Arastehfar et al forecasted...
that SHERLOCKv2 supplemented with an efficient DNA extraction tool holds promise as a portable platform to detect pathogenic fungal species, which also indicates that CRISPR-Cas system has potential for fungal diagnosis.74

### 3.4 CRISPR-Cas–based diagnostic test for spirochete

Spirochete usually refers to any of bacteria with a spiral shape, some of which are serious pathogens for humans. Among the diseases, leptospirosis, caused by leptospira, is a systemic influenza-like illness related to water contaminated by animal urine.75 The diagnosis of leptospirosis is primarily serologic. Leptospires can be separated from the blood or urine, but people are rarely attempted to culture because the organisms require a unique medium and weeks to grow.76 Also, the standard serologic test is limited to reference laboratories. Warwick Team designed to create a paper-based sensor for diagnosis of leptospira (The International Genetically Engineered Machine Competition: Warwick, [http://2016.igem.org/Team:Warwick/Description](http://2016.igem.org/Team:Warwick/Description), accessed March 2020). The detection system for infectious agents is based on CRISPR-Cas9 technology. The dCas9 enzyme possesses a binding domain, which takes the dCas9 handle on sgRNAs as the target, thus producing a strong binding with high affinity. The 20-nt target region at the 5’ end of sgRNAs can be recognized by the dCas9, which then guides it to the position near a PAM. After that, RNA-binding protein (RBP) is merged into an effector, which usually upregulates the transcription of adjacent genes through interacting with suitable promoters. The binding domain is connected to the effector domain by a serine-glycine linker, amino acids with a flexible length. When RBP binds to the appropriate stem loop structure on the sgRNAs, the effector is allowed to swing freely by the linker. The fusion protein can be combined with the complementary binding region of sgRNAs once it is expressed. The dCas9 will be guided by the CRISPR-Cas9 system to combine the transcription start site upstream for the reporter gene. The fusion of RBP-effector identifies the stem loop structure and is combined with dCas9. When RNA polymerase is recruited by the fusion protein, which is an RNA polymerase recruiter, the protein is located in an ideal position to start transcription. The reporter gene is expressed indicating gene activation, and it can be recognized with ease. After that, the sensor is put on the paper holder by freeze-drying for easy use. This gene circuit with modularity can identify RNA from a source of infection, such as Leptospira and Borrelia and output a signal of fluorescence. Consequently, a paper-based sensor for the diagnosis of leptospira, used in a low-tech environment, is created.

### 3.5 CRISPR-Cas–based diagnostic test for chlamydia

The chlamydiae, stain Gram-negative with an ovoid shape, are bacterial phylum and class whose members are obligate intracellular bacteria.77 Chlamydia, caused by *Chlamydia trachomatis* (*C. trachomatis*), is the universal sexually transmitted disease of bacteria in the world, affecting millions of people each year.78 A new diagnostic test system for *Neisseria gonorrhoeae* (*N. gonorrhoeae*) and *C. trachomatis* was developed by Columbia NYC Team (The International Genetically Engineered Machine Competition: ColumbiaNYC, [http://2018.igem.org/Team:ColumbiaNYC](http://2018.igem.org/Team:ColumbiaNYC), accessed March 2020). In order to detect *C. trachomatis*, they designed a ratio-reading system using CRISPR-Cas13a as the first cleavage activity. The detection system consists of two varied chromoproteins, which express and degrade at the same rate. The codon of one chromoprotein is optimized to contain many motifs favorable for the cleavage of cas13a, while the codon of another protein is optimized to contain few motifs, so it is not vulnerable to be cut. After the detection of interested genes, Cas13a showed the activity of collateral cleavage on mRNAs of the chromoproteins, which led to the color transition from the intermediary. In addition, using this cheap, field-deployable, CRISPR-based diagnostic tool, *C. trachomatis* and *N. gonorrhoeae* can be diagnosed concurrently.

### 3.6 CRISPR-Cas–based diagnostic test for parasite

The parasite lives on or in another organism, which is called the host. For humans, parasites can lead to parasitic diseases such as malaria, amebiasis, and trichomoniasis vaginals, among which, malaria is one of the deadliest diseases in history.79 At present, 2.5 billion people live in malaria epidemic areas, of which about 500 million are infected when exposed to malaria.80 Every year, between one and three million people, mainly children, die of malaria infection. Meanwhile, *Plasmodium falciparum* is one of the most lethal malaria parasite, which is found that resist to several antimalarial drugs. The increasing resistance of malaria vector to insecticides with low toxicity and low price has led to the reduction of control programs. Therefore, the emerging drug resistance is a core challenge for malaria. To solve this problem, FLASH (low abundance sequence found by hybridization) was developed by Quan et al.81 The system uses Cas9 gRNAs to cut the targeting sequence into fragments of appropriate size for Illumina sequencing. The input genomic DNA or cDNAs were first blocked by phosphatase treatment, and
then combined with Cas9 to enter the gRNAs for digestion. The resulting product can then be connected to the universal sort adapter. During subsequent amplification, the sequence was enriched and prepared to bind to the sequencing stream cells (Figures 11A and B). By using FLASH, the variant of malaria strain was identified in the mixed infection. They selected six *P. falciparum* drug-resistant genomic loci, 25 genomic loci with high diversity, and 17 microsatellite sites for FLASH-NGS. Dried blood spots indicating the mixtures of three strains adapted to *P. falciparum* were sequenced with FLASH-NGS and NGS. They consequently found 85.6% successful target reads by FLASH-NGS on average, compared with less than 0.02% using NGS. For the samples, when three experiments from all strain mixtures are averaged, the 31 windows targeted by them were sequenced with FLASH-NGS and NGS. 84 Cas9 proteins have promising potential in DNA and RNA quantification especially for multiplexed mutant detection.85 In addition to the genomic mutations, significance has also been attached to the detection of tumor secretions and some none coding RNAs, which have been proved to have close relationships with cancer for promoting invasion and metastasis.86

