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# CRISPR/Cas technology as a promising weapon to combat viral infections

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## Abstract

The versatile clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system has emerged as a promising technology for therapy and molecular diagnosis. It is especially suited for overcoming viral infections outbreaks, since their effective control relies on an efficient treatment, but also on a fast diagnosis to prevent disease dissemination. The CRISPR toolbox offers DNA- and RNA-targeting nucleases that constitute dual weapons against viruses. They allow both the manipulation of viral and host genomes for therapeutic purposes and the detection of viral nucleic acids in "Point of Care" sensor devices. Here, we thoroughly review recent advances in the use of the CRISPR/Cas system for the treatment and diagnosis of viral deleterious infections such as HIV or SARS-CoV-2, examining their strengths and limitations. We describe the main points to consider when designing CRISPR antiviral strategies and the scientific efforts to develop more sensitive CRISPR-based viral detectors. Finally, we discuss future prospects to improve both applications. Also see the video abstract here: https://www.youtube.com/watch?v=C0z1dLpJWl4

**KEYWORDS**
antiviral, biomedicine, CRISPR, CRISPR-associated protein, diagnosis, genome editing, therapy

## INTRODUCTION

The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas (CRISPR-associated proteins) system was described in 2005[1] as an adaptive immune mechanism against viral and plasmid infections present in many bacteria. Depending on the number of components and their mechanism of action, CRISPR/Cas systems can be classified as Class 1 or Class 2. While Class 1 includes multi-subunit-protein complexes, Class 2 comprises single-effector proteins. The Class 2 CRISPR/Cas system consists of a Cas nuclease in complex with a guide RNA (crRNA) that can cleave a complementary target nucleic acid (DNA or RNA) (Figure 1, Table 1).[2] The specificity of the system can be then easily modulated by just modifying the crRNA sequence, enabling the controlled modification of DNA/RNA targets.[3] The potential of CRISPR Class 2 as a programmable gene-editing tool was first reported in 2013.[4] CRISPR system discovery and its development as a gene editing tool obtained the Nobel Prize in Chemistry in 2020.

Up to now, three Class 2 subtypes (II, V, and VI) have been used as biotechnological tools in numerous applications.

*Class 2, type II* systems are characterized by the presence of a Cas9 effector nuclease and a dual RNA-guide (sgRNA), including a trans-activating crRNA and a crRNA (Figure 1A). The Cas9-sgRNA complex or ribonucleoprotein (RNP) recognizes a G-rich 3ť end-located protospacer adjacent motif (PAM) on a double-stranded DNA (dsDNA) target. PAM recognition leads to the complementary annealing of the sgRNA and the DNA and the activation of the nuclease to induce a blunt-ended double-stranded break (DSB) on the target (Figure 1B).[3]

*Class 2, type V* CRISPR/Cas system is mainly represented by a group of single RNA-guided dsDNA-targeting effector proteins known as Cas12 (Cas12a or Cpf1 and Cas12b) (Figure 1A). Upon Cas12-crRNA complex formation, the recognition of a T-rich 5ť end-located PAM enables crRNA pairing with a dsDNA target. Active Cas12 produces a site-specific dsDNA cleavage (*cis*-cleavage activity) and generates a DSB with staggered ends.[5] Furthermore, Cas12-crRNA binding to the

  

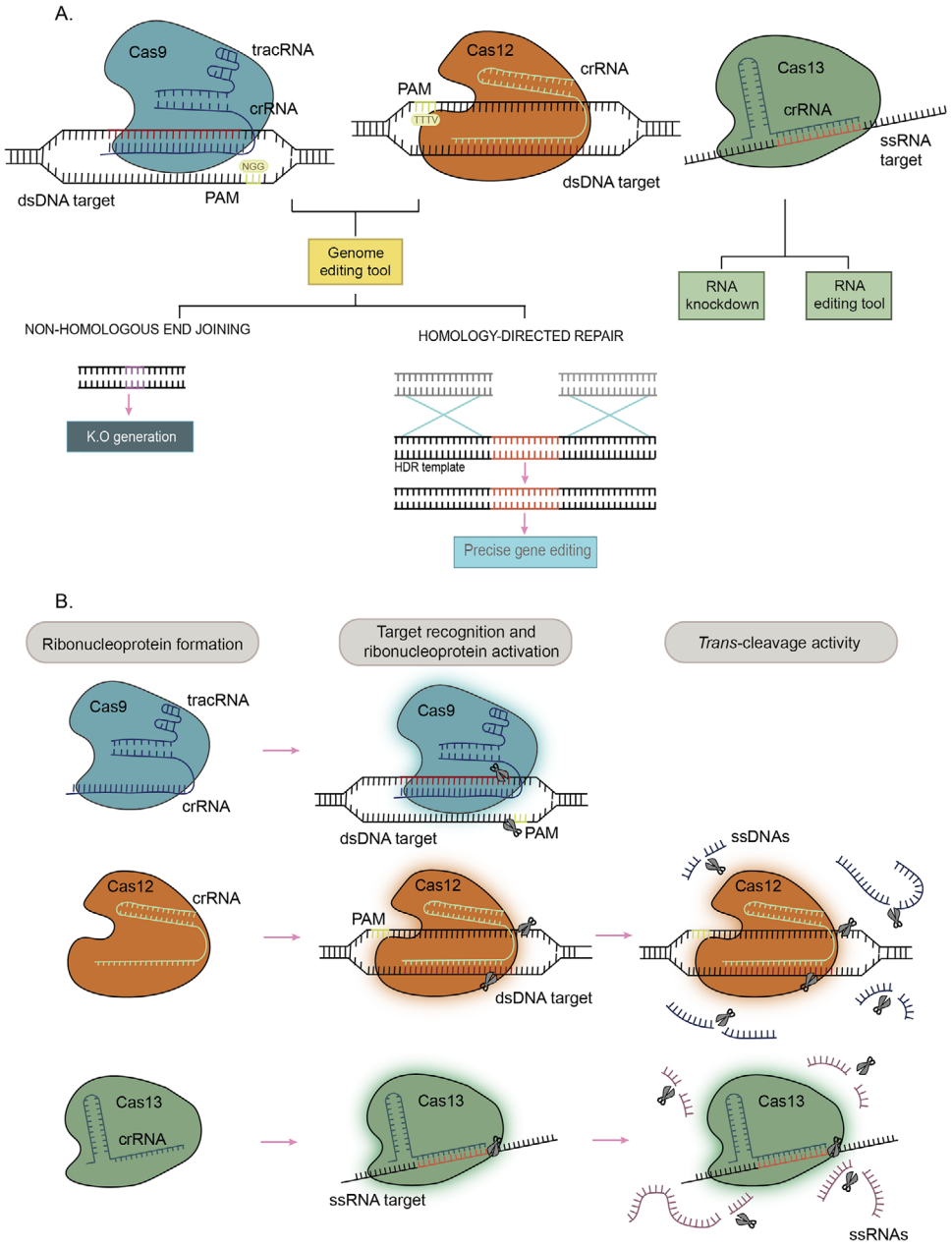


**FIGURE 1** Class 2 main CRISPR/Cas effector proteins, features, and mechanism. (A) CRISPR/Cas9, CRISPR/Cas12, and CRISPR/Cas13; Cas9 and Cas12 are used as genome editing tools. Both produce DSBs that can be repaired through two different pathways: NHEJ and HDR. CRISPR/Cas13 is used for RNA knockdown or as a RNA editing tool; (B) CRISPR/Cas mechanisms and *trans*-cleavage activity

target strand triggers multiple-turnover non-specific single-stranded DNA (ssDNA) *trans*-cleavage (Figure 1B).[6]

