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Review

Recent advances in rolling circle amplification-based biosensing strategies-A review

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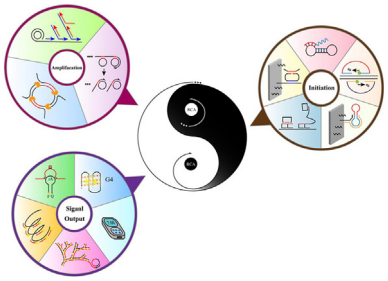
HIGHLIGHTS

- This review introduces the fundamental principles of RCA technology.
- The review summarizes the application of RCA technology in recent years.
- The perspectives of RCA technology are discussed.

GRAPHICAL ABSTRACT



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ABSTRACT

Rolling circle amplification (RCA) is an efficient enzymatic isothermal reaction that using circular probe as a template to generate long tandem single-stranded DNA or RNA products under the initiation of short DNA or RNA primers. As a simplified derivative of natural rolling circle replication which synthesizes copies of circular nucleic acids molecules such as plasmids, RCA amplifies the circular template rapidly without thermal cycling and finds various applications in molecular biology. Compared with other amplification strategies, RCA has many obvious advantages. Firstly, because of the strict complementarity required in ligation of a padlock probe, it endows the RCA reaction with high specificity and can even be utilized to distinguish single base mismatches. Secondly, through the introduction of multiple primers, exponential amplification can be achieved easily and leads to a good sensitivity. Thirdly, RCA products can be customized by manipulating circular templates to generate functional nucleic acids such as aptamer, DNAzymes and restriction enzyme sites. Moreover, the RCA has good biocompatibility and is especially suitable for *in situ* detection. Therefore, RCA has attracted considerable attention as an efficient and potential tool for highly sensitive detection of biomarkers. Herein, we comprehensively introduce the fundamental principles of RCA technology, summarize it from three aspects including initiation mode, amplification mode and signal output mode, and discuss the recent application of RCA-based biosensor in this review.

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1. Introduction

In recent years, people have witnessed the rapid development of medical diagnostics technology with the discovery of protein biomarkers such as specific antigen, surface proteins from cancer cells, and nucleic acid biomarkers such as circulating tumor derived DNA, microRNA [1]. The highly sensitive detection of various biomarkers not only plays an essential role in elucidating molecular mechanisms of life processes and diseases, but also promotes the early diagnosis of diseases and personalized medicine [2]. Therefore, achieving highly sensitive detection is an important goal in the development of novel biosensing methodologies for disease-related biomarkers. Taking polymerase chain reaction (PCR) as an example, this versatile molecular diagnostic method can sensitively detect nucleic acid biomarker with low starting abundance through nucleic acids amplification [3]. However, most types of target molecules (such as protein, small molecules) cannot be directly amplified exponentially, just as nucleic acids are amplified by PCR. Therefore, these types of target molecules need to be identified and bound in advance to trigger the signal amplification. As the alternative method of PCR, isothermal nucleic acids amplification also transforms target binding events into signal booming through copy number amplification and probe recognition [4,5]. And it is carried out at a constant temperature without thermocycling and has wide target tolerance [6]. These isothermal replacements such as rolling circle amplification (RCA), loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA) and nucleic acid sequence-based amplification (NASBA) attract attention and are applied in various fields [7].

Among them, due to its excellent efficiency, simplicity and versatility, RCA technology has become an attractive tool for biological detection and analysis. In the mid-1990s, as a simplified derivative of natural rolling circle replication, which synthesizes copies of circular nucleic acid molecules such as plasmids, RCA technique was established firstly to detect genomic sequences or transcripts [8,9]. A typical RCA reaction system consists of five parts including unique DNA/RNA polymerase (*e.g.*, phi29 DNA polymerase, T7 RNA polymerase), a short DNA or RNA liner single-stranded primer, a circle template (named padlock probe, with 3′-hydroxy and 5′-phosphate), ligase for the specific circularization of the padlock probe, and deoxynucleotide triphosphates (dNTPs)/nucleotide triphosphates (NTPs) [10,11]. The general process of RCA can be roughly divided into ligation reaction and amplification reaction. It begins with the hybridization of the linear primers and the padlock probes. If properly hybridized, the padlock probe can be cyclized to form a closed loop by enzymatic ligation, and then primers will extend under the catalysis of polymerase in 5′-to-3′ direction. Different from other isothermal amplification techniques, this technique has a series of unique features. Firstly, the RCA reaction can obtain long products (RCPs) with a large number of tandem repeat sequences which are complementary to the circular template. Therefore, by elaborately manipulating sequences of template, the RCPs can be customized with functional nucleic acid sequences such as DNA aptamers [12,13], DNAzymes [14–16] and restriction enzyme sites [17,18]. Accordingly, functions such as specific recognition, cleavage, and catalysis can be flexibly integrated into RCPs. Meanwhile, by rational design, RCPs can also self-assemble into organized nanostructures, which is the key to efficient delivery and high cargo capacity [19,20]. Therefore, reasonable sequence design or adding some spacer sequences is very necessary, because long-stranded products may produce steric hindrance when folded into functional moieties. Secondly, because of the good adaptability of RCA, it can be carried out in most solid (cells and tissues) and liquid (*e.g.,* blood, urine) clinical samples, providing a great convenience for target *in situ* analyses [21,22]. Thirdly, the good compatibility of the RCA greatly expands the types of target, which makes it not only be used to detect nucleic acid, but also be used to detect proteins, cells analyses via immuno-RCA or through *in situ* proximity ligation assays [23,24]. It has been successfully applied in the detection of multiple biosensing targets, including DNA [25,26], RNA [27,28], single nucleotide polymorphism (SNP) [29,30], proteins [31,32], pathogens [33,34], cells [35,36] and so on.

In this review, we summarized the research progress of RCA-based sensing strategies in recent years, and roughly divided the reaction into three aspects: the specific recognition mode in the initial stage, the signal amplification pattern in the intermediate stage and the signal output way in the final stage, focusing on the application characteristics in each step.

2. Specific recognition mode

The molecular recognition module is the basis for the selective assay of biosensors. In typical ligation step, the padlock probe is a uniquely designed single-strand oligonucleotide, and its 3′-end and phosphorylated 5′-end each contains complementary parts with the linker sequence. After proper hybridization, the circular template can be synthesized enzymatically or chemically through the intramolecular ligation of phosphate and hydroxyl end groups. This ligation process not only provides a template for the following amplification, but also improves specificity of the biosensing system by this additional hybridization process. In an alternative approach, padlock probe can also be designed as cyclized dumbbell type which is able to connect by its own sequence without linker sequence [37]. Subsequently, primers were adopted to start the amplification reaction under the action of polymerase. In a typical design, the linker sequence can be designed as a universal primer or it can be served by the target [10,11]. According to the different functions of the identified target molecules in the reaction, we summarized the specific recognition mode of RCA reaction into three ways: (1) the identified target molecules are used as the RCA primers, and the prepared circular RCA template is needed. (2) the identified target molecules are used as the linker sequence to connect the circular template, and then additional RCA primers are added to start the amplification reaction. (3) the identified target molecules play both the role of linker sequence to connect the circular template and the role of primer to start the amplification reaction.

2.1. Targets play the role of RCA primers

In this model, nucleic acid targets act as primers directly to initiate the reaction after the addition of a circle template. While in most cases, where non-nucleic acid targets are detected, targets indirectly initiate the RCA reaction by generating the primers after being recognized [38–40]. On the one hand, primers can be modified with multiple recognition elements such as aptamers [41–43], antibodies [44–46], which greatly expanded the target range of RCA reaction including nucleic acid, proteins, cells, enzymes activity and small molecules. For instance, in order to identify the epithelial cell adhesion molecule (EpCAM) positive tumor cells, we attached the RCA primers to EpCAM aptamers to designed bifunctional probes (Fig. 1A). When target tumor cells were specifically bound by aptamer, the primers at the tail of the bifunctional probes will trigger RCA after the addition of the linked circular template. The amplified products contained a large number of G-rich sequences, which can bind with hemin to form DNAzyme with horseradish peroxidase activity, catalyzing the substrate to produce colorimetric signal. Therefore, rare disseminated tumor cells in multiple types of clinical body fluid samples could be directly identified in this way, which benefited from the high efficiency of RCA [35]. On the other hand, for low abundance nucleic acid, it can be first amplified to generate a large number of new fragments, which then participate in the downstream RCA reaction. As shown in Fig. 1B, the target microRNA (miRNA) let-7b was specifically identified by a hairpin probe and triggered the formation of a stable Y-shaped DNA junction structure with the help of assistant probe, which generated numerous trigger sequences based on toehold-mediated strand displacement reaction. Then, the generated trigger sequences could serve as both the linker for template circularization and the primers for RCA polymerization reaction. This pattern greatly improves the specificity to discriminate miRNA from high-homology sequences and can also interact with other amplification methods to produce better sensitivity [47]. Moreover, this pattern is especially suitable for the detection of non-nucleic acid target. In this case, the reaction chains will be released by target-binding and amplified in advance to participate in the RCA reaction subsequently, which can further improve the sensitivity of the method. As shown in Fig. 1C [33], when the well-designed aptamer-probe containing a specific aptamer of *S. aureus* and a probe sequence recognized the target, the probe was released, which would start the nicking enzyme amplification reaction and produce a large number of short oligonucleotides. And then, the short oligonucleotides acted as the primers to trigger RCA reaction, converting the detection of *S. aureus* to the investigation of oligonucleotides.