Cancer is a disease involving unusual growth of cells which may invade other portions of humans.62 It is characterized by the accumulation of various epigenetic and genetic alterations in all genome, resulting in a group of mutative cells forming a lump which may be distributed diffusely.83 Initially, cancers are recognized due to the appearance of symptoms or screening. However, due to the high false positive rates and lack in sensitivity and specificity, a controversy has been aroused about whether this procedure can ensure a definitive diagnosis.84 Cas proteins have promising potential in DNA and RNA quantification especially for multiplexed mutant detection.85 In addition to the genomic mutations, significance has also been attached to the detection of tumor secretions and some none coding RNAs, which have been proved to have close relationships with cancer for promoting invasion and metastasis.86

It is showed that many cancer types might evolve by accumulation of genetic mutations. Jia et al proposed an assay based on CRISPR-Cas9 to detect mutations in DNA.87 It is demonstrated that CRISPR-Cas9 can cut wild-type genomic DNA but fails to cut mutant completely. Combining with blocking oligosaccharide (blocker), PCR can enhance the mutant DNA and inhibit the amplification of wild-type DNA. A common epidermal growth factor receptor (EGFR) mutation is associated with nonsmall cell lung cancer, the deletion in exon 19, E746-A750 del. So they used Cas9 to analyze a long deletion site (E746-A750 del, a 15-bp del) in the EGFR gene. The sgRNAs targeting EGFR exon19 del were designed for gene enrichment in vitro which suit wild-type templates to a large extent while are not suitable for mutant-type ones. It is proved that CRISPR-Cas9 cleavage is supposed to enlarge low-frequency DNA mutation, which makes efficient cancer in vitro diagnosis available. Gootenberget al also introduced the method of detecting low-frequency cancer mutations in cell-free DNA (cfDNA) segments.16 SHERLOCK could detect single-nucleotide polymorphism (SNP) alleles with a background DNA level of 0.1%. Then they revealed two unlike cancer mutations in simulated cfDNA samples with alleles as low as 0.1%, including EGFR L858R (L, Leu; R, Arg) and BRAF V600E (V, Val; E, Glu).

Eukaryotic cells can produce membrane-bound extracellular vesicles termed as exosomes, which can exist in blood, urine, and so on.88 They can be excluded into urine and have the potential to be employed as a tool for in vitro diagnosis.89 Zhao et al reported a way for detecting exosome by exploiting CD63 aptamer and CRISPR-Cas12a system, especially for cancer applications.90 CD63 is a common transmembrane protein usually utilized as markers for exosome and can convert the number of exosomes into nucleic acid detection.91 The CD63 aptamer is employed to capture CD63-bearing exosomes due to complementary DNA strands serve as its blocker. The complex of CD63 aptamer and exosomes results in a conformational transformation of the aptamer to release the complementary DNA strands. Then the CRISPR-Cas12a system will recognize the released blocker, which latter triggers the transcleavage of the TaqMan probe reporter. Consequently, the reported probe labeled with fluorescent chromium and the corresponding quench groups at both ends is split. The latter fluorescence signal is related to the outer body captured by the magnetic bead. Since CD63 has the potential of not being overexpressed in all exosomes, this method might fail to work each time. To some extent, it brings a novel idea for in vitro diagnosis in cancer.

Chen et al proposed a novel method to diagnose cancer by means of detecting human VEGF, together with human IL-6 and so on, which are regarded to be of great significance in cancer development.64 They employed the Cas13a system which have the top transcleavage efficiency together with a short ssRNA reporter of Black hole quencher (BHQ) and 6-Carboxyfluorescein (6-FAM) groups at the 3′- and 5′- ends, respectively, to enhance a fluorescence after cleavage. Before the CRISPR-Cas13a assay, in order to improve the efficiency and stability, they also utilized the transcription process by employing T7 promoter tagged DNA when incubating and washing. Next, for forming the “antibody–antigen–antibody” complex, they continuously diluted the human IL-6 antigen with detecting antibody (Figure 12A). Then, streptavidin and biotinylated DNA amplification templates can lead to the binding of DNA amplification templates with an “antibody–antigen–antibody” complex. After washing out the unbound template, they used T7 RNA polymerase
to amplify the template. All above were performed at 37°C. They also compare this assay with another way (Figures 12B and C). It has proved that the sensitivity of this novel method is drastically boosted and allows for quick cancer in vitro diagnosis.92

