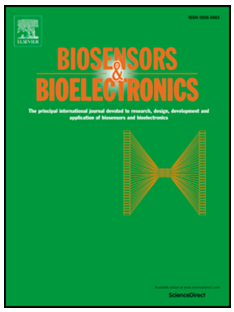

Nonlinear hybridization chain reaction-based functional DNA nanostructure assembly for biosensing, bioimaging applications

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Nonlinear hybridization chain reaction-based functional DNA nanostructure assembly for biosensing, bioimaging applications.

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ABSTRACT:

Hybridization chain reaction (HCR) can be divided into two categories: linear HCR and nonlinear HCR. In traditional linear HCR, the relatively slow kinetics and less sufficient sensitivity largely limit its scope of application. In the nonlinear HCR system, under the trigger of the initiator, the judicious designed substrate sequences (hairpin or hairpin-free) will self-assembly to dendritic or branched DNA nanostructures with exponential growth kinetics. Given the advantages of its enzyme-free, high-order growth kinetic, high sensitivity, and simple operation, nonlinear HCR is regarded as a powerful signal amplifier for the detection of biomarkers by integrating with versatile sensing platforms in the past few decades. In this review, we describe the basic features of nonlinear HCR mechanism and classify the nonlinear HCR into several categories based on their self-assembly mechanisms: the branched HCR, dendritic HCR, hydrogel-based clamped HCR, and other types of HCR. Then, we summarize the recent development of nonlinear HCR in biosensing, such as nucleic acid, protein, enzyme activities, and cancer cell detection, *etc*., and we also review the current applications of nonlinear HCR in bioimaging (mRNA in situ imaging). We choose several representative works to further illustrate the analysis mechanisms *via* various detection platforms, such as fluorescence, electrochemical, colorimetric, *etc*. At last, we also review the challenges and further perspectives of nonlinear HCR in the use of bioanalysis.

KEYWORDS: nonlinear hybridization chain reaction, biosensor, isothermal amplification, detection, nucleic acid, biomarker

1. Introduction

DNA, as functional biomacromolecule, its ability of self-assembly not only essential for maintaining normal life processes of living organisms, but also has the potential to artificially construct functional DNA dynamic nanostructures (Vybornyi et al., 2019; Pinheiro et al., 2011; Kallenbach et al., 1983). DNA nanostructures, can utilize the flexibility and rigidity of deoxynucleotide strands to create any desired functional nucleic acids through sophisticated design and assembly (Pinheiro et al., 2011; He et al., 2008). Moreover, its excellent biocompatibility makes DNA molecular an ideal basic nanomaterial which is widely used in bioimaging, bioanalysis, and drug delivery (Roh et al., 2011; Li et al., 2008). DNA dynamic nanotechnology is one of the important components of DNA nanotechnology, that mainly uses toehold mediated strand displacement (TMSD) to construct an automated synthesis of oligomers (Simmel et al., 2019). TMSD was a process which two partially complementary single-strand hybridize to each other first; then, the toehold domain (unhybridized part) can be hybridized with the third strand, replacing former strand from a hybridized product, thereby branch migration occurred (Zhang and Seelig, 2011).

Recently, TMSD based Nucleic acid amplification has been wildly used in the field of bioanalysis. In particular, the most typical example of TMSD is Hybridization chain reaction (HCR), an enzyme-free isothermal amplification strategy proposed by Dirks and Pierce in 2004 (Dirks and Pierce, 2004). Hybridization chain reaction breaks the previous inherent impression of nucleic acid amplification methods, as it discards complicated thermocycling steps, achieves PCR (Polymerase Chain Reaction)-like ultra-high detection sensitivity without enzymes involved, and simplifies the detection system greatly. Nevertheless, HCR-based amplification still confronts several problems: (1) At present, most HCR reactions are only at the one-dimensional level; linear growth HCR alone may not provide sufficient sensitivity when it comes to identifying and tracking targets (Yang et al., 2017). (2) Initiating the HCR reaction by designing multiple hairpin structures requires time-consuming process and critical principle.

Additionally, there is still a lack of systematic guidelines for hairpin design standard (Bi et al., 2017; Srinivas et al., 2017). (3) In the absence of target molecules, signal leakage is unavoidable because of transient DNA melting or the "breathing" phenomenon between hairpins (S. Li et al., 2020). Sensitivity needs to be improved if it is to be applied to clinical diagnosis in the future (Srinivas et al., 2017). Nonlinear HCR, as a highly dimensional HCR, tends to improve its sensitivity by forming multiple branches DNA nanostructure on the basis of HCR. Under the trigger of the initiator sequence, the hairpin structure will be opened to self-assembly dendritic or branched DNA nanostructures with high-order growth kinetics. Compared with one-dimensional HCR, Nonlinear HCR has greatly improved the detection performance which needs to be solved at present, especially the detection sensitivity and selectivity (Li et al., 2018). Hsing's Group developed a hairpin-free Nonlinear HCR protocol. In this protocol, two double strand DNA substrate were designed with loop and toehold, and its excellent sequence specificity resulted in a dendritic DNA nanostructure formation under a triggered process. This Nonlinear HCR can readily realize exponential growth, and the signal leakage can also be well-controlled, which greatly reduces the background signal (Xuan and Hsing, 2014). Inspired by Hsing's work, Ding's group improved and constructed a labeled-free electrochemical biosensor for detecting nucleic acid and small molecules (Ding et al., 2017b). By designing different primers and nonlinear methods, various Nonlinear HCR has been reported, including bHCR (branched HCR) (Tang et al., 2017), dendritic HCR (Xuan and Hsing, 2014) , and clamped HCR (Jianbang Wang et al., 2017), etc. In addition to biomarker detection, fluorescence reporter molecules conjugated nucleic acid probes have enabled the realization of biomolecule in situ imaging and signal localization in living cell through the combination of nonlinear HCR amplifier. Liu's group proposed a branched HCR circuit for amplified imaging of mRNA in a living cell, which demonstrated the higher amplification efficiency than conventional HCR (Liu et al., 2018). The traditional FISH (fluorescence in situ hybridization) strategy can only provide compromised sensitivity and specificity, especially for point mutation.

With the in situ nonlinear HCR amplification method, Jiang and co-workers designed bHCR method which offered multiple visible fluorescence spot on the hybridization product, and significantly enhanced FISH specificity (Tang et al., 2017). Due to the properties of enzyme-free, isothermal signal exponential amplification and readily achieved dendritic nanostructure, Nonlinear HCR-based amplification strategy has great promise in the development of ultrasensitive detection platform for biosensing(Zhou et al., 2014, Zhao et al., 2017; Xu and Zheng, 2016) (especially for low-abundance biomarker detection), bioimaging (Shang et al., 2020; Wei et al., 2018; Huang et al., 2018) and molecular diagnostics field, given its advantages in ultra-high sensitivity, excellent sequence specificity, and simplistic operation (G. Xu et al., 2019).

In this review, we first introduce the fundamental principles and classification of Nonlinear HCR. Then we discuss the representative application of Nonlinear HCR directed amplification in biosensing and bioimaging. Finally, the conclusions and future perspectives of Nonlinear HCR will be proposed.

2. Basic features of Nonlinear HCR

After proposing the idea of the hybridization chain reaction, which autonomously forms an one-dimensional DNA nanostructure for amplification. Pierce and coworkers came up with several DNA dynamic assembly proposals on the basis of HCR. For example, a four-way branch migration (which is achieved by the exchange of the bases at the junction of the four helices) (Venkataraman et al., 2007) can program biomolecular self-assembly pathways, in which hairpin sequence can catalyze biomolecular self-assembly for dendritic growth of DNA nanostructures under the trigger of initiator (Yin et al., 2008). However, this kind of dendritic growth of DNA can hardly be considered as a formal Nonlinear HCR, due to the non-self-sustained process. During this molecular assembly pathway, additional hairpins need to be added in each step to continue the dendritic formation process. Afterwards, Chandran *et al.* developed a DNA

detection system, in which a target DNA triggered two-loop structure hairpins to form a dendritic nanostructure without external intervention, resulting in exponential growth of DNA dendrimers (Chandran et al., 2013). Nevertheless, the drawbacks of this method are obvious: temperature needs to be controlled; the spontaneously opened hairpin structure is a relatively long stem which may cause linear growth instead of dendritic growth; inevitable leakage of the signal causes the false positive result, which largely decreases the sensitivity of the reaction. Additionally, it is doubted that the exponential kinetic growth happened as no solid evidence (morphology, the kinetics of the formation of DNA nanostructures) is mentioned. Therefore, great improvements were remaining urgently for direction self-assembly of nanostructures with high efficiency and controllability. In recent years, various novel exponential enzyme-free amplification methods based on HCR have been developed. Here, we summarized several categories of Nonlinear HCR and demonstrated the amplification process well in Figure 1, including branched HCR, dendritic HCR, and clamped HCR. In addition, the AFM images showed in Figure 2 intuitively revealed the morphology of final hybridized products and further confirmed that the hybridization event for the polymerization of strands into giant polymeric structures of each approach could proceed efficiently as anticipated.