Cas9 and Cas12-induced DSBs can be repaired by the cells through two different pathways: non-homologous end joining (NHEJ) and homology directed repair (HDR). The error-prone NHEJ repair mechanism consists in the random insertion or deletion of base pairs and can generate gene knock-outs. HDR, on the other hand, enables precise gene editing and knock-in generation. This pathway requires an exogenous DNA template that contains the sequence of interest flanked by regions that are homologous to those surrounding the DSB. Homologous recombination mediates the insertion of the sequence of interest in the genome (Figure 1A). Although both repair mechanisms occur naturally in cells, NHEJ is much more frequent than HDR.[7]

*Class 2 type VI* CRISPR/Cas system includes a group of single RNA-guided nucleases known as Cas13 (Cas13 a, b, c, and d) that target single-stranded RNA (ssRNA) (Figure 1A). Although Cas13 lacks PAM requirements, some orthologs are activated upon protospacer flanking site (PFS) recognition.[8] Following PFS (when required) and ssRNA target recognition, the Cas13-crRNA complex becomes active, cleaving not only guide-complementary ssRNAs (*cis*-cleavage activity), but also other surrounding ssRNAs (*trans*-cleavage activity) (Figure 1B).[9] CRISPR-Cas13 can be programmed to mediate efficient and specific

**TABLE 1** Main features of the most used Class 2 CRISPR/Cas effector proteins

| Effector protein | Cas9 | Cas12 | Cas13 |
|---|---|---|---|
| Target | dsDNA | dsDNA | ssRNA |
| *trans*-Cleavage | – | dsDNA/ssDNA | ssRNA |
| PAM | G rich | T rich | – |
| PFS | – | – | Ortholog dependant |
| Guide type | Dual (tracRNA and crRNA) | Single (crRNA) | Single (crRNA) |

RNA knock-down[8,10] and also RNA editing by using catalytically inactive Cas13 (dCas13) (Figure 1A).[10]

CRISPR/Cas system's versatility and specificity have broadened its range of applications, including molecular diagnosis and therapy. One of the most promising goals of this technology is the detection and treatment of viral infections. Viruses are the underlying cause for numerous acute and chronic disorders, and although some produce minor diseases, many others induce severe conditions, like the human immunodeficiency virus (HIV) and the recent SARS-CoV-2 pandemic. An early diagnosis and effective treatment are critical to prevent their spread and reduce their prevalence. CRISPR technology is expected to play a prominent role in this context and its use as a sensor or as a therapeutic agent against viral infections is currently a growing field. In fact, the first CRISPR/Cas-based sensor for SARS-CoV-2 has been recently approved by the FDA.

Here, we review the most relevant existing and emerging applications of the CRISPR/Cas system for the treatment and diagnosis of viral infections.

## THERAPEUTIC APPROACHES AGAINST VIRAL INFECTIONS BASED ON THE CRISPR/Cas TECHNOLOGY

The CRISPR/Cas system was discovered in bacteria as an in-built mechanism against viral infections, so it is only natural that this technology should be explored as an antiviral therapy. CRISPR/Cas-based strategies have the potential to treat both acute and chronic infections, but they are especially interesting for the latter. Many clinically relevant viral diseases often become chronic due to viral latency, a phase in which viral activity is minimal. Current treatments frequently lack efficacy in these cases. For instance, highly active antiretroviral therapy, the treatment of choice against HIV-induced acquired immunodeficiency syndrome, fails to target latent virus reservoirs.[11] The CRISPR/Cas system constitutes a promising tool for the definitive cure of these patients, who normally require life-long treatments. Although most research efforts have so far targeted HIV and hepatitis B virus, the CRISPR/Cas system has also demonstrated its therapeutic potential against herpesviruses,[12] human papilloma virus (HPV)[13] and dengue virus,[14] among others (Table 2). Additionally, this technology has allowed to identify novel druggable antiviral targets[15] and contributed to the generation of disease models.[16,17]

The versatility of the CRISPR/Cas toolbox is one of its major advantages. So far, researchers have mostly focused on the well-characterized Cas9 protein. However, recent approaches have shown the antiviral potential of other Cas nucleases (i.e., Cas13 and Cas12) (Table 2). While Cas9 and Cas12a can be used for DNA manipulation, the Cas13 family allows to target RNA sequences. This is of great interest in antiviral therapy since viruses can have RNA or DNA as genetic material. Cas12a and Cas9 are useful against DNA viruses and RNA viruses with dsDNA intermediaries in cells. Cas13, on the other hand, can directly target RNA viruses. Moreover, it constitutes a safer alternative for in vivo applications as it cannot induce permanent genetic alterations in host cells. Recent as this field is, several groups have used different Cas13 orthologs to target dengue,[14] influenza A, lymphocyte choriomeningitis, and vesicular stomatitis viruses.[80] Interestingly, Cas13's applicability as a prophylactic for SARS-CoV-2 infections has been investigated too.[79] Besides the intrinsic versatility of the CRISPR/Cas system, these nucleases can be combined with antiviral drugs[58,20] and other biomolecules,[82] greatly expanding therapeutic possibilities.

Table 2 summarizes the main studies conducted to date, reflecting the interest of the scientific community in exploring the antiviral applications of the CRISPR/Cas technology. However, it should be noted that most research has been performed in vitro and exhaustive in vivo studies confirming CRISPR's antiviral potential are required. This is especially limiting for infections lacking good animal models, such as hepatitis B.[22] In this section, we review the main aspects to consider when designing CRISPR-based antiviral strategies. The choice of adequate target genes and crRNAs is a key determinant not only for the effectiveness of antiviral therapies but also for their safety, since these two elements are closely related to off-target effects and the emergence of viral resistance. In addition, as mentioned above, the CRISPR/Cas system enables the generation of both knock-outs and knock-ins in specific *loci* and the transcriptional regulation of sequences of interest. This adaptability can be exploited to design strategies tailored to different therapeutic scenarios. Finally, we discuss different approaches for in vivo delivery, one of the main bottlenecks for CRISPR-based therapeutic applications.