It should be noted that nonspecific amplification may also occur when RCA is applied to cell-related environments, such as *in situ* imaging or cell lysate detection. Therefore, in addition to reasonable design of circular template sequence, some special ways also can be used to avoid this nonspecific amplification. The most noteworthy one is the proximity-mediated RCA based on the proximity ligation assays (PLAs), which is designed to detect proteins, protein-protein interactions and post-translational modifications [48]. In PLA, a pair of proximity probes consisting of affinity reagents (antibodies or aptamers) equipped with oligonucleotide are required. Upon binding of two such proximity probes to interest target, a connector oligonucleotide will hybridize to both proximity probes and then serve as a template for PCR to obtain powerful signal amplification [49]. Based on these principles, the proximity-mediated RCA technique has been developed by guiding the connector oligonucleotide to form a circular DNA template with the help of ligase. Then DNA circles in turn served as templates to initiate the RCA to generate localized RCA-products which can be visualized by hybridization of fluorescently labeled detection probes, especially for *in situ* detection of receptor protein interaction and nucleic acid in cells and tissues [49–51]. Especially, Söderberg and co-workers have been devoted to the research of the proximity-mediated RCA and developed a series of interesting works to realize the detection of proteins or more complex targets [52–58]. For example, they demonstrated a novel approach to measure the BCR-ABL1 fusion protein in chronic myeloid leukemia by combining the *in situ* PLA (Fig. 2A). By employing a pair of oligonucleotide-conjugated antibodies (PLA probes) to target of the BCR and ABL1 parts of the fusion protein, the oligonucleotides on PLA probes will guide the ligation of connector oligonucleotides into DNA circles. Then, the DNA circles were locally amplified through RCA reaction to create strong fluorescent signals by addition of complementary fluorophore-labeled oligonucleotides. This method used flow cytometry as readout, allowing sensitive detection of cells harboring BCR-ABL1 fusions as low as one in 10,000 [56]. Another proximity-mediated RCA method was developed to profile RNA and protein expression in a single cell [50]. Only when proximity probes synergistically bind to an RNA transcript can they form a nicked circular insert/backbone DNA molecule, which can be closed by ligase and then amplified via RCA using phi29 DNA polymerase. By introducing unique insert sequences similar to nucleotide barcode into RCA, this measurement provides a multiplexing routine to specifically detect each transcript (Fig. 2B).

Furthermore, toehold-initiated RCA (TIRCA) is also a common type of method which enables accurate sequence identification even at single base resolution [59]. In TIRCA, the specific recognition of toehold-mediated strand displacement (TMSD) and effective amplification of RCA are integrated to guarantee specificity and sensitivity. It uses a dumbbell-shaped sealing template with a tunable toehold on its loop, which acts as both the probe of TMSD and the template of the subsequent RCA. Compared with traditional RCA, the background signal it produces is much lower, thereby increasing the signal-to-noise ratio and showing better sensing performance [38]. Many researchers have established related

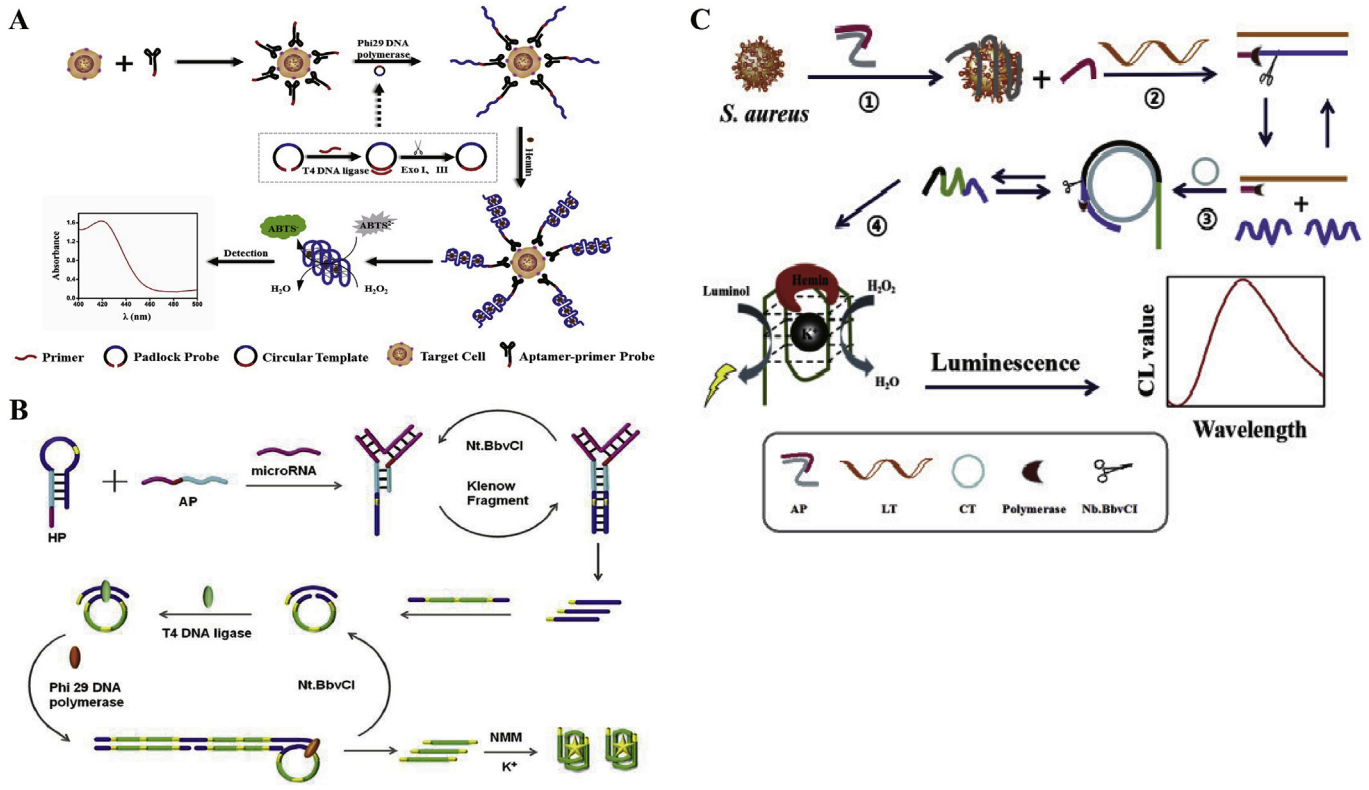

Fig. 1. Schematic representation of targets that play the role of indirect RCA primers. (A) A colorimetric assay of tumor cells by aptamer-induced RCA on cell surface. Reprinted with permission from Ref. [35]. Copyright 2017 Elsevier B.V. (B) A miRNA detection strategy based on the combination of target recycling and RCA process. Reprinted with permission from Ref. [47]. Copyright 2016 Elsevier B.V. (C) RCA-based chemiluminescent aptamer sensor for *S. aureus* detection. Reprinted with permission from Ref. [33]. Copyright 2018 Elsevier Inc.