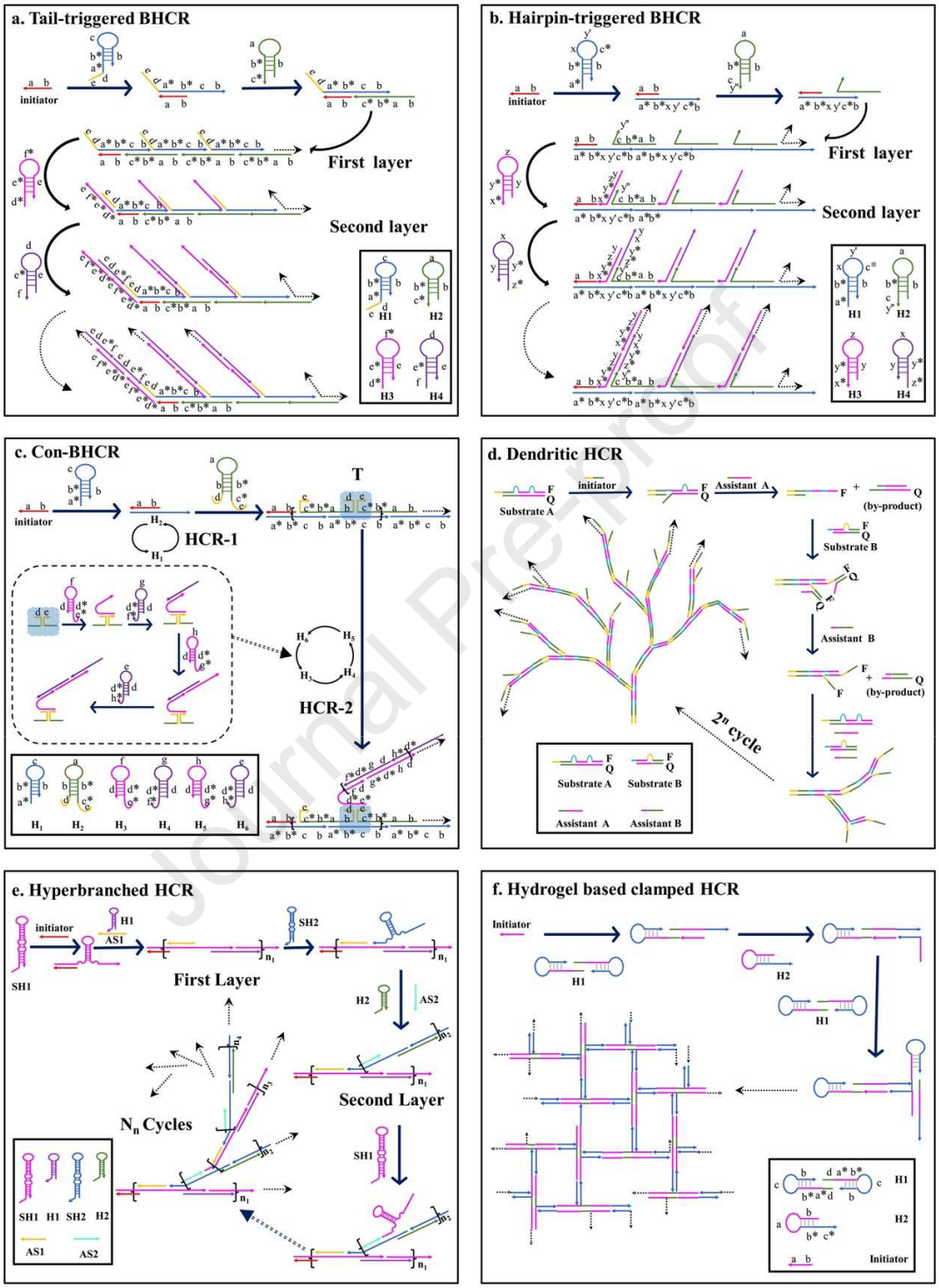

Fig. 1 Fundamental of nonlinear HCR. **a.** Schematic illustration of Tail-triggered HCR. In the first layer, initiator sequence(a-b) hybridized with a*-b* segment of hairpin H1 and thus unfolded H1 to further expose segment c-b, which enabled the open up of H2 via strand displacement interaction. The exposed domain on H2 was identical with initiator sequence (a-b) and thus propagted a long nicked dsDNA as traditional HCR. Subsequently, the hairpin H3 was added, and the yellow segment e-d overhang at each terminus of the first layer product acted as a new initiator for the sencond layer reaction, thus opened H3 and H4 formed the branched dsDNA products in the same manner. **b.** Schematic illustration of Hairpin-triggered HCR. In the first layer, a long nicked double strand polymer was formed under the hybridization event between initiator, H1 and H2. Consequensely, the formation of first layer product brought two spilt initiator segment (x, y' at the H1, y'' at the H2) together, activting the second layer HCR with the cross-opening of H3 and H4. **c.** Schematic illustration of Con-BHCR. In the first layer, the initiator a-b triggered the HCR assembly by cross-opening H1 and H2. During the assembly process, the yellow domain d and e were brought proximty to form a "T" structure. The mutiple "T" on the HCR-1 backbone acted as new initiator for the HCR-2, and opened up the H3, H4, H5, H6 *via* strand displacement interaction, resulting in branched dsDNA concatamer. **d.** Schematic illustration of Dendritic HCR. The initiator was complementary with Substrate A and opened the left loop. The assistant A was then introduced to remove Q strand from Substrate A to produce by-product via TMSD reaction and opened the right loop. Two Substrant Bs could combine to the two new exposed toeholds on the Substrate A, and two Assistant Bs were added to dissociated Q strands in the same manner. The new exposed four toehold rigions were idendital to initiator sequence, thus triggered new round cycle. **e.** Schematic illustration of Hyperbranched HCR. The initiator first hybridized with 5' SH1 and opened the bottom two loops. Then the H1 and AS1 were added to open the upper loop in the same way and finished the first layer reaction. The red segment on the first layer product might be an initator and opened the bottom two loops of SH2 *via* branch migration. After the introducation of H2 and AS2, the second layer was complished and the loop on the SH2 was totally opened up, the newly exposed segment was idential with initiator sequence in the first layer reaction, and would extend forward to propagate branched-growth nanostructure. **f.** Schematic illustration of Clamped HCR. H1 owned a unique dimer structure (ten palindromic bases at the 5' end before annealed). The introduce of initiator first opened the right loop of H1 and its exposed segment c-b would further open up H2 through DNA strand displacement reaction. The opened rigion on H2 was identical with initiator sequence. One H1 had two branches, which were able to hybridize with both initiator and H2 simultaneously, forming the three-arm conjunction, or hybridize with two H2 to construct the four-arm conjunction, thus the 3D hydrogel nanostructure was successfully constructed.

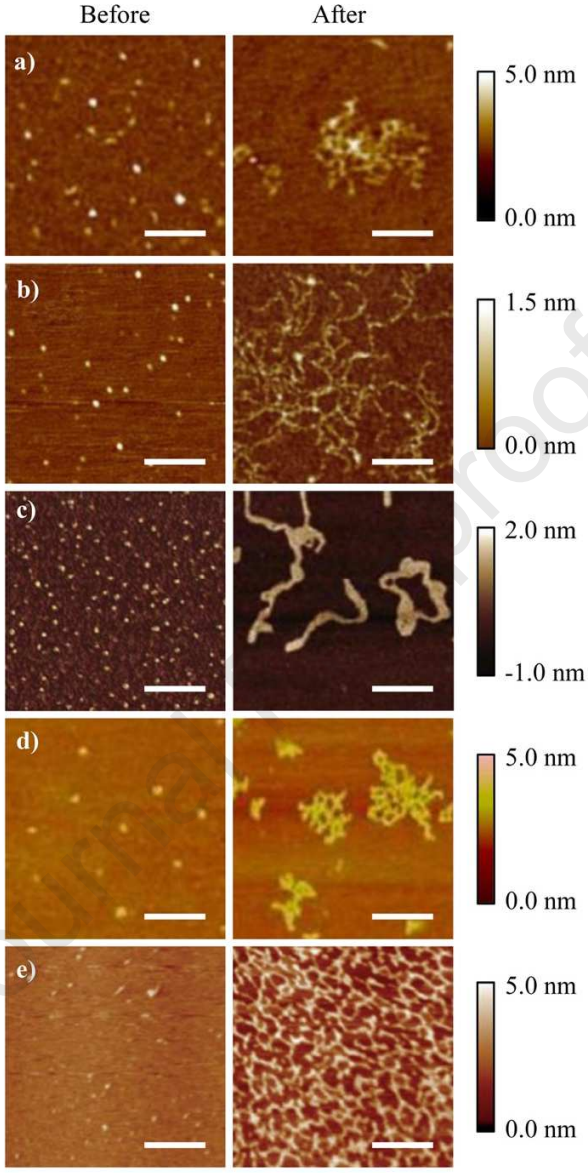

Fig. 2 AFM images of the assembled products. **a)** Tail-triggered HCR. **b)** Hairpin-triggered HCR. **c)** Con-BHCR. **d)** Dendritic HCR. **e)** Hyperbranched HCR. In the absence of target molecule, only some small spots can be observed (Left panels). After the target-triggered self-assembly of nonlinear HCR, the multi-branched products both polydisperse in size and morphology with different shapes (Right panels). Scale bars: 200nm. Adapted with permission from Ref. (Xu and Zheng, 2016), (Tang et al., 2017), (Wei et al., 2018), (Xuan and Hsing, 2014), (Bi et al., 2015).

2.1 Branched hybridization chain reaction

Branched hybridization chain reaction (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 hybridization chain reaction strategies in a sequential and continuous manner, leading to the formation of branched DNA nanostructure. The component unit of B-HCR is two layers of successful linear HCRs. The first layer HCR is used as an intermediate that recognizes the target sequence, and the second layer HCR acts as the amplified transducer. Typically, B-HCR requires three to six functional hairpin probes to continue the self-assembly process. The reactant is well-designed and coexists metastably in the absence of initiator. After triggered by the initiator, the domain on the backbone chain of DNA hybridized product might act as a new initiator, yielding the formation of a branched double-stranded nanostructure.

