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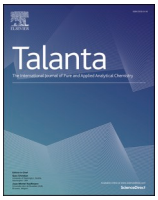

Recent advances in cascade isothermal amplification techniques for ultra-sensitive nucleic acid detection

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ABSTRACT

Nucleic acid amplification techniques have always been one of the hot spots of research, especially in the outbreak of COVID-19. From the initial polymerase chain reaction (PCR) to the current popular isothermal amplification, each new amplification techniques provides new ideas and methods for nucleic acid detection. However, limited by thermostable DNA polymerase and expensive thermal cycler, PCR is difficult to achieve point of care testing (POCT). Although isothermal amplification techniques overcome the defects of temperature control, single isothermal amplification is also limited by false positives, nucleic acid sequence compatibility, and signal amplification capability to some extent. Fortunately, efforts to integrating different enzymes or amplification techniques that enable to achieve intercatalyst communication and cascaded biotransformations may overcome the corner of single isothermal amplification. In this review, we systematically summarized the design fundamentals, signal generation, evolution, and application of cascade amplification. More importantly, the challenges and trends of cascade amplification were discussed in depth.

1. Introduction

Nucleic acids are essential for organism, which not only store genetic information, encode proteins, but also are used as important biomarkers in the fields of medical diagnosis, food safety testing, and forensic identification [1]. Usually, the initial nucleic acid concentration of samples is too low to be detected directly. Therefore, nucleic acid amplification is important to increase the concentration of nucleic acid exponentially to reach detectable level [2].

Polymerase chain reaction (PCR) is the first and remained to be the most popular amplification techniques for amplifying and detecting low-abundance nucleic acids. However, PCR depended on the thermostable DNA polymerase and expensive thermal cycler, which limited its wide applications in point-of-care testing (POCT). Isothermal amplification is a simple, rapid, and efficient process for nucleic acid amplification at constant temperature without thermal cycler [1,3]. Compared with conventional PCR, isothermal amplification techniques avoid the requirements of strict high-temperature denaturation, annealing, and extension to simulate *in vitro* amplification [4,5]. In addition, it does not require special equipment [4,6].

With the discovery of numerous isothermal polymerases, a series of enzyme-mediated amplification isothermal nucleic acid amplification techniques have been developed, for example, loop mediated isothermal amplification (LAMP), rolling circle amplification (RCA), and strand-displacement amplification (SDA). The emergence of isothermal amplification techniques has improved the speed of detection, the miniaturization of instruments, the cost of reagents, and the visual interpretation of results than PCR. However, there are still some problems in isothermal amplification techniques, such as lack of standard primer design process and industrialization of biological reagents is far less than PCR [7–9].

In addition, enzyme-free nucleic acid amplification techniques, such as hybridization chain reaction (HCR), and catalytic hairpin assembly (CHA), are used as promising analytical methods based on the unique Watson-Crick base-pairing interaction [10]. These methods showed unique advantages of easy operation, time-saving, and low cost [11,12]. Unfortunately, although only a simple probe for chemical synthetic is needed and the preservation of the probe is relatively easy, the detection method driven by chemical balance is insufficient in the detection of trace samples [13–15].

Thus, efforts to integrating different isothermal amplification techniques that enable to achieve intercatalyst communication and cascaded

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biotransformations may overcome the corner of single isothermal amplification.

Cascade amplification technique is a process in which receptors receive external signals and finally make a comprehensive response. It is also a very common metabolic regulation mode of cells or organisms. In addition to amplifying the signal, the cascade amplification can also play the role of information transmission and signal transduction. For isothermal amplification, the key requirement of the isothermal cascade amplification technique is that the product of the upstream method acts as a trigger for the downstream method, that is, acts as a "bridge" connecting these modules.

Practically, a series of work have focused on cascade amplification, although this concept is not explicitly proposed in nucleic acid detection. In this review, the design signal generation, evolution, and application of cascade isothermal amplification-based biosensors were systematically summarized (Fig. 1). Finally, the challenges and trends of cascade amplification were discussed in depth. The purpose of the review was to highlight the importance of developing advanced cascade amplification-based biosensors and promote the application in the fields of biological research, clinical diagnosis, food safety, and environment monitoring.

2. Isothermal enzyme-activated cascade amplification

Enzymes play an important role in the functioning of organisms, such as replication, ligation, symmetrical or nonsymmetrical cleavage and nicking a DNA duplex structure. The unique features of multienzyme biocatalytic or biosynthetic pathways increase reaction rates, eliminate cross-talks between different pathways, and achieve thresholding or feedback mechanisms [16]. During this process, the signal was gradually accumulated and enhanced. Polymerases and endonucleases are the most widely used enzyme in isothermal amplification. This subsection will address the different applications of polymerases or endonucleases dominated cascade amplification.

2.1. Polymerases-dominated cascades amplification

2.1.1. Self-cascade amplification based on polymerases

RCA is a circular replication of the DNA template proceeding hundreds and even thousands of times by isothermal extension through hybridization of template and primer [17–19]. In RCA, targets are generally used as primers [20] or linkers [21] of padlock probe, which can hinder the improvement of sensitivity to some extent [22]. Dual primers (forward primer and reverse primer) can greatly improve the reaction efficiency with exponential amplification process, contributable to branched rolling circle amplification (BRCA) [23] and hyperbranched rolling circle amplification (HRCA) [24]. Nevertheless, compared with double stranded DNA (dsDNA) products, single stranded DNA (ssDNA) products of RCA are preferable depending on the design of circular DNA template [22], which is affected by false-positive results caused by off-template polymerase products, contaminants, and side-reactions [1,25].

Circle-to-circle amplification (C2CA) is a typical RCA-based cascade amplification that has been used for nucleic acid analysis and single molecule detection [26–28]. As shown in Fig. 2A, in C2CA, amplicons of the first round RCA are converted into multiple circles by digestion and ligation, which are then used as templates for next round. The limit of detection (LOD) for C2CA is at the level of femtomolar [29,30]. However, primers of the second round RCA can change the first round RCA to hyperbranched-RCA, moreover, the process of monomerization and ligation are incompatible, so C2CA can only be performed step-by-step, which limit its wide application in biosensing.

Tian et al. [31] demonstrated a homogeneous and isothermal nucleic acid quantification strategy based on C2CA and optomagnetic analysis of magnetic nanoparticle (MNP) assembly for SARS-CoV-2 detection (Fig. 2B). The proposed homogeneous circle-to-circle amplification (HC2CA) strategy consisted of padlock probe ligation followed by homogeneous amplification and detection. Intermediate amplicons can be directly used as primers for next round RCA without any additional monomerization and ligation. Final amplicons of HC2CA hybridized with detection probes grafted onto MNPs and lead to the assembly of MNPs. Then, an optomagnetic sensor was utilized to analyze the state of MNPs, achieving a real-time detection. LOD of this method was 0.4 fM,

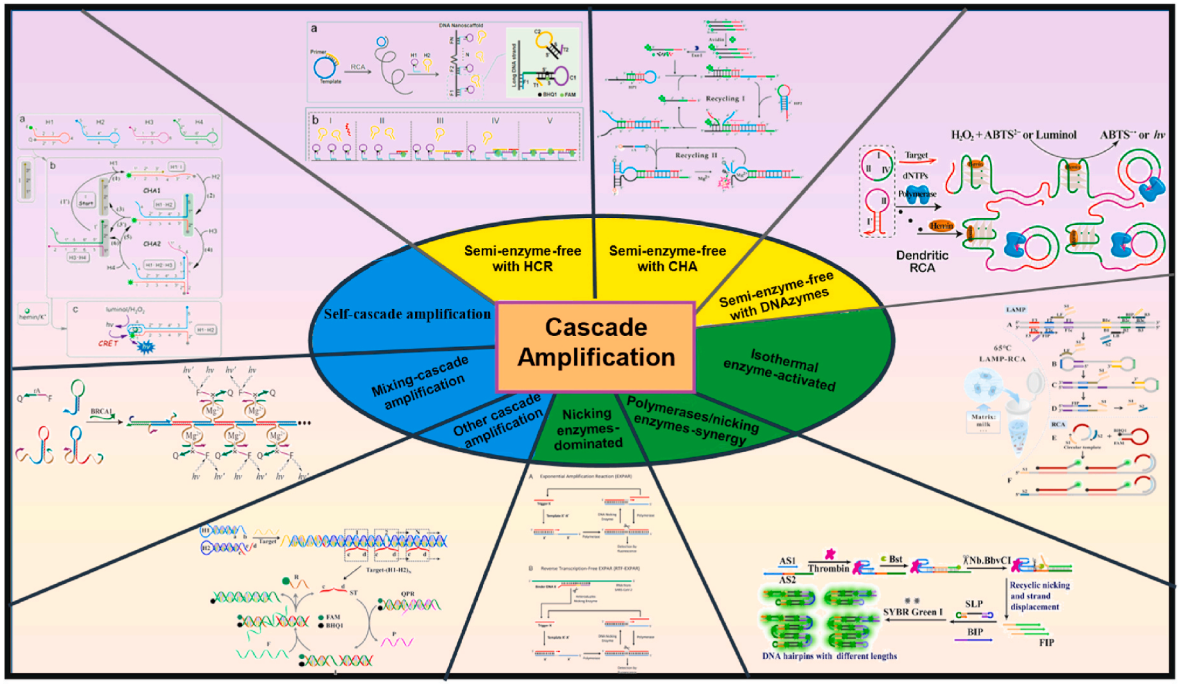

Fig. 1. The design fundamentals, signal generation, evolution, and application of cascade amplification.