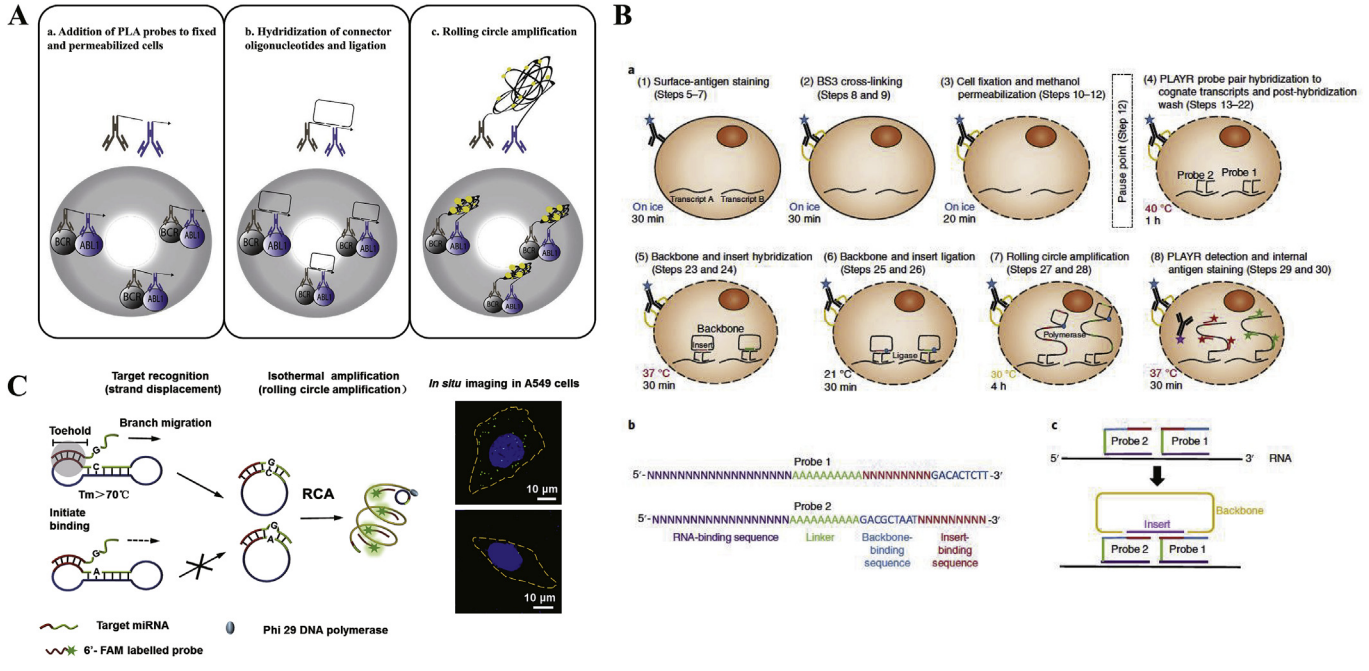

Fig. 2. (A) A novel approach to measure the BCR-ABL1 fusion protein in chronic myeloid leukemia by combining the *in situ* PLA. Reprinted with permission from Ref. [56]. Copyright 2017 Springer Nature. (B) In situ proximity-mediated RCA for staining of intracellular mRNA. Reprinted with permission from Ref. [50]. Copyright 2019 Springer Nature. (C) Toehold-initiated RCA for *in situ* visualization of individual miRNA in a single cell. Reprinted with permission from Ref. [60]. Copyright 2014 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

sensing platforms to detect biomarkers based on the specific recognition properties of TIRCA [37,40,60]. Deng and co-workers have achieved highly specific detection of miRNAs in single cells [60]. As depicted in Fig. 2C, when the target miRNA bound to the

toehold domain of the dumbbell-shaped template, a spontaneous branch migration converted the "closed" template into an "activated" circular form, thus activating RCA, and abundant bright spots appeared inside the cells. By contrast, due to the resistance of the stable dumbbell structure, the miRNA with single base mismatch failed to migrate through, resulting in faint signal.

2.2. Targets play the role of linker sequence for circularization template

In the second strategy which is more widely used, the primers involved in the RCA reaction are added additionally, and the target molecules (usually nucleic acid) only participate in the ligation reaction to bridge the two ends of the padlock probe, hence forming a closed circular template with the action of ligase (typically T4 DNA ligase). This strategy exhibits several unique merits. First of all, by introducing the external oligonucleotides (RNA and DNA) as RCA primers, the target nucleic acid can be released to participate in another ligation reaction of circle templates or to catalyze the interaction of ligation template with padlock probes [61,62], which increases the sensitivity of the method to a certain extent. Secondly, the target-templated cyclization reaction ensures good specificity. When the target does not exist or there is a mismatched target, the template circularization and subsequent amplification reaction cannot be carried out. Thirdly, non-specific signal induced by primers artifacts can be overcome by designing unique primers. Therefore, this ligation-RCA enables high sensitivity at ultra-trace levels and also has the high specificity even with single base resolution. Furthermore, the ligation-RCA is compatible with various types of interfaces including functional particles and cells [63,64], *etc*. For instance, Huang's groups first demonstrated the integration of ligation-RCA with the quantum dots-encoded microbead array (Qbeads) for triplex detection of G-quadruplex (G4) G4-forming sequences (Fig. 3A) [63]. In this system, three kinds of padlock probe were specifically designed with complementary sequence to target at two ends, and the corresponding three primers were conjugated to the Qbeads. When the corresponding target exists, the target would trigger the circularization of the padlock probe by T4 DNA ligase. Subsequently, the primers connected to the Qbeads promoted the RCA reaction, resulting in a large number of products containing G4-forming sequence on the beads. With the help of G4/Zinc phthalocyanine (ZnPc) fluorescent label, this Qbead-RCA assay was capable of detecting three G4-forming sequences by flow cytometry in a high-throughput manner and also successfully demonstrated to analyze the clinical sample. Moreover, RCA is an attractive assay to detect targets that strictly prohibit thermal amplification, such as in vivo detection of bio-analytes and protein analysis. For intracellular mRNA detection, Li's group introduced an extra short DNA sequence to act as the primers to perform *in situ* RCA for direct detection of mRNA and used Splint R, a new type of ligase, to ligate the padlock probe by target mRNA linker efficiently (Fig. 3B). Taking advantages of the high specificity of the ligation-based method and the high efficiency of RCA *in situ* amplification, mRNA in single cells can be visualized with near-single-molecule resolution [64].

RCA reaction provides a powerful tool to analyze single nucleotide variants in target nucleic acid based on the specificity of the padlock probes ligation reaction. Particularly, to further improve the specificity of the ligation, there is an alternative version of the padlock probes where two arms of the probes hybridize to the target with a certain number of nucleotide gaps between 5′ and 3' terminal, named gap padlock probes [30,65]. Gap, ranging from one nucleotide to the entire length of an exon, can be filled by enzymatic polymerization and ligation between the two target-complementary arms to circularize the padlock gap probes. Theoretically, the strict requirement of polymerization and ligation events can improve the specificity compared to the single ligation event required in classical ligation-based assays. Recently, our group developed a low abundance B-type Raf kinase (BRAF) V600E mutation detection strategy by using a pair of single-base gap padlock probes, which hybridized with the template at the region of BRAF V600E mutation site (Fig. 3C) [30]. When BRAF V600E mutation existed, a pair of gap padlock probes was perfectly matched to target mutation gene and the gaps between 5′ and 3′ terminals of padlock probes would be filled with the help of Taq DNA polymerase. As a result, circularized padlock probes ligated by the Taq DNA ligase acted as the template for subsequent hyperbranched rolling cycle amplification after adding the two excess primers with Bst polymerase. In this work, the design of dual gap padlock probes showed superior specificity owing to its ligase and polymerase-based dual discrimination of mutation. By utilizing the clever design of dual gap padlock probes and the excellent amplification efficiency of hyperbranched rolling circle amplification, this strategy showed ultra-high sensitivity and specificity for mutation discrimination, where as low as 0.01% genomic DNA with BRAF V600E mutation in 40 ng wild-type genomic DNA (~1 copy) can be detected.

Rolling circle transcription (RCT) is a modified strategy developed by the RCA reaction, which is suitable for the analysis of RNA. It uses T7 promoter as the primer to trigger circular template amplification under the catalysis of T7 RNA polymerase. It has been successfully applied to generate multimeric transcripts and short interfering RNA [20,66,67]. RCT route is not affected by short length of RNA, and this prominent merit makes RCT an attractive substitute to directly analyze miRNA *in situ*. For example, target miRNA let-7a specifically ligated 5′ end and 3′ termini of padlock probes by T4 DNA ligase (Fig. 3D), and then introduced the T7 promoter to initiate an isothermal RCT at its 3' end under the help of T7 RNA polymerase [68]. When transcribing to the target site, the strand displacement function of T7 RNA polymerase would release the target sequence. And then, the released target let-7a can hybridize to a new template to initiate work repeatedly, resulting in a product composed of many repeated copies of the circle.

2.3. Targets play the role of both the linker sequence and RCA primer

Compared with the aforementioned ways, the last one might provide relatively easier experimental operation and lower background signal. The target material (usually nucleic acid type) not only participates in the circularization of padlock probes, but also acts as primers to directly initiate the subsequent extension reaction [22,69–72]. Hence, this strategy benefits from both the specificity of ligation reaction and the sensitivity of extension reaction. For example, Hildebrandt et al. reported a one-step and sensitive assay for quantifying miRNA by combining the RCA reaction and Förster resonance energy transfer (FRET) [70]. As shown in Fig. 4A, the miRNA targets guided the efficient ligation of the DNA padlock probe with the help of SplintR ligase and then acted as a primer for RCA reaction to synthesize a long single-stranded DNA (ssDNA) product. This assay has successfully achieved the detection of very low amounts of miRNAs even with single nucleotide variation by hybridizing the RCT with FRET pair probes. Moreover, they extended the same strategy for multiple detection of four different DNA targets by placing FRET pairs including single-color excitation and two-color dyes on amplified RCT (Fig. 4B). This RCA-FRET was the first method that combing spectral and temporal multiplexing into highly sensitive detection of DNA with the limitation to 40 zmol [72].