Generally, in the first layer of HCR, 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 difference of second layer HCR triggering, B-HCR can be divided into 3 types.

2.1.1 Tail-triggered B-HCR (Fig. 1a)

Hairpin H1 itself modified with extra single strand overhang at the 5’ end created a hybridization tail (e d). Upon the involvement of the initiator a-b, it hybridized with segment a*-b* on hairpin H1 and thus unfolded H1 to further expose segment c-b. The new exposed c-b then opened up H2 *via* strand displacement interaction. The opened H2 now revealed segment a-b that was identical with initiator sequence. Therefore, segment a-b of H2 could trigger another hybridization event with the formation of long-nicked dsDNA polymer. Now the hybridization tail e-d was already overhang at each terminus of the first layer product, it acted as a new

initiator for the second layer of HCR process that hybridized with H3, H4 in the same manner. In briefly, the first layer HCR was triggered with recognition event of initiator sequence, activating cross-open of H1 and H2 through toehold mediated strand displacement. After the formation of a nicked double strand, the hybridization tail overhang at each terminus triggered the second layer of HCR process that further hybridized with H3 and H4 to program self-assembly of branched nonlinear HCR (Xu and Zheng, 2016).

2.1.2 Hairpin-triggered B-HCR (Fig. 1b)

In the presence of initiator (a-b), H1 was opened and exposed domain x-y'-c*-b thought TMSD event, then the domain c*-b enabled the cross-opening of H2 that further led to the release of domain a-b of H2, accessible for the subsequent TMSD event to accomplish the first layer hybridization. As the hairpin structure is opened up in the formation of first layer procedure that bought two spilt initiator domain (x, y' at the H1, y'' at the H2) together, the newly exposed domain (x y'') on the new produced double strand DNA turns into an initiator, activating the second layer linear growth assembly between H3 and H4. Thus, the exponential growth of branched DNA nanostructure process can be readily achieved.

Zheng's group reported a 2-dimensional hybridizing chain polymerization for the sensitive detection of RNA, by forming several sandwich like complexes on the solid phase; and SYBR green was introduced to generate fluorescence signal (Xu and Zheng, 2016). Similarly, Jiang's group developed a fluorescence FISH strategy based on B-HCR for the detection of a single mRNA mutation in a single cell; and this strategy had the potential for quantifying the mRNA expression(Tang et al., 2017). Moreover, the same group successfully developed a bHCR circuit to localize imaging of mRNA in a living cell with fluorescent resonance energy transfer (FRET) organic group (donor and acceptor) labeled in the probe H3, which generated super-strong fluorescent signals from target mRNA when the probes were transfected into cells. A highly dimensional multiplexed HCR system was well fabricated by Reboun's group, which used a

three-arm branching structure to realize multiplexed biomolecular detection in a label-free and reagent-less manner (G. Xu et al., 2019).

2.1.3 Concatenated B-HCR (Fig. 1c)

The concatenate branched hybridization chain reaction (Con-B-HCR) was proposed in the same manner. With the elaboration sequence design of six hairpin structure, the toehold mediated strand displacement between H1 and H2 brings domain d and e together to reconstitute the "T" structure, leading to the formation of long dsDNA nanowires with tandemly repeated "T" on the backbone of first layer hybridized product. Subsequentially, each of reconstituting "T" structure enables the triggering of downstream HCR-2 and propagates the hybridization between H3, H4, H5, and H6. The fabric of Con-B-HCR make it readily adopted in a new target detection through simply re-designed of base pairing between strands, this enables to utilize the functional amplification strategies in various sensing platform for biomarker detections.

Wang's group utilized this cascaded self-assembly method with an amplified FRET signal readout. In the HCR-2 process, the hairpin of whom labeled with donor/acceptor were brought in close proximity to each other, thus generating strong FRET signals. This method showed great promise for analyzing various biological target, such as intracellular imaging of miRNA (Wei et al., 2018), polynucleotide kinase (Shang et al., 2020), the detection of UDG (Uracil-DNA glycosylase) and its inhibitors (Jing Wang et al., 2017), *etc*. Additionally, a Concatenated B-HCR-DNAzyme circuit could be introduced into the HCR-2 step for the following generation of DNAzyme branched nanowires to achieve amplified signal readout, which was systematically investigated *in vitro* for the distinguish of a biomarker in cancer diagnosis (Wu et al., 2019). Furthermore, based on the Con-B-HCR, Fuan's group developed a polynucleotide kinase (PNK) sensing platform for intracellular imaging. Hairpin Hp and λ exonuclease (λ Exo) were used as PNK capture and recognition element (Shang et al., 2020). Compared with traditional linear

HCR assay, the PNK response of Concatenated B-HCR and detection limit is significantly improved.

2.2 Dendritic hybridization chain reaction

Dendritic hybridization chain reaction (D-HCR) can be described as a target-triggered formation of an exponentially growing dendritic nanostructure through recruiting hairpin or non-hairpin probes. It can be divided into two categories according to the number of starting points: single starting point, and multi-starting points.

A hairpin-free triggered self-assembly DNA dendrimers protocol is the typical single start point D-HCR (Fig. 1d). The reaction consists of two double strand probes (Substrate A and Substrate B), and two single strand probes (assistant A and assistant B). Substrate A and Substrate B, both are annealed by two partially complementary single strands Q and F, which formed a bulge loop structure in the middle of the F chain and exposed toehold domain at one end. Once the trigger is inputted into the system, it soon combines with the exposed toehold of Substrate A and open the first bulge loop on the F chain, initiating the first TMSD reaction. Subsequently, the exposed part of the Q chain, which is completely complementary with assistant A and spontaneously hybridizes together, releases from Substrate A, and generates the by-product. Thus, the exposed two identical toehold domains will recruit Substrate B and open its exclusive bulge loop. Similarly, assistant B has the responsibility to replace the Q chain from Substrate B. It sooner exposes identical two toeholds on the F chain which is regarded as a new trigger that continuously initiates the TMSD reaction, and the dendritic growth of DNA nanostructure will sustainably continue. Since the system does not require hairpin structures, there are few secondary structures in the DNA molecules participating in the reaction, which can greatly reduce non-specific cross-reactions between the chains and shows excellent exponential amplification efficiency.

The dendritic HCR amplification system (Xuan and Hsing, 2014) was successfully applied

in the construction of electrochemical and electrochemiluminescence biosensor platform for analyte recognition through optical and electrochemical method (Ding et al., 2017b; Zhao et al., 2017; Jia et al., 2016; Hun et al., 2017; Xu et al., 2017), which had achieved high sensitivity and outstanding specificity. The same group had developed an integrating DNA displacement circuitry based on the D-HCR with minor change of primer sequence to achieve arbitrary target detection. A new circuit was created before dendritic self-assembly, which released a single strand through TMSD reaction to trigger the programmed self-assembly procedures which obtained a high molecular-weight dendritic nanostructure. Nevertheless, there remained an unavoidable signal leakage problem, which was caused by the purification step, for the extra unreacted strand needed to be removed through the form of by-products. This sort of leakage might lead to false positive results.

A hairpin-based hyperbranched hybridization chain reaction (HB-HCR) strategy was reported with multiple start points (Fig. 1e). This HB-HCR strategy relies on the design of two super-hairpins (SH1, SH2), two hairpins (H1, H2), and two ssDNA helper (AS1, AS2) species. In the absence of the initiator, each probe could metastably coexist in the solution. Once the DNA initiator is introduced, the initiator hybridizes with SH1 thus open up two bugle loop in the middle of the hairpin, the newly exposed toehold region can trigger the first linear hybridization chain reaction between SH1 and H1 to propagate the first layer products, and the single strand AS1 is hybridized to the SH1 at the same time. Analogously, SH2 can bind to the toehold region on the first layer, cooperating with H2 and AS2 to active the second hybridization chain reaction, resulting in the generation of the second layer with branched growth of products. Theoretically, the HB-HCR will continue, as the product of each layer will expose a new toehold domain to trigger the next TMSD reaction to obtain exponential growth of HB-HCR products after many reaction cycles.

Compared with Hsing's work, the HB-HCR reaction owned multiple start points as the formation of a nicked double strand DNA, the hyperbranched strand could extend from each start

point. This HB-HCR method had exhibited ultrasensitive amplification efficiency and had provided an outstanding platform in the biosensor. Moreover, a photoelectrochemical biosensor for Human telomerase RNA detection was proposed based on the catalytic hairpin assembly (CHA) coupled with HB-HCR amplification strategy; and it showed excellent amplification efficiency (Chu et al., 2019).

2.3 Hydrogel-based clamped hybridization chain reaction

DNA hydrogel is a novel microscopic functional nanomaterial that can utilize nucleic acid to construct a 3D hydrophilic crosslink network with excellent biocompatibility, easy programmability, and stability (Lee and Mooney, 2001; Khajouei et al., 2020; (Ahmed, 2015). In recent years, this 3D polymer scaffold has been wildly exploited in drug delivery, biosensors, imaging, and tissue engineering (Willner, 2017). The clamped hybridization chain reaction (Clamped HCR) strategy was reported to form a 3D network hydrogel under the guidance of small amount initiator by stimuli-triggering hybridize events between hairpin probes (Jianbang Wang et al., 2017) (Fig. 1f). Unlike typical linear or dendritic HCR, the construction of Clamped HCR is enabled by bridging and locking the DNA branches, forming the 3D network nanostructure, and realizing the solution-gel transition. In the clamped HCR, two hairpin (H1, H2) and an initiator are prepared 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 the dsDNA. In the presence of initiator, one bugle loop of H1 is opened and exposed to the toehold domain (b and c), which activates the H2 unfolded in the same manner. Due to the dimer structure, one H1 owns two branches which are able to hybridize with both initiator and H2 simultaneously, forming the three-arm conjunction, or hybridize with two H2 to construct the four-arm conjunction. In this case, the gel growth can be controlled by the stimuli-triggered Clamped HCR with the formation of expanded 3D network.

