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Review

Overcoming the Short-Length Barrier: Molecular Engineering Strategies for Cas12a-Based miRNA Detection

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Abstract: The CRISPR/Cas12a system has emerged as a powerful platform for nucleic acid diagnostics owing to its programmable target recognition and collateral cleavage–mediated signal amplification. In recent years, extensive molecular engineering efforts have expanded the applicability of Cas12a-based sensing systems to the detection of microRNAs (miRNAs), short non-coding RNAs that regulate gene expression at the post-transcriptional level and serve as important biomarkers for a wide range of diseases. In this review, we summarize recent advances in Cas12a-based miRNA sensing strategies, with a particular emphasis on two major design paradigms: activator-centered engineering, which translates miRNA recognition into Cas12a-activating molecular inputs, and crRNA-centered engineering, which modulates the structure and function of the Cas12a–crRNA complex to enable miRNA-responsive activation. We discuss representative implementations and underlying molecular design principles to illustrate how these approaches expand the design space of CRISPR-based diagnostics. Collectively, these developments highlight the growing potential of Cas12a-based platforms as versatile tools for next-generation miRNA diagnostics.

Key words: CRISPR/Cas12a, microRNA detection, activator engineering, crRNA engineering, nucleic acid diagnostics

1. Introduction

MicroRNAs (miRNAs), short non-coding RNAs of approximately 19–23 nucleotides, regulate gene expression at the post-transcriptional level by binding to target mRNAs thereby playing critical roles in

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diverse physiological processes.[1,2] The regulatory landscape of miRNAs is remarkably extensive, with more than half of human protein-coding genes estimated to be subject to miRNA-mediated regulation.[2,3] Consistent with their widespread regulatory influence, aberrant miRNA expression has been reported in a wide range of diseases, including cancer, neurological disorders, and cardiovascular diseases.[4-6] Notably, miRNAs are detectable in body fluids such as blood and serum, highlighting their potential as minimally invasive molecular biomarkers for disease diagnosis, prognosis assessment, and monitoring of therapeutic responses.[7-9]

Despite this significant diagnostic potential, reliable quantitative analysis of miRNAs remains technically challenging.[10] These challenges primarily arise from the intrinsically low abundance of miRNAs in biological samples, their extremely short sequence length, and the high sequence similarity among members of the same miRNA family.[11,12] Collectively, these features impose stringent requirements on detection platforms, which must simultaneously achieve high analytical sensitivity and single-nucleotide discrimination capability. To address these challenges, various miRNA detection platforms have been developed. Early approaches relied on hybridization-based techniques such as Northern blotting and microarray analysis.[13,14] Subsequently, enzyme-dependent amplification strategies —including quantitative reverse transcription PCR (qRT-PCR), rolling circle amplification (RCA), exponential amplification reaction (EXPAR), strand displacement amplification (SDA), and loop-mediated isothermal amplification (LAMP)—were introduced to enhance detection sensitivity.[15-20] However, these amplification-based methods typically require specialized instrumentation or precise temperature control and often involve multistep reaction processes, which increase the risk of contamination and complicate the implementation of one-pot analytical systems.[21]

Emerging as a promising alternative, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)–associated protein (Cas) system has attracted considerable attention as a next-generation nucleic acid diagnostic framework.[22,23] Derived from the adaptive immune systems of bacteria and archaea, CRISPR–Cas systems enable programmable, sequence-specific nucleic acid recognition through RNA-guided Cas effectors.[24] In select Cas effectors, including Cas12 and Cas13, target recognition induces a conformational activation that triggers nonspecific *trans*-cleavage (or collateral cleavage) activity toward nearby single-stranded nucleic acids. This property has been widely exploited in diagnostic platforms to generate detectable signals for nucleic acid sensing, with representative examples including DNA endonuclease-targeted CRISPR trans reporter (DETECTR) and Specific High-Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK), which have achieved attomolar-level detection sensitivity.[25,26]

Compared with Cas13-based systems that employ RNA reporters, Cas12a utilizes single-stranded DNA (ssDNA) reporters, offering several advantages, including reduced susceptibility to RNase degradation, greater chemical stability, and improved compatibility with diverse DNA-based biosensing platforms.[27] However, in the context of miRNA detection, Cas12a presents inherent limitations. As a DNA-targeting effector, Cas12a typically cannot directly recognize RNA substrates. Moreover, the extremely short length of miRNAs is insufficient to simultaneously accommodate both the protospacer adjacent motif (PAM) sequence required for Cas12a activation and the full protospacer length (~20 nt). We refer to this intrinsic limitation arising from sequence length as the "short-length barrier."