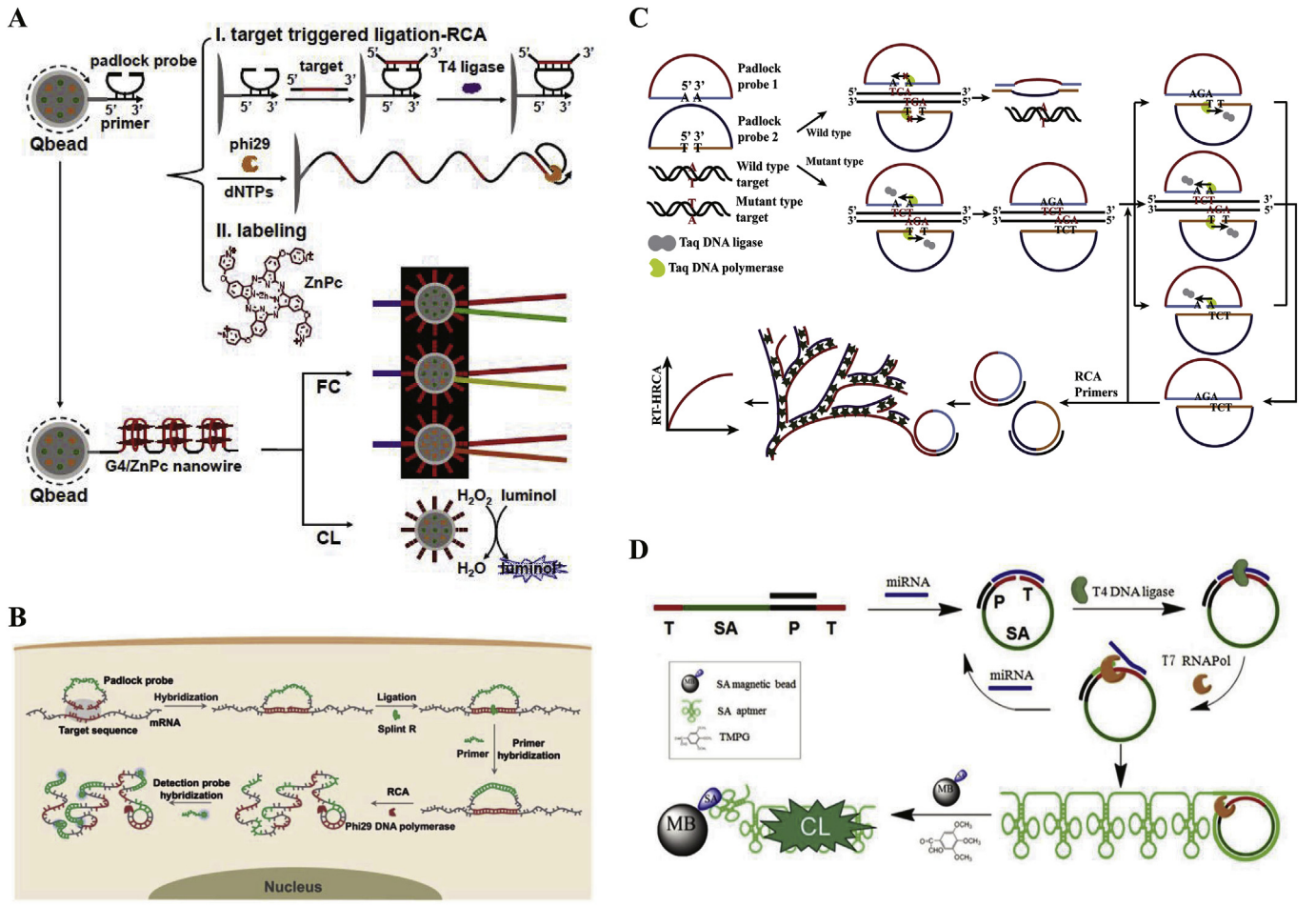

Fig. 3. Schematic representation of targets that play the role of linker sequence for circularization template. (A) A proposed Qbead-RCA assay for triplex detection of G4-forming sequences. Reprinted with permission from Ref. [63]. Copyright 2018 American Chemical Society. (B) A target-initiated RCA for the direct detection of intracellular mRNA. Reprinted with permission from Ref. [64]. Copyright 2017 Royal Society of Chemistry. (C) A biosensing strategy based on dual gap-padlock-based RCA for the detection of point mutation. Reprinted with permission from Ref. [30]. Copyright 2019 Elsevier B.V. (D) An RCT-based label-free chemiluminescence method for miRNA detection. Reprinted with permission from Ref. [68]. Copyright 2016 American Chemical Society.

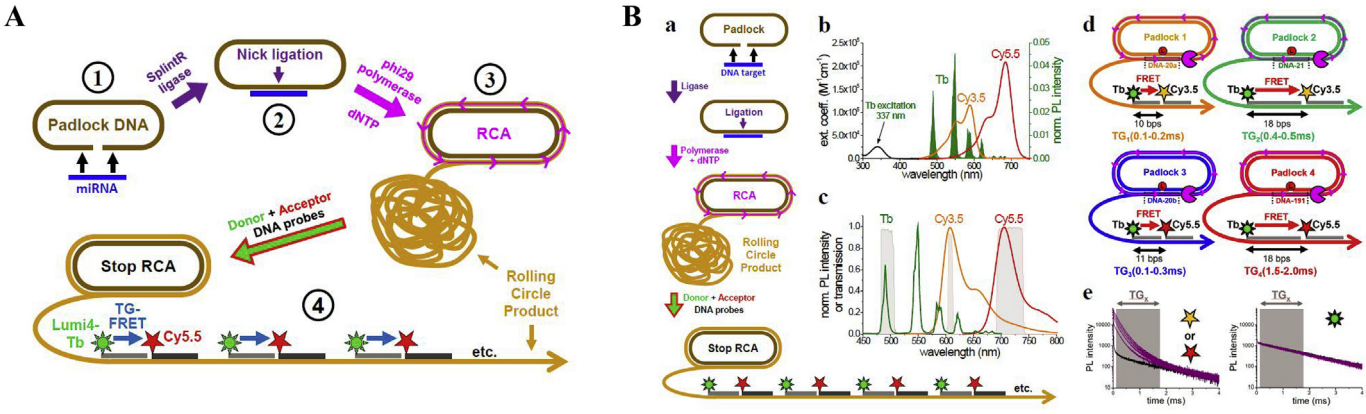

Fig. 4. Schematic representation of targets that play the role of both linker sequence and RCA primer. (A) Principle of miRNA detection by amplified TG-FRET. Reprinted with permission from Ref. [70]. Copyright 2018 The Royal Society of Chemistry. (B) The principle of multiplexed TG-RCA-FRET. Reprinted with permission from Ref. [72]. Copyright 2018 American Chemical Society.

3. Signal amplification mode

New methods and technologies which introduce effective signal amplification are of great significance to improve the performance of biosensors and achieve ultra-sensitive detection of various biomolecules. In this section, we discussed RCA-based linear amplification methods and several versions of RCA-based exponential amplification schemes.

3.1. RCA-based linear amplification

In linear RCA (LRCA) format, only one primer is involved in the reaction and catalyzed by phi29 DNA polymerase to produce a long ssDNA, which minimizes the interference of nonspecific amplification. If RNA polymerase is used instead of DNA polymerase [68], LRCA reaction can transcript a long RNA chain with repetitive sequence. Detection of non-nucleic acid substances is mainly based on the special recognition of the double-antibody sandwich or aptamer-ligand complex [73,74]. Some fast, convenient and high-throughput approaches have been proposed to detect ions [75,76], monitor the glycosylation status on cells [77], and explore the interactions between proteins [45,46,78].

3.2. RCA-based exponential amplification

Exponential RCA is an improvement over LRCA and is undertaken by multiple primers or templates, resulting in multiple RCA products, which mainly includes HRCA and primers generation rolling circle amplification (PG-RCA). By this way, the signal from a single recognition event can be amplified in an exponential manner.

As to HRCA, its principle is similar to that of LRCA, except that a second new primer (completely consistent with the circle template sequence) is added to the amplification system [79]. As depicted in Fig. 5A, the first primer triggered linear RCA and generated a long single-stranded-product in a few minutes; then, multiple second primers bound to the amplified products and enzymatically extended to replace the downstream extension chain; further, the substituted extension products acted as the template of the first primers for another round of amplification [80]. After continuous linear extension, strand displacement and ramified extension, numerous double-stranded products of different lengths were obtained exponentially in a short time [81,82]. In comparison with the linear amplification of RCA (10^5-fold), HRCA has much higher amplification efficiency (10^9-fold) [79]. If LRCA products are vividly regarded as a trunk, then HRCA is more like a tree structure with many branches on the trunk [83]. It integrated primer extension and strand displacement into one tube to achieve cubic amplification. Specifically, the result of the HRCA reaction is a rapid accumulation of double-strand (ds) DNA, which can be easily detected by intercalating molecules with extremely high affinity, such as SYBR Green I (act as fluorescent indicators) [84,85], $Ru(phen)_3^{2+}$ (act as electrochemiluminescence indicators) [86,87], Methylene blue (act as electrochemical indicators) [88]. To date, this HRCA technique shows great potential to serve as an ultrasensitive approach and has been successfully applied to detect diverse targets.