Based on the controllability character of Clamped HCR, Song's group further designed a scheme for an aptamer-triggered Clamped HCR (atcHCR) which enabled controllable growth of

patterned hydrogels on the cell surface, and had successfully applied in suit circulating tumor cells (CTCs) for live cell analysis (Song et al., 2017). Moreover, a pH-triggered hydrogel based on clamped HCR strategy had also been proposed and this macroscopic hydrogel showed great potential in drug delivery, tumor therapy and biosensing with less loss of cell viability and integrity (Y. Li et al., 2020).

2.4 Other Nonlinear hybridization chain reaction

In order to establish a cancer cell detection platform with an enzyme-free isothermal amplification strategy, avoiding tedious lysis step, a re-engineered Multi-branched HCR (mHCR) with multiple branched arms and binding sites was proposed (Zhou et al., 2014). The biotin-labeled aptamer could hybridize with both initiator and EpCAM (Epithelial cell adhesion molecule), which provided multiple binding sites of HCR product in the cell surface. Initiated by target sequence, mHCR reaction autonomously formed multiple branched arms of HCR products on the cancer cell. Additionally, Chen's group reported a target-triggered DNA assembly of multibranched DNA nanostructure composed of self-assembly DNA concatamers and G-quadruplex wires through the biotin-avidin system (J. Xu et al., 2019).

Other shapes of nucleic acid self-assembly nanostructure based on nonlinear HCR can also be effectively used for cell detection or molecular biomarkers analysis. Zhang's group proposed a cross-like structure nonlinear HCR, which involved the cross-open of DNA hairpins, producing micro-net-like nanostructure automatically for the *in situ* determination of protein expression on bone marrow cells (Li et al., 2018). Based on specific recognition of aptamer, a net-like hybridization chain reaction (nHCR) method for the detection of cytokine IFN-γ (interferon-gamma) was reported by Xiang's group (Shen et al., 2018) (Fig. 4a). Hairpin probe HA and dumb-bell DNA hairpin probe HF were coexisted metastably in the solution. HA probe consisted of two functional domains, one was hybridized with the complementary region of IFN-γ, and the other triggered polymerization of the dumb-bell hairpin probe. In the presence of

IFN-γ, it sooner bound the complementary region in the HA probe and exposed initiator region to hybridize with dumb-bell HT probe to liberate a single strand sequence which could further hybridize with another dumb-bell HF, leading to the net-like structure between HF and HT and resulting in an amplified FRET signal for the detection of IFN-γ. The nHCR based sensor platform could detect IFN-γ with the limit of detection (LOD) of 1.2 pM. Yang's group had also reported a DNA nanoprobe conformational change for lead(II) ion assay to form a reticulated dsDNA nanostructure on the electrode surface based on a four-way junction probe (Yang et al., 2019).

3. Applications of nonlinear HCR in biological sensing

Compared with traditional HCR, the nonlinear growth kinetic of HCR reaction has satisfactory sensitivity and its amplification efficiency has proved to be a facile tool in the build of biological sensing platform, making it more suitable for point-of-care (POC) diagnosis. In particular, the endogenous nucleic acids (especially those with highly identical sequence between family members) can easily be detected and discriminated based on the nonlinear HCR amplified biosensor protocol. Moreover, the other biological metabolites and heavy metal ion can also be sensitively detected upon introducing of aptamer technology. In this review, we summarized several representative nonlinear HCR biosensor protocols and classified them into five categories (Table 1).

Table 1. Nonlinear HCR based biosensing strategies.

Target	Method	Signal output	Limit of detection	Specificity (Target/Blank)	Detection time	RSD (%)	Ref.
DNA	chemiluminescence	Hemin/G-quadruplex HRP-mimicking DNAzyme	7.0×10^{-12} M	43.5	30min	N/A	(Hun et al., 2017)
BCR/ABL fusion gene	Electrochemiluminescence	$Ru(phen)3^{2+}$	5.49fM	61.5	60min	3.33	(Xu et al., 2017)
MicroRNA	Electrochemiluminescence	Dopamine and terbium (II) organic gel	0.18fM	3.3	6h	2.4	(Li et al., 2019)
DNA	Colorimetric	Peroxidase-mimicking DNAzyme	0.8fM	5.5	2h	N/A	(Ravan et al., 2018)
MicroRNA	Colorimetric	TMB	13aM	36	5min	2.41	(Tang et al., 2019)
MicroRNA	Colorimetric	Hemin /peroxidase mimicking G-quadraplex DNAzyme	1pM	1.7	150min	N/A	(Hosseinzadeh et al., 2020)
microRNA	Electrochemical	interfacial electrons transfer resistance between DNA nanostructure and $[Fe(CN)_6]^{3-/4-}$	0.3334fM	3.14	52min	3.9	(Zhou et al., 2019)
Circulating tumor DNA	Electrochemical	streptavidin-alkaline phosphatase (ST-ALP) catalyze the substrate α-naphthol (α-NP) to an electroactive product	3pM	22	30min	N/A	(Huang et al., 2020)
DNA	Electrochemical	TMB	0.4fM	31.5	N/A	5.1	(Jia et al., 2016)
MicroRNA	Fluorescence	Fluorophore	0.3pM	4	5h	N/A	(Wu et al., 2019)
RNA	Fluorescence	SYBE Green I	1pM	15	N/A	N/A	(Xu and Zheng, 2016)
microRNA	Fluorescence	Zinc (II)-protoporphyrin IX (ZnPPIX)	0.7fM	40	90min	N/A	(Xue et al., 2018)
MicroRNA	Fluorescence	Fluorophore	2.14pM	9	N/A	N/A	(Wang et al., 2019)
DNA	Photoelectrochemical	ZnO flower-rod architectures (ZnO FRs)	3.7fM	6.6	N/A	1.2	(Han et al., 2017)
Human Telomerase RNA	Photoelectrochemical	4-chloro-1-naphthol (4-CN) is deposited on the PEC electrode to reduce photocurrent	17fM	12	N/A	2.1	(Chu et al., 2019)
PML/RARα gene	SPR	Macromolecule DNA dendrimer deposits on the SPR gold chip	0.72pM/ 0.65pM	48	60min	3.1/1.5	(Guo et al., 2017)

Table 1. continued

KRAS gene	QCM	frequency response of amplified QCM biosensor	1.5nM	8.7	60min	4.2	(Zhao et al., 2017)
DNA	Acoustic	HCR product propagating SAW on the sensor surface	25nM	N/A	N/A	N/A	(G. Xu et al., 2019)
TRPV4	Fluorescence	Fluorophore	2.8pg/mL	N/A	50min	N/A	(Li et al., 2018)
VEGF	Colorimetric	AuNPs aggregation	185pM	1.7	25min	N/A	(Chang et al., 2016)
PSA	SERS	The formation of Au@Ag core-shell on the gold electrode surface with SRES signal output.	0.94fg/mL	7.5	2h	5.3	(Wang et al., 2020)
CA125	Electrochemical	molybdophosphate	50μU.mL^{-1}	N/A	2h	4.5	(Nie et al., 2018)
IFN-γ	Fluorescence	Fluorophore	1.2pM	5.2	3h	5.1	(Shen et al., 2018)
IFN-γ	Electrochemical	hemin/G-quadruplex DNAzyme catalyze H2O2 reduction of thioine	0.6fM	5.5	2h	2.8	(Bao et al., 2019)
UDG	Fluorescence	Forster resonance energy transfer (FRET)	0.00011 U.mL^{-1}	100	65min	N/A	(Jing Wang et al., 2017)
PNK	Fluorescence	Forster resonance energy transfer (FRET)	9.59x10-4 U.mL^{-1}	16	40min	N/A	(Shang et al., 2020)
DNA Methyltransferase	Fluorescence	Forster resonance energy transfer (FRET)	0.015 U.mL^{-1}	15	60min	N/A	(Q. Wang et al., 2017)
Cancer cell	Fluorescence	SYBR Green I	4.4x10^{3} cells/mL	5	60min	6.1	(Zhao et al., 2017)
Cancer cell	Electrochemical	TMB	4 MCF-7 cells	11	15min	N/A	(Zhou et al., 2014)
Ochratoxin A	Fluorescence	Fluorescent perylene probe	0.1pM	10	N/A	2.9	(Wang et al., 2016)
Kanamycin	Photoelectrochemical	CuS NPs is deposited on the photoactive matrix rGO-Bi2WO6-Au to reduce photocurrent	0.78pM	14	180min	1.02	(Zeng et al., 2019)
Polychlorinated biphenyl	Electrochemical	methylene blue	0.001 ng mL^{-1}	11	90min	1.1	(Han et al., 2020)
ATP	Electrochemical	streptavidin-alkaline phosphatase (ST-AP) catalyze the substrate α-naphthol (α-NP) to an electroactive product	5.8nM	7	60min	5.6	(Ding et al., 2017b)

Table 1. continued

Lead (II)	Electrochemical	Silver nanoparticle (AgNPs)	0.24pM	3	120min	2.1	(Xie et al., 2018)
Lead (II)	Electrochemical	methylene blue (MB)	8.4pM	35	120min	N/A	(Yang et al., 2019)
Mercury (II)	Colorimetric	AuNPs aggregation	2.8pM	5	12h	N/A	(Shao et al., 2020)

Specificity: The ratio of the target molecule to the blank signal

RSD: Relative standard deviation

3.1 Nucleic acid detection

Sequence-specific DNA and RNA represent ideal candidates for biomarkers in therapy (Abi et al., 2018). Therefore, the sensitive detection of nucleic acid has great potential in disease diagnosis (especially for cancer) and clinical analysis. Here, we introduce nonlinear HCR based nucleic acid detection by using optical or electrochemical methods.