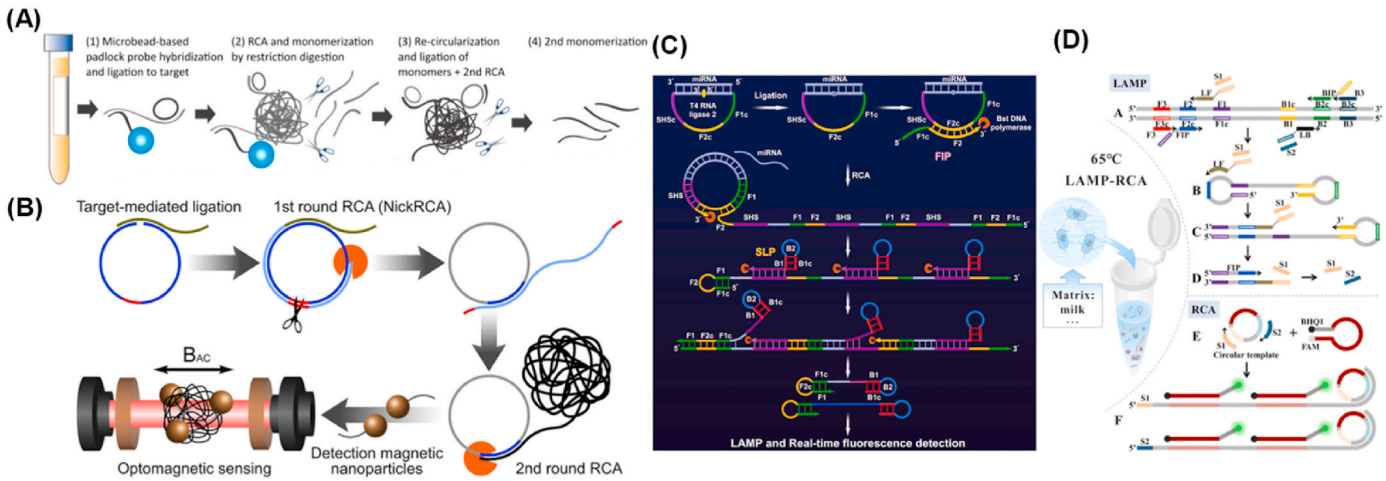

Fig. 2. Polymerases-dominated cascade amplification. (A) Schematic illustration of C2CA protocol [29]. Copyright © 2015, American Chemical Society. (B) Schematic illustration of homogeneous C2CA [31]. © 2020 Elsevier B.V. (C) Mechanism of real-time LAMP-RCA for fluorescence detection of bacteria [32]. Copyright © 2018 Elsevier B·V.(D) Mechanism of RCA-LAMP fluorescence detection [22]. Copyright © 2021 Elsevier B·V.

which was 750 times lower than that of single-round RCA based DNA quantification.

Self-cascade amplification based on polymerase is generally performed under the same conditions, which avoids repeated capping, operational complexity, and the possibility of aerosol contamination. However, since the same enzyme and substrate are used in two layers, the dosage must be optimized.

2.1.2. *Mixing-cascade amplification based on polymerases*

LAMP is another attractive isothermal amplification strategy with high efficiency. In conventional LAMP, an extremely long template including at least six pre-defined sequences [33] and four primers (or six primers) are prerequisites for producing double stem-loop DNA structures with elegantly designed sequences, which render the design of probe extremely stringent and sophisticated. Besides, one molecule target can only produce one molecule of double stem-loop DNA, resulting in the low formation efficiency of the LAMP starting structures [34].

Tian et al. [32] proposed a facile and ultrasensitive method for the detection of miRNA let-7a by elegantly integrating the distinct advantages of RCA and LAMP (Fig. 2C). In the strategy of RCA-LAMP, miRNA directly templated the ligation of a rationally designed padlock probe to initiate RCA, producing long ssDNA products with multiple tandem repeats. A rationally designed stem-loop primer bound with the formed tandem repeats, and initiated cascading extension and displacement reactions to generate double stem-loop DNAs. By using the proposed RCA-LAMP, 10 aM of target miRNA can be unequivocally detected, which was more sensitive than that of RCA or LAMP assay separately. Some studies have also suggested that the addition of RNase in RCA-LAMP can reduce the presence of nonspecific products and improve the specificity [35].

Chen et al. [22] reported another idea for the cascade of RCA and LAMP (Fig. 2D). After LAMP reaction, two primers are released to initiate downstream RCA reactions. Molecular beacons (MB) underwent a conformational change and "turned on" fluorescence signal after recognizing amplicons produced in RCA. Compared with LAMP [36] or RCA [37], the sensitivity of LAMP-RCA was enhanced 7.8 times with the LOD of 32 CFU/μL. Polymerases-dominated cascade amplification for biomarkers detection are compared and summarized in Table 1.

Mixing-cascade amplificationbased on polymerase could make full use of the characteristics of enzymes. However, due to different optimal conditions of enzymes, the mixing-cascade amplification method usually requires multiple steps.

With the powerful advantages of polymerase, polymerase-dominated isothermal amplification techniques have been widely used either as the first layer amplification to generate sufficient intermediates or as the second layer to generate the output signal.

2.2. *Endonucleases-dominated cascade amplification*

At present, the endonucleases used in cascade amplification mainly include two categories: nicking enzyme developed by NEB Company and CRISPR/Cas system. Unlike conventional restriction enzymes, nicking enzyme recognizes a specific sequence and makes a notch on only one strand of DNA, exposing the free 3 'end, which can also be used as a template to extend. As a defense mechanism for bacteria, CRIPSR has become an unparalleled genome editing tool. In recent years, following the discovery of its *trans*-cutting activity, numerous members of the CRISPR family have also been embarked on the journey of nucleic acid diagnostics. This section mainly introduces the application of endonuclease based on nicking enzyme and CRISPR for nucleic acid detection.

Table 1
Comparison of sensitivity enhancing effect of polymerases-dominated cascade amplification based on different principles.

Methodology	Signal	Target	LOD	Sensitivity enhancement	Time	Reference
C2CA	Fluorescence	ATP7B gene	25 nM	/	1h	[26]
C2CA	Colorimetric	CCHF	10^3copies/mL	/	/	[27]
C2CA	Paramagnetic	*Pseudomonas aeruginosa*	1 aM	/	1h	[28]
C2CA	Optomagnetic	bacteria	1.4 pM	7 times	4h	[29]
C2CA	Fluorescence	Stand DNA	0.48 aM	1000 times	3h	[30]
C2CA	Optomagnetic	SARS-CoV-2	0.4 fM	an order of magnitude	100min	[31]
RCA-LAMP	Fluorescence	miRNA let-7a	10 aM	an order of magnitude	90 min	[32]
RNase RCA-LAMP	Fluorescence	Standard RNA	1 pM	100 times	3 h	[35]
LAMP-RCA	Fluorescence	*S. typhimurium*	32 CFU/mL	7.8 times	60 min	[22]

2.2.1. Nicking enzymes-dominated cascade amplification

A typical template design of exponential amplification reaction (EXPAR) greatly identified the ideal of nicking enzymes-dominated cascades amplification method. As shown in Fig. 3A, the two layers of EXPAR templates are composed of X–Y and Y–Y, respectively. When the target binds to the first layer template and completes the amplification, the cleavage is achieved with the help of nicking enzyme, releasing Y′ to participate in the second layer amplification. The cleavage products in the second layer were back to the circulation to produce stronger signals [38]. As an alternative isothermal amplification technique that combines polymerase strand extension and single-strand nicking, EXPAR has been successfully used in the detection of miRNAs [39,40], proteins [41], enzyme activity [42,43], and metal ions [38].

With the help of EXPAR, a nicking enzyme mediated product recycling idea, and has been proposed a lot of interesting research has been carried out. Li et al. [44] reported a target-induced circular amplification strategy for miRNA21 detection. SsDNA template was hybridized with target miRNA-21 to trigger cyclic polymerization via Klenow Fragment and nicking enzyme (Fig. 3B). The products were used to open the hairpin structure HAP1 and initiate another polymerase-aided process. With the help of SYBR Green I fluorescent dye, this method could achieve the detection of 5.4 fM miRNA21. Compared with Exo III-assisted target amplification, the sensitivity is improved nearly 200 times. Zhou et al. [45] constructed RNA aptamer involved cascade transcription amplification method (termed RACTA) for miRNA detection (Fig. 3C). Plenty of ssDNA was produced by miRNA-initiated strand-displacement amplification, which triggered the subsequent transcriptional amplification of spinach RNA aptamers. Consequently, fluorophore DFHBI d with transcribed tremendous spinach aptamers for miRNA quantitative analysis with the LOD of 5.12 aM. The sensitivity was 50 times higher than that of previously reported.

Compared with dsDNA, ssDNA is more versatile in the design of cascade amplification. With the ability to accumulate large number of ssDNA products, nicking enzymes-dominated cascade amplification will be more widely used in different cascade amplification strategies.

2.2.2. CRISPR-dominated cascade amplification

CRISPR is an adaptive immune mechanism, which widely existed in bacteria and archaea to defend against viruses and foreign nucleic acids [48]. Cas-protein can be guided by its crRNA to recognize complementary ssDNA or dsDNA with protospacer-adjacent motif (PAM) sequences. Cas12 cleaves target DNA through a single RuvC domain [49]. In addition, target DNA leads to the activation of the unique *trans*-cleavage activity to cleave ssDNA nonspecifically. Therefore, efficient *trans*--cleavage can be used as an excellent signal amplification way compared with molecular beacons [50,51]. In recent years, many efficient nucleic acid detection platforms have been developed based on *trans*-cutting activity of CRISPR, such as SHERLOCK [52] (specific high-sensitivity enzymatic reporter unlocking), HOLMES [53] (one HOur Low cost Multipurpose highly Efficient System), DETECTR [54] (DNA endonuclease-targeted CRISPR trans reporter), and the improved version of HOLMES system [55] (namely HOLMESv2).

However, the capability of CRISPR to cleave probes was at the pM level, which was simply not enough for sensitive detection. Based on classic CRISPR system, cascade CRISPR system has been developed to improve the sensitivity of nucleic acid detection.

Sha et al. [46] developed a cascade CRISPR system via the trans cutting activity of Cas13a and Cas14a with the LOD of 6.25 fM for miRNA 17 detection without target amplification (Fig. 3D). Cas13a was activated by target to collaterally cleave the locked-trigger for Cas14a. As a result, the activated Cas14a cut substrate probe and produced a large number of readable signals. Another method [47] based on Cas13 and Csm6 realized the detection of SARS-CoV-2 with the sensitivity comparable to PCR (Fig. 3E). The CRISPR-dominated cascade amplification improves sensitivity by nearly 2–3 orders of magnitude, suggesting that this cascade is a very meaningful work. However, most CRISPR systems are now self-expressed and purified by researchers, there may not be universal between different groups, while commercial

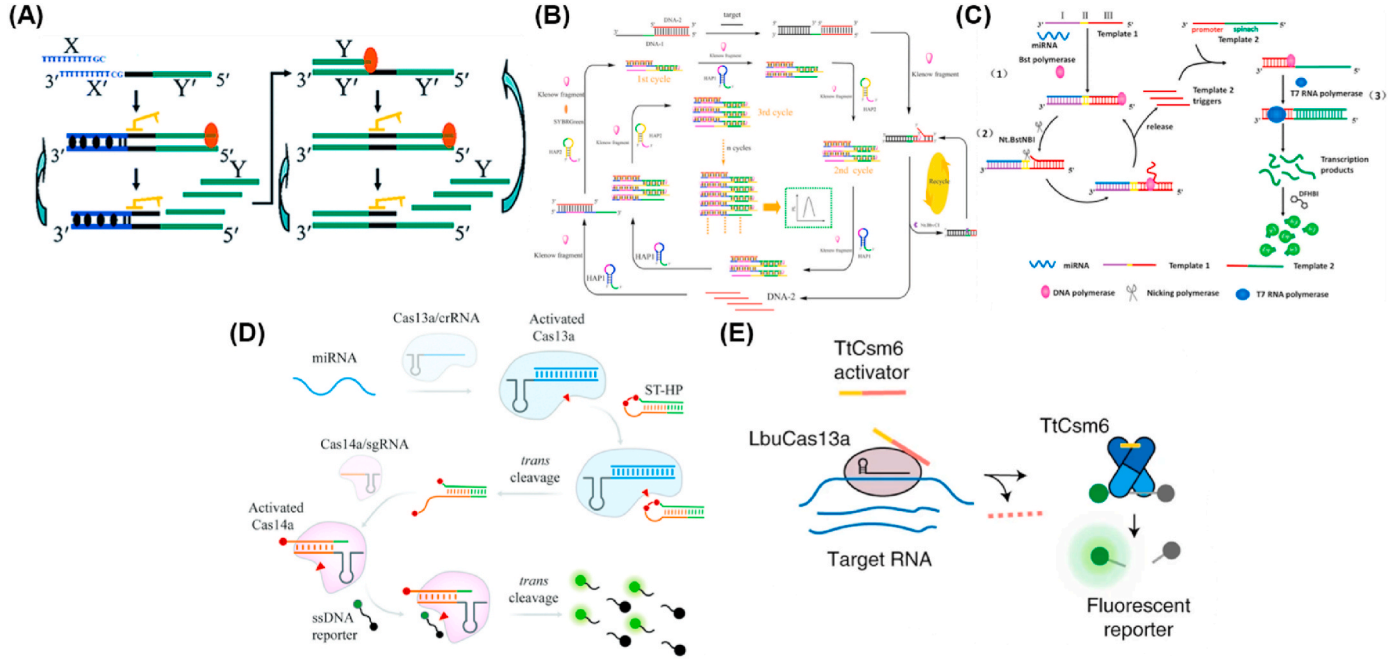

Fig. 3. Endonucleases-dominated cascade amplification. (A) Mechanism illustration of EXPAR [38]. Copyright © The Royal Society of Chemistry 2014. (B) Schematic illustration of the fluorescence biosensing system based on the DNA network nanoarchitecture by a target-induced cyclic amplification strategy [44]. Copyright © 2021 Elsevier B.V. (C) Schematic illustration of RNA aptamer-based cascade transcription amplification (RACTA) assay for miRNA analysis [45]. Copyright © 2019, American Chemical Society (D) Schematic illustration of the cascade Cas13a–Cas14a (casCRISPR) system for miRNA detection [46]. Copyright © The Royal Society of Chemistry 2021. (E) Schematic illustration of TtCsm6 activation by LbuCas13a assembled with a crRNA [47]. Copyright © 2021, The Author(s), under exclusive licence to Springer Nature America, Inc.

CRISPR systems are expensive. Endonucleases-dominated cascade amplification for biomarkers detection are compared and summarized in Table 2.

2.3. Polymerases/endonucleases-synergy cascades amplification

Polymerases and endonucleases cooperate with each other to realize nucleic acid detection is an ideal way to signal amplification. According to the different endonucleases used, there are generally discrepancy in the combination order. In the cascades amplification with nicking enzyme involving, the first layer is nicking enzyme cutting, and the produced ssDNA is used as primer for isothermal amplification of polymerase. In contrast, in cascades amplification with CRISPR involving, the nucleic acid is typically amplified before the CRISPR system recognizing the product and cutting the substrate probe.

2.3.1. Polymerases/nicking enzyme-synergy cascades amplification

SDA is one of the most popularly used primary level for isothermal cascade amplification for miRNA detection [56–59]. Especially when nicking enzyme was discovered by New England Biolabs (NEB), the problem of requiring resultant hemiphosphothioate *Hinc*II site in SDA has been well solved [9].

Wang et al. [60] developed a multistep isothermal amplification fluorescence strategy for 17β-Estradiol (E_2) detection (Fig. 4A). E_2 aptamer and cDNA were hybridized and modified on the magnetic beads. The binding of E_2 to aptamer caused the release of cDNA, which then combined with the template DNA to initiate the reaction of SDA-RCA-MRCA. After specifically recognized by the formed long ssDNA products, the fluorescence of the molecular beacons could be restored, and the concentration of E_2 can be quantitatively detected according to the fluorescence intensity

Zhang et al. [61] developed an ultrasensitive and label-free aptamer-based assay for thrombin detection through the target-induced polymerization nicking reaction (TIPNR, Fig. 4B). The presence of thrombin caused spontaneous assembly of two distinct aptamer probes to form a thrombin/G-quadruplex structure to initiate SDA, which was capable of producing a large amount of primer sequences to trigger LAMP, yielding numerous long-stem hairpin products. The resulting hairpin products can bind with SYBR Green I organic dyes to exhibit significantly enhanced fluorescence intensity to sensitively and efficiently detect thrombin at the level of femtomolar. Compared with traditional LAMP, TIPNR-LAMP strategy generated numerous trigger sequences for LAMP through the repeated interaction of the nicking enzyme and polymerase. With this design, the numbers of primers for traditional LAMP needed to be precisely designed were reduced, and the sequences of the primers were no longer constrained by the target, which made TIPNR-LAMP suitable for a wide range of targets.

The synergistic effect of the polymerase and nicking enzyme is conducive to cascade amplification. On the one hand, Bst DNA polymerase and phi 29 DNA polymerase used in LAMP or RCA with high efficiency polymerization and chain replacement can greatly improve the efficiency of DNA synthesis compared with traditional Klenow fragment. On the other hand, the nicking enzyme can also help the polymerization product to better trigger the second layer reaction, avoiding the steric hindrance of the product secondary structure in the first layer.

2.3.2. Polymerases/CRISPR-synergy cascades amplification

Wang et al. [62] reported RCA-assisted CRISPR/Cas9 cleavage (RACE) method for highly specific detection of multiple extracellular vesicle miRNAs (Fig. 4C). The amplified long ssDNA produced by RCA contained a large number of repetitive target sequences and PAM structures for specific recognition by Cas9 nuclease. Then the TaqMan probe hybridized with ssDNA can be completely cleaved by the robust exonuclease activity of Cas9, and "turn on" fluorescence change can be conveniently measured. Moreover, multicolor TaqMan probes complementary with distinctive target miRNAs can be introduced for multiplex detection.

The *trans*-cleavage of CRISPR has been integrated with nucleic acid isothermal amplification techniques, including RCA [25,69,70], LAMP [71–73], RPA [74,75], and RAA [76–78] to realize various cascade amplifications.

Polymerases/endonucleases dominated cascade amplification for biomarkers detection are compared and summarized in Table 3. The limited signal amplification capacity of CRISPR was overcome by polymerase-dominated isothermal amplification. It is worth mentioning that different amplification methods can produce the same PAM structure for CRISPR to achieve the standardized detection mode.

3. Semi-enzyme-free cascade amplification

Multi-enzyme-mediated cascades demonstrate excellent signal amplification and broaden the application of nucleic acid isothermal amplification techniques. However, different enzymes need different optimal conditions, so the cascade steps have to be carried out separately, which enhances the complexity of the operation. Meanwhile, repeatedly open the centrifuge tube is easy to produce aerosol pollution.

As mentioned above, the problem of multi-enzyme reaction is mainly caused by the incompatibility of optimal reaction conditions between different enzymes. Therefore, it may become an excellent idea to cascade enzyme-free isothermal amplification with enzyme-mediated isothermal amplification to achieve signal amplification. Hence, this section focuses on enzyme-mediated isothermal amplification cascading with the toehold-mediated strand displacement reactions such as HCR and CHA, and DNAzymes, and the outstanding representative of functional nucleic acids (FNAs).

3.1. Semi-enzyme-free cascade amplification involving HCR

3.1.1. Semi-enzyme-free cascade amplification involving polymerase-dominated isothermal amplification and HCR

Traditional LAMP amplification is usually based on pyrophosphate turbidity or intercalation of dyes into dsDNA, which is lack of specificity. Instead, a series of thermostable, toehold-mediated, and strand exchange devices called OSD (short for one-step strand displacement) probes was proposed to allow real-time sequence detection [79–81]. Unfortunately, OSD was incapable of signal amplification, the signal-to-noise ratios or the changes in the observable signals may be not significant enough to satisfy the requirement of practical application to some extent. Dong et al. [63] replaced OSD by HCR for detection of contagious norovirus (NoV) (Fig. 5A). LAMP products were no longer bound to fluorescent probes. Instead, a single loop in LAMP products specifically recognized and opened H1 probes, leading to the subsequent cascade of hybridization reaction. By labeling invertase on the harpin

Table 2
Comparison of sensitivity enhancing effect of endonucleases-dominated cascade amplification based on different principles.

Methodology	Signal	Target	LOD	Sensitivity enhancement	Time	Reference
EXPAR	Fluorescence	MiRNA 21	5.4 fM	200 times	2 h	[44]
Termed RACTA	Fluorescence	miRNA let-7a	5.12 aM	50 times	3 h	[45]
Cas 13a/14a	Fluorescence	miRNA 17	6.25 fM	3 orders of magnitude	1.5 h	[46]
Cas 13a/csm6	Fluorescence	SARS-CoV-2	30 copies	3 orders of magnitude	20 min	[47]

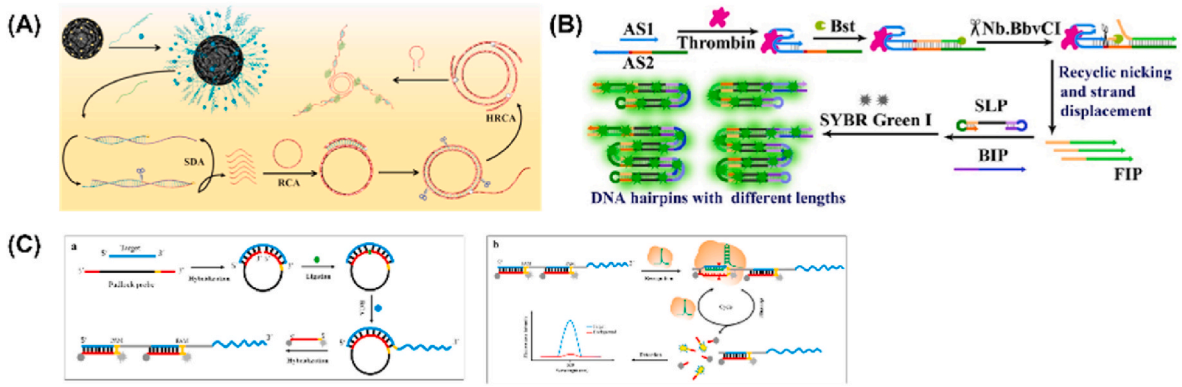

Fig. 4. Polymerases/endonucleases-synergy cascades amplification. (A) Schematic illustration of multistep isothermal amplification in the detection of E_2 [60]. Copyright © 2021 American Chemical Society. (B) Schematic illustration of the TIPNR-LAMP-based, highly sensitive and label-free fluorescent thrombin assay [61]. Copyright © 2019 Elsevier B.V. (C) Schematic illustration mechanism of RACE [62]. (a) Replication step of PAM domain in RCA process. (b) Detection step for the sgRNA/Cas9 complex recognizes the PAM domain in the dsDNA assembly. Copyright © 2019 American Chemical Society.

Table 3
Comparison of sensitivity enhancing effect of polymerases/endonucleases -dominated cascade amplification based on different principles.

Methodology	Signal	Target	LOD	Sensitivity enhancement	Time	Reference
SDA-RCA-MRCA	Fluorescence	E2	63.09 fM	100 times	3 h	[60]
TIPNR-LAMP	Fluorescence	Thrombin	3.6 fM	100 times	2.5 h	[61]
Cas13a-RCA	Fluorescence	EV miRNA	90 M	3 orders of magnitude	3 h	[62]
Cas12a-RCA	Fluorescence	DNA	342aM	20 times	60 min	[69]
Cas12a-LAMP-G4	colorimetric	*V.parahaemolyticus*	6.1 × 102 CFU/mL	3 times	/	[70]
Cas12a-RPA	Fluorescence	*Candidatus Liberibacter asiaticus*	0.1 fM	10^4 times	3 h	[74]
Cas12a-RAA	Fluorescence	HIV-1	20 copies/ml	Similar sensitivity	30 min	[76]

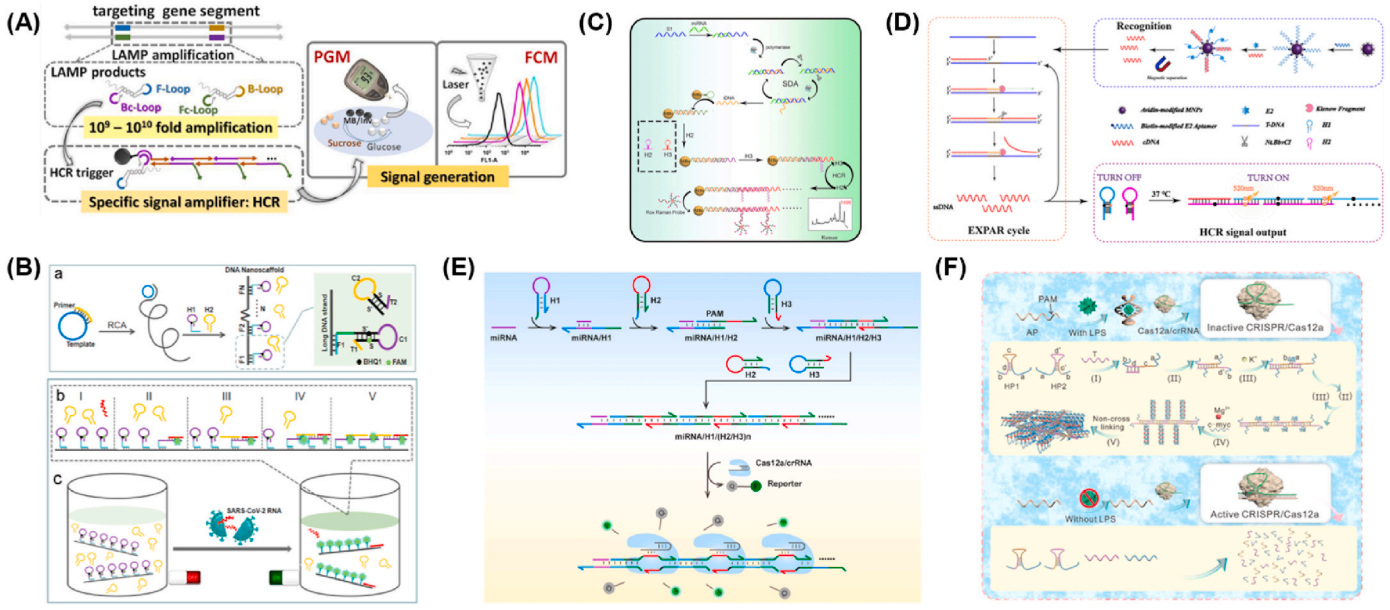

Fig. 5. Semi-enzyme-free cascade amplification involving HCR. (A) A versatile molecular diagnostic method by coupling LAMP with HCR for norovirus gene detection [63]. Copyright © 2019 Elsevier B.V. (B) A DNA nanoscaffold hybrid chain reaction (DNHCR)-based method assay for the detection of SARS-CoV-2 RNA [64]. Copyright © 2020 Elsevier B.V. (C) Schematic illustration of the proposed strategy for miRNA detection [65]. Copyright © 2018 Elsevier Inc.(D) Schematic illustration of the detection procedure of EXHCR-FL for E_2 [66]. Copyright © 2020 Elsevier B.V. (E) Schematic illustration of CRISPR-HCR for miRNA detection [67]. Copyright © 2022 Elsevier B.V. (F) Schematic illustration of GWancHCR amplified CRISPR/Cas12a system for LPS detection by RRS [68]. Copyright © 2022 American Chemical Society.

probe, products could be detected by using the commercial personal glucose meter (PGM). Compared with LAMP-OSD, sensitivity of LAMP-HCR improved nearly 10 times.

Another interesting study, DNA nanoscaffold based on RCA-HCR was proposed by Jiao et al. [64] (Fig. 5B). H1 probe could be specifically bound to universal RCA products through toehold to form nano scaffold. Subsequently, the target specifically recognized and opened H1 probes, to trigger the subsequent cascade of hybridization reaction. As the

reaction progressed, the fluorescence signal enclosed in H2 was recovered. It was worth noting that the DNA nano scaffold could effectively stabilize the HCR products and facilitate the extension. The LOD was approximately 0.96 pM, which was 2 orders of magnitude lower than that of conventional HCR.

3.1.2. Semi-enzyme-free cascade amplification involving nicking enzyme-dominated isothermal amplification and HCR

HCR is mainly used for the amplification of short single-stranded DNA (about 20 nt) [82,83], especially for miRNA [84–86]. The isothermal amplification involving nicking enzyme could generate a large amount of ssDNA which was an ideal upstream for HCR. At present, studies based on nicking-HCR have covered many aspects such as genotyping [87,88], disease diagnosis [89] and small molecule detection [90–92].

Wu et al. [65] reported a miRNA triggered cascade amplification based on SDA and HCR. After SDA, a large amount of ssDNA was produced for triggering downstream HCR reaction (Fig. 5C). Sufficient tails in HCR products corresponding were used to bind the Raman probe. The quantitative detection was achieved by the intensity of the Raman signal. Compared with SDA alone, SDA-HCR improved sensitivity by two orders of magnitude.

Wang et al. [66] reported a cascade amplification based on EXPAR and HCR for E_2 detection. E_2 and cDNA competed to bind with E_2 aptamer modified on the magnet bead (Fig. 5D). After magnetic separation, the remaining cDNA started EXPAR, following by HCR process. With the progress of HCR reaction, the fluorescence signal was recovered. Compared with HCR alone, EXPAR-HCR improved sensitivity by two orders of magnitude.

3.1.3. Semi-enzyme-free cascade amplification involving CRISPR/Cas and HCR

As a special DNA circuit, HCR is a triggered self-assembled and non-enzyme process, in which single nucleotide input initiates a cascade hybridization reaction to form a long nicked DNA duplex. In this process, the formed sequence repeats of HCR provide numerous recognition sites for crRNA.

Jia et al. [67] designed a versatile and sensitive miRNA detection platform based on CRISPR/Cas12a system combined with HCR circuit (Fig. 5E). In this design, the HCR circuit as the signal transducer converted each miRNA into DNA duplexes containing protospacer adjacent motif (PAM) and protospacer sequence, which can be recognized by the Cas12a/crRNA complex. Then, the FQ-labeled nucleic acid reporter was cleaved by the activated Cas12a/crRNA complex, and realized the fluorescence output with an LOD of 6.3 pM. In addition, the designing flexible and programmable detection platform can be used for different miRNAs detection by simply changing the sequence of the first hairpin without the limitation of sequence dependence.

Another CRISPR/Cas-HCR method was based on resonance Rayleigh scattering (RRS). In general, RRS is a special elastic scattering, which is generated when the wavelength of Rayleigh scattering is located at or close to the molecular absorption band [93]. Consequently, the intensity of RRS will rapidly be enhanced when the absorption band of nanoparticles is close to that of the wavelength of the incident beam [94,95]. As a label-free, sensitive, and rapid analytical technique, RRS has been used for investigating the interaction of biological macromolecules and the molecular recognition [96–99]. Recently, a guanine nanowire (G-wire)-based RRS amplification strategy has been described as a novel assay for detection of heavy metal ions [100,101], small molecules [102], and nucleic acid [103] in an excellent sensitive and selective way. The G-wire structure is specifically produced using a parallel-stranded G-quadruplex with Mg^{2+} [104].

Gao et al. [68] reported a novel CRISPR-derived RRS amplification strategy and logical circuit based on G-wire assisted non-cross-linking HCR (GWancHCR) for label-free detection of lipopolysaccharide (LPS, Fig. 5F). In the presence of LPS, the protospacer-adjacent motif-inserted aptamer was rationally designed to specifically combine with LPS rather than Cas12a, suppressing the *trans*-cleavage activity of CRISPR/Cas12a and retaining the reporter probes to trigger non-cross-linking aggregation. Owing to the automatic HCR, in the presence of Mg^{2+}, the released G-quadruplex sequence was aggregated to assemble the G-wire superstructure. As a result, a dramatically amplified RRS intensity was observed, allowing for LPS detection at LOD of 0.17 pg/mL.

Semi-enzyme-free cascade amplification integrated with HCR for biomarkers detection are compared and summarized in Table 4. HCR has strong adaptability, which has no strict requirements on pH and ionic strength. Hence, it can be cascaded with a variety of enzyme-mediated isothermal amplification techniques. However, the length of HCR product cannot be extended indefinitely, so the signal amplification ability is relatively weak.

3.2. Semi-enzyme-free cascade amplification involving CHA

3.2.1. Semi-enzyme-free cascade amplification involving polymerase-dominated isothermal amplification and CHA

RCA and LAMP, two main polymerase-assisted NACs, have been integrated with CHA for sensitive detection.

In the integration of RCA with CHA, two common ways were chosen, including CHA product as RCA primer (CHA-RCA) [105–108] and RCA product as CHA trigger (RCA-CHA) [109,110]. In addition, a nicking enzyme was used in RCA-CHA to deal with the problem of hindering the initiation of CHA by RCA products [111]. Recently, Wang et al. [112] developed a label free fluorescent biosensor based on target-driven, RCA-activated and multisite-CHA for Y-shaped DNA nanotorches (Y-DNTs, Fig. 6A) construction to achieve ultrasensitive detection of ochratoxin A (OTA). OTA specifically bound to aptamer resulting in the release of circular template to actuate RCA process. Numerous Complex II probes can anneal with the first-generation RCA product with multiple sites to activate the CHA process. The LOD was 0.2 pg/mL, which was at least 10 times lower than that of previous reported [113,114].

CHA was also coupled with LAMP as simple molecular transducers. In the LAMP-triggered CHA, extended loops after LAMP reaction acted as an initiator of dsCHA [123] and three way-CHA [124].

3.2.2. Semi-enzyme-free cascade amplification involving nicking enzyme-dominated isothermal amplification and CHA

Endonucleases was widely used in the cascade with CHA, including endonuclease IV [125], exonuclease III [126,127], λ exonuclease [128], exonuclease I [129], and ribonuclease H [130].

Tang et al. [115] developed a universal protein detection method based on a protein-to-DNA signal transducer, by cascading EXPAR and CHA (Fig. 6B). When antibody was conjugated with DNA1 (hybridized with DNA3) and DNA2 with bound with target protein, strand displacement was occurred between DNA3 and DNA2 because of the increasing local concentration. The releasing DNA3 initiated EXPAR reaction and a large number of ssDNA were produced, which were further amplified by CHA to generate dsDNA. The dsDNA products were detected by lateral flow assay within 5 min. The LOD of the proposed approach was 9.9 fg/mL, which was 2 orders of magnitude better than that of antibody-based immunoassay [131,132].

3.2.3. Semi-enzyme-free cascade amplification involving CRISPR/Cas and CHA

Recently, more attention has been paid to the combination of a CRISPR-Cas system with CHA for sensitive detection. The fundamental principle is that the CRISPR-Cas system cleaved nucleic acid to produce an initiator for CHA reaction [116,133], or CHA products are used as sgRNA recognition sequences to activate the CRISPR system [118]. As shown in Fig. 6C, an electrochemical biosensor platform based on CRISPR-Cas13a-mediated CHA was established for miRNA-21 detection with a LOD of 2.6 fM [116]. Nie's group [117] reported two-layer NACs by integration of CRISPR-Cas12a with CHA circuit (Fig. 6D). In the

Table 4
Comparison of sensitivity enhancing effect of semi-enzyme-free cascade amplification integrated with HCR based on different principles.

Methodology	Signal	Target	LOD	Sensitivity enhancement	Time	Reference
LAMP-HCR	Fluorescence	norovirus (NoV)	30 copies	10 times	1h	[63]
RCA-HCR	Fluorescence	SARS-CoV-2	0.96 pM	7.2×10^3 times	10min	[64]
EXPAR-HCR	Fluorescence	BRCA1	74.48 aM	7×10^4 times	60min	[87]
EXPAR-HCR-G4	Electrochemistry	H7N9	9.4 fM	2×10^3 times	90min	[88]
EXPAR-HCR-G4	Electrochemistry	Thrombin	33 fM	500 times	5h	[89]
HCR-SDA	Fluorescence	ricin.	26.31 pM	270 times	120min	[90]
HCR-DNAzyme	Fluorescence	miRNA	7.9 fM	10-100 times	3h	[91]
EXPAR-HCR-DNAzyme	Electrochemistry	Cu2+	10pM	100 times	7h	[92]
SDA-HCR	SERS	miRNA let-7a	0.5 fM	/	/	[65]
EXPAR-HCR	Fluorescence	E2	0.37 pg/mL	300 times	6h	[66]
CRISPR/Cas12a-HCR	Fluorescence	lipopolysaccharide (LPS)	1 fM	6-300 times	20min	[67]
CRISPR/Cas12a-HCR	RRS	miRNA21	0.17 pg/mL	100 times	60min	[68]

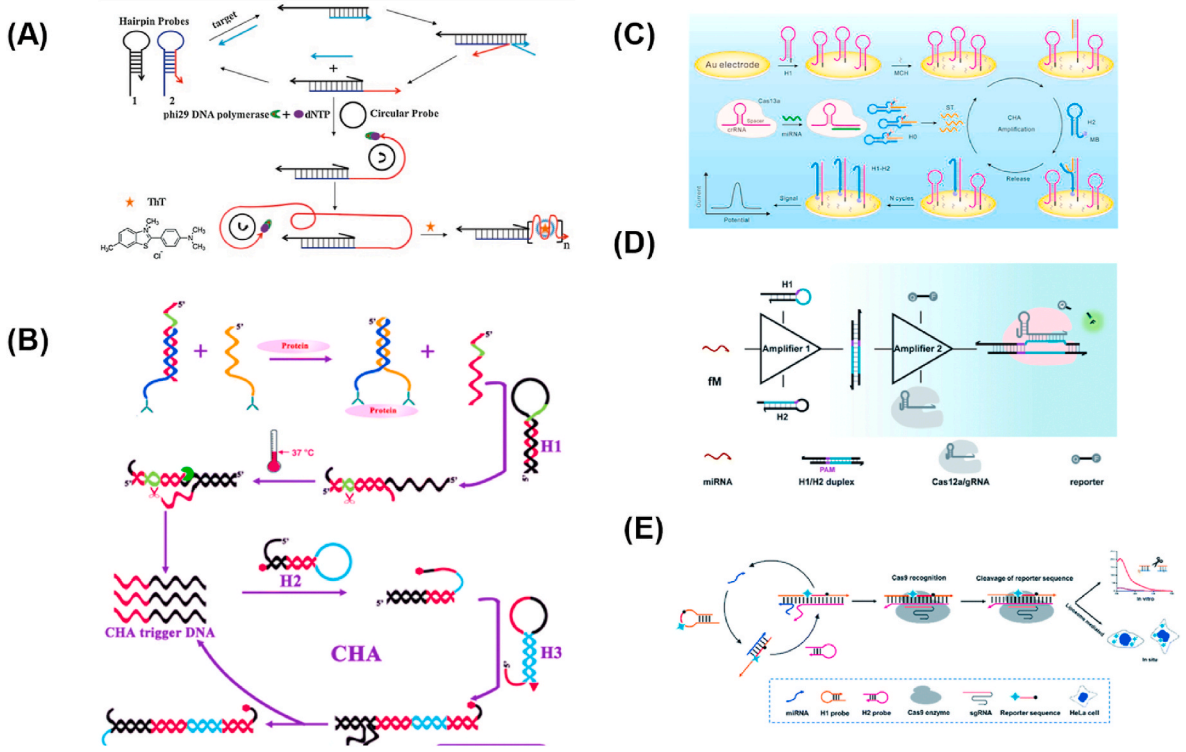

Fig. 6. Semi-enzyme-free cascade amplification involving CHA. (A) Schematic illustration of the label-free fluorescence signal-on sensing platform for ultrasensitive detection of OTA based on a target-driven primer remodeling RCA-activated multisite-CHA dual signal amplification concurrent strategy [112]. Copyright © The Royal Society of Chemistry 2019. (B) Schematic illustration for protein detection based on proximity hybridization-mediated isothermal exponential amplification [115]. (C) Schematic illustration of electrochemical assay for miRNA based on CRISPR-Cas13a system and CHA reaction [116]. (D) Schematic illustration of amplified detection of miRNA by the CRISPR-CHA method [117]. (E) Schematic illustration for microRNA detection and in situ imaging based on CHA-CRISPR method [118].

presence of target miRNA, CHA was initiated, the formed products included a protospacer adjacent motif and a protospacer sequence targeted by Cas12a gRNA. Thus, CRISPR-cas12a recognized the formed dsDNA and efficiently digested Taqman probes to generate fluorescent signals. 3–4 order of magnitude signal amplification for different miRNA detection can be obtained. Liu's group [118] combined CHA and CRISPR-Cas9 for miRNA detection with a LOD of 23 fM (Fig. 6E). In addition, this strategy can realize in situ imaging of miRNAs in living cells.

Semi-enzyme-free cascade amplification integrated with CHA for biomarkers detection are compared and summarized in Table 5. CHA is a typical target-recycling amplification mode, which can be regarded as an endless amplification technique with infinite circulation. Of course, given the rapid detection, amplification cannot be carried out indefinitely. More importantly, unlike HCR, leakage expression of CHA is more serious, which may lead to severe false positives.

3.3. Semi-enzyme-free cascade amplification involving DNAyzme

There are two main types of DNAzymes, RNA-cleaving DNAzymes (RCD) and peroxidase mimicking DNAzyme (PMD). RCD can bind to target sequences and achieve a specific cleavage reaction [134]. PMD is a class of DNA structures with peroxidase activity, catalyzing oxidation-reduction reaction [135]. DNAzymes can be used as signal transduction elements to amplify detection signals in combination with RCA [136,137], LAMP [138], and other isothermal amplification techniques.

3.3.1. Semi-enzyme-free cascade amplification involving PMD

By elegant design, RCA products can form tens of thousands of DNAyzme structures, which makes RCA an ideal semi-enzyme-free cascade amplification technique. Currently, RCA-PMD system has become the mainstream in semi-enzyme-free cascade amplification

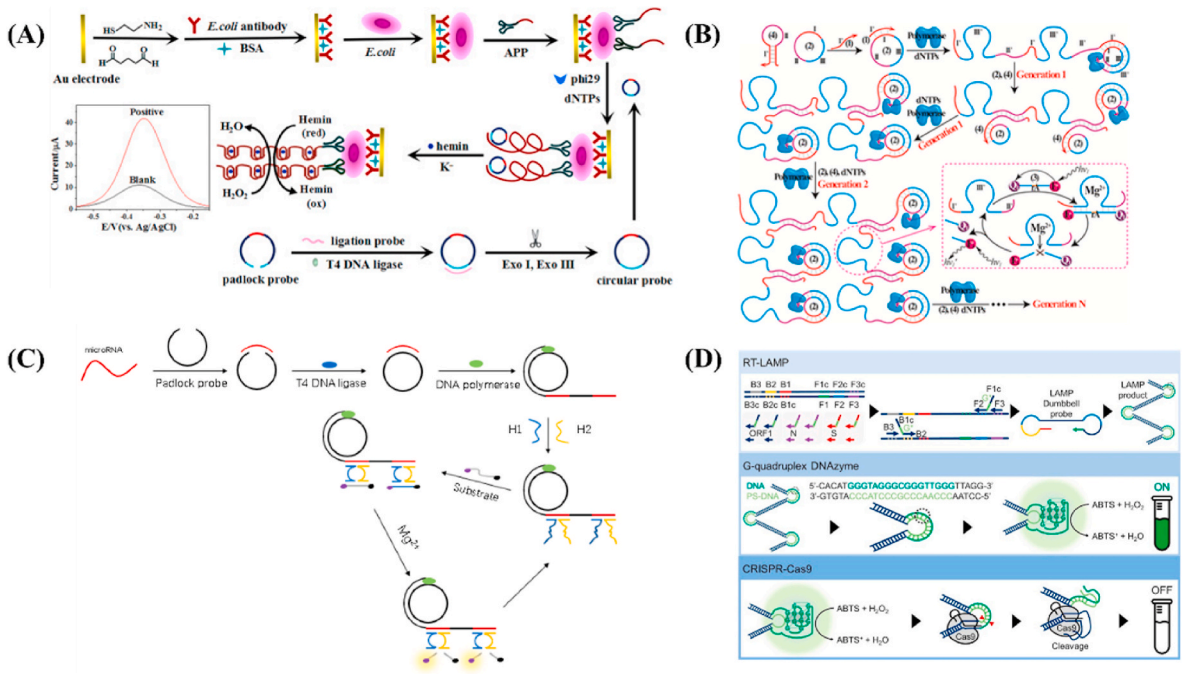

Fig. 7. Semi-enzyme-free cascade amplification integrated with DNAyzme. (A) Schematic illustration of the label-free electrochemical assay for highly sensitive detection of *E. coli* based on RCA and DNAzyme [119]. (B) Schematic illustration of the target by the dendritic RCA-stimulated synthesis of DNA chains and the amplified generation of the Mg^{2+}-dependent DNAzyme units [120]. (C) Schematic illustration of the fluorescence assay for the detection of miR-21 using RCA and DNAzyme [121]. (D) Schematic illustration of DAMPR assay based on RT-LAMP, G-quadruplex DNAzyme, and CRISPR-Cas9 reactions [122].

Table 5
Comparison of sensitivity enhancing effect of semi-enzyme-free cascade amplification integrated with CHA based on different principles.

Methodology	Signal	Target	LOD	Sensitivity enhancement	Time	Reference
CHA-RCA-G4	electrochemical	ssDNA	13.5 fM	7.2×10^4times	4h	[105]
LAMP-CHA	fluorescence	Nerve growth factor-beta (NGF-β),	10 copies/μL	20 times	6h	[123]
EXPAR-CHA	lateral flow biosensor (LFB)	miRNA-21	0.74 fM	700 times	90min	[115]
Cas13a-CHA	electrochemical	adenosine triphosphate/glutamate dehydrogenase	2.6 fM	5 times	1h	[116]

[139–142].

Guo et al. [119] designed an electrochemical biosensor based on the coupling of RCA with PMD to detect *E. coli* (Fig. 7A). The circular probe designed with two G4 units started RCA reaction and generated repetitive G4 DNA molecules when *E. coli* was captured by aptamer immobilized on the electrode surface. The generated G4 unit bound to the heme molecule and folded into an active HRP mimicking DNAzyme structure. A dramatically strong current signal was achieved owing to the electro-reduction of the H_2O_2 reaction catalyzed by PMD. Under optimal conditions, the proposed biosensor exhibited ultrahigh sensitivity toward *E. coli* with a detection limit of 8 CFU/mL.

3.3.2. *Semi-enzyme-free cascade amplification involving RCD*

Wang et al. [120] introduced a highly sensitive method for DNA detection using the dendritic RCA and RCD (Fig. 7B). The analytical platform included a circular DNA and a structurally tailored hairpin structure. The analyte activated the RCA process, leading to DNA chains formation consisting of the Mg^{2+}-dependent DNAzyme. The DNAzyme-catalyzed cleavage of a substrate probe led to a fluorescence readout signal. The method enabled the analysis of the target DNA with a LOD 1 aM.

Fang et al. [121] developed a detection platform for miRNA-21 based on RCA and an allosteric deoxy ribozyme system (Fig. 7C). With the presence of target miRNA, RCA was initiated and a long chain ssDNA with numerous repeating units was formed. In the presence of single-stranded DNA (H1 and H2), multi-component nucleic acid enzymes (MNAzymes) were formed, which possessed the ability to cleave substrates and achieve amplified fluorescence intensity.

Song et al. [122] proposed a colorimetric DNAzyme reaction triggered by cascading LAMP and CRISPR (DAMPR) for SARS-CoV-2 detection (Fig. 7D). LAMP reaction produced dumbbell structured intermediate amplicons, which could be denatured easily to become active DNAzymes to produce colorimetric signal, due to phosphorothioate-modification [138,143,144]. Meanwhile, CRISPR system was introduced to check the false-positive products. Only correctly amplified LAMP products could be recognized PAM site, decreasing the colorimetric signal. In contrast, the false-positive LAMP products maintained the colorimetric signal. Semi-enzyme-free cascade amplification integrated with DNAzymes for biomarkers detection are compared and summarized in Table 6.

According to the principle of DNAzymes, PMD is often used in the last layer to generate colorimetric signals, while RCD can be used in both first layer to produce intermediate, and the last layer to generate signal. However, the dominant way for RCD is still to be used for signals genaration.

4. Fully-enzyme-free cascade amplification

4.1. *Self-cascade amplification*

4.1.1. *CHA-self cascade amplification*

Several exciting demonstrations have shown that CHA can be

Table 6
Comparison of sensitivity enhancing effect of semi-enzyme-free cascade amplification integrated with DNAzymes based on different principles.

Methodology	Signal	Target	LOD	Sensitivity enhancement	Time	Reference
RCA-RCD	Fluorescence/colorimetric output	Tay-Sachs	3 nM	3-10 times	40min	[139]
RCA-PMD	Colorimetric/chemiluminescent	DNA	1aM	10-1000 times	3h	[120]

connected with itself to construct two-layer CHA, cross-CHA, and self-replicating CHA.

The first version of two-layer CHA reaction was constructed by Chen et al. [145] in 2013 (Fig. 8A). In order to realize minimum leakage, the initiator of the second layer CHA was blocked by hairpin of the first layer CHA. Meanwhile, two-layer CHA yielded 7000-fold fluorescence signal amplification. With the increase of the numbers of CHA layers, the signal amplification effect became more significant. In addition, Zhu et al. [146] reported a two-layer three arm-based CHA. Two unrelated CHA systems were linked by the DNA substrate of named dimer-H2 to form a dimer for signal amplification.

Cross-CHA is an exponential amplification method for rapid detection [153,147]. As shown in Fig. 8B, the CHA product in the first layer made the toehold and branch-migration region close into proximity to trigger the second layer of CHA. The product of the second layer CHA can further initiate the first layer CHA. Interestingly, because the cross-CHA was performed in a cascade manner, this system can be considered as multi-level molecular logic circuits with a feedback mechanism [154].

However, both two-layer CHA and cross-CHA contain at least four hairpin DNAs, enhancing the design complexity. Xiao group simplified the system to construct self-replicating CHA only using two DNA hairpins [148,155,156] (Fig. 8C). With the presence of the target, a pair of harpin probes formed a complex with a replica, which could be cyclically used as a new initiator of harpin probes. The self-replicating CHA has been successfully used to detect DNA and small molecules [157, 158].

CHA-self cascade amplification can greatly improve the condition of leakage expression, but CHA-self cascade generally needs a longer reaction time.

4.1.2. *HCR-self cascade amplification*

HCR-self cascade amplification can be traced back to the nonlinear HCR. Different from traditional linear HCR, nonlinear HCR shows high-order growth kinetic, which makes products own more hybrid structure. Therefore, nonlinear HCR has been regarded as a powerful signal amplifier by integrating with versatile sensing platforms in the past few decades. Based on the self-assembly mechanisms, nonlinear HCR was classified into branched HCR (B-HCR), dendritic HCR (D-HCR), and hydrogel-based clamped HCR (C-HCR).

B-HCR can be described as a hierarchical coupled reaction of duplex HCR circuits. It can achieve a significant signal by integrating two cascaded traditional linear HCR in a sequential and continuous manner, leading to the formation of branched DNA nanostructure. As shown in Fig. 8D, hairpin H1 and H2 alternate a nicked double strand helix after triggered by the target DNA. The initiator of the second layer always comes from the backbone of the first layer hybridized product. The main difference among these methods is how to trigger the second layer HCR reaction. Based on the discrepancy in second layer HCR triggering, B-HCR can be divided into tail-triggered B-HCR [149], hairpin-triggered

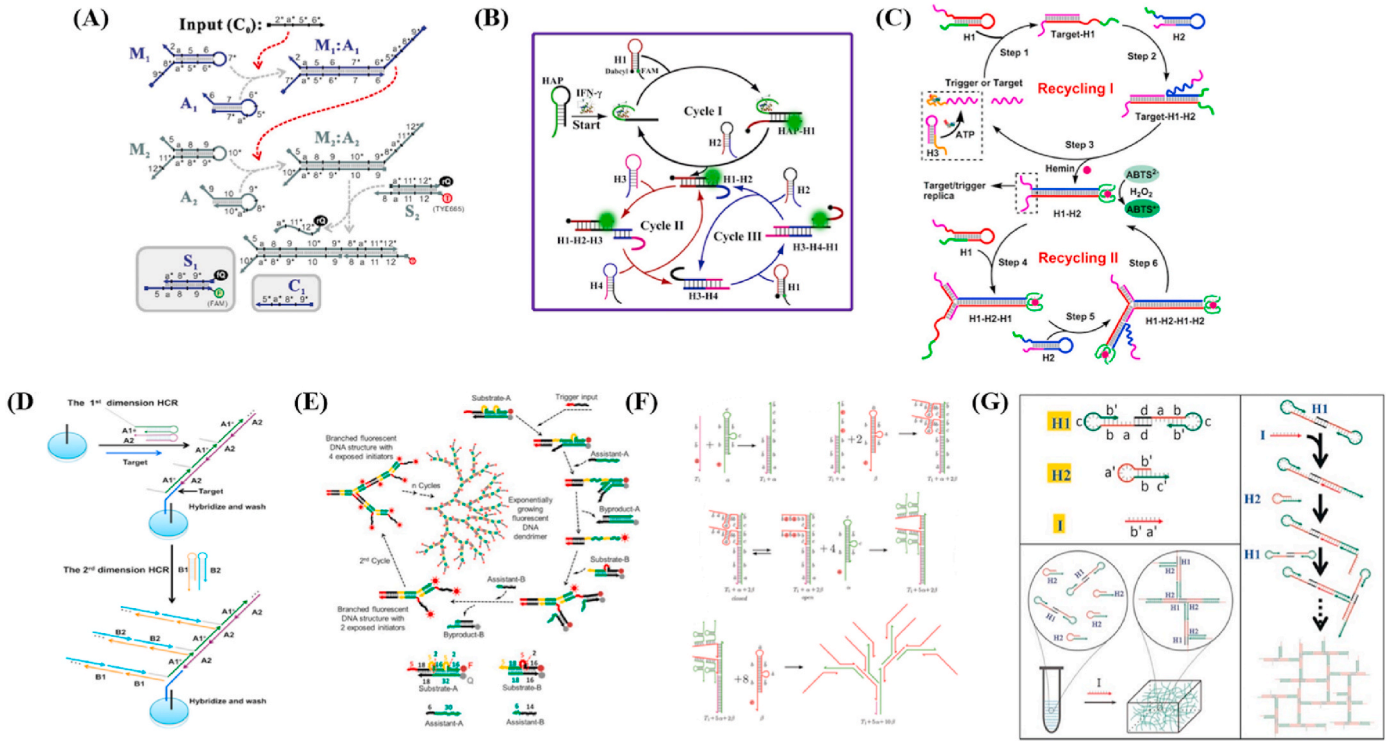

Fig. 8. Self-cascade amplification. (A) Schematic illustration of a two-layer linear CHA cascade composed of chemically synthesized hairpins from different purification processes [145]. (B) Schematic illustration of the principle for cascaded CHA amplification strategy for fluorescently sensing of the target IFN-γ [147]. (C) Schematic illustration of the rapid signal amplification based on the self-replicating catalyzed hairpin assembly (SRCHA) [148]. (D) Schematic illustration of tail-triggered-BHCR [149]. (E). Components and reaction pathway for the triggered chain-branching growth [150]. (F) Schematic illustration of dendritic nanostructure formation upon target (T1) detection [151]. (G) Schematic illustration of the DNA initiator triggered self-assembly process. a) Structures of the hairpin strands H1-dimer and H2, and initiator strand I. b) Details of the C-HCR process initiated by I. c). Representation of the gelation process [152].