PG-RCA, also known as nicking enhanced RCA (Nick-RCA), could achieve quadratic amplification without exogenous primers, thereby avoiding primers dimerization or non-specific amplification [89]. In PG−RCA, the circular template contains a hybridization sequence to the target nucleic acid and a recognition sequence of the nicking enzyme. When the circular templates first generated long concatenated repeat sequence with numerous nicking sites, the subsequent LRCA products can hybridize to multiple circular templates and then be recognized and cleaved by thermostable strand-specific nicking enzymes [89]. During the reaction, LRCA reaction and nicking reactions occur successively. Thus, the signal can be amplified in an exponential mode simply by mixing circular ssDNA templates, DNA polymerase and nicking enzyme (Fig. 5B). Under the optimum reaction conditions, PG-RCA has the comparable sensitivity to PCR for the detection of low copy number nucleic acid sequence and enables different applications in a variety of molecular diagnostic analysis [90−92]. For example, using the telomerase-triggered strand displacement amplification (SDA)-mediated PG-RCA, Zhang et al. developed a simple “mix-and-detection” method for the sensitive detection of telomerase from

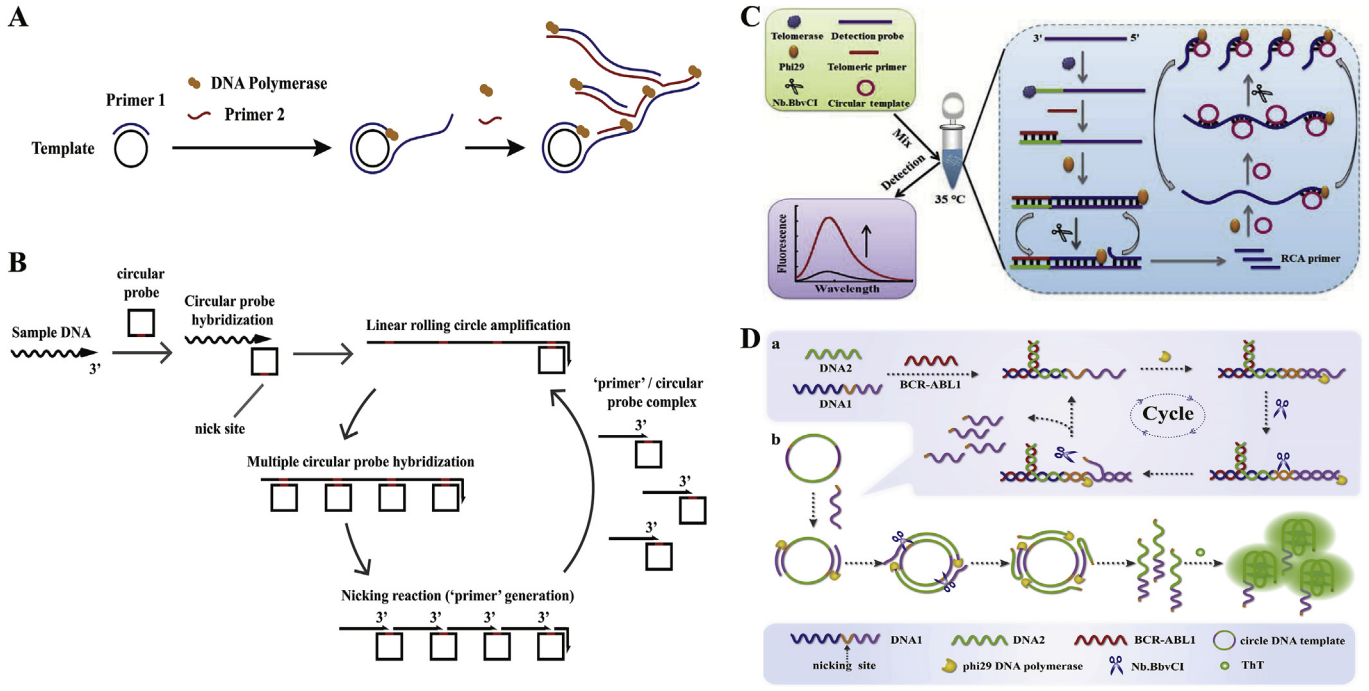

Fig. 5. Schematic representation of the principle of RCA-based exponential amplification. (A) The principle of HRCA. (B) The principle of PG-RCA. Reprinted with permission from Ref. [89]. Copyright 2008 Oxford University Press. (C) The principle of an exponential strategy based on LRCA and PG-RCA. Reprinted with permission from Ref. [93]. Copyright 2018 Royal Society of Chemistry. (D) The principle of a novel fluorescence strategy based on Nick-RCA. Reprinted with permission from Ref. [94]. Copyright 2018 Royal Society of Chemistry.

cancer cells. The amplification RCPs were cleaved by thermostable strand-specific nicking enzymes and generated numerous nicked fragments, which can initiate next round of PG-RCA reactions (Fig. 5C) [93]. In addition, another expanded variation of Nick-RCA is constructed by introducing more nicking endonuclease recognition sites into a circular DNA template, which is engineered in multiple amplicon monomers. Moreover, based on this polymerase/nicking endonuclease DNA machine and multiple primer-like RCA, we designed a one-step, rapid fluorescence biosensing method for ultrasensitive detection of the BCR-ABL1 fusion gene (Fig. 5D). In the strategy, the BCR-ABL1 fusion gene can be specifically identified by using a dual probe to form a three-way junction structure and then a large number of triggers were generated by SDA reaction, which initiated downstream nicking-mediated RCA reaction [94]. The introduction of two nicking endonuclease recognition sites into a circular DNA template makes RCA occur in a multiple primer-like manner, the developed method generates a wide linear response from 10 fM to 1 nM with a low detection limit of 5.52 fM.

3.3. Cascade amplification combined with RCA

Occasionally, using a single amplification technology alone is difficult to detect the target with extremely low abundance. To overcome these limitations, great efforts are made by integrating two or more signal amplification means into one assay. This kind of method is called cascade amplification. In such design, the product of the upstream method acts as "bridge" to trigger the downstream reaction, achieving a cumulative sensitivity by connecting the multiple amplification techniques. RCA technology is often given priority in combination with other amplification methods due to its flexibility and simplicity. Recently, the biosensors of RCA-combined cascaded amplification have been widely established to further improve the detection efficiency.

Incorporating RCA and enzyme-assisted amplification into a sensing system is an important way [95–98]. Li et al. rationally combined RCA with efficient LAMP to establish a rapid and ultrasensitive method for the detection of miRNAs [96]. In this RCA-LAMP (Fig. 6A), the target let-7a miRNA acted as the linker sequence of padlock probes to trigger RCA reaction directly, producing long product with tandem repeats. This RCA-products then served as template to generate dumbbell-shaped DNA with functional sequences, which was the essential materials to initiate downstream LAMP reaction. Through the combination of ligation-mediated RCA with LAMP, the end products can be fluorescently monitored in a real-time manner which is in proportion to the initial miRNA concentrate. Compared with conventional RCA-based miRNA assays, the combination of RCA with LAMP significantly improved the amplification efficiency and the detection sensitivity with 10 aM detection limit. Recently, a CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated nucleases) system has attracted much interest for nucleic acid detection owing to its high efficiency. Luo and co-workers demonstrated a highly specific platform for extracellular vesicle (EV)-derived miRNAs quantification, named RCA-assisted CRISPR/Cas9 cleavage (RACE) [98]. As illustrated in Fig. 6B, RCA reaction was triggered by target acting as both linker and primer. After the TaqMan probes hybridized with the ssDNA RCA products to form a long dsDNA assembly, a sgRNA/Cas9 complex would cleave the dsDNA assembly and release fluorescence to the solution. Therefore, by using the multicolor TaqMan probes multiplex detection can also be achieved in this "turn-on" RACE strategy.

The combination of RCA and several enzyme-free reactions also provides an attractive way, in which DNAzyme-assisted and self-

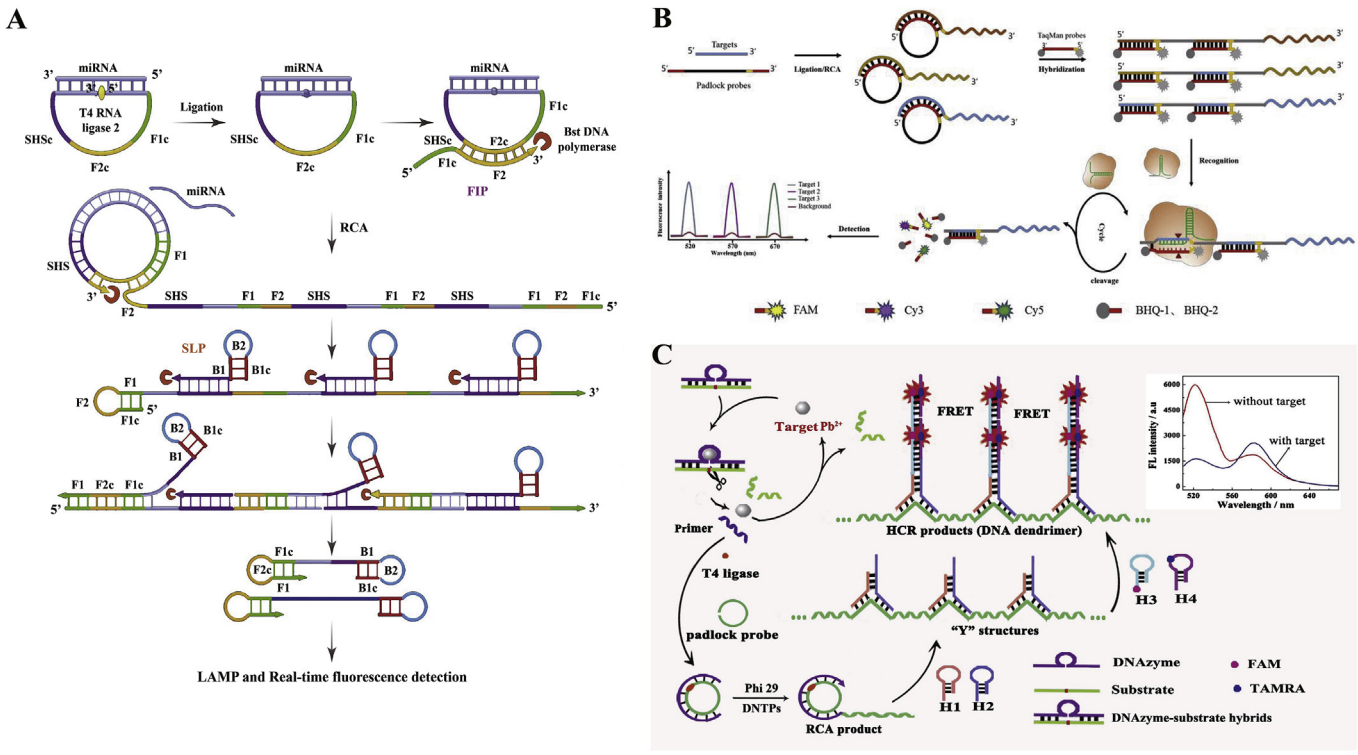

Fig. 6. Schematic representation of the principle of RCA-related cascade signal amplification. (A) A cascade amplification strategy based on the combination of RCA and LAMP for miRNA detection. Reprinted with permission from Ref. [96]. Copyright 2018 Elsevier B.V. (B) A highly specific platform based on RACE for the detection of EV-derived miRNA. Reprinted with permission from Ref. [98]. Copyright 2019 American Chemical Society. (C) A fluorescence strategy based on the combination of RCA, HCR and DNAzyme for the detection of Pb^{2+}. Reprinted with permission from Ref. [99]. Copyright 2019 Springer Nature.

assembly methods such as HCR and CHA are frequently reported [99–101]. As shown in Fig. 6C, Hu and co-workers constructed an ultrasensitive platform for the detection of Pb^{2+} by combining RCA, HCR and DNAzyme [99]. Firstly, the target Pb^{2+} was specifically recognized by DNAzyme, hence triggering the enzymatic cleavage of substrate and the release of Pb^{2+} and DNAzyme probe. Then, the released target Pb^{2+} entered another round of recognition process, while the released DNAzyme probes hybridized with padlock probes to initiate RCA reaction with the assistance of phi29 DNA polymerase and dNTPs. RCA products were generated to open hairpin 1 and hairpin 2 to form considerable "Y" structures which further provided multiple sites for triggering HCR by opening hairpin 3 and hairpin 4 in succession. As a result, a DNA dendrimer was constructed with signal boost upon loading with fluorescence resonance energy transfer. This method obtained a 0.3 pM detection limit, and showed satisfying performance in the detection of intracellular Pb^{2+}.

4. Signal output

The programmability of the RCA process and the customization of products make it flexible to detect target through various signal readout techniques. According to the different mechanism of signal output, we summarize the signal detection methods into three types.

4.1. Functionalized signal probes-based signal output

A long LRCA product with repeated tandem sequence provides enough sites for the binding of complementary signal probes. Thus, the most common way to analyze the RCA products is their hybridization with complementary oligonucleotides, which are tethered to various indicators including fluorophores [78,102,103], nanoparticles [104,105], quantum dots (QDs) [73,75,106] and ferrocenes [107,108] and so on. These functional modified signal probes hybridize with RCA product, resulting in a long DNA duplex with a periodic arrangement of signal indicators.

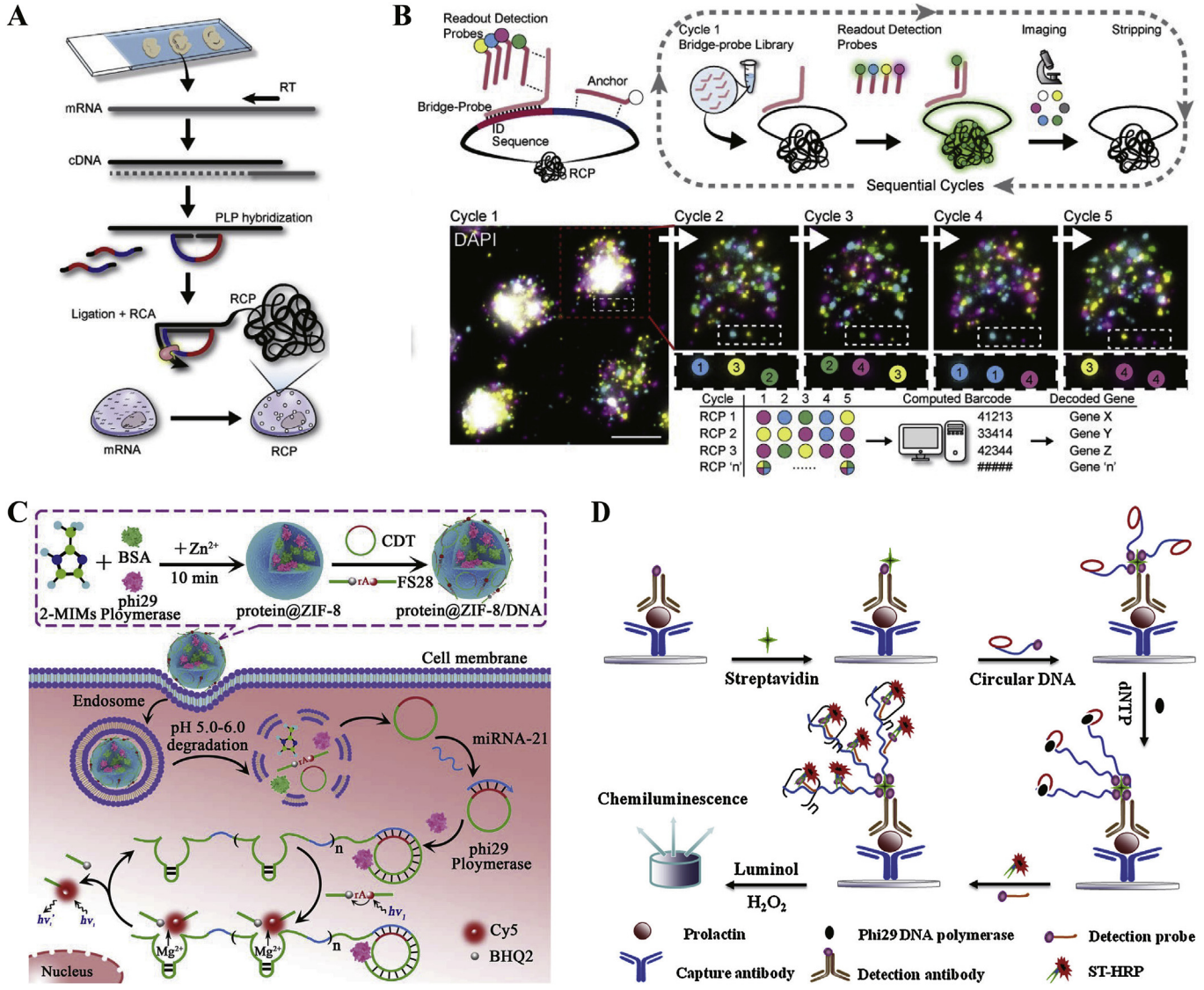

Fig. 7. Schematic representation of functionalized signal probes-based signal output. (A) Overview of ISS. Reprinted with permission from Ref. [115]. Copyright 2020 Oxford University Press. (B) HybISS method overview, hybridization-based *in situ* sequencing. Reprinted with permission from Ref. [115]. Copyright 2020 Oxford University Press. (C) A fluorescence strategy for miRNA imaging in living cells using nucleic acid probes. Reprinted with permission from Ref. [119]. Copyright 2019 American Chemical Society. (D) A chemiluminescence immunoassay strategy for protein detection using biotinylated detection probes and streptavidin-HRP. Reprinted with permission from Ref. [123]. Copyright 2015 Elsevier B.V.

Among them, the fluorescence-dependent signal output is the most widely used. By labeling multicolored fluorescence group, the detection of multiple targets can also be realized [109]. Fluorescence signal can be generated directly by labeling fluorescent groups, or indirectly by opening or shearing the signal molecule labeled with both fluorophores and quenchers group (such as TaqMan probes and molecular beacons) to recover fluorescence [62,110–112]. In particular, fluorescence-dependent signal output provide a powerful tool for RCA-based *in situ* imaging in analyzing RNAs, DNAs and special epigenetic modifications in cells and tissues [113–115]. For example, based on RCA and direct signal output of fluorescent labeled probe, Nilsson's group established *in situ* sequencing (ISS) technology. The first step was reverse transcribing mRNA to cDNA, and then the cDNA would specifically hybridize and ligate with both juxtaposed ends of the padlock probes. This technology had successfully applied to detect RNA isoforms [116], transcriptomic distribution [117], and cell typing across tissue sections [118]. Subsequently, the enzyme-catalyzed RCA reaction was triggered to generate RCPs which can be visualized by fluorescence microscope after hybridizing with fluorophore conjugated readout detection probes (Fig. 7A). Recently, they further improved ISS method and reported a hybridization-based *in situ* sequencing (HybISS) strategy for investigation the spatial mapping of larger gene panels across entire tissue sections (Fig. 7B). Similar to previous established ISS methods, target specific cDNA sequences from reversed transcribed mRNA guided the ligation of the padlock probes which were amplified through RCA and generated RCPs. Every cycle consists of hybridizing bridge-probes to RCPs which can combine with fluorophore conjugated probes to be read out. For sequential cycles, bridge-probes were then stripped off to allow for rehybridizing next round of bridge-probes. Therefore, with different bridge-probe libraries, target transcripts within a cell can be decoded [115]. In addition, the indirect fluorescence signal output mode by turning on the signal molecule (such as Molecular beacon) is also suitable for cell analysis. As depicted in Fig. 7C, with the carrier of metal-organic framework nanoparticles, phi29 DNA polymerase and nucleic acid probes were delivered into the cells [119]. After being taken into cells, the materials of RCA reaction were released from the capsule, and then the RCA reaction was triggered by the target miRNA-21. The resulting products contained a large amount of Mg^{2+}-dependent DNAzymes which cleave the fluorogenic substrates and yield signal recovery.

What's more, introducing biotin-streptavidin system into the RCA reaction can further enhance the sensitivity through the multivalent connection. Usually, the first step is to introduce biotinylated probes into the RCA reaction products, and then streptavidin-labeled catalytic enzyme (*e.g.,* horseradish peroxidase (HRP), alkaline phosphatase) can be anchored there through the high affinity of biotin-streptavidin system, generating strong signals [120–123]. Based on this mode, our group reported a chemiluminescence immunoassay strategy for protein detection (Fig. 7D). After specifically recognition of target protein through sandwich immunoassay, circular DNA was immobilized onto the electrode via biotin-streptavidin system for subsequent RCA, generating thousands of repeated sequences for probe hybridization. Thus, the streptavidin-HRP can be included into the reaction to catalyze the oxidation of luminol by H_2O_2 and yield an enhanced chemiluminescence signal [123].

4.2. DNA intercalating agent-based signal output

First of all, DNA fluorescent intercalating dyes are common signal molecules, including specific double-stranded dyes (*e.g.,* SYBR green I) [69,85,96,124] and single-stranded dyes (*e.g.,* SYBR

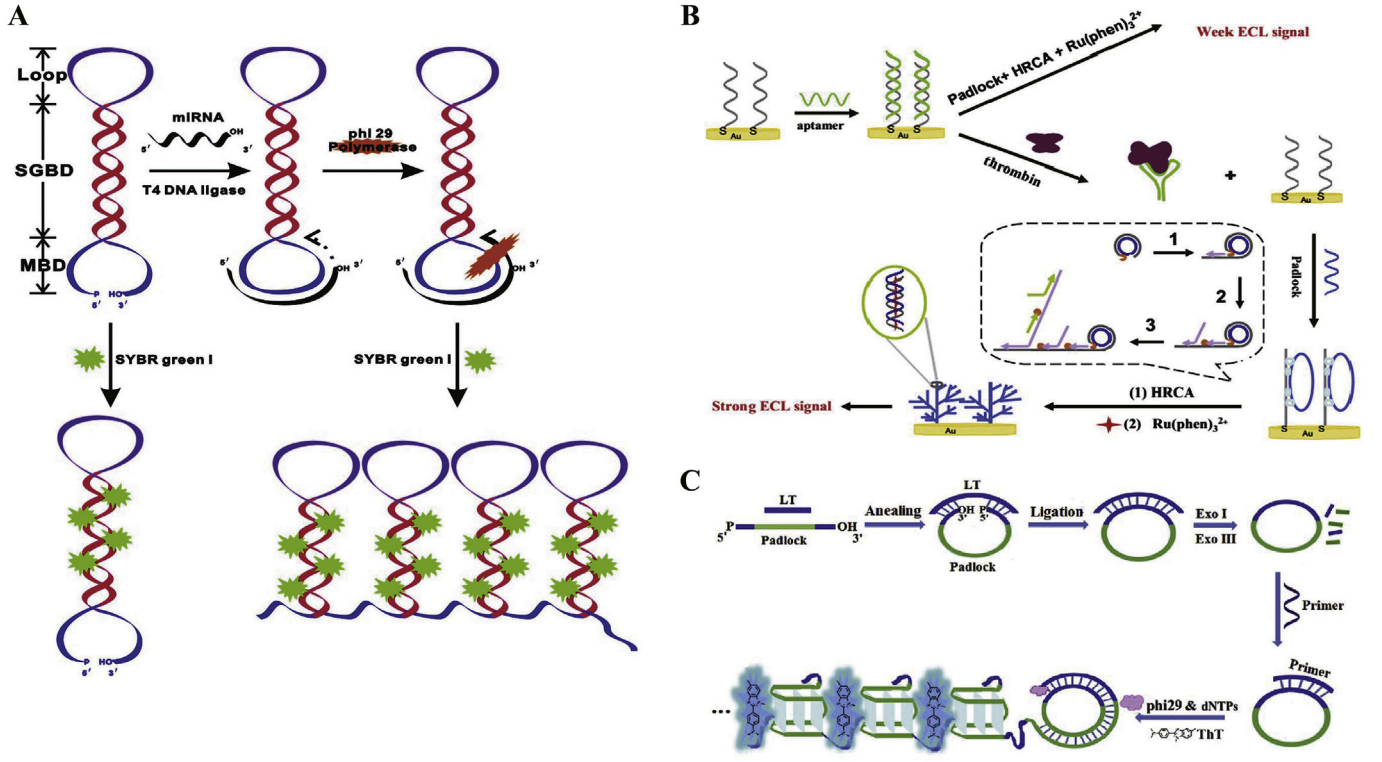

Fig. 8. Schematic representation of signal output mode based on DNA intercalating agent. (A) Quantification of miRNAs by fluorescent signal from SYBR Green I. Reprinted with permission from Ref. [124]. Copyright 2010 Oxford University Press. (B) Detection of thrombin by electrochemiluminescence signal from ECL indicator $Ru(phen)3^{2+}$. Reprinted with permission from Ref. [86]. Copyright 2014 Elsevier B.V. (C) A real-time monitoring strategy utilizing the specific fluorescence response of thioflavin T to G4. Reprinted with permission from Ref. [136]. Copyright 2016 Elsevier B.V. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

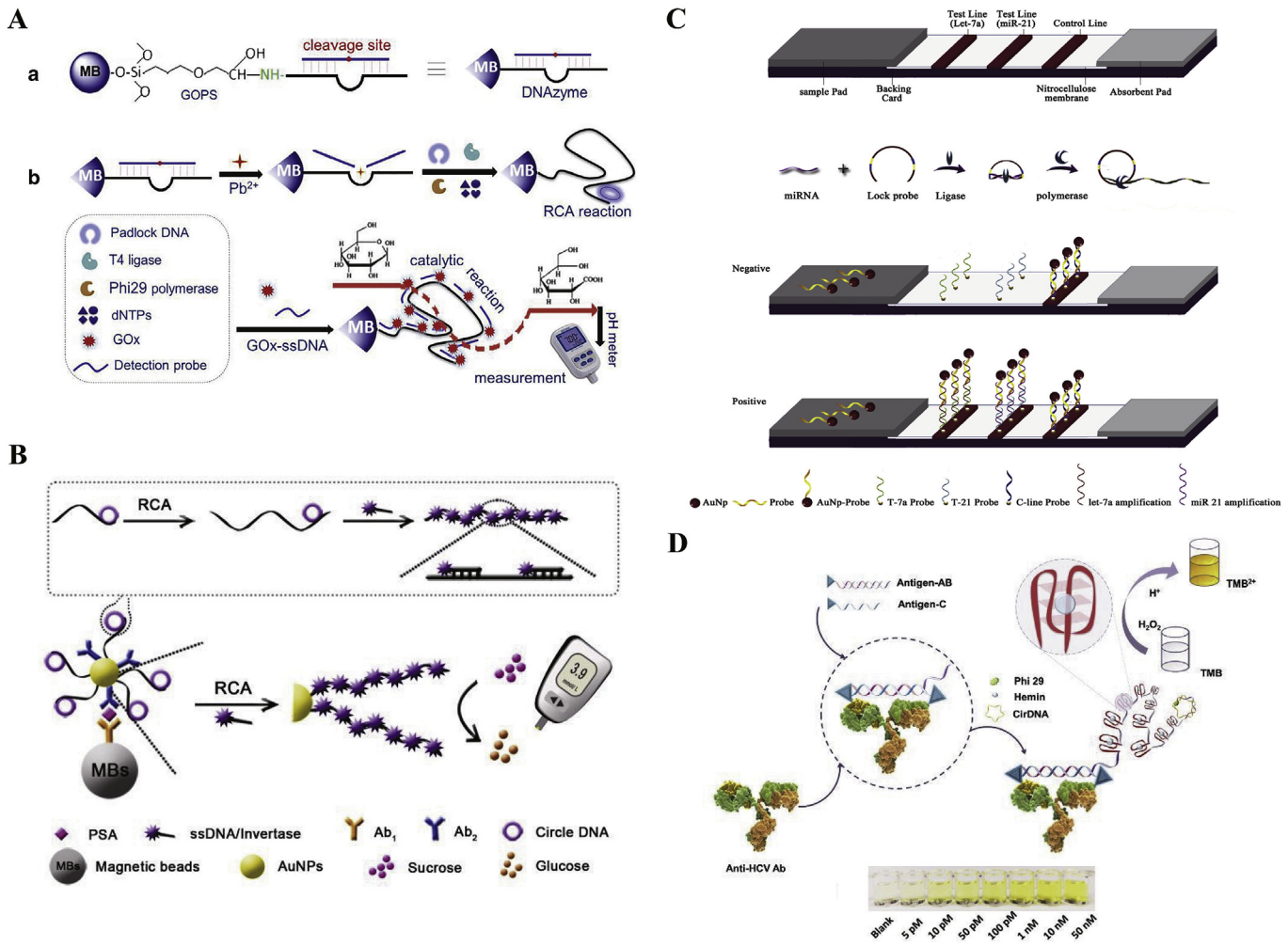

Fig. 9. Schematic representation of POCT-based signal output. (A) A portable pH meterbased magnetic detection method for lead ion (Pb^{2+}). Reprinted with permission from Ref. [138]. Copyright 2019 Springer Nature. (B) A portable glucose meter-based immunosensor for protein detection. Reprinted with permission from Ref. [139]. Copyright 2019 Royal Society of Chemistry. (C) A detecting platform combining RCA with AuNPs-based lateral flow strips for simultaneous detection of two miRNAs. Reprinted with permission from Ref. [143]. Copyright 2018 Elsevier B.V. (D) A new colorimetric assay method for the detection of anti-HCV Ab. Reprinted with permission from Ref. [148]. Copyright 2019 Royal Society of Chemistry.

Green II) [125]. In addition, aggregation-induced emission (AIE) has provided a new idea to real-time quantitative RCPs. The basic principle is that such non-emissive fluorescent dyes aggregates on single-stranded RCA products and then emits fluorescence as an AIE signal [126]. With intercalating dyes in the reaction system, the process of RCA reaction can be monitored dynamically in real time. As illustrated in Fig. 8A, target miRNA helped circularization of the dumbbell-like probe and started RCA. SYBR Green I preferably intercalated into the grooves of repeated dsDNA loops in RCA product, hence leading to strong fluorescent signals. Thus, this dumbbell-like probe-mediated RCA strategy realized the highly sensitive quantification of microRNA via fluorescence from SYBR Green I [124]. Similarly, $(Ru(phen)_3^{2+})$ can specifically intercalate into dsDNA as electrochemiluminescent (ECL) indicator [86,127]. In Fig. 8B, Lin et al. developed an ultrasensitive ECL aptamer sensor for thrombin detection based on HRCA [86]. In the presence of thrombin, ssDNA (CDNA) can be released from the original aptamer/complementary duplex strands due to the formation of aptamer–thrombin bio-affinity complexes. The linear padlock probe would hybridize with the free CDNA on the electrode surface and be circularized to act as a template for HRCA reaction. In the process of HRCA, a large amount of dsDNA was generated, whose grooves can be intercalated by a lot of $Ru(phen)^{2+}$ to generate ECL signal. Taking advantages of powerful signal amplification of HRCA and high sensitivity of ECL, the established aptamer biosensor achieved a thrombin detection limit of 1.2 aM. As another frequently used electroactive dye, methylene blue (MB) [128] can also stably bind to DNA molecules through the electrostatic attraction of negatively charged phosphate backbone and the guanine bases [129]. Owing to these characteristics, it is widely used to detect RCA products as electrochemical redox indicator [130,131].

What's more, some intercalating agents have also structural selectivity, including N-methyl mesoporphyrin IX [47,132], protoporphyrin IX [133,134], thioflavin T and its derivatives [38,92,135–137]. These dyes can specifically combine with G4 structures. Therefore, by introducing G4 structures into RCA products, only when these structure-selective organic dyes specifically bind to the G4 structure, a strong fluorescent signal can be generated (Fig. 8C) [136]. Through the special response between these dyes and G4 structure, a series of label-free molecular sensors with low background noise has been constructed.

4.3. POCT-based signal output

Except to the methods mentioned above, RCA products can also be detected based on point-of-care testing (POCT) methods, such as pH meter [138], glucometer [139–141], test strip [142–144] and microfluidic device [42,145]. For example, when RCTs hybridized with glucose oxidase-labeled ssDNA (GOx-ssDNA), the linked GOx molecules could oxidize glucose, resulting in a drop in local pH, which could also be monitored by using a portable pH meter (Fig. 9A) [138]. While complementary signal probes with multiple DNA-invertase conjugations hybridized with RCTs, it could catalyze the hydrolysis of sucrose to generate abundant glucose, which can be detected by glucometer (Fig. 9B) [139]. Moreover, Rasheed and co-workers developed a platform for simultaneous detection of miRNA-21 and miRNA let-7a by virtue of combining RCA with AuNPs-based lateral flow strip (Fig. 9C). In the presence of RCTs, complexes of AuNPs-RCA products moved along the nitrocellulose membrane under the action of capillary force to bind specific probe on the test line, and gave a positive signal as red color [143].

It is noteworthy that encoding the G4 complementary sequence into the RCA circular template, a long DNA sequence with a large number of tandem repeating G4 sequences are obtained, which can be combined with hemin to form peroxidase-mimicking DNAzyme [146,147]. The DNAzyme, commonly in $ABTS^{2-}$-H_2O_2 system and TMB-H_2O_2 system, can be used to catalyze a series of colorimetric reactions with visible color changes [40,100,148]. As shown in Fig. 9D, numerous G4 sequences fragments were generated during the RCA process and formed stable complexes with hemin to gradually change the TMB solution from colorless to blue, which further amplified measurable colorimetric signals. This method exhibited high sensitivity for detection anti-hepatitis C virus antibody with a detection limit of 0.998 pM and can clearly distinguish different target concentrations by the color change of the reaction solution [148]. Utilizing this RCA-based colorimetric strategy, the change of target concentration can be evaluated by the naked eye, which is suitable for point-of-care diagnosis. Coupling these simple and rapid signal output ways with miniaturized readout equipment, RCA reaction is expected to be an excellent point-of-care diagnostic method.

5. Summary and outlook

To sum up, the strict connection requirements of the circle template and the highly efficient polymerization process give RCA technology unique advantages in specificity and sensitivity compared with other amplification methods. By rationally designing the sequence of the circular template, the products can be endowed with multiple functions moieties such as aptamers and DNAzymes, which greatly expanded the application scope of RCA reaction. For instance, aptamer sequences encoded in the RCA products can be utilized to recognize multiple types of target, including nucleic acids, small molecule, proteins and cells. The DNAzymes with shear or catalytic function formed in RCA products can further amplify the signal. At present, RCA technology has shown great application value and potential in the detection of whole genome, SNP, protein, and single cell. It can also be used to detect pathogens and disease-related genes, avoiding time-consuming and complex operation in traditional clinical testing methods. Given the simplicity, excellent performance and high adaptability, the RCA techniques will become an important strategy in molecular diagnostic, particular in the field of rapid diagnosis and POCT. However, up to now the commercial application of RCA technology has not been fully realized and still has some shortcomings. For instance, purifying circular templates in large quantities and with high quality remains a great challenge. In the enzymatic ligation step, because the ligation efficiency is affected by the length of the circular template, it is difficult to control the ligation efficiency with poorly purified circular template. Additionally, due to the large molecular weight of RCA products, it may affect the recognition ability of aptamers and restriction sites in the RCA products for cell targeting, which will increase the corresponding nonspecific binding. In the future, further exploration will be needed in probe design and laboratory quality control. We believe that with the continuous development of RCA technology, this powerful tool will be more widely utilized in the diagnosis and treatment of diseases and provide technical support for elucidating the mechanism of related diseases, which will help to further deepen the understanding of diseases.

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.

Acknowledgments

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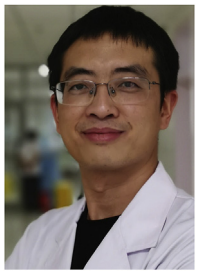

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