In this review, we focus on engineered strategies that have been developed to overcome the short-length barrier and enable high-performance Cas12a-based miRNA detection. First, we discuss activator-centered engineering strategies, which reconstruct miRNA molecules into activation triggers recognizable by Cas12a. Subsequently, we introduce CRISPR RNA (crRNA)-centered engineering strategies, highlighting design approaches that enable precise regulation of Cas12a activation through crRNA engineering. For each category, we discuss the underlying design principles, representative implementations, analytical performance, and remaining challenges, ultimately

presenting an integrated framework for advancing Cas12a-based miRNA diagnostics toward clinical applications.

2. Mechanistic Framework of Cas12a-Based miRNA Detection

Cas12a (Cpf1) is a Class 2, Type V CRISPR nuclease that forms a ribonucleoprotein (RNP) complex with a single crRNA to recognize target nucleic acids, primarily double-stranded DNA (dsDNA).[24] Cas12a first recognizes a PAM, induces local DNA unwinding, and forms an R-loop structure through base pairing between the crRNA spacer and the target sequence. During this process, the target DNA is cleaved in *cis*, and the enzyme simultaneously undergoes a conformational transition into an activated state. The activated Cas12a exhibits robust *trans*-cleavage activity toward non-target ssDNA, and this collateral cleavage reaction enables catalytic signal amplification by cleaving labeled ssDNA reporters.[28,29] This dual cis–trans cleavage property constitutes the fundamental mechanistic basis for extending Cas12a into a nucleic acid sensing platform.[30,31]

Because Cas12a intrinsically recognizes PAM-containing dsDNA, miRNA cannot serve as a direct activator under native conditions. Therefore, miRNA is typically converted into a Cas12a-activating form (DNA activator) through reverse transcription (RT) and amplification reactions such as RPA or LAMP.[25,26,32] The resulting DNA activator then activates Cas12a via the canonical target recognition pathway, generating a *trans*-cleavage–based signal. This architecture resembles conventional methods such as qRT-PCR in that it relies on RNA-to-DNA conversion and amplification. Furthermore, the short length of miRNAs results in limited hybridization stability during the initial recognition and conversion stages, thereby affecting the yield and structural integrity of the resulting DNA activator and, consequently, Cas12a activation efficiency.[33,34] High sequence similarity among miRNA family members further complicates the discrimination of single-nucleotide mismatches, and nonspecific activation may be amplified through *trans*-cleavage, increasing background signals.[35,36] Additionally, RT/amplification reactions and Cas12a cleavage often require distinct enzymatic conditions (temperature, ionic composition, accessory proteins), making their integration into a unified reaction system challenging.[37,38] Thus, to fully leverage the catalytic signal amplification capability of Cas12a for miRNA sensing, redesign of both activator input and guide RNA structure is required beyond simple DNA conversion strategies. The following sections examine activator-centered strategies and crRNA-centered engineering approaches developed to address these challenges.

3. Activator-Centered Engineering Strategies for miRNA Detection

A central design challenge in Cas12a-based miRNA detection is to connect the miRNA target to a molecular state capable of activating Cas12a. Early studies primarily employed RT- and polymerase-based amplification strategies to transform miRNA into DNA activators, achieving high sensitivity.[39,40] Subsequent efforts moved beyond enzyme-dependent amplification toward circuit-based strategies that generate activators through DNA dynamic reactions or structural transitions. In parallel, direct activation strategies were proposed in which miRNA itself or RNA-containing hybrid structures directly establish the activation condition for Cas12a. Accordingly, the concept of activator formation has evolved from DNA-mediated conversion to broader structural and molecular interface engineering.

3.1. Indirect activation via RNA-to-DNA conversion

Indirect strategies activate Cas12a through DNA activators generated downstream of miRNA recognition, with early RT- and polymerase-based amplification approaches representing the canonical implementation. To mitigate limitations associated with enzyme-dependent template amplification, DNA dynamic circuit–based strategies were subsequently introduced (*Fig.* 1).