3.1.1 Fluorescence-based detection

Nonlinear HCR based fluorescence method was described by using fluorescence spectra as the amplified responses and can be classified into three types: (1) Fluorophore is labeled on the hairpin structure or substrate flue chains to produce the fluorescence signal during the assembly process. (2) Fluorescent dye such as SYBR Green or fluorescent indicator (e.g. Protoporphyrin IX zinc) is added that further intercalates into the specific binding site of nonlinear HCR products. (3) The FRET signal can be detected, due to the acceptor group and doner group labeled on the substrate flue strands getting into proximity during the assembly process.

Zheng's group proposed a label-free fluorescent biosensor platform by using several oligonucleotide probes to form a sandwich-like hybridization product that aims to capture target miRNA on the solid phase (Xu and Zheng, 2016). The capture stage was constructed by hybridization even between series of oligos (capture probe, CEs, LEs), then the adaptor probe contained initiator sequence that was introduced and combined to the sandwich-like product, triggering the formation of 2-dimensional branched DNA nanostructure on the solid surface. SYBR Green I was added as amplification indicator. In this study, they replaced the tedious modification step of immobilizing target miRNA on the solid surface with a series of oligonucleotides, which greatly reduced the experimental cost. However, the non-specific binding of SYBR Green I might enhance background signal and decreased the selectivity and sensitivity. To overcome this problem, Xue's group proposed the high-selectivity ZnPPIX (zinc

(II)-protoporphyrin IX)/G-quadruplex fluorescence system-mediated D-HCR to achieve low background detection (Xue et al., 2018). The two ends of the substrate chain were cleverly designed with split G-quadruplex sequences for nonlinear HCR self-assembly. After the introduction of target miRNA, the hairpin-capture probe would open and the exposed fragments acted as an initiator to trigger the D-HCR reaction. What's more, during the assembly process, the divided G-quadruplex fragments gradually approached each other to obtain a macromolecule dendrimer that contained multiple G-quadruplex structures. In the end, the ZnPPIX was selectively inserted into the multiple G-quadruplex to generate exponential growth of fluorescent signals.

In addition to fluorescent indicators, Kong's group reported a quadrivalent tetrahedral DNA nanostructure (qTDN)-mediated hyperbranched HCR protocol for the detection of miRNA via FRET signal output (Wang et al., 2019). Unlike the traditional HCR reaction, the hairpin H1 (labeled with doner) and H2 (labeled with acceptor) could be connected to the four vertices of qTND, respectively. Therefore, multiple direction self-assembly would occur on each terminal vertex of qTND. The possibility of intermolecular collision was greatly increased because the HCR amplification could be carried out at different directions, finally forming a hyperbranched nanostructure with FRET signal output. On the contrary, Wang's group demonstrated extracellular vesicles-associated miRNAs discrimination assay by destroying the FRET system, apart the acceptor and doner away, via the cleavage of Mg^{2+}-dependant DNAzyme in the presence of Mg^{2+} ions (Wu et al., 2019).

3.1.2 Electrochemical-based detection

Electrochemical biosensor has implemented into various biomarkers detections due to the advantage of flexible instrumentation step, high sensitivity, low cost, and less consumption of reagent. Nonlinear HCR based electrochemical strategy plays an increasingly crucial step in the establishment of electrochemical biosensor with outstanding signal amplification capability,

which can be divided into three varieties: (1) Based on the employment of enzymes (e.g. HRP, alkaline phosphatase) immobilized on products to catalyze substrates and produce electroactive substances. (2) Immobilized electroactive indicator on the electrode surface. (3) Immobilized-free strategies, which introduce peptide nucleic acid probe with its natural neutral backbone.

Wang's group developed a DNA detection biosensor based on the measurement of the electrocatalytic current of 3, 3′ , 5, 5′ -tetramethylbenzidine (TMB), which was generated by avidin-HRP in the presence of H_2O_2 (Jia et al., 2016). In this method, the capture probe (CP) was hybridized with auxiliary DNA 1(A1) to form a dsDNA (with two bugle loop structures) on the gold electrode via Au–S bond. In the presence of target DNA (tDNA), it soon combined to the unhybridized domain of CP and opened the first bugle loop. Then the auxiliary DNA 2 (A2), which was completely complementary with A1, spontaneously hybridized together with A1 to open the second loop, and generated the by-product. Up to then, two identical toehold domains were exposed, which were able to recruit two HRP labeled H1 to form two branched hybridization products. Then the H2 spontaneously hybridized with H1 which were readily for the new round reaction. The formation of multi-HRP labeled dendritic nanostructure on the gold electrode made it readily catalyzed by H_2O_2 and the reduction current could be monitored by the catalytic oxidation of TMB. The detection limit of this strategy was as low as 0.4fM and it was also capable to distinguish single-nucleotide mutation between accompanying DNA sequences.

Similarly, three different well-designed dumbbell DNA probes were designed to generate nest-like DNA nanostructures by Wang's group (Huang et al., 2020). In the presence of target DNA, one segment of the target DNA could be hybridized to the capture probe on the electrode surface and the other end combined with the HCR products to form a stable hybridization. According to the avidin-biotin system, the biotinylated nonlinear HCR products reacted with the streptavidin-alkaline phosphatase (ST-ALP), and the ALP could catalyze the irreversible conversion of the substrate α-naphthol (α-NP) to an electroactive product for the quantitative

detection of target DNA. Additionally, another multibranched DNA nanoarchitecture was ingeniously designed by using linear HCR product − DNA concatamer, as the main body and multiple G-quadruplex wires constructed by TdT (terminal deoxynucleotidyl transferase)-promoted polymerization were used as branched arms. The final added hemin then combined to the G-quadruplex with electrochemical redox signal (Xu et al., n.d.).

Another competitive label-free electrochemical method for the detection of miRNA was reported by Chen's group (Zhou et al., 2019) (Fig 3a). The author immobilized the Y-shaped DNA structure (consisting of three oligo DNA Y1, Y2, Y3) as the probe on the electrode, and the miRNA was the target to compete for the probe binding site, thus released Y3 from Y-shape structure, thereby triggering the nonlinear HCR reaction on the electrode surface. To our acknowledge, most of the electrochemical based detection requires a specific probe to be immobilized on the electrode surface, and laborious optimization methods are used to increase sensitivity and accuracy. However, the steric hindrance effect still cannot be avoided, thus results in the reduction of hybridization kinetics and lower sensitivity. To overcome the above limitations, an immobilization-free and kinetically-controlled strategy was developed by Hsing's group for the nucleic acid sensing (Xuan et al., 2015) (Fig. 3b). In the absence of target DNA, the freely diffusible ferrocene-labeled peptide nucleic acid probe (Fc-PNA) in the solution was access to the negative charge indium tin oxide (ITO) electrode surface because of its neutral backbones, and a strong electrochemical signal could be detected (signal-on). When the target DNA was introduced in the solution, Fc-PNA and elaborate designed DNA strands were automatically assembled into dendritic nanostructures through a series of toehold-mediated strand displacement reactions. The DNA/PNA complex was thus formed in the solution. Due to electrostatically repulsion, the DNA/PNA complex was hard to be in close proximity to the ITO electrode, and the signal was weakened (signal-off). This immobilized-free detection limit of this system could be down to 100fM.

3.1.3 Colorimetry-based detection

The colorimetric biosensor has come into notice due to the advantage of easy operation, instrument-free and cost-effective (Song et al., 2011). Recently, a functional nanomaterial with catalytic properties was proposed by Tang's group for signal amplification (Tang et al., 2019). Two hairpin H1 and H2 were the substrate flue chains of linear HCR reaction. Magnetic Fe_3O_4 nanosheet was modified with four identical ssDNA which had fragments that were complementary to H1 and H2. In the presence of target miRNA, it initiated the hybridization chain reaction, and each of the ssDNA on the Fe_3O_4 nanosheet was able to hybridize with H1 and H2, the magnetic nanosheet and dendritic dsDNA structure were thus conjugated to each other, thereby resulting in the formation of 2D DNA/ Fe_3O_4 network. As the aforementioned property, the peroxidase-liked 2D DNA/ Fe_3O_4 network was able to catalyze TMB in the presence of H_2O_2, thus generated a blue product that could be read out by naked eyes and the sensitive UV-vis signal could be detected. In this study, the background signals could be mostly reduced by magnetic separation and the LOD could be as low as 13aM. In another study, Pourghadamyari's group used DNAzyme fragments modified hairpin to construct peroxidase mimicking G-quadraplex DNAzyme structure through bHCR circuit for miRNA-21 detection. In the presence of hemin, the gathered split DNAzyme fragments could catalyze the oxidation of colorless $ATBS^{2-}$ (2, 2-azino-bis, 3-ethylbenzothiazoline-6-sulfonic acid) into green color $ATBS^{-}$, with a wide linear range from 1pM to 1nM (Hosseinzadeh et al., 2020).