B-HCR [159,160], and concatenated B-HCR [161–164].

D-HCR can be described as a target-triggered formation of an exponentially growing dendritic nanostructure through recruiting hairpin or non-hairpin probes. According to the number of input sequences, it can be divided into single input D-HCR and multi-input D-HCR. In single input D-HCR [150] (Fig. 8E), target DNA hybridized with substrate A, exposing the whole sequence of D-HCR H1 with the help of assistant A. Then D-HCR H1 hybridized with multiple D-HCR H2. In multi-input D-HCR [165,151] (Fig. 8F), just like the single-input D-HCR, the assistant chain 1 is needed to help the target complete the opening process of D-HCR H1. However, in the process of opening H2, it is difficult to complete the arduous task by relying on H1 alone. Therefore, the assistant chain 2 is needed to complete the complete opening process of D-HCR H2. Recently, multi-input D-HCR based on regular tetrahedral structures has also been proposed [166,167]. By assembling DNA hairpins at the vertexes of tetrahedral DNA nanostructures, the reaction kinetics of HCR is greatly accelerated due to the increased collision probability and the enhanced local concentrations caused by the synergetic contributions of multiple reaction orientations [168].

Unlike B-HCR or D-HCR, the construction of C-HCR is enabled by bridging and locking the DNA branches to form a 3D network nanostructure [152]. In C-HCR (Fig. 8G), two hairpin (H1, H2) and an initiator are used to drive the self-assembly process. H1 probe is designed with a dimer structure (ten palindromic bases at the 5' end before annealed) to bridge dsDNA. Due to the dimer structure, one H1 probe owns two branches, which are able to hybridize with both initiator and H2 simultaneously to form the three-arm conjunction, or hybridize with two H2 probes to form the four-arm conjunction. In this case, the gel growth can be controlled by the stimuli-triggered C-HCR with the formation of expanded 3D network. The C-CHR system has been widely used in drug delivery [169], tumor detection [170,171], and in situ analysis [172,173]. Fully-enzyme-free of self-cascade amplification for biomarkers detection are compared and summarized in Table 7.

HCR-self cascade amplification can make up for the sensitivity problem of single HCR. Unfortunately, with the increase of the layer of cascades amplification, the structure of the product becomes more complex, which is more difficult to be carried out efficiently.

4.2. Mixing-cascade amplification

4.2.1. CHA/HCR cascade amplification

HCR-triggered CHA (HCR-CHA) circuits were reported for the first time by the group of Ellington in 2012 [174] (Fig. 9A). In this design, a toehold domain was designed at both ends of the HCR probe. When the target triggered HCR reaction, the probe hybridization brought the two sequences close together to trigger the CHA reaction. The signal was produced by the combination of CHA products with reported probes. In order to simplify the HCR-CHA system, Wei et al. [175] removed the original reporting probes. Instead, two harpin probes of CHA was modified with fluorescent and quenched groups. Fluorescence could be restored with the progress of CHA reaction.

The first version of CHA-HCR circuits was also reported by the group of Ellington [176] (). The formed product of CHA exposed the whole initiate domain of HCR, and the second layer HCR was triggered. There was a polyT18 tail on HCR probes, which could hybridize to a polyA18 modified DNAzyme to produce signal. On this basis, a fluorescent based CHA-HCR cascade circuit was designed [182] (Fig. 9B). It is worth mentioning that four hairpin probes were used for alternating hybridization rather than two hairpin probes in HCR process, which effectively avoided the interference between fluorescence and quenching groups while reducing the cost of modification. At present, the CHA-HCR circuit was widely used in protein [183], miRNA [184], and small molecule [185] detection.

4.2.2. CHA-DNAzyme cascade amplification

Lu's group [177] reported a DNAzyme-CHA biosensor for Na^+ imaging inside cells for the first time (Fig. 9C). In the presence of Na^+, DNAzyme cleaved its substrate strand and released ssDNA, which could trigger CHA reaction. Based on this principle, the DNAzyme-catalyzed CHA biosensor was successfully used for viruses [186] and bacteria [187] detection, and intracellular imaging [188,189].

CHA activates DNAzymes (CHA-DNAzymes) as signal amplifiers for fluorescent biosensors. Jiang's group [178] reported a CHA-DNAzyme-based fluorescent biosensor for mRNA imaging and specific gene silencing (Fig. 9D). Part sequences of intact DNAzyme were blocked on the stem of hairpins. CHA was initiated via GalNAc-T mRNA to activate the modified Mg^{2+}-dependent DNAzymes to cleave EGR-1 mRNA.

Split DNAzyme provided a more effective strategy to reduce the background comparing with intact DNAzyme. In this strategy, DNAzymes were divided into two subunits that were spatially separated on the hairpin substrates of CHA. CHA allowed the two subunits of DNAzyme to be close into proximity to make DNAzyme turn into an activated state, cleaving rA containing Taqman probes [129,190,191]and MB [192–195].

4.2.3. HCR-DNAzyme cascade amplification

Two main forms of DNAzyme, RCD and PMD, have been integrated with HCR to realize cascade amplification.

When DNAzyme was used for signal recognition, RCD-HCR was the preferred method. Target activates DNAzyme to realize the cleavage of DNA substrates, and the resulting fragments can further initiate HCR (Fig. 9E). However, this mode was mostly used to detect micromolecules, such as $Cu2^+$ [179], $Pb2^+$ [196], $Uo2^+$ [197] and histidine [198,199].

In 2011, Wang et al. [180] firstly developed HCR-RCD for nucleic acid detection (Fig. 9F). In this study, target triggered autonomous cross-opening of functional DNA hairpin structures, and yielded polymer DNA wires consisting of Mg^{2+}-dependent DNAzyme. DNAzyme recognized and cleaved rA sites of DNA substrates to generate signal. This technique can be used for both enzyme activity analysis [200] and intracellular imaging [201].

HCR-PMD based cascade methods amplification were commonly coupled with electrochemical [181,202], chemiluminescence [203–205], fluorescence [206], and colorimetric [207] to realize

Table 7
Comparison of sensitivity enhancing effect of fully-enzyme-free of self-cascade amplification integrated with DNAzymes based on different principles.

Methodology	Signal	Target	LOD	Sensitivity enhancement	Time	Reference
CHA-CHA	fluorescence	dsDNA	9 nM	7000 times	1h	[145]
CHA-CHA	electrochemical	miRNA-34a	3 nM	3 times	10min	[146]
CHA-CHA	fluorescence	Standard DNA	2.5pM	240 times	4h	[153]
SRCHA	colorimetric	malaria	48 nM	120 times	20min	[148]
BHCR	fluorescence	Standard DNA	1 pM	800 times	4h	[149]
BHCR	electrochemical	Uracil-DNA glycosylase (UDG)	25 nM	/	10h	[159]
CHCR	fluorescence	Standard DNA	0.11 mU/mL	10 times	50min	[161]
BHCR	fluorescence	Standard DNA	45 nM	60 times	30min	[150]
CHCR	hydrogels	Standard DNA	2pM	400 times	1h	[152]

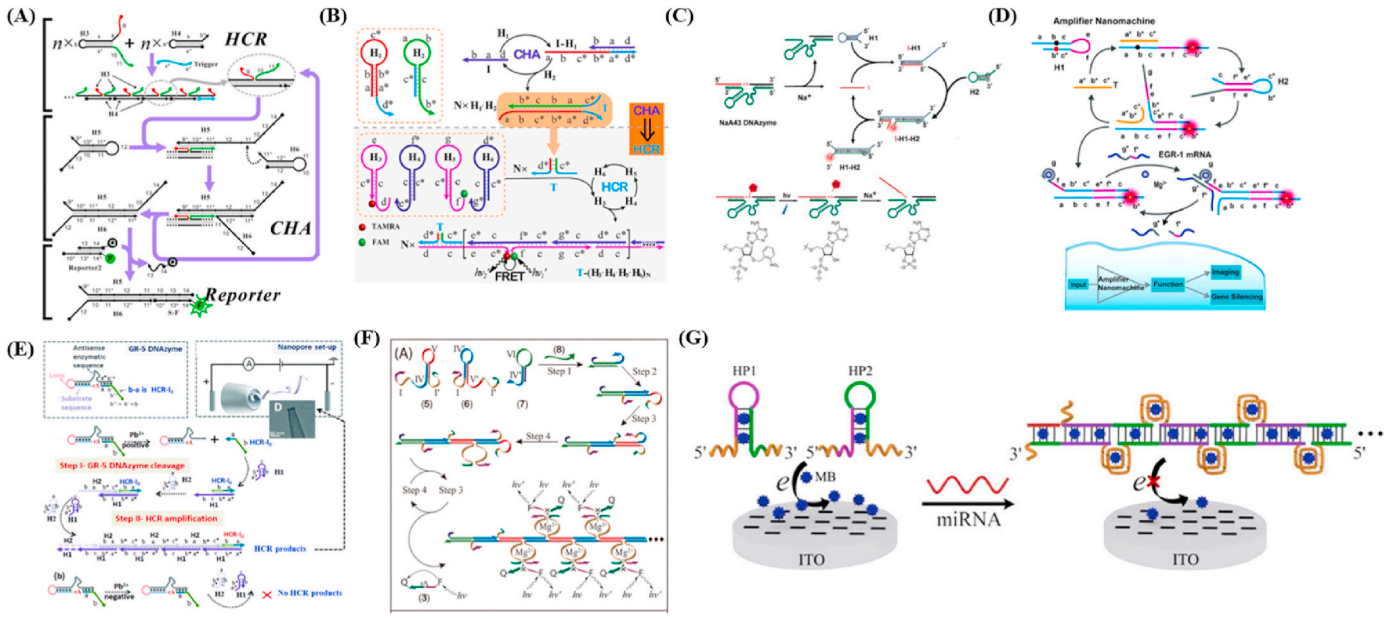

Fig. 9. Mixing-cascade amplification (A) Schematic illustration of the CHA-HCR based circuit [174]. (B) Schematic illustration of CHA-HCR two-layer DNA amplifier [176]. (C) Schematic illustration of a DNAzyme-catalytic hairpin assembly (DzCHA) probe for detection of endogenous Na^+ in living cells [177]. (D) Schematic illustration of the DNAzymatic amplifier nanomachine for mRNA imaging and gene silencing [178]. (E) Schematic illustration of Pb^{2+}recognition based on DNAzyme-HCR transition [179]. (F) The "two-sided" Mg^{2+}-dependent DNAzyme subunits polymer wires for the BRCA1 oncogene analysis [180]. (G) Schematic illustration of the label-free and enzyme-free homogeneous electrochemical strategy based on HCR amplification for miRNA detection [181].

sensitive detection. For example, Hou et al. [181] designed a label-free HCR-based homogenous electrochemical method for miRNA detection (Fig. 9G). MiRNA initiated HCR probes with split G-quadruplex sequences assembly, and multiple G-quadruplexes formed long dsDNA chains. MetB molecules were intercalated into the formed dsDNA chains and G-quadruplexes, subsequently blocking the access to the electrode surface, and greatly decreasing the diffusion current. The LOD of miRNA let 7a detection was 1 pM. Fully-enzyme-free of mixing-cascade amplification for biomarkers detection are compared and summarized in Table 8.

4.3. Other mixing cascade amplification

EDC reaction was first proposed by Yurke and co-workers in 2007 [208], which was mainly driven by the augmentation enthalpy to form stable dsDNA structures without increasing base pairs.

Xing et al. [209] developed an EDC-HCR cascade circuit for sensitive detection of ATP (Fig. 10A). The cascade amplification circuit started with the upstream EDC, where the initiator strands bound with the exposed toehold domain of the premade substrate complex to displace the assistant strand through TMSD, releasing the initiator of HCR. Correspondingly, Huang et al. [210] developed HCR-EDC cascade circuit for highly sensitive and selective detection of standard DNA target (Fig. 10B). The target DNA initiated HCR, generating polymeric dsDNA nanowires with numerous tandem reconstituted triggers (STs). Then, STs bound to the terminal of three-stranded DNA complex QPR. With the help of fuel strand, STs were cyclically reused, and large amounts of reporter strands were released to produce a significantly amplified fluorescence signal.

Xiong et al. [211] developed an EDC-triggered CHA (EDC-CHA) for a dual signal amplification fluorescent biosensor (Fig. 10C). Initiator of CHA was blocked in the as part of substrate dsDNA of EDC. Assembly of EDC promoted the release of substrate DNA and the exposure of intact CHA initiator, which triggered CHA. Based on the similar principle, several modified EDC-CHA strategies have been developed for Hg^{2+} [212], MUCI [213] and miRNA21 [214] detection.

Fully-enzyme-free of other mixing-cascade amplification for biomarkers detection are compared and summarized in Table 9.

5. Conclusions and perspectives

In this review, the design fundamentals, signal generation, evolution, and application of cascade amplification methods in recent years are summarized. By combining different isothermal amplification, cascade amplification achieves ultra-sensitive detection while overcoming the problems existing in the original single isothermal amplification techniques, such as false positives, nucleic acid sequence compatibility, and limited signal amplification capability. In the past decade, cascade amplification techniques have been widely used in the fields of disease diagnosis, environmental monitoring, and food safety.

Table 8
Comparison of sensitivity enhancing effect of fully-enzyme-free of mixing-cascade amplification integrated with DNAzymes based on different principles.

Methodology	Signal	Target	LOD	Sensitivity enhancement	Time	Reference
HCR-CHA	Fluorescence	Standard DNA	5 pM	300 times	16 h	[174]
HCR-CHA	Fluorescence	miRNA 141	0.3 fM	2.3×10^5 times	3 h	[175]
CHA-HCR	Fluorescence	Standard DNA	5 nM	450 times	3.5 h	[176]
CHA-HCR	Fluorescence	miRNA 21	4 pM	360 times	5 h	[182]
RCD-CHA	Fluorescence	Na^+	14 μm	3.5 times	3 h	[177]
CHA-RCD	Fluorescence	mRNA	9 pM	160 times	8 h	[178]
HCR-RCD	Fluorescence	Standard DNA	10 fM	7.2×10^4 times	8 h	[180]
HCR-PMD	Electrochemistry	miRNA let-7a	1 pM	1.45×10^3 times	2 h	[181]

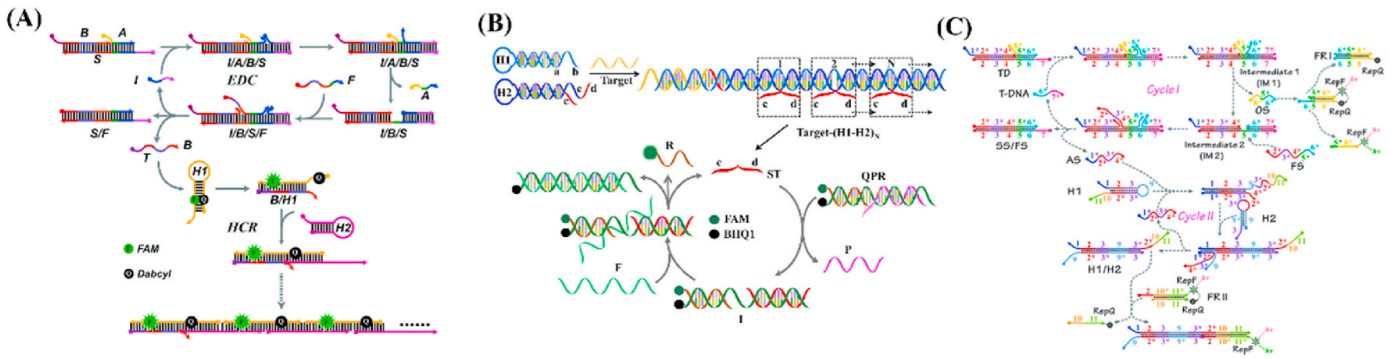

Fig. 10. Other mixing cascade amplification. (A) Schematic illustration of EDC-HCR circuit for accurate DNA detection [209]. (B) Schematic illustration of HCR-EDCR dual-amplification strategy for DNA detection [210]. (C) Schematic illustration of EDC-CHA [211].

Table 9
Comparison of sensitivity enhancing effect of fully-enzyme-free of other mixing cascade amplification integrated with DNAzymes based on different principles.

Methodology	Signal	Target	LOD	Sensitivity enhancement	Time	Reference
EDC-HCR	Fluorescence	Standard DNA, ATP	7 pM, 38 nM	190 times	45 min	[209]
HCR-EDC	Fluorescence	Standard DNA	87 fM	1.2×10^4 times	45 min	[210]
EDC-CHA	Fluorescence	T-DNA	15.6 fM	4.7×10^4 times	2 h	[211]

Despite the remarkable progress, there are still two key issues needing to be further considered in the cascade amplification.

On the one hand, it is necessary to reconcile the number of cascading layers with the cascading reaction time. The main purpose of cascade amplification is to improve the detection sensitivity through multistep signal amplification. However, with the increase of cascade layers, the required detection time is also prolonged exponentially. Thus, it is of significance to establish the optimal efficiency curve for cascade amplification.

On the other hand, it is necessary to design more efficient cascading order between various isothermal amplification. The current cascade amplification is more about the possible cascade attempt, which combines any two isothermal amplification methods and then switches the order to make a new attempt. However, for different length and sequence information of nucleic acids, the process of recognition and reaction is different, even the same isothermal amplification method. Thus, it is of significance to consider the optimal combination of cascades, including a target adapting first-level cascade amplification, corresponding second and sometimes third-level cascades amplification.

In the future, cascade amplification may be further improved from the following four aspects.

First, cascade amplification from enzyme-mediated to enzyme-free. Indeed, enzyme-mediated isothermal amplification techniques show strong signal amplification capability, which owns a tendency to surpass PCR. Especially in the COVID-19 epidemic, isothermal amplification techniques have shown good detection performance, and some isothermal amplification products have gradually occupied the market. However, it must be pointed out that enzyme-mediated isothermal amplification has three fatal disadvantages compared with enzyme-free amplification, which makes it unable to prevent enzyme-free amplification from becoming the mainstream in the future. As an extremely delicate biological sample, enzymes must be strictly controlled in the process of transportation, storage, and use, which is not conducive to the application in resource-constrained scenarios. Meanwhile, the optimum conditions of different enzymes are not the same, which makes the one-pot reaction difficult to carry out effectively. The multi-step reaction increases the complexity of the operation, prolongs the reaction time and may inevitably cause aerosol pollution. More importantly, it is difficult to meet the application conditions of enzymes under physiological conditions, which greatly limits the application of enzyme-mediated isothermal amplification *in vitro* even *in vivo*.

Second, cascade amplification from multi-step to single-step. Most of the cascaded amplification techniques developed were multi-step reactions, which meant the cover of tube needing to be repeatedly opened between each layer of amplification. This undoubtedly increased the workload of the operators, time consuming of detection, the risk of health care workers contacting suspected infected samples as well as the aerosol pollution. In contrast, one-pot reactions can save time and resources and improve efficiency by avoiding lengthy separation and purification of intermediate compounds during post-treatment. Of course, when multi-step reaction is unavoidable, microfluidic chip can be considered as a friendly reaction platform to automate adding the sample and reduce the pollution caused by repeated opening of the cover.

Third, introducing functional nucleic acids such as aptamers to expand the range of targets. Unlike nucleic acids, non-nucleic acid analytes such as proteins and small molecules cannot be amplified, so improving the sensitivity of such analytes has been a hot issue. By competitive binding of aptamer complementary chains and analytes with aptamers, the non-nucleic acid detection can be converted into nucleic acid detection, thus cascade amplification techniques can be used to significantly improve the sensitivity of non-nucleic acid analytes.

Finally, taking full advantage of nanomaterials in cascade amplification. Nanomaterials have been widely used in isothermal amplification over the past 2 decades. However, this application is usually in the form of $1 + 1$, where the nanomaterials only act as downstream signal donors of isothermal amplification, rather than participate in the signal amplification. Instead, the structural and functional properties of nanomaterials may be used to increase the local reaction concentration, improve the reaction efficiency, and reduce the non-specific reaction. In addition, by using the size and charge characteristics of nanomaterials, the substrates of cascade amplification can be effectively delivered into cells to improve endocytosis efficiency, reduce the risk of nucleic acid degradation, and effectively realize intracellular detection.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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