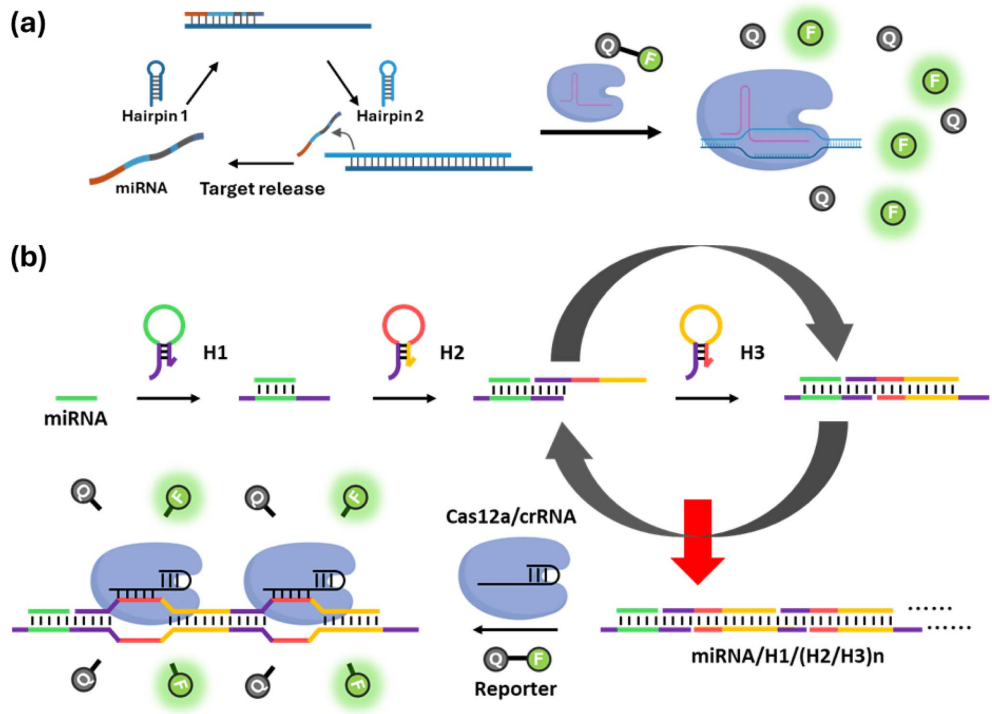

Fig. 1. Representative examples of indirect miRNA-to-DNA conversion strategy. (a) Catalytic hairpin assembly (CHA)-mediated target recycling and DNA activator formation. (b) Schematic illustration of a hybridization chain reaction (HCR)-based detection strategy. Created with BioRender.com

Representative examples include catalytic hairpin assembly (CHA) (*Fig.* 1(a)) and hybridization chain reaction (HCR) designs (*Fig.* 1(b)), in which miRNA serves not merely as a template for conversion but as a catalyst that initiates structural reactions to generate multiple activator structures.[41,42] Peng *et al.* achieved sub-femtomolar sensitivity for various miRNAs using a CHA-based strategy and reported a 3–4 orders-of-magnitude improvement in detection limits compared with conventional CRISPR-Cas diagnostics.[43] The platform enabled precise detection of miR-21 in cancer cell lines and clinical serum samples.

Similarly, HCR-based approaches have been developed to couple enzyme-free nucleic acid circuits with CRISPR detection. In a representative example, an HCR circuit was employed as a signal transducer to convert each miRNA molecule into multiple DNA duplex activators capable of triggering Cas12a *trans*-cleavage activity.[44] In this design, the HCR-generated duplex structures serve as sequence-defined DNA activators, enabling sensitive detection of different miRNAs without altering the spacer sequence of crRNA. The resulting platform demonstrated high sensitivity for miRNA detection and showed strong agreement with RT-qPCR analysis of miRNAs extracted from different cell lines, highlighting its potential for clinical cancer diagnostics.

3.2. Direct activation via RNA recognition

Direct strategies are designed such that miRNA neither generates an independent DNA activator nor relies on pre-existing DNA scaffolds to transmit signals. Instead, miRNA itself or RNA-containing hybrid structures directly participate in Cas12a activation.[45-48] In such designs, miRNA functions not merely as an input signal but as part of the activator domain, directly contributing to target recognition and *trans*-cleavage activation (*Fig.* 2).