At the same time, microRNAs profiling can be employed for numerous clinical variables of cancer diagnosis since their expression patterns are supposed to have a relationship with cancer and cell-free microRNAs are definitely stable in blood quantifiable for in vitro diagnosis.93 Qiu et al developed a CRISPR-Cas9-based microRNA detection for diagnosing multiple diseases like cancer.94 First, via RCA microRNAs were isothermally amplified and combined with in vitro dead Cas9 (dCas9), Cas9 mutants with inactivated nuclease activity, which is used to for introducing specific genome loci with a great range of application like editing, imaging, and so on. Second, the split-horseradish peroxidase activity assessment will be performed. A color change from light yellow to blue of the 3,3’, 5,5’-tetramethylbenzidine (TMB) substrate can indicate the presence of specific miRNAs (Figures 12D-F).

5 | CONCLUSIONS AND FUTURE PERSPECTIVES

It is fundamental to make the right diagnosis for disease management and hence the development of effective diagnostic tools is permanent goal pursued by all biomedical researchers. Over the past two decades, remarkable achievement has been made considering the development of the CRISPR-Cas system based diagnostic methods and engineering these modules to suit or enable various pathogens detection. As discussed, it opened a new window in the clinical laboratory with remarkable convenience, sensitivity, and specificity and has the potential for detection of not only pathogen during daily practice or pandemic, such as novel coronavirus disease outbreak in 2019, but also cancer and genetic diseases. This review provides a comprehensive overview of CRISPR-Cas biological mechanism and several in vitro diagnostic platforms based on it, in order to guide the improvement of in vitro diagnosis. Despite many successful advances of the CRISPR-Cas system based on in vitro diagnostic platforms that have been reported, there is still a long way to take this technology from the bench to the bedside and offer effective diagnostic capability. The following recommendations are put forward to serve as hints for future research, making the field of CRISPR-Cas–based diagnostic more translational:

1. The studies of the biological mechanism of CRISPR-Cas systems have shown the diversity and complexity of the prokaryotic acquired immune system. Even though a series of researches in the past decades have established a framework for our learning, the understanding of these defense systems still needs more comprehensive illustrations.
2. The plasticity of bacteria and viruses may lead to genetic polymorphism of gRNAs targets, which makes
CRISPR-Cas–based treatment and diagnosis ineffective. Mutations in PAM sequences have also been revealed that phages are able to keep away from the CRISPR-Cas systems. It remains to be seen if the problem can be solved by delivering and packaging diverse gRNAs with different targeting sequences.

3. The technical problems of storage and activation of the necessary biomolecules in the toolkit are still inescapable on the road to commercialization. RNA is fragile on account of the widespread existence of RNA enzymes; the detection of target nucleic acids is easily affected. Herein, it is essential to ensure that the longer sgRNAs used for signal amplification in CRISPR-Cas systems are not truncated or degraded by the ubiquitous RNase, leading to negative false. In addition, the target drug delivery of CRISPR-Cas systems in vivo is still a challenge whether there is obvious toxicity on target or off target.

4. With distinct characteristics of identifying particular nucleic acid sequences, designing CRISPR-Cas–based “smart” materials that respond to more specific changes in DNA or RNA is also the future direction of development. Moreover, these “smart” materials have applications as controlled-release coatings, responsive biomaterials, self-assembly hydrogels, shape-memory agents, and sensing platforms, used for biosensing, medical implant, drug release, and treatment. For example, a specific circulating tumor DNA responsive antitumor drugs loaded hydrogel nanosphere could be fabricated with cross-linking of designed gRNAs which is precisely disassembled in response to target tumor DNA in circulation and controls the wide metastasis of tumor cells. Moreover, a smart flexible dressing with specific pathogenic nucleic acid sequences sensor and color indicator integrated onto flexible masks could also be fabricated that provides intuitive and sensitive protective equipment for respiratory infectious diseases like COVID-19. More specifically, on the presence of target nucleotide sequences, the cleavage function of Cas protein is triggered, generating the activation or release of fluorescent reporter and producing a visible color change.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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ORCID
Wenguo Cui https://orcid.org/0000-0002-6938-9582

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AUTHOR BIOGRAPHIES

Zhen Wang received his M.D. degree from the Central South University of Clinical Medicine. Since then, he has continued to pursue his Ph.D. degree at Ruijin Hospital, Shanghai Jiao Tong University School of Medicine under the supervision of Prof. Wenguo Cui. His scientific interests currently lie in the development of nanomedicines for biomedical and healthcare applications, particularly for skeletal regeneration.

Wenguo Cui is a full professor at Ruijin Hospital, Shanghai Jiao Tong University School of Medicine. He is currently the group leader of Regenerative Biomaterials. His current research is focused on the development of functionalized biomaterials and tissue engineering scaffolds using electrospun fibers and hydrogel for tissue regeneration, drug delivery, and disease treatment.

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