### Target genes: Viral genomes versus host factors

The success of antiviral CRISPR therapy strongly relies on the choice of optimal target genes. Although research has mostly focused on viral nucleic acids, targeting host factors can also constitute a valuable approach (Figure 2).[83] Anti-HIV Cas9 strategies constitute a prime example of how CRISPR/Cas technology can be exploited to target either viral genomes of host factors. Numerous studies have used the Cas9 endonuclease to directly block or eliminate HIV in acute and latent infections,[58,46,54,50] either alone or in combination with antiviral drugs.[58] Most of them are directed against the long terminal

**TABLE 2** Overview of the main studies on the therapeutic applications of the CRISPR/Cas system against human viral infections

| | Virus | gRNA target | Delivery method | In vivo | Ref. |
|---|---|---|---|---|---|
| Cas9 | Hepatitis B | X, C, P | Nucleofection/tail injection | x | [18] |
| | | S, X | Transfection | | [19] |
| | | S, C, RT | Lentiviral transduction | | [20] |
| | | C, P, S, X | Transfection/hydrodynamic injection | x | [21] |
| | | C, P, S, X | Transfection/hydrodynamic injection | x | [22] |
| | | PreS, S, X, preC, C, EnhI | Transfection | | [23] |
| | | C, P, S, X | Transfection/hydrodynamic injection | x | [24] |
| | | S, P, X | Transfection/hydrodynamic injection | x | [25] |
| | | S, X, C | Transfection | | [26] |
| | | S, X, P | Transfection/hydrodynamic injection | x | [27] |
| | | C, P, S, X | Lipid like nanoparticles | x | [28] |
| | | repeated core region | Transfection | | [29] |
| | | S | Adeno-associated viral transduction | | [30] |
| | | C, P, S, X | Transfection/hydrodynamic injection | x | [23] |
| | | C, P, S, X | Adeno-associated viral transduction | x | [31] |
| | | RT, P, XCp | High capacity adenoviral transduction | | [32] |
| | | pre S1, pre S2/S | Transfection | x | [33] |
| | | S | Transfection | | [34] |
| | Herpesvirus | Viral miRNAs (EBV) | Transfection | | [35] |
| | | ICP0/4/7 (HSV) | Transfection | | [36] |
| | | UL54/57/70/105/86/84 (HCMV); UL8/29/52 (HSV); viral miRNAs (EBV) | Lentiviral transduction | | [37] |
| | | UL122/123 (HCMV) | Lentiviral transduction | | [38] |
| | | EBNA1, OriP, W repeats (EBV) | Transfection | | [39] |
| | | UL29, UL30, UL54/ICP27, RS1/ICP4 (HSV) | Lentiviral transduction | | [40] |
| | | LANA (Kaposi's sarcoma virus) | Adenoviral transduction | | [41] |
| | | UL30, UL54 (HSV) | Adeno-associated viral transduction | x | [12] |
| | Human immunodeficiency virus | | | | |
| | Viral elements | LTR, gag, env, pol, vif, rev | Transfection and lentiviral transduction | | [42] |
| | | LTR, pol, rev | Nucleofection | | [43] |
| | | LTR, gag | Adeno-associated viral transduction (tail injection) | x | [44] |
| | | LTR | Transfection and lentiviral transduction | | [45] |
| | | LTR | Transfection and lentiviral transduction | | [46] |
| | | LTR, gag, pol | Lentiviral transduction | | [47] |
| | | LTR, gag, env, pol, rev, tat | Transfection and lentiviral transduction | | [48] |
| | | gag, pol, env, rev | Lentiviral transduction | | [49] |
| | | LTR, gag, env, pol, tat, rev | Lentiviral transduction | | [50] |
| | | LTR, gag, pol | Lentiviral transduction | | [51] |
| | | LTR, gag, pol | Lentiviral transduction | | [52] |

(Continues)

**TABLE 2** (Continued)

| | Virus | gRNA target | Delivery method | In vivo | Ref. |
|---|---|---|---|---|---|
| | | LTR, gag, pol | Adeno-associated viral transduction (IV injection) | x | [53] |
| | | LTR | Adeno-associated viral transduction + synthetic surface peptide | | [54] |
| | | TAR | Lentiviral transduction | | [55] |
| | | tat, rev | Lentiviral transduction | | [56] |
| | | LTR, gag, pol, vif, env, rev, tat | Lentiviral transduction | | [57] |
| | | LTR, gag | Adeno-associated viral transduction | x | [58] |
| | Host factors | CXCR4 | Lentiviral transduction | | [59] |
| | | APOBEC3G, APOBEC3B | Transfection and lentiviral transduction | | [60] |
| | | CCR5 | Lentiviral and adenoviral transduction | | [61] |
| | | CCR5/CXCR4 | Lentiviral transduction | | [62] |
| | | CXCR4 | Lentiviral transduction | | [63] |
| | | TRIM 5 | Lentiviral transduction | | [64] |
| | | CXCR4 | Transfection | | [65] |
| | | USP18 | Nucleofection | | [66] |
| | | mir146a | Lentiviral transduction | | [67] |
| | | CCR5/CXCR4 | Lentiviral transduction | | [68] |
| | | CCR5 | Transfection | | [69] |
| | Latency reversal | LTR | Lentiviral transduction | | [70] |
| | | LTR | Transfection | | [71] |
| | | LTR | Transfection | | [72] |
| | | LTR | Transfection/nucleofection | | [73] |
| | Papillomavirus | E7 | Transfection | | [74] |
| | | E6, E7 | Transfection | x | [13] |
| | | E6, E7 | Transfection | | [75] |
| | | E6, E7 | Adeno-associated viral transduction | x | [76] |
| | | E7 | Self-assembled micelle | x | [77] |
| | | E6 | Adeno-associated viral transduction | x | [78] |
| Cas13 | Coronavirus | RdRP, nucleocapsid | NA (stable cell line expressing Cas13d) | | [79] |
| | Dengue virus | Capsid, PrM, NS1, NS2a/b, NS3, NS4a/b, NS5, 3′UTR | Transfection | | [14] |
| | Influenza A virus | Viral mRNA (and complementary RNA) | Transfection/electroporation | | [80] |
| | Lymphocyte choriomeningitis virus | L and S segment | Transfection/electroporation | | [80] |
| | Vesicular stomatitis virus | N, P, L, G, M | Transfection/electroporation | | [80] |
| Cas12a | HIV | LTR, gag, env, pol, tat, rev, vpr, nef | Lentiviral transduction | | [81] |

repeat (LTR) promoter regions. LTRs flank retroviruses integrated genetic material and regulate pro-viral transcription, constituting thus a specially suited target for therapy. Nevertheless, viral genomes can be targeted for purposes other than gene disruption. In the case of latent infections, CRISPR/Cas technology allows to reverse viral quiescence by specifically activating viral promoters using catalytically dead versions of Cas nucleases.[71,73] These mutant proteins lack endonuclease activity but are able to interact with complementary DNA targets, and if coupled with regulating factors, can activate or repress transcription at specific DNA *loci*. Latency reversal can boost host's immune response and improve the efficacy of antiviral treatments.