Zhao *et al.* systematically demonstrated that LbCas12a can be activated by fragmented ssDNA activators

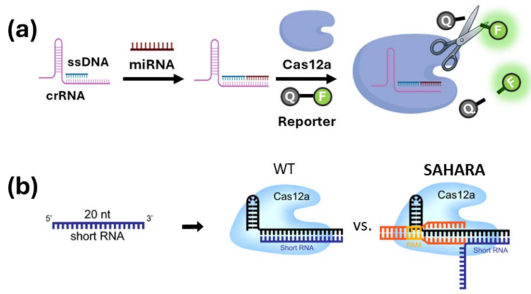

Fig. 2. Direct activation via RNA recognition strategies for Cas12a-based miRNA detection. (a) Fragment Complementary Activation Strategy (FCAS)-based direct detection strategy through miRNA-dependent activator formation and reporter cleavage. Created with BioRender.com. (b) Schematic illustration of Cas12a activation by short RNA, highlighting the role of a PAM-proximal DNA activator in the Split Activator for Highly Accessible RNA Analysis (SAHARA) system compared to the wild-type (WT) condition. Adapted from Ref [47], licensed under CC BY 4.0.

and further showed that DNA fragments in the crRNA distal domain can be replaced by target miRNA sequences (*Fig.* 2(a)).[45] Based on this finding, they proposed the Fragment Complementary Activation Strategy (FCAS), constructing a CRISPR-Cas12a platform in which miRNA serves directly as an activator without pre-amplification. The system achieved a detection limit of 5.53 fM and enabled quantification of miRNA-10b in clinical serum samples, as well as in situ imaging of tumor cells.

Similarly, Lou *et al.* reported that LbCas12a can be directly activated by miRNA. By integrating an Au-nanobeacon reporter, they developed a CRISPR-Cas12a biosensor capable of direct miRNA detection without DNA conversion or amplification.[46] The system achieved attomolar-level sensitivity within 5 minutes and maintained high selectivity in complex biological samples.

Additionally, direct RNA detection using Cas12a has been achieved through engineered activator strategies. Rananaware *et al.* reported the Split Activator for Highly Accessible RNA Analysis (SAHARA) system, which exploits the distinct target-recognition regions of Cas12a (*Fig.* 2(b)).[47] In this design, the PAM-proximal seed region of the crRNA spacer is hybridized with a short complementary DNA oligonucleotide to fulfill the DNA recognition requirement for Cas12a activation, while the PAM-distal region recognizes the RNA target through RNA–crRNA base pairing. Together, the DNA and RNA components cooperatively reconstruct a functional activator, forming a hybrid R-loop that triggers the *trans*-cleavage activity of Cas12a. By supplying a short DNA sequence complementary to the seed region, SAHARA enables direct detection of RNA targets without reverse transcription, amplification, or strand-displacement reactions. Using this strategy, the authors demonstrated picomolar-level RNA detection and multiplexed analysis of different RNA and DNA targets through programmable crRNA/Cas12a arrays.

4. crRNA-Centered Engineering Strategies for miRNA Detection

While activator-centered design strategies focus on constructing molecular mediators that transmit miRNA recognition into Cas12a activation, crRNA-centered engineering strategies directly manipulate the crRNA itself as an active design variable to control Cas12a activation. This perspective treats the Cas12a–crRNA complex not as a fixed system but as a structurally and conformationally tunable module. Particularly for short and weakly binding targets such as miRNA, conventional protospacer complementarity alone may be insufficient for robust Cas12a activation. Accordingly, strategies involving crRNA structural reconfiguration or sequence/conformational modification have been developed to enhance stability and activation efficiency.[49,50]

4.1. Structural reconfiguration: Split-crRNA architectures

Split crRNA strategies control Cas12a activation by dividing the crRNA into two or more fragments. This approach conceptualizes crRNA not as a single continuous guide but as a modular architecture composed of a handle (for protein binding) and a spacer (for target recognition) (*Fig.* 3).[49-52] Structural reconfiguration allows tuning of activation thresholds and kinetics, particularly mitigating instability and