3.1.4 Electrochemiluminescence-based detection

Electrochemiluminescence (ECL) biosensor has received a lot of attention thanks to its low background interference and wild linear ranges. In this protocol, an intermediate produced by electrochemistry undergoes a highly exothermic reaction and emits light to be detected (Forster et al., 2009). Li's group developed an electrochemiluminescence detection *via* introducing self-assembly DNA high-weight molecular on the terbium organic gels (TOGs) modified

electrode (Li et al., 2019). TOGs, as a newly emerged electroluminescent material that can generate superior ECL emission under the negative potential scanning. In this study, four hairpins were introduced in the system. One of the hairpins was labeled with biotin, the other three were labeled with dopamine which could quench the ECL signal produced by TOGs. The nonlinear HCR reaction was initiated by the target miRNA, assembling a branched nanostructure on the Streptavidin-AuNPs/TOGs electrode surface *via* biotin-streptavidin interaction, and successfully constructed target-triggered nanostructure biosensor platform in a one-step approach. Therefore, it could be observed that the ECL signal of TOGs was significantly reduced, allowing the detection of microRNA-141 with a 0.18fM LOD. In another ECL-based strategy, Ding's group proposed a target-triggered hairpin switch for self-assembly of dendritic nanostructure, using $Ru(phen)_3^{2+}$ as ECL indicators, and produced an exponential signal for the sensitive detection of BCR/ABL fusion gene (Xu et al., 2017). Similarly, Hun and coworkers constructed the hemin/G-quadruplex DNAzyme structure on the basis of dendritic HCR by redesign the substrate sequence. As a result, the ECL signal could be detected in the presence of H_2O_2 (Hun et al., 2017).

3.1.5 Other detecting signals

Surface plasmon resonance (SPR) is a label-free analytical method capable of real-time detection of low abundance of biomolecules quickly and efficiently (Nguyen et al., 2015). By using nonlinear HCR as a signal amplification method, a large number of macromolecular dendritic DNA nanostructure can be deposit on the sensing ship surface and generates a strong SPR response signal readout. Ding's group proposed a label-free and enzyme-free SPR based biosensor for the detection of DNA and small molecules (Ding et al., 2017a). The gold electrode surface was immobilized with a capture probe *via* Au-S bound that could further recognized the target DNA, activating the autonomous self-assembly of DNA dendrimer. In another SPR study, Xu's group improved the aforementioned method by reducing the assembly sequence of

dendritic nanostructures at the SPR interface and simplifying the analysis steps to detect PML/RARα fusion gene (Guo et al., 2017). Recently, the photoelectrochemical (PEC) analysis, has emerged as a powerful tool in the field of molecular detection and environmental analysis. PEC analysis uses light as the excitation signal and photocurrent as the detection signal, which involves the charge and energy transfer reaction (Zang et al., 2017). Accordingly, Chu's group established a detection technology based on CHA (catalytic hairpin assembly) and hyperbranched HCR amplification strategy in the photoelectrochemical platform (Chu et al., 2019). In the presence of target DNA, it activated the CHA-hyperbranched HCR to form a dendritic DNA nanostructure on the electrode surface. The amplified product would further form hemin/G-quadruplex DNAzymes structure, generated insoluble substance 4-chloro-1-naphthol under the catalysis of H_2O_2 and formed an insulating layer on the electrode surface to suppress photocurrent. Since the photocurrent signal was inversely proportional to target concentration, it could achieve high sensitivity and stability detection of Human Telomerase RNA with 17fM LOD. In another study, photoactive substance ZnO flower-red architectures (ZnO FRs) was added to releases photocurrent, and the current can be inhibit once the formation of DNA dendrimer on the electrode allow a detection limit of fmol (Han et al., 2017).

By using acoustic biosensor, Xu's group proposed a highly dimensional amplification product coupled with interdigitated transducers (IDT) for multiplexed detection of DNA (G. Xu et al., 2019). Moreover, DNA dendrimer was utilized as a functional signal amplifier probe for the establishment of quartz crystal microbalance (QCM) biosensing platforms by Liu's group (Zhao et al., 2017).

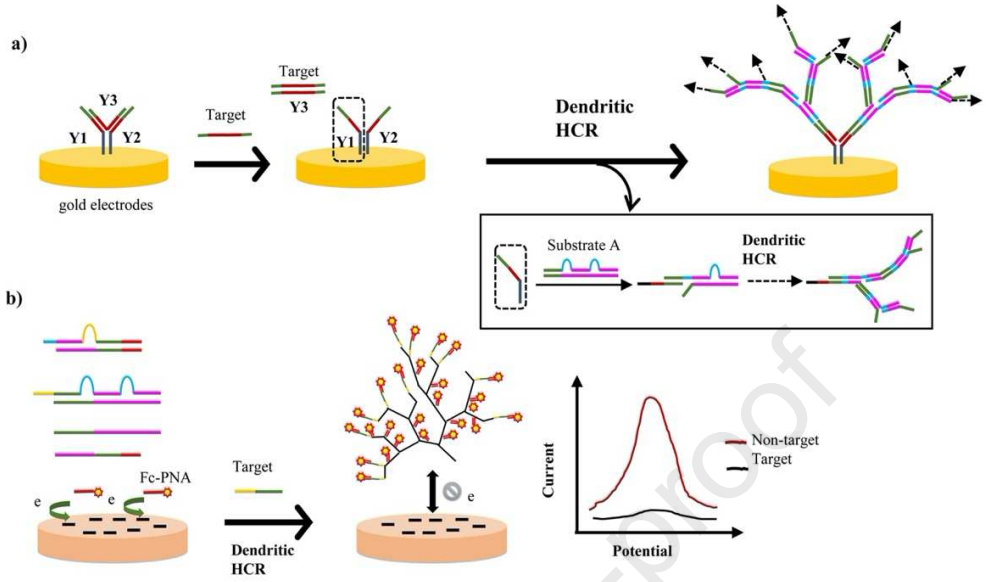

Fig. 3 Nonlinear HCR based nucleic acid sensing. **a)**. Schematic illustration of a Y-shape DNA structure based electrochemical biosensor to detect microRNA. Reproduced with permission from Ref. (Zhou et al., 2019). **b)**. Schematic illustration of immobilization-free dendritic DNA/PNA nanostructure based nucleic acid sensing. Reproduced with permission from Ref. (Xuan et al., 2015).

3.2 Protein detection

Protein, as an important material participates in the execution and regulation of major life activities. The abnormality of protein molecular structure and functional deviations in the human body is an important cause of disease. Therefore, protein detection is very important potential biomarker for the diagnosis of diseases (Jayanthi et al., 2017). However, at present, there are certain difficulties in detecting and identifying of low-abundance proteins. In this case, the biological detection platform can be used to detect proteins with high sensitivity through signal amplification. Nonlinear HCR as a highly selective and highly sensitive amplification method can be used to detect and monitor low-abundance proteins.

3.2.1 Antibody based approaches

Using specific antigen-antibody interaction as a recognition strategy, the nonlinear HCR signal amplification and fluorescent indicator as signal output can achieve the highly sensitive detection of transient receptor potential vanilloid 4 (TRPV4) on cell surface (Li et al., 2018). In this study, an initiator was first labeled on the antibody, after the immune interaction between TRPV4 and its antibody, the initiator subsequently opened the hairpin 1 to expose new toehold that further hybridized with hairpin 2 and opened up the hairpin structure. The hairpin 3, and hairpin 4 was unfolded in the same way and formed a micro-net like nanostructure on the cell surface, the fluorophore labeled on the hairpin would generate strong fluorescence signal which could be easily detected. This method could also perform *in situ* cell detection, monitor protein expression without damaging cells.

3.2.2 Aptamer based approaches

Based on the aptamer-linker DNA, Huang's group had constructed an antibody-antigen-aptamer hetero-sandwich structure-based SERS (Surface Enhanced Raman Scattering) immunoassay for the detection of prostate specific antigen (PSA) (Wang et al., 2020). One ends of the aptamer bound to the antigen, and the other end acted as a trigger, initiating the Con-BHCR to form a macromolecular nanostructure on the SRES platform. The newly formed DNA nanostructure combined with ST-ALP *via* the biotin-avidin system, and the ST-ALP could hydrolyze the 2-phospho-$_L$-ascorbic acid trisodium salt (APP), result in the formation of strong reducing ascorbic acid (AA), which could restore $AgNO_3$ and settle the silver element on the gold electrode surface to form Au@Ag core-shell with excellent SRES signal output. Nie's group proposed immunodetection of carbohydrates by using gold nanoparticles to enhance the signal amplification of HCR (Nie et al., 2018). After the formation of the antibody-antigen-aptamer linker DNA structure, the hairpin labeled AuNPs sooner hybridized with aptamer sequence and then initiated the dendrimer like chain reaction. The phosphate groups of DNA reacted with molybdate to form a redox-active molybdophosphate, which could detect strong electrochemical

current on the electrode surface. Another AuNPs based colorimetric sensing strategy was proposed by Chang's group (Chang et al., 2016). In this study, AuNPs acted as signal indicator, upon the introduction of VEGF (Vascular endothelial growth factor), it unfolded the capture probe and triggered the branch growth assembly of dsDNA, leading to the AuNPs salt-induced aggregation with the red to blue color changes. This sensing platform allowed the limitation lower to the femtomole level. Similar target-responsive nHCR aptamer-based sensor platform was built to detect IFN-γ with fluorescence assay with the detection limit of 1.2pM (Shen et al., 2018). In another study, AuNCs-GR@ZIF-8 (graphene@zeolitic imidazolate framework-8 hybrids anchored gold nanoclusters) electrochemical platform was developed for the detection of IFN-γ based on layered-branched HCR reaction, and the detection limit was as lower as 0.3fM (Bao et al., 2019).