However, other anti-HIV approaches have opted for targeting host elements. Cas9 has been used to prevent viral cell entry by knocking-out the CCR5 and CXCR4 co-receptors[57,65,59,69,61,68,62] and also to

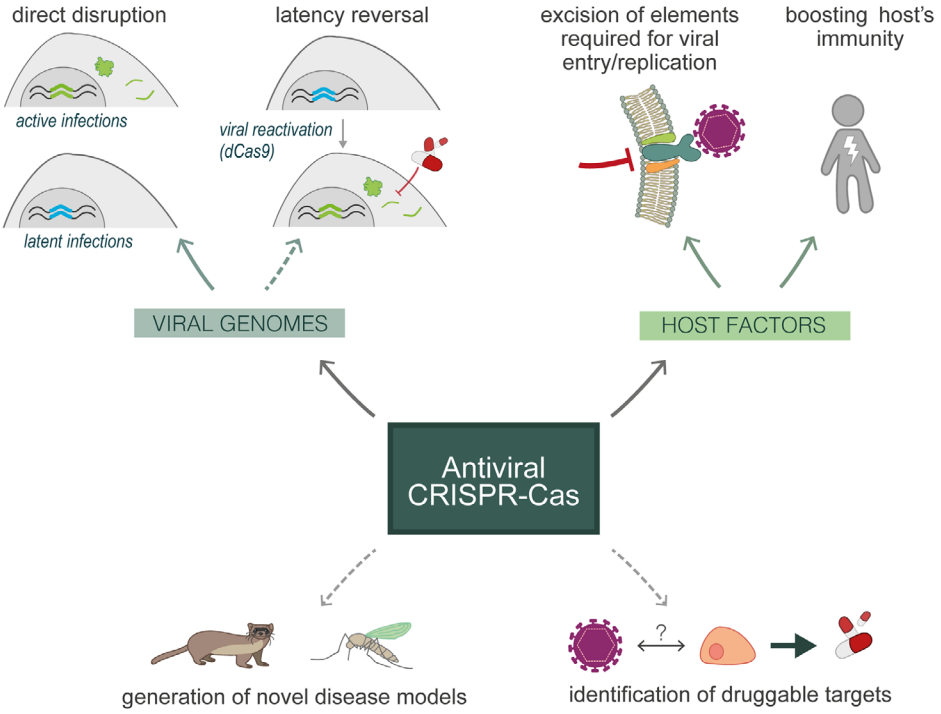


**FIGURE 2** CRISPR-based strategies to combat viral infections. Antiviral CRISPR/Cas approaches target either viral genomes or host factors. Virus clearance can be achieved through viral DNA/RNA disruption; however, "shock and kill" strategies may be used in quiescent infections to induce latency reversal. Host factors can be targeted either to boost host's immunity or to ablate those elements required for viral entry or replication. This technology has also enabled identification of novel druggable targets through the unravelling of virus–host interaction factors. The genetic engineering of non-model organisms that act as reservoirs has allowed the generation of novel disease models

enhance host's antiviral response by inducing restriction factors that prevent viral replication[60] and inactivating elements that repress the immune response against HIV.[67] Host targeting approaches have been mostly limited to HIV infections, however, they may be useful against many other viruses. Different host factors, generally involved in viral entry or replication, have already been proposed as targets for CRISPR-based therapy against several infections.[83] Since genetic modifications are irreversible, host targets must be carefully selected to avoid deleterious side effects. In fact, the CRISPR/Cas system has contributed to identify potential host therapeutic targets, required for viral entry and replication but nonessential for the host.[84]

Generally, targeting viral genomes is the preferred strategy as it constitutes a more straightforward and simple approach. Host-based therapies offer a valuable alternative and can contribute to overcome resistance issues: host genes are much less likely to experience mutations affecting crRNA recognition. However, targeting host factors requires a thorough evaluation of the chosen genes to ensure that they are dispensable for the host. Moreover, the irreversible modification of the host's genome may give rise to ethical concerns.

## Guide RNA design: Viral escape mutants and the importance of multiplex approaches

The design of crRNAs for the recognition of the chosen target gene(s) is also a key aspect to consider. Guide RNA's must simultaneously display high on-target activity and minimize off-target recognition. Although this is a general requirement for CRISPR/Cas designs, it becomes particularly important when applying this technology in vivo. Off-target cleavage might have severe and unpredictable consequences and constitutes a major concern regarding the safety of CRISPR-based therapies. Antiviral CRISPR studies typically test numerous guides to find optimal sequences. In a recently published study on the use of CRISPR/Cas technology against SARS-CoV-2, Abbott et al. designed more than 3000 crRNAs in silico and evaluated the efficacy of 20 crRNAs per target gene. Eventually, they narrowed them down to a minimal pool of 6 crRNAs able to target 90% of all coronaviruses.[79]

The use of individual crRNAs has generally been ineffective due to the emergence of viral escape mutants. Multiplex designs combining several crRNAs have consistently proven to be the best strategy to prevent viral resistance to CRISPR therapy.[52,85,86] This kind of designs typically involve combinations of at least three different crRNAs.[26,32,38,53]

Alternatively, the problem of viral resistance can be circumvented by targeting conserved viral genome regions where mutations are poorly tolerated.[79] crRNA design is often limited to predicted on-target and off-target scores and fails to consider the genetic robustness or fragility of the chosen site, which can crucially determine the efficacy of the editing strategy. In the case of Cas9, NHEJ in less conserved targets typically results in a three-nucleotide insertion.[48] When this short insertion occurs in a protein-coding region, the open reading frame and the coding capacity are likely to be preserved.

However, these minor nucleotide alterations can severely affect critical non-coding regions. For instance, Mefferd et al. were able to avoid viral resistance by targeting HIV *cis*-acting elements. Interestingly, they designed two different crRNAs against HIV's transactivation response element but only one of them prevented viral escape, which further highlights the importance of thorough target site selection.[55]

The multiplexing of highly specific crRNAs designed against conserved relevant viral genome sites constitutes the most promising strategy against viral escape mutants.

## Beyond gene knockout: Gene activation and knock-in-based approaches

The vast majority of CRISPR-based antiviral approaches opt for the straightforward inactivation of genes contributing to the progress of viral infections through the knock-out or knock-down of viral genes or relevant host factors. As previously mentioned, the double-stranded breaks on target DNA sequences induced by Cas9 or Cas12a may result in gene knock-outs when repaired via NHEJ. Cas13, on the other hand, can be used for the knock-down of viral RNA sequences both therapeutically and prophylactically.[14,79] In addition, CRISPR-mediated knock-out generation has proven useful for the study of virus–host interactions and the identification of new therapeutic targets. These approaches use Cas9 in combination with multiplexed pools of crRNAs comprising the entire human genome to detect host factors involved in viral replication.[15]

The highly versatile CRISPR/Cas system can also be applied to transcriptional regulation. Engineered catalytically inactivated dead Cas9 (dCas9) can be coupled with regulating factors to activate or repress transcription.[7] Several anti-HIV CRISPR studies have used dCas9-mediated transcriptional activation with different goals. For example, the coupling of dCas9 with a synergistic activation mediator (SAM) has proven useful to induce the expression of restriction factors that block HIV infection.[60] This kind of transcriptional regulation has also been exploited in the context of latency reversal, an alternative anti-HIV approach consisting in the reactivation of latent virus reservoirs to induce recognition and clearance by the immune system. Ji et al., for instance, used the dCas9-SunTag-VP64 system to reactivate latent HIV transcription in latently infected T-cell lines.[73]

DNA DSBs repair through the homology directed pathway in presence of a donor template may also be exploited for the generation of knock-ins and precise gene editing. Unarguably, this strategy is infrequent among antiviral CRISPR approaches. Nonetheless, it has shown relative effectiveness in certain scenarios. Dufour et al., for example, used Cas9 to introduce two amino acid substitutions in the type I interferon-inducible human restriction factor TRIM5$\alpha$ to prevent HIV infection of human cells.[64] Interestingly, although they were able to edit and isolate several clones, none of them had all the alleles corrected, which illustrates the challenges faced by this kind of approaches.

The knock-out of viral genes is, by far, the most common strategy for antiviral CRISPR/Cas applications. This approach is generally more direct, efficient, and easier to design. CRISPR-mediated transcriptional regulation may also become a key instrument in the development of antiviral therapies as it is a versatile tool that can be adapted to different targets and therapeutic needs. Knock-in generation via HDR, on the other hand, possibly constitutes an unnecessarily complicated alternative. Since the HDR pathway occurs much less frequently than NHEJ, this kind of strategies show very low efficiency. Knock-in generation through the CRISPR/Cas system, however, has contributed to the generation of novel animal models enabling a better understanding of viral infections, and therefore, the development of improved therapies.[16,17]

## Delivery strategies with in vivo translatability

A major limitation for the therapeutic application of the CRISPR/Cas system is the delivery of its components. Residual non-edited infected cells may act as virus reservoirs hindering treatment's efficacy. The choice of optimal vehicles is determined by the location and the cell type. This is specially challenging in the case of infections affecting circulating cells, such as HIV, and those occurring in the central nervous system, due to the blood–brain barrier.

Two main aspects should be considered regarding in vivo CRISPR delivery (Figure 3). First, Cas nucleases and their guides may be delivered as RNPs, mRNA, or DNA, typically in the form of plasmids. Due to DNA's long life-time, plasmid delivery can increase off-target cleavage and poses additional risks such as DNA residual integration. The use of mRNA, on the other hand, is limited by its low stability. Direct RNP delivery is thus the preferred strategy for therapeutic applications.[87] Nevertheless, to our knowledge, in vivo RNP delivery has not yet been studied for antiviral applications. Second, the delivery method for CRISPR effectors must be safe and efficient. Electroporation, nucleofection, and Lipofectamine-based transfection yield good results in vitro, but they are unsuitable for therapeutic purposes, with the exception of ex vivo treatments.[69] Viral-based strategies have been extensively tested for CRISPR delivery (Table 2). However, they are mainly limited to mRNA and DNA delivery and they are known to be immunogenic, which hinders their in vivo potential. Widely used adeno-associated virus vectors show less immunogenicity, but they have two major drawbacks. On the one hand, they display strong tropism for specific organs and tissues, being thus unsuited for the targeting of certain cell types such as lymphocytes.[88] On the other hand, their low loading capacity is highly limiting considering the large size of Cas nucleases.[89] Therefore, non-viral delivery systems able to evade the immune system are of great interest. Nanotechnology constitutes a promising tool for the non-viral delivery of Cas-RNPs as it has the potential to overcome the main drawbacks of viral vectors: safety concerns and low loading capacity. A number of nanomaterials have already been used as Cas-RNPs carriers in other fields[90,91] and their in vivo applicability has been demonstrated.[92] The surface of these nanomaterials is highly tuneable and can be modified for the binding of different cargo, while the wide range of available materials of very different nature provides a choice of solutions to suit every

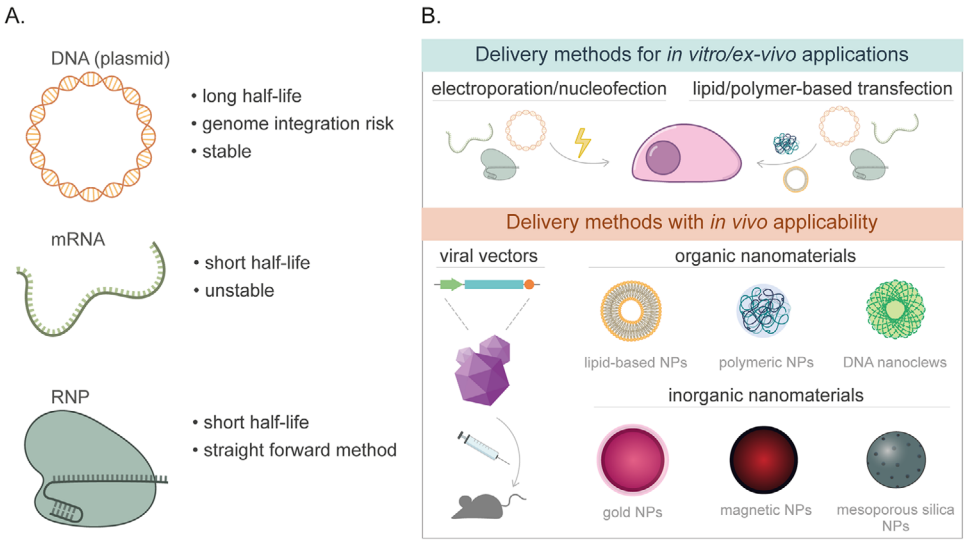


**FIGURE 3** Delivery methods for antiviral CRISPR/Cas strategies. (A) Cas nucleases and their guides may be delivered as DNA (plasmid), mRNA, or RNP. RNP delivery is a more direct approach with lower off-target risk. (B) Efficient delivery methods such as electroporation or nucleofection and lipid/polymer-based transfection can only be applied in vitro and ex vivo. Viral vectors and nanoparticles are more suited carriers for in vivo delivery, and therefore, for therapeutic applications

application. Furthermore, nanoconjugates may include aptamers or other targeting molecules to improve their specificity and favor their accumulation in the tissues or cell types of interest.[93] The use of nanostructures as vehicles in antiviral CRISPR approaches has been limited to DNA and mRNA delivery.[28,94,77] For instance, Kaushik et al. developed a Cas9 delivery system based on magnetic nanoparticles to eradicate HIV infection.[94] Interestingly, the resulting nanoconjugates were able to cross an in vitro model of blood–brain barrier, showing their potential to reach the central nervous system in in vivo settings. In conclusion, the development of suitable RNP carriers could mean a crucial boost for antiviral CRISPR-based therapeutics.

## CRISPR/Cas-BASED VIRAL BIOSENSING SYSTEMS

Nucleic acid detection has been extensively used for molecular diagnosis of viral infections, mainly by quantitative polymerase chain reaction (qPCR). This is a highly specific and sensitive method, but requires sample preparation, high-cost equipment and materials, and specialized technicians, being unfeasible as point of care (PoC) diagnosis or in developing countries. Pandemics like the current SARS-CoV-2 outbreak highlight the need for fast, sensitive, and specific PoC virus detection methods. In this regard, the CRISPR/Cas system has great potential as an alternative sensing tool, due to its high sensitivity and specificity (Figure 4). Cas9, Cas12, and Cas13 have been used for viral nucleic acids sensing. Cas9 detection read-out is based on target recognition and cleavage, while Cas12 and Cas13-based sensors use their *trans*-cleavage activity that is activated upon target recognition. The three CRISPR nucleases are especially suited for viral genotypes discrimination, due to the high specificity of the crRNA target can be abrogated just by changing a small number of nucleotides. However, some limitations hamper its general use for genotype discrimination. Cas9 and Cas12 need the presence of PAM sequences in the target for further recognition (Figure 1).[3,5] The genotype differences must then be located close to PAM sequences. Some Cas13 orthologs, like *Leptotrichia wadei* (Lwa) Cas13a, completely lack this kind of sequence requirements.[8] However, Cas13 recognition is highly dependent on targets' secondary structure. Target RNA secondary structure must be carefully studied to design successful crRNAs and several guides must be tested for optimal detection. Despite these limitations, many relevant CRISPR-based sensors have been developed to detect viral nucleic acids (Table 3).

### Cas9-based sensors

Cas9's target recognition and cleavage were combined with isothermal amplification to develop two detection methods: CRISPR-Cas9-triggered nicking endonuclease-mediated strand displacement amplification (CRISDA)[96] and NASBA-CRISPR.[95]

The CRISDA method uses a pair of engineered Cas9 nickases that produce single-stranded breaks (nicks) on the target dsDNA. The cleavage triggers the strand displacement isothermal amplification of the target. This isothermal DNA in vitro amplification method uses a DNA polymerase together with restriction enzymes and different probes. The resulting amplicon is detected by fluorescence measurement, using a peptide nucleic acid (PNA) labeled with biotin and Cy5, which is complementary to the middle region of the amplicon. The complex is isolated by streptavidin-coated magnetic beads and the fluorescence is recorded. This method presents attomolar (aM) sensitivity and single-nucleotide specificity.[96]

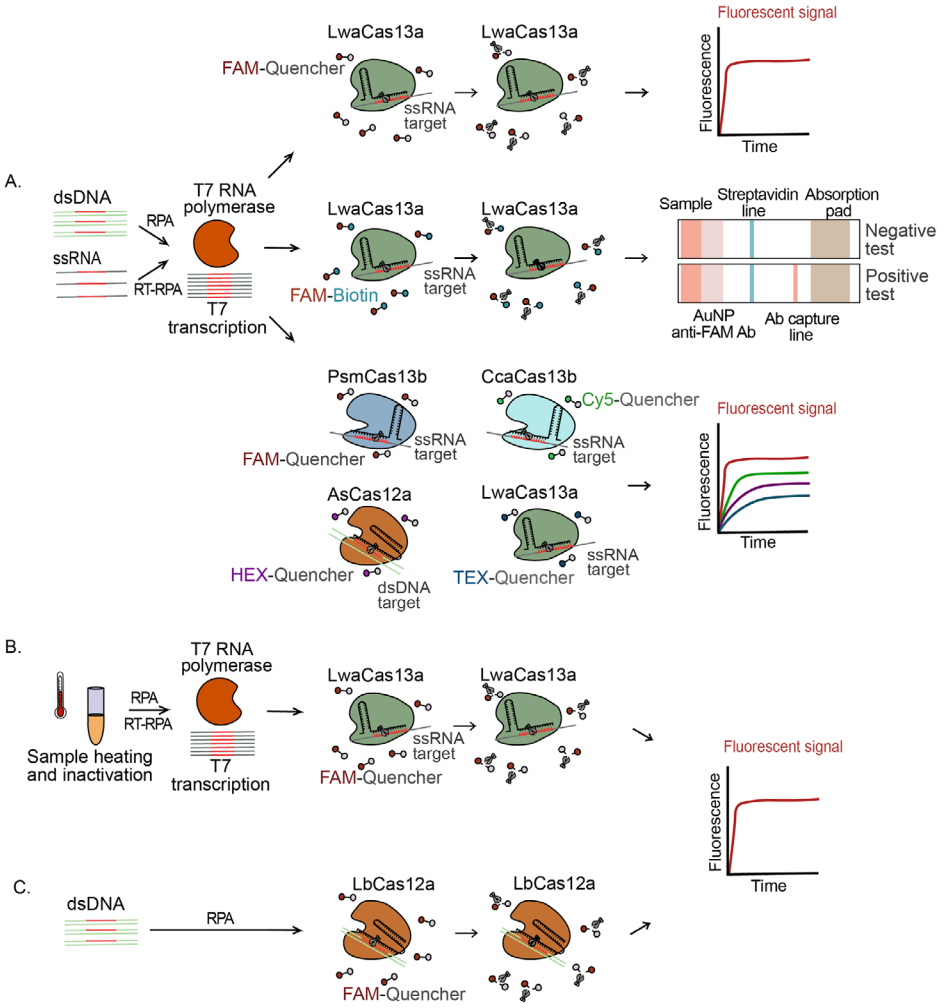


**FIGURE 4** Most relevant CRISPR-based biosensing platforms. (A) SHERLOCK system scheme with different readouts: a single fluorescent reporter, lateral flow that provides a colorimetric readout using a FAM-Biotin reporter, and multiplex detection that uses a combination of Cas effector proteins and different fluorescent reporters. (B) HUDSON in combination with SHERLOCK to bypass the nucleic acid isolation step. (C) DETECTR system scheme

NASBA-CRISPR is a low-cost detection system for ZIKV and DENV, two deleterious ssRNA viruses present in developing countries. The system is assembled in a paper-based platform and combines isothermal RNA reverse amplification, the ability of Cas9 to cleave specific target DNA and toehold switch sensors.[95] Toehold switch sensors are programmable synthetic RNAs that repress gene translation. The binding of a *trans*-acting trigger RNA, complementary to the toehold, reverts this repression. When they are coupled with the translation of the LacZ enzyme, the RNA trigger interaction can be detected by the change in color of a yellow substrate. In the NASBA-CRISPR, the isothermal amplification of the viral RNA produces a dsDNA sequence comprising a trigger sequence, a T7 promoter, and a PAM sequence. The toehold switch sensors are responsible for virus detection, while Cas9's specificity allows discriminating between different virus strains because the specific DNA cleavage generates a truncated reverse transcribed RNA product that lacks the trigger RNA and is unable to activate the toehold switch sensor, lacking then the substrate color change.[95]

Both methods are sensitive, specific, and suitable for PoC viral nucleic acids detection. The use of isothermal amplification instead of PCR increases their portability since a thermocycler is no longer required. Furthermore, fluorescence and color changes can be detected by benchtop fluorometers or colorimeters. However, these methods are highly dependent on the presence of PAMs in the target and on complicated designs of PNAs and toehold switches. In consequence, Cas9 detectors have been overtaken by Cas13 and Cas12-based sensors.

## Cas12 and Cas13-based sensors

The success of Cas12 and Cas13-based sensors lays on their multiple-turnover *trans*-cleavage activity. It is specifically activated by target recognition and allows signal amplification by adding reporter oligonucleotides that are substrates for the *trans*-activity. The recognition of one target molecule activates then the cleavage of many reporter

**TABLE 3** Overview of the main studies on CRISPR-based biosensing platforms

| | Method | Target | Amplification | Sens | Readout | T | Virus | Ref. |
|---|---|---|---|---|---|---|---|---|
| Cas9 | NASBACC | RNA | NASBA | fM | Colorimetric | 3 h | ZIKV DENV | [95] |
| | CRISDA | DNA | SDA | aM | Fluorescent | 2–3 h | N/S | [96] |
| Cas12 | DETECTR | DNA | RPA | aM | Fluorescent | 2 h | HPV | [6] |
| | Cas-gold | DNA | RPA | aM | Colorimetric Fluorescent | 2 h | ASFV | [97] |
| | CRISPR-responsive hydrogel | DNA/RNA | RPA/RT-RPA | aM | Colorimetric | 1 h | Ebola | [98] |
| | Naked-eye detection platform | DNA/RNA | PCR/RPA | aM | Colorimetric | 1 h | ASFV, miRNAs | [99] |
| | STOPCovid | RNA | RT-LAMP | aM | Fluorescent Colorimetric | 1—2 h | SARS-CoV-2 | [100] |
| | AIOD-CRISPR | DNA/RNA | RPA | aM | Fluorescent | 0.5–1 h | SARS-CoV-2 | [101] |
| | Poly (A)- AuNPs | DNA | RPA | – | Naked-eye | – | ASFV | [102] |
| | SHERLOCK | DNA/RNA | RPA | aM | Fluorescent | 1–5 h | ZIKV. DENV, KPC, NDM1 | [103] |
| Cas13 | SHERLOCK v2 | DNA/RNA | RPA | zM | Fluorescent Colorimetric | 0.5–3 h | ZIKV, DENV | [104] |
| | HUDSON + SHERLOCK | DNA/RNA | RPA | aM | Fluorescent | 2 h | ZIKV, DENV, WNV, YFV | [105] |
| | CARMEN | DNA/RNA | PCR/RPA | aM | Fluorescent | – | HCV, HIV, ZIKV, DENV, influenza, SARS | [106] |
| | CRISPR microfluidic | RNA | – | fM | Fluorescent | 5 min | Ebola virus | [107] |

oligonucleotides. Both Cas12 and Cas13-based sensors are experiencing impressive and parallel development since 2017. Additionally, the existence of a dsDNA detector (Cas12) and an ssRNA detector (Cas13) expands sensing possibilities. Cas12-based sensors are more economically viable since reporter ssDNA oligonucleotides are cheaper than ssRNA. Furthermore, isothermally amplified samples do not need to be transcribed to RNA. However, Cas12 target recognition depends on the presence of PAM sequences in the target. The use of LwaCas13a, which has not sequence restriction recognition, allows the potential detection of any ssRNA target. Nevertheless, the secondary structure of the target RNA must be studied to design suitable crRNA guides.

The first developed Cas13 and Cas12 detectors, named Specific High-Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK)[103] and DNA endonuclease-targeted CRISPR *trans* reporter (DETECTR)[6] are based on the specific cleavage of previously amplified viral nucleic acid. This recognition triggers the *trans*-cleavage of quenched ssRNA or ssDNA fluorescent reporters, respectively. The fluorophore is then released, resulting in an increase in fluorescence (Figure 4). Both sensors have been used for the detection of several viruses with aM sensitivity[103,108–111] (Table 3), including SARS-CoV-2.[108,111] In fact, in May 2020 the FDA authorized the use of SHERLOCK for COVID-19 detection, and it has recently been clinically validated.[112] SHERLOCK is a DNA and RNA detection platform based on the *trans*-cleavage activity of LwaCas13a. It requires previous isothermal recombinase polymerase amplification (RPA) or RT-RPA of the extracted nucleic acids and T7 RNA transcription to convert amplified DNA to RNA. Then, LwaCas13a RNP recognizes the amplified RNA target (Figure 4A). Similarly, DETECTR involves prior RPA isothermal amplification but does not need T7 RNA transcription, as the dsDNA is directly recognized by *Lachnospiraceae bacterium* (Lb) Cas12a RNP (Figure 4C). In both cases, the fluorescence readout of the signal hampers their use as PoC detectors, especially for developing countries where benchtop fluorometers may not be affordable. However, their strength lies in multiplexed nucleic acid detection, combining RNPs with different specificities and fluorescent probes. SHERLOCKv2[104] took advantage of the large diversity of dinucleotide cleavage motif preferences among Cas13 orthologs to perform four-channel single-reaction multiplexing detection. They combined PsmCas13b, CcaCas13b, LwaCas13a, and AsCas12a RNPs that interact with different targets, with reporter oligonucleotides labeled with four different fluorophores, each of them specific to one type of nuclease

(Figure 4A). Recently, Sabeti's group developed a new SHERLOCK-based multiplexed detection platform called Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic acid (CARMEN). CARMEN uses arrays of nanoliter droplets containing the amplified sample, LwaCas13a, and detection reagents. Fluorescence microscopy allows to detect aM concentrations of more than 4500 nucleic acids from different strains of ZIKV, DENV, HPV, HIV, influenza virus, or SARS-CoV-2.[106]

SHERLOCK and DETECTR have also been coupled to lateral-flow readout systems, to develop fully portable PoC detectors.[104,97] In SHERLOCKv2, the ssRNA reporter is labeled with FAM and biotin, while the paper strip contains gold nanoparticles (AuNPs) coated with anti-FAM antibodies and two retention lines: a first streptavidin line for biotin binding and a second protein A line for antibody recognition. Reporter cleavage results in the retention of AuNPs on the protein A line instead of in the streptavidin line (Figure 4A). The Cas-Gold system relies on the same readout, but using LbCas12a and ssDNA oligonucleotides.[97] Interestingly, Barnes et al. have recently developed a mobile phone application that enables the quantification of the viral load from the paper strip based on the band intensity.[110]

Although fluorescence and colorimetric lateral flow are the most used readout methods for Cas12 and Cas13 target recognition, other approaches for signal detection have been explored. English et al. combined microfluidics and CRISPR to detected RPA-amplified Ebola virus RNA with aM sensitivity. They used Cas12a, DNA hydrogels, and a microfluidic paper-based analytical device. The DNA hydrogel, formed by acrylamide polymers crosslinked by ssDNA linkers, is located in the microfluidic path, obstructing the flow. The presence of Cas12a RNP and the target DNA triggers ssDNA linkers' cleavage, opening the flow path. The use of colored fluids allows for naked-eye detection and can also be detected measuring electrical conductivity.[98]

Despite the impressive and fast development of this kind of sensors, some obstacles must be circumvented to produce even more suitable viral nucleic acid PoC sensors: the dependence on nucleic acid extraction and amplification. Recent studies show the efforts to improve Cas13 and Cas12-based detectors. Sabeti's group implemented heating unextracted diagnostic samples to obliterate nucleases (HUDSON) coupled to SHERLOCK. It enables rapid and sensitive (aM) DNA or RNA detection from body fluids, avoiding the nucleic acid isolation step. It inactivates the viral particles and the RNases in urine, saliva, whole blood, or serum samples with heat and chemical reduction. Those samples can be directly added to RPA reactions.[105] Interestingly, two recent publications show that it is possible to unify the isothermal amplification and target detection steps in one-pot, preventing cross-contamination in sample manipulation. The first one, STOPCovid (SHERLOCK testing in one-pot), uses the thermostable *Alicyclobacillus acidophilus* (Aap) Cas12b. The system is able to combine viral particles lysis and RNA extraction with one buffer in a short time, followed by RNA concentration using magnetic beads. Then, sample amplification is performed straight from the lysate by RT-LAMP, and Cas12b detection is performed in one-pot at 60°C. The use of thermostable nucleases like AapCas12b is then the key point of this kind of methods. Besides, LAMP primers and crRNAs must be carefully designed. Lateral-flow or fluorescence can be used as readout, allowing to detect aM concentration of SARS-CoV-2 N gene, similar to qPCR.[100] The all-in-one dual CRISPR-Cas12a combines RPA isothermal amplification and Cas12a RNP target recognition in one-pot. It is performed at 37°C, so the better studied LbaCas12a is applied.[101] This method uses two LbaCas12a-crRNA RNPs, which interact with two different sites within the amplicon, very close to the RPA primers recognition sites. The RPA amplification and the Cas12a-crRNAs cleavage occur in parallel, together with the *trans*-cleavage activity. The system lacks PAM sequence restriction because Cas12a lacks PAM requirements if the target sequence is already unwound.[113] The amplification reaction unzips Cas12a RNP target sites, allowing its PAM-free interaction with the DNA. Furthermore, the use of two Cas12a RNPs per target increases the specificity. Coupled to a fluorescent read-out, this method has been used to detect few copies of SARS-Cov-2 N gene in clinical samples, presenting aM sensitivity.[113]

Undoubtedly, the most needed development of CRISPR-based sensors is to extend their detection limit to avoid prior sample isothermal amplification, as this implies an extra-step that must be optimized for every target. Qin et al.[107] used LwaCas13a, an automated multiplexed microfluidic chip for sample concentration, and a custom integrated benchtop fluorometer to circumvent isothermal amplification. This method detects Ebola virus with 100 fM sensitivity in only 5 min. Although less sensitive than qPCR detection, this work showed the promising future of multidisciplinary approaches to develop better CRISPR-based sensors. In this regard, English et al. detected unamplified samples combining Cas12a, DNA hydrogels, microfluidics, and electric conductivity detection.[98] Both methods combine microfluidics to increase sample concentration and improve sensitivity.

Recently, Jennifer Doudna and colleagues have developed an LbuCas13a detection system for SARS-CoV-2 directly from viral RNA, with no need for previous amplification. They combine various crRNAs carefully designed to improve target recognition. The readout is conducted using a mobile phone camera with laser illumination. The mobile phone works then as a fluorometer, with an app for RNA quantification, allowing for PoC detection. This work shows that sensitivity can be highly increased by just combining various well-designed crRNAs to recognize the same target. Thus, it should be underlined the importance of the crRNA design step, which can be crucial for effective and sensitive target recognition.[114]

The recent coupling of CRISPR-based viral sensors with nanotechnology has opened a novel field with great potential. Bao et al. developed a Magnetic Bead-Quantum Dot (MB-Qdot)-based sensing system able to detect nM concentrations of ASFV's nucleic acids. The system uses streptavidin-coated MBs and two complementary ssDNA probes tagged with biotin and Qdots, respectively. The biotin probe can bind to MB and interact with the complementary ssDNA labeled with QDot. When the MBs are separated using a magnetic field, the Qdots are pulled down, resulting in a colorless solution. When LbCas12a RNP and its target are previously added to the biotin-labeled probes, the target-activated RNP cleaves the biotin probe and the Qdot-probes cannot bind the MBs, so the solution remains colored after magnetic MB separation.[115] This is a highly portable system due to the naked-eye

readout. Its low sensitivity could be improved by coupling it with prior sample amplification. Hu et al. discovered a fast and efficient AuNPs coating method using poly (A) linkers, based on which they developed a diagnosis system. It relies on the same kind of read-out than Bao et al. but using AuNPs instead of Qdots. This method was able to detect ASFV from serum samples, after RPA isothermal amplification, although the limit of detection is not provided. They also designed an RNA detection system using AuNPs coated with ssRNA-FAM oligonucleotides and LbuCas13a. Upon target recognition, the FAM is released and can be detected by fluorescence. This alternative method was used to detect bacteria strains, but not viruses.[102]

## CONCLUSIONS

The CRISPR/Cas system has an enormous potential as a novel therapy against viral infections. The use of Cas nucleases not only enables the direct disruption of viral DNA and RNA in a highly specific manner but also allows to act on relevant host genes. Remarkably, CRISPR-based approaches have proven useful for targeting latent virus reservoirs, one of the main drawbacks of current antiviral treatments. The success of CRISPR therapeutic applications strongly relies on overcoming key limitations. Future antiviral CRISPR designs must combine several guide RNAs (multiplexing) and preferentially target conserved viral genome regions to prevent viral resistance. Additionally, the specificity of CRISPR/Cas systems must be improved to ensure the safety of therapeutic applications. This will be achieved through the delivery of RNPs instead of DNA, and the generation of improved high-fidelity Cas variants. The development of suitable RNP carriers is the most promising path to enhance the safety and efficiency of CRISPR delivery.

CRISPR-based viral detection systems are rapid, versatile, specific, able of self-signal amplification, and sensitive. They are also especially suited for multiplex detection.[106] Many of the platforms here reviewed do not require expensive machinery, reagents, or specialized staff, making them an ideal tool for PoC applications. The future of this technology relies on the design of more sensitive and portable sensors. Some studies have already shown that direct nucleic acid detection, without previous sample amplification, is possible. Further research to enhance Cas *trans*-cleavage activity and improve crRNA design will help increase sensitivity. Moreover, the combination of the CRISPR system with nanotechnology and nanofluidics will further enhance both the sensitivity and portability of diagnostic devices.

In conclusion, CRISPR/Cas technology constitutes an important tool to combat viral infections. It has a great therapeutic potential and could be applied soon, once safety and delivery issues are sorted out. Moreover, its use for viral detection is an actual fact.

### DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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