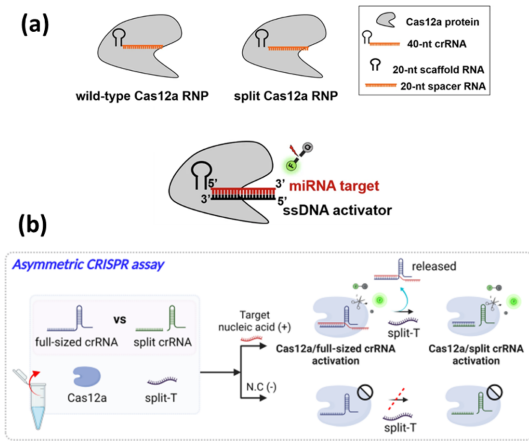

Fig. 3. Split-crRNA architectures for structural reconfiguration of Cas12a activation. (a) Comparison of wild-type and split-crRNA Cas12a ribonucleoprotein (RNP) complexes and principle of the miRNA detection in the split-crRNA-Cas12a system. Adapted from Ref [50], licensed under CC BY 4.0. (b) Asymmetric CRISPR assay based on sequential activation by full-sized crRNA and split crRNA. Adapted from Ref [53], licensed under CC BY 4.0.

background signals associated with short targets such as miRNA.

The most intuitive split strategy involves binary fragmentation of handle and spacer components. Collins *et al.* demonstrated that Cas12a crRNA can be separated into functional modules and that substantial portions of the handle can be removed, or the handle and spacer can be supplied independently, while preserving target DNA cleavage and collateral ssDNA cleavage activity.[49]

Chen *et al.* extended this structural flexibility to miRNA detection by designing a split Cas12a system (SCas12a) in which target miRNA functions as the spacer component (*Fig.* 3(a)).[50] Without pre-amplification, the system enabled detection of miRNA and long RNA targets, discrimination between mature and precursor miRNA, single-nucleotide variation analysis, and quantification in clinical samples. Zeng *et al.* further optimized binary split-crRNA structures for short RNA detection, achieving attomolar-level sensitivity without reverse transcription or pre-amplification.[51]

Subsequent work expanded spacer reconfiguration strategies. The "Splice-at-will" concept demonstrated that Cas12a spacers can be split at nearly arbitrary positions, enabling conditional guide reconstitution only when specific RNA complements missing spacer segments.[52] This enabled quantification of ultra-short RNAs (6–8 nt), which are difficult to detect using conventional Cas systems.

Split crRNA strategies have also been applied to kinetic control. Moon *et al.* proposed an Asymmetric CRISPR strategy leveraging differential binding affinities between full-length and split crRNAs (*Fig.* 3(b)).[53] Following initial activation by full-length crRNA, reactivation by split crRNA generated sequential *trans*-cleavage cascades, achieving 856 aM sensitivity without pre-amplification and enabling detection of miR-19a in bladder cancer patient plasma samples.

4.2. Sequence and conformational engineering of crRNA

Whereas split-crRNA strategies reconfigure crRNA topology to regulate Cas12a activation, the approaches discussed here maintain crRNA as a single continuous molecule while employing sequence composition and conformational modulation to fine-tune functional properties (*Fig.* 4).[54-57] Because target recognition by Cas12a is governed by the hybridization between the crRNA spacer and the target sequence, such adjustments modulate hybridization thermodynamics, mismatch tolerance, and activation efficiency of the Cas12a–crRNA complex.

Sequence-level engineering of crRNA provides a versatile strategy to modulate target recognition and expand the detectable target range of Cas12a-based diagnostic systems. For example, shortening the spacer region of crRNA has been shown to reduce mismatch tolerance by lowering hybridization stability, thereby enhancing discrimination of highly homologous miRNA sequences.[54] Such truncated crRNA designs enable improved differentiation of single-nucleotide variants within closely related miRNA families, such as the *let-7* family, where conventional CRISPR–Cas systems often suffer from off-target activation.

Modified crRNA architectures have been further explored to improve target recognition and enable

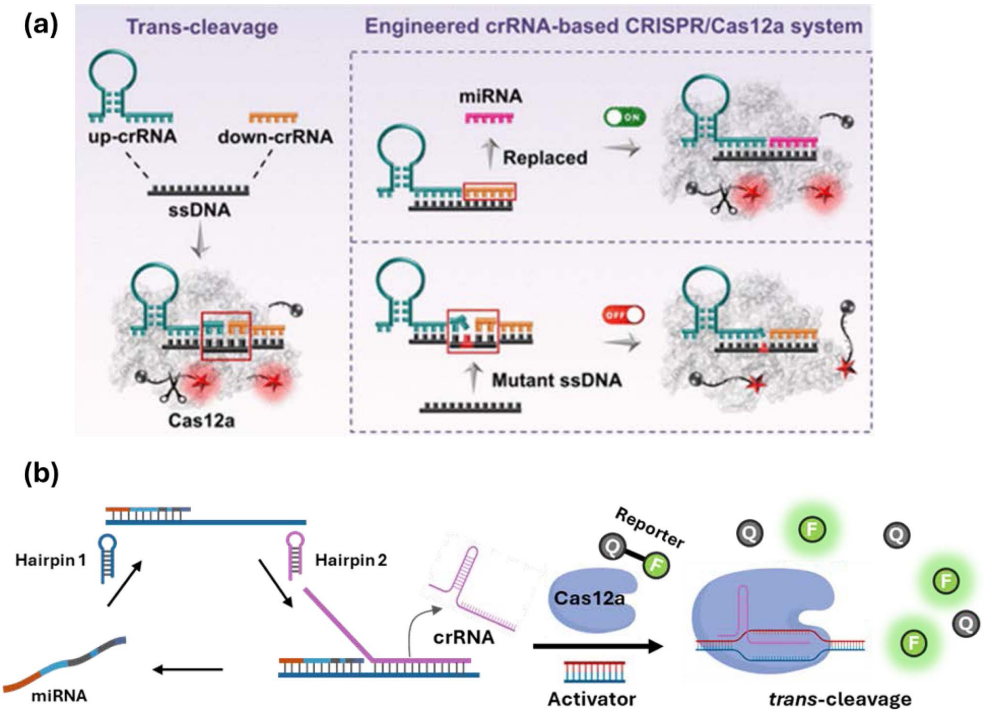

Fig. 4. Sequence and conformational modification strategies of crRNA for miRNA-responsive Cas12a activation. (a) Shortened spacer design for miRNA-assisted crRNA reconstruction and Cas12a activation. Reproduced from [55], Copyright © 2025 American Chemical Society (b) miRNA-triggered conformational rearrangement of hairpin-structured crRNA into an active configuration enabling Cas12a activation. Created with BioRender.com

RNA-responsive activation of Cas12a.[55] In these systems, the crRNA spacer is strategically shortened and reconfigured to facilitate direct RNA target recognition, enabling amplification-free detection of miRNAs and DNA point mutations while maintaining *trans*-cleavage activity comparable to that of wild-type crRNA (*Fig*. 4(a)). These engineered constructs preserve the essential scaffold required for Cas12a binding while expanding their applicability to molecular diagnostics and bioimaging.

Conformational modulation strategies regulate Cas12a activity by inducing structural transitions in crRNA in response to target binding or external stimuli. Liu *et al.* developed a light-activated CRISPR–Cas12a (LAC12a) system using an *ortho*-nitrobenzyl phosphate ester–caged nucleic acid hairpin structure coupled to the Cas12a complex.[56] In this design, the caged hairpin maintains the system in an inactive state until light irradiation removes the cage group, triggering structural activation of the Cas12a machinery. Upon photoactivation, the system enables amplified fluorescence detection of miRNA-21. In addition, the LAC12a platform was further applied to intracellular imaging of miRNA-21 across different cell lines and cell-cycle phases. Integration with two-photon laser confocal microscopy enabled spatiotemporally controlled activation of the CRISPR–Cas12a system at the single-cell level, allowing visualization of miRNA-21 expression dynamics and mapping of cell-cycle phase distributions within cell populations.

Additional conformational control strategies have also been explored to regulate crRNA accessibility (*Fig*. 4(b)). For instance, Chen *et al.* further designed spacer-blocked crRNA to integrate RNA-based CHA circuits with CRISPR-Cas12a in a one-step isothermal system.[55] Target miRNA triggered structural conversion of blocked crRNA into pre-crRNA, which was subsequently processed into mature crRNA by intrinsic

Cas12a RNase activity, enabling femtomolar miRNA detection without RPA.

5. Challenges and Perspectives

Activator-based designs and crRNA-centered engineering strategies have advanced rapidly; nonetheless, fundamental challenges remain in the application of Cas12a to miRNA detection. First, the introduction of molecular design elements such as activator engineering and crRNA modification substantially increases the design complexity at the protein–RNA–DNA interface. In particular, miRNAs present a narrow design window due to their short length, limited hybridization stability, and high sequence homology among family members. These properties make single-nucleotide discrimination critically important.[10-12] Consequently, converting miRNAs into a Cas12a-activating input requires careful optimization of multiple parameters, including the geometry of the activator structure, seed-region stability, and hybridization thermodynamics. When strategies such as split-crRNA or conformationally modified crRNA are employed, additional optimization of the structural and kinetic properties of the crRNA itself becomes necessary. As a result, robust detection requires multilayered molecular engineering that goes beyond simple guide-target complementarity.

Second, DNA dynamic circuit strategies such as CHA and HCR have been introduced to bypass reverse transcription–based amplification.[43,44] Although these approaches enable signal amplification without enzymes, they still rely on multistep reaction architectures. In typical designs, miRNA recognition, circuit initiation, activator generation, and Cas12a activation occur through sequential molecular reactions. During this process, compounding interactions between design parameters and reaction conditions may introduce additional variability. In particular, when circuit reactions and Cas12a activation occur simultaneously within a single reaction environment, imbalances in reaction kinetics or accumulated background leakage may affect signal precision and reproducibility. Therefore, while circuit-based strategies provide an alternative to enzyme-driven amplification, significant challenges remain in achieving fully simplified one-step platforms.

Despite these challenges, Cas12a-based miRNA detection platforms continue to evolve owing to the high flexibility of molecular design. Addressing the issues outlined above will require advances across multiple fronts, from simplifying molecular design to expanding detection capability toward multiple targets. As the understanding of the Cas12a activation mechanism continues to deepen, recent studies have increasingly attempted to control activation thresholds and reaction kinetics by adjusting design parameters such as fragment length, seed-region stability, and component concentration ratios.[47,50,56] As these design variables accumulate, empirical tuning alone will become insufficient. Future efforts will likely shift toward more systematic and predictive design frameworks. By integrating binding thermodynamics with reaction kinetics, it may become possible to narrow the design space in advance while simultaneously improving reproducibility and scalability.

Furthermore, future platform development may benefit from reducing the number of reaction steps. Precise control of individual reaction kinetics is equally important to stabilize the overall reaction flow. In practical molecular diagnostic systems, multiple reaction steps are often connected in cascade[53,58] Therefore, coordinating reaction rates across different stages will be essential to minimize cumulative errors and nonspecific signals. From this perspective, molecular design strategies that structurally isolate interfering steps or clearly define reaction pathways may provide important directions for realizing more robust one-step platforms.

Beyond simple activation architectures that rely on a single molecular input, increasing attention is also being given to logic-based reaction networks incorporating AND, OR, and cascade operations.[59] Such designs can structurally suppress nonspecific activation by conditioning Cas12a activation on specific combinations of molecular inputs or structural requirements. Furthermore, logic-based molecular networks offer the potential to more accurately reflect disease-specific miRNA signatures that involve

complex expression patterns.

Finally, for practical clinical applications, it will be important to move beyond single-target proof-of-concept demonstrations toward systems capable of simultaneously analyzing multiple miRNAs.[60] While a single miRNA may sometimes provide sufficient diagnostic information, in many cases, the combined interpretation of multiple miRNA expression changes offers a more accurate representation of disease states. Accordingly, multiplex detection platforms will play a key role in improving diagnostic accuracy and interpretability. Achieving this goal will require orthogonal guide–activator sets and signal separation strategies that remain interference-free even in the presence of Cas12a *trans*-cleavage activity.

6. Conclusions

In this review, we examined how Cas12a-based sensing platforms can be adapted for miRNA detection through extensive molecular engineering. In particular, we discussed two major design paradigms developed to overcome the short-length barrier inherent to Cas12a-based miRNA detection: activator-centered engineering strategies that translate miRNA recognition into Cas12a-activating DNA structures, and crRNA-centered engineering strategies that modulate the structure and function of the Cas12a–crRNA complex itself. Together, these approaches have significantly expanded the design space of CRISPR-based diagnostics and enabled sensitive detection of short RNA targets that were previously difficult to address using conventional Cas12a systems.

As programmable nucleic acid engineering and CRISPR-based biosensing technologies continue to advance, the integration of rational molecular design with a deeper mechanistic understanding of Cas12a activation is expected to yield more robust, multiplexed, and point-of-care-compatible platforms. Such developments will broaden the applicability of Cas12a-based sensing to diverse clinical contexts and contribute to the realization of next-generation molecular diagnostics capable of accurate and accessible disease detection.

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