3.3 Enzyme activity detection

The enzyme is an indispensable biocatalyst with catalytic ability for metabolism in organisms. The accurate evaluation of enzyme activities (e.g. Uracil-DNA glycosylase(Jing Wang et al., 2017), Polynucleotide kinas (Shang et al., 2020), and DNA methyltransferase (Q. Wang et al., 2017) et.al) and screen for its inhibitor will be of great significance in clinical treatment and diagnosis. Polynucleotide kinase (PNK) plays an important role in the DNA ligation reaction and its abnormal expression is often related to various diseases. The reported nonlinear HCR based platforms for enzyme activity analysis are mainly based on fluorescence method. When the target enzyme appears, it will release the pre-designed initiator nucleic acid through different approaches, and triggers the con-BHCR event to get a strong FRET signal after amplification. Wang's group established an ultrasensitive PNK biosensor based on the Con-B-HCR and λ exonucleases (λ-Exo) mediated DNA cleavage reaction (Fig. 4b). In the presence of PNK, the 5' hydroxyl end of the hairpin Hp could be converted into a phosphate group under the action of ATP, then the Hp carried with phosphate group was recognized and

cleaved by λ-Exo. The exposed I sequence would initiator the first layer HCR-1 process. In the step of HCR-2, the TAMRA (accepter) and FAM (donor) labeled on hairpins got into proximity, resulting multiple “off” fluorescence signal caused by FRET. In the absence of PNK, the λ-Exo could not recognize and cleave Hp and was unable to initiate the DNA self-assembly event. The Epigenetic process changes caused by DNA methyltransferase (MTase) defection are often associated with the occurrence and development of tumors. The same group had also proposed the MTase (Dam) sensing platform based on Con-BHCR, via introducing a specific designed Y-shape DNA initiator, and had the potential for screening MTase inhibitors (Fig. 4c). On the contrary, in the presence of target MTase, the Con-B-HCR was blocked, and it was attributed to the recognition and methylation of MTase to the Y-shape DNA initiator. Then, under the cleavage of DpnI endonuclease, the Y-shape DNA was converted into split oligonucleotides, which were failure to initiate subsequent amplification event. When there was no target MTase, the Y-shape initiator could easily trigger the Con-B-HCR, with the generation of efficient FRET signal.

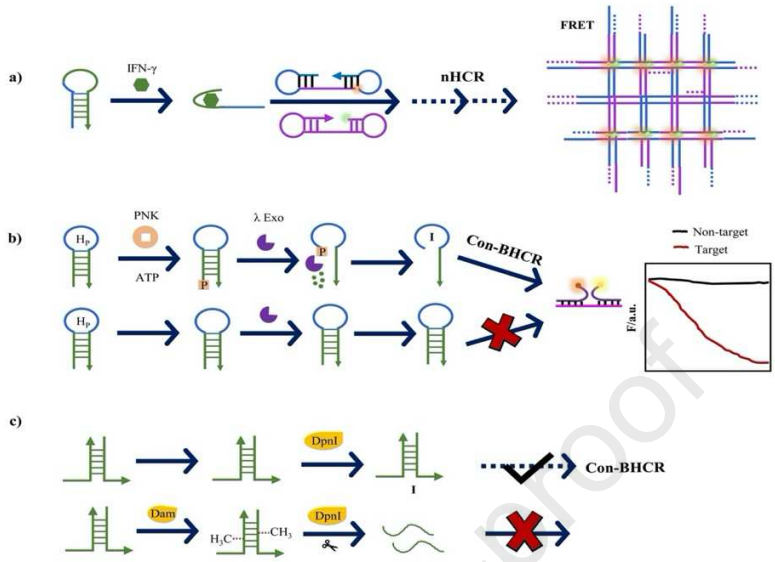

Fig. 4 Nonlinear HCR based protein and enzyme activity sensing. **a)** Schematic illustration of netlike HCR for amplified detection of IFN-γ. Reproduced with permission from Ref. (Shen et al., 2018). **b)** Schematic illustration of Con-BHCR PNK assay. Reproduced with permission from Ref. (Shang et al., 2020). **c)** Schematic illustration of Con-BHCR for the DNA Methyltransferase Activity detection. Reproduced with permission from Ref. (Q. Wang et al., 2017).

3.4 Cancer cell detection

Malignant epithelial cell tumors, also called cancers, are the most serious diseases that endanger human health. Circulating tumor cell (CTC) is a kind of cell that sheds from primary tumors and then is transported into the peripheral blood stream (Pantel and Speicher, 2016). Capturing of living CTC has been investigated as a crucial tool for disease diagnosis and prognosis. There is various detection technology for cancer cell sensing, including electrochemical platform, QCM (quartz crystal microbalance) fluorescence platform and plasmon resonance adsorption signal. An aptamer triggered clamped HCR for in suit capture and the release of CTC *via* DNA hydrogel was developed by Zou's group (Song et al., 2017), and it had the ability to determine low amount of cancer cell in whole blood (Fig. 5a). In this method, the aptamer-initiator probe was particularly bound to the EpCAM on the cell surface, it then

triggered the clamped HCR reaction with the generation of DNA hydrogel. Afterward, the newly formed porous hydrogel could cloak and capture cells for further sensitive clinical diagnosis. Due to the design of ATP stimulus-response sites in the hydrogel, the conformation of the hydrogel could be destroyed and decloak cells without the loss of integrity when ATP was added. Interestingly, the plasmon resonance adsorption of AuNPs was used as a visual indicator for the quantitative detection of circulating tumor cells, and up to five cancer cells could be detected using this method. In another study, Zuo and co-workers proposed a multi-branch HCR (mHCR) method by capturing the cancer cell to the gold electrode surface. Large amount of HRP was applied for signal output, and it would attach to the mHCR product on the cancer cell, thus efficient electrochemical signal could be well detected on the electrode surface (Zhou et al., 2014).

Another label-free cell sensing platform based on QCM (quartz crystal microbalance) technology and self-assembly DNA dendrimer nanocomplex was developed by Liu and coworkers (Zhao et al., 2017). In this study, the DNA dendrimer-SA nanocomplex, composed by the dendritic HCR products (DNA dendrimer) and streptavidin (SA), was regarded as a fluorescent probe by coupling with an aptamer reporter and SYBR Green I. Under the specific interaction between sgc8 aptamer and the PTK7 protein on the HeLa cell surface, the aptamer capture probe, which was immobilized onto the quartz glass slides, subsequently captured the cell on a solid surface. After that, the fluorescent probe was final added with strong fluorescence signal output.

3.5 Metal ion and chemical compound detection

Heavy metal ion contamination of food and crops is closely related to human health. However, the current analytical methodology (e.g. spectrometry) is rather expensive and immature (Saidur et al., 2017). DNA based biosensors are expected to solve the issue of heavy metal detection. Yang's group have demonstrated a conversion strategy by introducing four-way

conjunction nanoprobe and Au@Fe_3O_4 scaffold, transforming the input Pb^{2+} into output oligonucleotides thus initiating the nonlinear HCR process with generation of electron current signal (Yang et al., 2019). The four-way nanoprobe was hybridized with four oligonucleotides (S1, S2, hD, P) and immobilized on the Au@Fe3O4 to construct the conversion system (Fig. 5b). When the Pb^{2+} was input in the system, the nanoprobe collapsed due to the interaction between P (consist of Pb^{2+} aptamer sequence) and Pb^{2+}, resulting in the release of two outputs ssDNA probe S1 and S2. The solution that contained S1 and S2 then dropped into the H1 immobilized electrode and hybridized, respectively. Branched hybridization chain reaction occurred between the output DNA and four hairpins (H2, H3, H4, H5), yielding the reticulated dsDNA nanostructure on the electrode surface which provided multiple embedding site for methylene blue (MB) to generate electrochemical current. In another study, Xu's group took the metal-mediated base-pairs into consideration for the establishment of Hg (II) ions sensing platform (Shao et al., 2020). In the first reaction, the Hg (II) ion triggered exponential amplification reaction (EXPAR) between oligo X'Y' and X that produced massive oligo Y assistant by the DNA polymerase and endonuclease. In the second reaction, the oligo Y released from the first circuit was used to initiate the clamped HCR reaction and generated three-dimensional DNA hydrogel. The porous hydrogel had the ability to accept and trap AuNPs into its network, and the assembly of massive AuNPs-hydrogel structure turned the solution into red color which could be measured and quantified by UV–vis absorption spectra.

Antibiotic residues in water and agricultural products are a new type of environmental pollutant in recent years. Tang's group employed the ternary nanocomposite—rGo-Bi_2WO_6-Au, coupled with bHCR amplification strategy to produce remarkable decreased PEC signal (Zeng et al., 2019). As a new photocatalytic material, rGo-Bi2WO6-Au had superior electron transfer efficiency and photocurrent output signal due to the involvement of graphene oxide (rGo) and gold nanoparticles (Au). In the presence of Kanamycin, many branched products would be formed on the surface of rGo-Bi2WO6-Au due to the bHCR, and the deposition of CuS

nanoparticles (labeled on hairpin probe) would greatly promote the consumption of hole-trapping agent and remarkably reduce the photoelectric response signal. This photoelectrochemical bioanalysis platform showed excellent detection performance with a LOD of 0.78pM. In addition, by using fluorescent perylene probe and branched HCR strategy, ochratoxin A (OTA) could be detected with the limit detection of 0.10pM (Wang et al., 2016). Moreover, the DNA microcapsules based dendritic HCR with electrochemical catalytic reaction was used for the polychlorinated biphenyl (PCB) detection, the specific binding between aptamer and target enabled the dissociation of DNA microcapsules, and thus released the methylene blue molecules to interact with the newly formed dendritic nanostructure (Han et al., 2020).

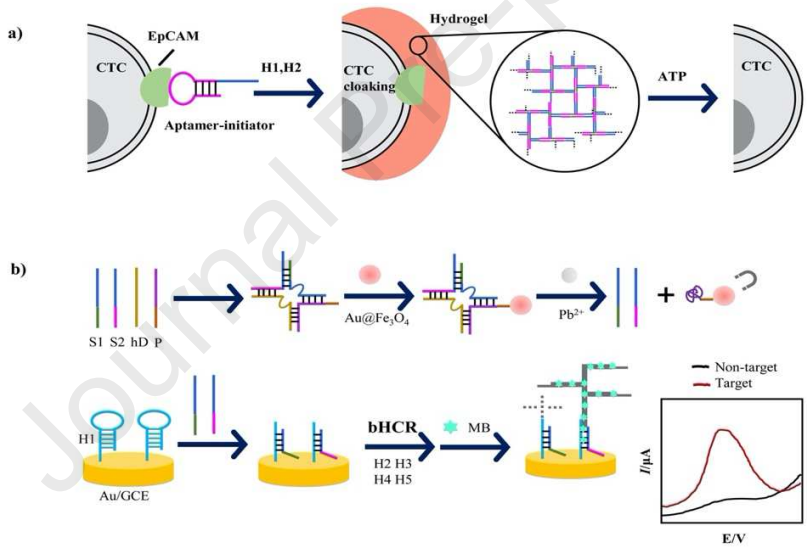

Fig. 5 Nonlinear HCR based CTC cell and metal ion sensing. **a)** Schematic illustration of a aptamer triggered clamped HCR for in suit capture and the release of CTC *via* DNA hydrogel. Reproduced with permission from Ref. (Song et al., 2017). **b)** Schematic illustration of four way branched nanoprbe based bHCR for Pb^{2+} detection. Reproduced with permission from Ref. (Yang et al., 2019).

4. Applications of nonlinear HCR in bioimaging

Nucleic acid, as the genic information carrier, shows great potential in biological applications; and the monitoring of abnormal expression and mutation of RNA in living cell offers an approach for disease diagnosis and clinical analysis. However, the low-abundance RNA and complex intracellular environments create hindrance to the development of biomolecular *in suit* imaging (Visvader, 2011). Although the standard HCR has offered more options for intracellular assay, the poor amplified efficiency, low kinetics, and less biostability of hairpin probes remain to be solved. In recent years, nonlinear HCR based FISH strategy was applied for *in situ* imaging of mRNA in genotyping point mutation in single cell level. In this study, Jiang's group reversely transcribed the target mRNA into cDNA with locked nucleic acid (LNA), and also protected the complementary sequence from degradation by RNase H. Then the DNA ligase was added to produce ligate product that contained bHCR initiator sequence, Under the perfect match of cDNA and oligo probe, the ligate product triggered the nonlinear HCR amplified process, generating bright spot-like signals for individual mRNA molecules (Fig. 6a). The hairpin labeled with different colors was used to amplify and localize the wild-type and mutant mRNA, which was able to discriminate single nucleotide mutation in a single cell (Tang et al., 2017). However, its multiple and tedious washing steps made it hard to apply in living cells. Therefore, the author proposed another wash-free branched HCR based localizable imaging in living cells (Liu et al., 2018). The HCR reaction necessary hairpin was transfected into the cell and propagated brush like nanostructure on every single mRNA. In another study, Wang developed Con-BHCR amplified system that could also be applied in the miRNA (Wei et al., 2018) and polynucleotide kinase (Shang et al., 2020) intracellular imaging by using FRET signal output.

In particular, tetrahedral DNA nanostructure has been developed for drug delivery carrier and its excellent cell membrane penetrability makes it readily fit to the complex intracellular environment, offering new possibilities for biomolecule imaging. Jiang's group constructed the protein scaffolded DNA tetrads for miRNA imaging based on crosslinking HCR (Fig. 6b). In this

process, the high-affinity of streptavidin and four biotin-labeled hairpin H1 or H2 constructed multiple robust protein scaffolded nanotetrads (Huang et al., 2018). More importantly, the nanotetrads could escape from the lysosome, which made the quick and fast entry to the cell without the help of any transfection regent. In the presence of miRNA, it unfolded the hairpin H1 and initiated cross-linking HCR reaction in four directions of nanotetrad, generating hydrogel like 3D nanostructure. Chain-hybridize reaction brought the doner (labeled in H2) and acceptor (labeled in H1) in close proximity and FRET signal could be detected along with the unfolded hairpin. Similarly, a tetrahedral DNA is able to accept four hairpin probes thus lead to the formation of macromolecule nanostructure with outstanding cell membrane penetrability. The hairpins based tetrahedral DNA nanostructure can not only easily enter the cell for *in situ* miRNA-21 imaging, but also can be regarded as drug deliver (e.g. methylene blue) for the photodynamic therapy treatment of cancer cells and has negligible cytotoxicity (Wang et al., 2019).

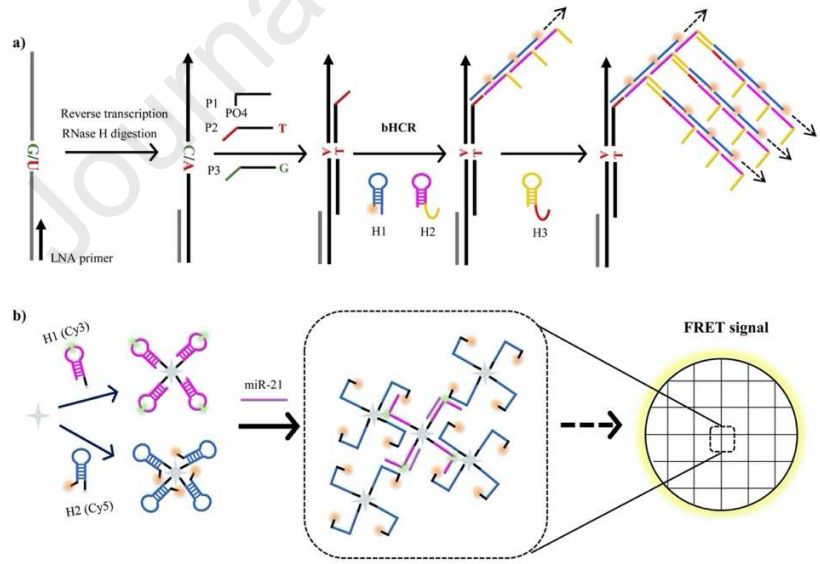

Fig. 6 Nonlinear HCR based bioimaging applications. **a)** Schematic illustration of bHCR based FISH strategy for in situ imaging of mRNA. Reproduced with permission from Ref. (Tang et al., 2017). **b)** Schematic illustration of protein scaffolded DNA tetrads for miRNA imaging. Reproduced with permission from Ref. (Huang et al., 2018).

5. Conclusions

In this review, we have introduced a novel enzyme-free nucleic acid amplification strategy — nonlinear hybridization chain reaction, classifying its amplification mechanism into four categories and holding several representative examples to clarify its biological applications in biosensor and bioimaging. It is not hard to notice that nonlinear HCR is more in line with the requirements of point-of-care testing (POCT) than traditional HCR, for its exponential scale of amplification, rapid test speed, and low detection limit. Nonlinear HCR strategy can integrate with optical techniques (e.g. fluorescent, colorimetric), electrochemical techniques, and other signal readout platforms (e.g. SPR, PEC) for ultrasensitive and specific detection of diseases biomarker. Nevertheless, some limitations of this methodology still need to be addressed. First, the self-assembly of DNA nanostructures largely relies on kinetics and are driven by free energy, sometimes even in the absence of catalyst. The reaction may proceed spontaneously, resulting in high background signals and false positive results. Therefore, it is crucial to enhance the robustness of the reaction system and the purity of the nucleotide. Second, the nonlinear HCR process is irreversible and hard to control. Consequently, it is difficult to monitor the change of reactant concentration over a long period of time. Moreover, the various shapes of amplification products make it rather difficult to achieve absolute quantification. Third, on account of surface charge, size, and biocompatibility, it is formidable to deliver nucleic acids directly into cells. Fourth, like traditional HCR, nonlinear HCR based biosensing technology is still at the laboratory level, because its polydisperse assembly and the uncontrollable size of hybridization product can only provide compromised reproducibility in complex sample matrix. Therefore, it is still a long way to go before volume production and rapid point-of-care applications.

6. Further perspective

In spite of these challenges, we have to admit that nonlinear HCR strategy is an innovation of isothermal amplification methods, which can achieve exponential amplification of targets

molecule under moderate experimental conditions. In the term of biosensor, nonlinear HCR acted as signal amplifier that can incorporate with versatile signal transduction platform for the sensitive detection of nucleic acids, proteins, metal ions and the activity of enzymes. It should be noted that nonlinear HCR had the same amplification potential as PCR and did not require temperature cycling, which was expected to become an emerging single molecule detection method just as ddPCR (digital droplet PCR) by combining with microfluidic technology. On the other hand, nonlinear HCR has provided new opportunities for *in situ* imaging of molecules in a living cell. On the whole, we assumed that, under judicious design, the synergy between nonlinear HCR strategy and signal transduction platform is expected to be a satisfactory tool in clinical diagnosis and point-of-care testing (POCT) in the future.

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Nonlinear hybridization chain reaction is regarded as a powerful signal amplifier for the detection of biomarkers by integrating with versatile sensing platforms.

The basic features of nonlinear HCR mechanism are summarized.

The recent development of nonlinear HCR in biosensing and bioimaging applications have been discussed.

The challenges and further perspectives of nonlinear HCR are highlighted.

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: