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Mini Review

Avenues Toward microRNA Detection *In Vitro*: A Review of Technical Advances and Challenges

Chu-shu Zhu [a,1], Lingyun Zhu [a,*,1], De-an Tan [b,1], Xin-yuan Qiu [a], Chuan-yang Liu [a], Si-si Xie [a], Lv-yun Zhu [a,*]

[a] Department of Biology and Chemistry, College of Liberal Arts and Sciences, National University of Defense Technology, Changsha, Hunan 410073, PR China
[b] Department of Clinical Laboratory, Hospital of National University of Defense Technology, Changsha, Hunan 410073, PR China

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ABSTRACT

Over the decades, the biological role of microRNAs (miRNAs) in the post-transcriptional regulation of gene expression has been discovered in many cancer types, thus initiating the tremendous expectation of their application as biomarkers in the diagnosis, prognosis, and treatment of cancer. Hence, the development of efficient miRNA detection methods *in vitro* is in high demand. Extensive efforts have been made based on the intrinsic properties of miRNAs, such as low expression levels, high sequence homology, and short length, to develop novel *in vitro* miRNA detection methods with high accuracy, low cost, practicality, and multiplexity at point-of-care settings. In this review, we mainly summarized the newly developed *in vitro* miRNA detection methods classified by three key elements, including biological recognition elements, additional micro-/nano-materials and signal transduction/readout elements, their current challenges and further applications are also discussed.

Contents

* Corresponding authors.
E-mail addresses: lingyunzhu@nudt.edu.cn (L. Zhu), zhulvyun@nudt.edu.cn (L. Zhu).
[1] These authors contributed equally to this work.

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1. Introduction

MicroRNAs (miRNAs) are small non-coding RNAs that contain approximately 22 nucleic acids, playing important roles on the post-transcriptional regulation of gene expression, and are involved in the regulation of many important biological events [1]. Circulating miRNAs can serve as a promising cancer biomarker because their expression pattern can be correlated with cancer type, stage, and other clinical variables, thus implying that miRNA profiling can be used as a tool for cancer diagnosis and prognosis [2]. Moreover, circulating miRNAs remain stable under some extreme conditions, such as RNase exposure, multiple freeze-thaw cycles, and extreme pH, thus making them strong candidates for low-cost detection and analysis [3,4]. However, detecting miRNAs is considerably challenging regarding to their intrinsic properties. Their small size (18–25 nt) presents challenges to be amplified, and sequence homology makes detection challenging in terms of selectivity [5]. Conventional methods for detecting miRNAs, including Northern blot [6], microarray [7], real-time polymerase chain reaction [8], and next generation sequencing [9], suffer from limitations, such as low detection sensitivity, costly equipment, time-consuming methods, and false positive signal [10–13]. Hence, the development of more efficient and low-cost detection methods is urgently needed to monitor miRNA levels in early clinical diagnosis.

Over the past decade, many efforts have been made to optimize conventional methods [14], and various novel methods have been developed for sensitive, selective, and high-throughput *in vitro* detection of miRNAs, such as electrochemical methods [15], optical methods [5], isothermal amplification [16], and nanoparticle-derived probes [17]. Several reviews dealing with the evolution of various analytical methods for miRNA determination have been published [15–20]. However, the fast-evolving, wide "miRNA sensing field" needs to be updated, and increasing number of cutting-edge technologies, such as CRISPR (clustered regularly interspaced short palindromic repeats) Cas (CRISPR-associated) system [21], have been applied to this. At the same time, a common feature of these newly developed methodologies is the combination of biological recognition modules with some sensitive signal transduction and readout assays; sometimes, additional micro- and nano-materials are also utilized. Herein, we mainly discussed some of those notable advances in the sensitive, selective and high-throughput *in vitro* detection of miRNAs over the past decade and summarized in groups of (1) biological recognition elements, (2) additional micro- and nano-materials, and (3) signal transduction and readout elements. The current challenges and future aspects in the further application of such approaches are also highlighted and discussed in this paper.

2. Biological Recognition Elements

2.1. Hybridization Only

2.1.1. Single-Stranded DNA/RNA (ssDNA/ssRNA) Probe

ssDNA/ssRNA probes are frequently used in the detectors for miRNA detection as the biological recognition element [22]. They are typically immobilized on the surface of various kinds of biosensors, such as on the surface of molybdenum disulfide (MoS_2) field-effect transistor biosensor [23] or on the electrode surface of electrochemical biosensor [24], to hybridize with the target miRNA to form the rigid DNA–RNA (or RNA-RNA) hetero-duplex structure, resulting in the signal change of corresponding biosensors (Fig. 1a). To further amplify the readout signals, the target miRNAs are bound with two different probes containing ssDNA. For example, ssDNA probes modified on the electrode were designed to hybridize with the miRNA at the 3' end, allowing the exposure of the 5' end of the miRNA to hybridize with another reporter probe [25] (Fig. 1b). Besides, this strategy was also used to selectively turn on the surface-enhanced Raman scattering (SERS) enhancement in the DNA-encoded Raman-active anisotropic nanoparticles modified origami paper analytical devices through a single piece of SERS probes, which comprises two different ssDNA probes to target the whole sequence of the miRNA [26]. Moreover, ssDNA/ssRNA probes labeled by fluorescent dyes have also received extensive attention. Usually, they are immobilized on the surface of quenching moiety as a signal

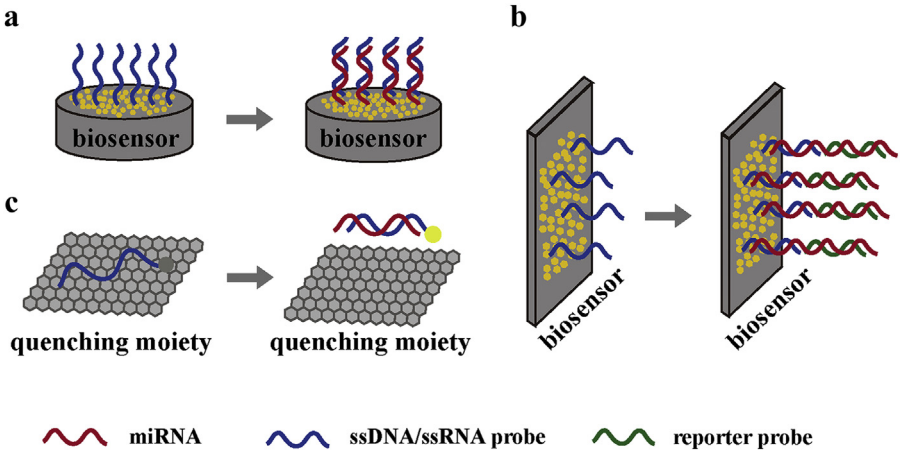

Fig. 1. Schematic illustration of the *in vitro* miRNA detection methods based on ssDNA/ssRNA probes. (a) ssDNA/ssRNA probes immobilized on the surface of biosensors are designed to hybridize with the target miRNA to form the rigid DNA–RNA (or RNA-RNA) hetero-duplex structure. (b) The shorter ssDNA probe and an additional reporter probe are designed to bind with two adjacent segments of the target miRNA, which can help to carry out subsequent signal amplification procedures. (c) ssDNA/ssRNA probes labeled by fluorescent dyes are immobilized on the surface of quenching moiety as signal molecules. Once hybridizing with complementary miRNA, a stable DNA-RNA heteroduplex could be formed and then be released from the surface of the quenching moiety, resulting in the fluorescence regeneration and signal readout.

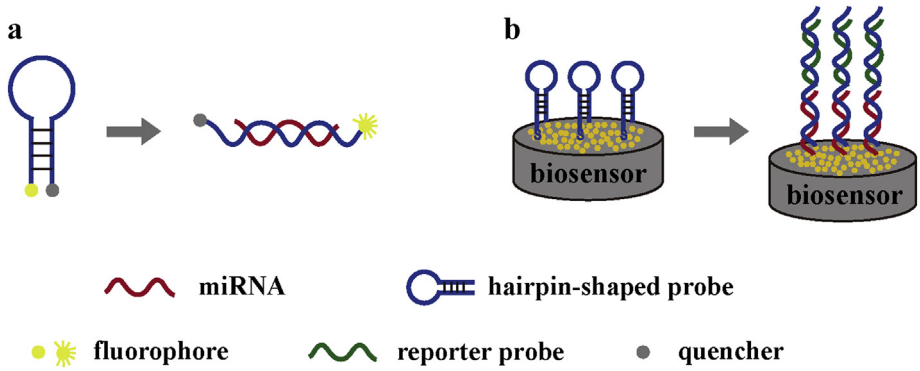

Fig. 2. Schematic illustration of the *in vitro* miRNA detection methods based on hairpin-shaped probes. (a) Mechanism of MB-based miRNA detection method. The fluorescence signal readout from MB can be occurred in the presence of complementary miRNA target. (b) Hairpin-shaped probes are immobilized on the surface of biosensors. Upon hybridizing with the target miRNA, the linearized hairpin structures could allow the binding of another reporter probe, which could result in a sensitive signal readout.

molecule, whose fluorescence would be restored when sensing the target miRNA [27] (Fig. 1c).

2.1.2. Hairpin-Shaped Probe

Molecular beacons (MBs) are widely used hairpin-shaped probes. They are usually labeled with a fluorophore at one end and a quencher at the other end which can be dissociated to linear forms in the presence of target nucleic acid sequence, thus restoring their fluorescence [28,29] (Fig. 2a). For example, simultaneous and multiplexed detection of miRNAs have been achieved by utilizing different fluorophores labeled MBs [30]. However, traditional MBs have the disadvantage of low quenching efficiency, which limits their further application. Thus, many kinds of nanomaterials, such as MoS_2 nanosheets [31], graphene oxide (GO) [32] and gold nanoparticles (Au NPs) [33], have been used as quenchers of MBs to reduce the fluorescence background and enhance the signal-to-background ratio.

In addition, hairpin-shaped probes have been widely used in the field of biosensors due to their high sequence specificity (Fig. 2b). Notably, a thiol group was usually labeled at its 5'-end for assembly on the surface of biosensors. After hybridization with target miRNA, the loop sequence formed a rigid duplex with the target, which broke the relatively shorter stem duplex. Then, another reporter probe would hybridize with the former stem sequence, resulting in sensitive signal readout. For example, strepavidinylated gold nanorods were used for the enhanced surface plasmon resonance (SPR) response [34], and oligonucleotide encapsulated silver nanoclusters (Ag NCs) were used as effective electrochemical label due to their high catalytic capability toward H_2O_2 reduction, resulting in a sensitive and label-free electrochemical miRNA biosensor [35].

2.2. Hybridization Followed by Molecular Amplification

2.2.1. Rolling Circle Amplification (RCA)

RCA describes an isothermal enzymatic process that utilizes a circular DNA template and special DNA or RNA polymerases to synthesize long ssDNA and RNA [36]. It was first applied in miRNA detection in 2006 in which target miRNA serves as template for the ligation of the padlock probe and primer in the RCA reaction [37] (Fig. 3a). However, this assay requires separation of RCA products by gel electrophoresis and radioactive-band measurements, which may cause high labor intensity and inherent safety problems, and the specificity needs to be improved [38]. To address this issue, the Li group utilized T4 RNA ligase 2 to improve the specificity for the ligation of padlock probes, and a second primer complementary to the RCA products was introduced, resulting in a branched RCA (BRCA) reaction, thus the RCA products can be sensitively determined by using SYBR (Synergy Brands) Green I as the fluorescence dye [38] (Fig. 3b). To further improve the specificity of RCA, Deng et al. utilized toehold-mediated strand displacement (TMSD) to initiate the RCA of specific miRNAs by constructing a structure-switchable seal probe, which serves as both the probe for TMSD and template for the subsequent RCA. It remains stable as a dumbbell-shaped structure until the target miRNA binds to the toehold domain, following the spontaneous branch migration, which switches the probe to a "activated" circular form, thereby initiating RCA. The specificity is guaranteed as the dumbbell-shaped status is "closed" for RCA (Fig. 3c) [39]. This assay termed toehold-initiated rolling circle amplification (TIRCA) is a potent strategy that achieves both stringent recognition and in situ amplification of the target miRNA [39].

2.2.2. Exponential Amplification Reaction (EXPAR)

EXPAR is an isothermal amplification reaction devised by Galas and coworkers in 2003 [40], which can rapidly amplify short oligonucleotides

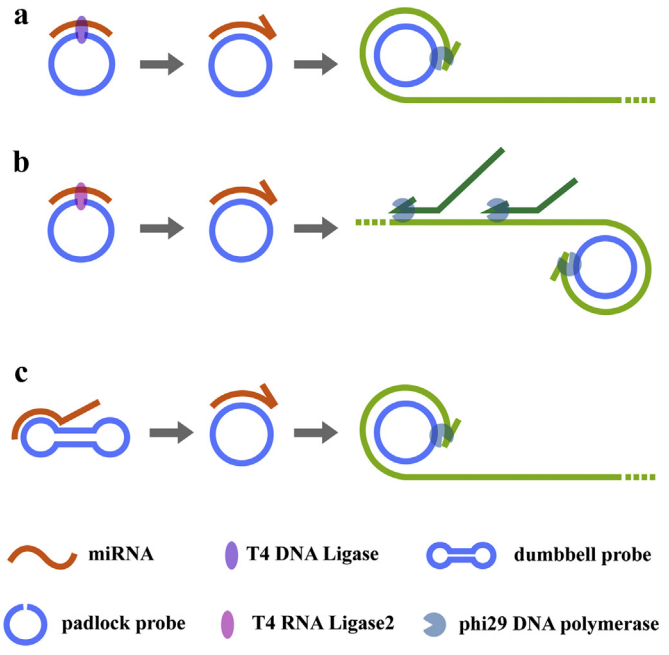

Fig. 3. Schematic illustration of the *in vitro* miRNA detection methods based on RCA. (a) Padlock probe-based RCA. Upon annealing to the miRNA, padlock probes could be circularized by DNA ligase, which subsequently initiate the RCA catalyzed by the phi29 DNA polymerase with the miRNA as primer. (b) Target-primed BRCA reaction. T4 RNA ligase 2 is used to ligate the padlock probe instead of DNA ligase after annealing. After the initiation of RCA similar to the Padlock probe-based reaction, a second primer, which is complementary to every tandem sequence on the RCA product, is used to prime the BRCA reaction as it can be extended to displace downstream growing strands. (c) TIRCA. After binding to the toehold domain of the dumbbell-shaped probe, the target miRNA switches the dumbbell-shaped probe to the "activated" circular form through a TMSD process, thereby initiating RCA.

(10^6–10^9 fold) within minutes by a combination of polymerase strand extension and single-strand nicking [41]. EXPAR method has been widely used for the miRNA detection owing to its rapid amplification kinetics, isothermal condition requirement, and high amplification efficiency and flexibility [40,42]. Jia et al. first applied EXPAR in miRNA detection in which miRNA served as a trigger and was detected by means of the real-time fluorescence detection of EXPAR products [42] (Fig. 4a). However, there is a significant drawback that the product of the EXPAR method contains a short DNA strand with the identical sequence with the miRNA target, which restrains the further improvement of the detection sensitivity through the combination of EXPAR with other amplification methods. To address this issue, the two-stage EXPAR method with two different templates and two-stage amplification procedures was proposed. In this assay, the first template enables the amplification of miRNA and the second one enables the conversion of miRNA to the reporter oligonucleotide [43] (Fig. 4b). To simplify the procedure and reduce the non-specific amplification, a special template was designed with two identical motifs to bind with target miRNAs and a third motif to produce reporter oligonucleotide [44] (Fig. 4c). In this way, exponential amplification and production of reporter oligonucleotide can be achieved in a one-stage EXPAR method. Moreover, with the development of a novel structure-switchable symmetric toehold dumbbell-template, the loss caused by the binding between the trigger and the 5' end of the linear symmetric template, which is inextensible, can be avoided. The detection specificity could also be significantly improved with the reason similar to the TIRCA (Fig. 4d) [45].

2.3. Hybridization Followed by Signal Amplification

2.3.1. Catalyzed Hairpin Assembly (CHA)

As a versatile strand displacement amplification (SDA) isothermal amplification technique, CHA is a typical enzyme-free DNA circuit developed by Pierce et al. [46] in which two hairpin-shaped probes, including hairpin detection probe (HDP) and hairpin assistant probe (HAP), hybridize to form a duplex when the target appears and then yield multiple signal outputs [16,47–49] (Fig. 5a). Recently, CHA has been combined with miscellaneous detection techniques such as chemiluminescence [50,51], colorimetry [52], electrochemistry [53], SERS [54,55], fluorescence [56,57] and SPR [58] to develop efficient miRNA detection platforms due to its high sensitivity and specificity. However, the background leakage in CHA circuit caused by the non-specific hybridization of two hairpin-shaped probes in the absence of target miRNA impedes the practical applications of CHA in trace miRNA analysis [59–61]. Accordingly, a novel heterogeneous CHA amplification strategy integrated with polydiacetylene (PDA) microtube waveguide system was proposed in which the target miRNAs were enriched adjacent to the PDA microtube, while most uncatalyzed double-stranded DNAs were excluded [60]. This methodology simultaneously enhanced the target signal but reduced the background leakage, making it sufficiently sensitive to be applied directly to analyze human serum miRNA at an ultralow concentration. With a further concern about the multiple detection of miRNA, Dai et al. reported a facile and sensitive miRNA detection strategy merely based on CHA system and conventional gel electrophoresis, and multiple miRNAs can be detected at the same time and distinguished by different lengths of the designed HDP/HAP pairs [62] (Fig. 5b). A novel double-hairpin DNA inducing dual circuit CHA strategy was also proposed for multiple targets ultrasensitive detection coupling with electrochemiluminescence biosensor [63] (Fig. 5c).

2.3.2. Hybridization Chain Reaction (HCR)

HCR is a kinetics-controlled amplification method via triggered cascade of DNA polymerization by target molecules or initiator [46,64–66],

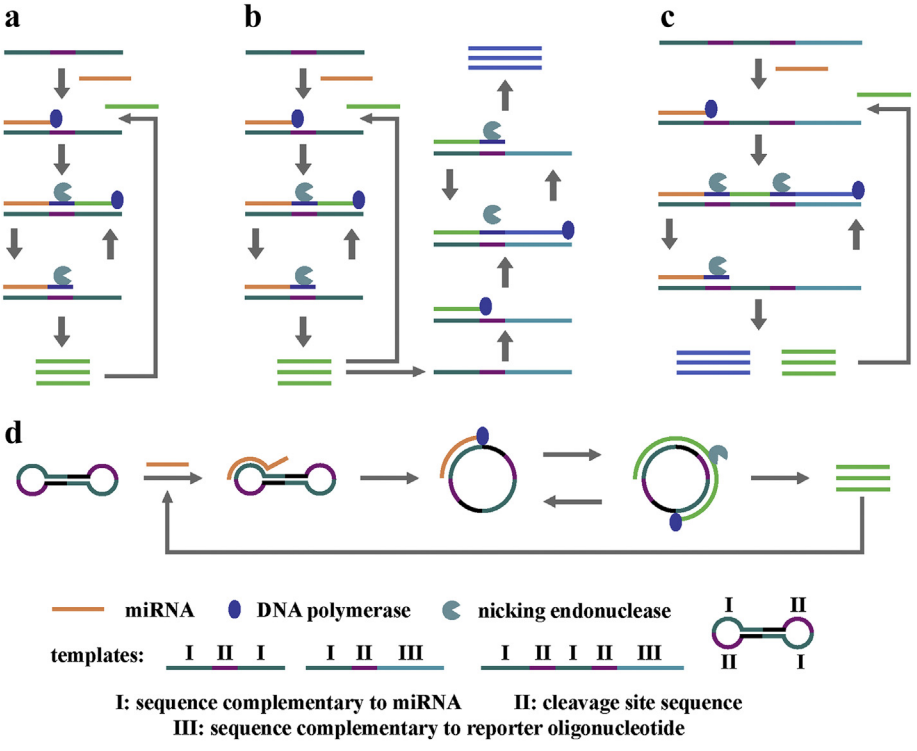

Fig. 4. Schematic illustration of the *in vitro* miRNA detection methods based on EXPAR. (a) The initial design of the EXPAR for miRNA detection. The target miRNA hybridizes with the 3' end sequence of the template and then extend to form a dsDNA helix containing a nicking-enzyme recognition site. After the recognition and cleavage by the nicking enzyme, the newly synthesized ssDNA strand will be removed, and the extension reaction is able to restart. Due to the identical sequences of the released ssDNA and the target miRNA, more EXPAR cycles will be carried out which leading to an exponential amplification of the miRNA signals. (b) Two-stage EXPAR. The two-stage EXPAR uses two different templates to trigger two sequential reactions. The newly synthesized ssDNA strand released from the first-step of EXPAR not only can participate in the exponential amplification of EXPAR cycles but also can initiate the subsequent reporter reaction using another reporter template. (c) Simplified EXPAR based on one template composed of two identical motifs complementary to target miRNA and a third motif to produce reporter oligonucleotide. (d) Improvement of the detection specificity of EXPAR based on structure-switchable symmetric toehold dumbbell-template. The target miRNA triggered TMSD process is necessary to initiate the EXPAR.

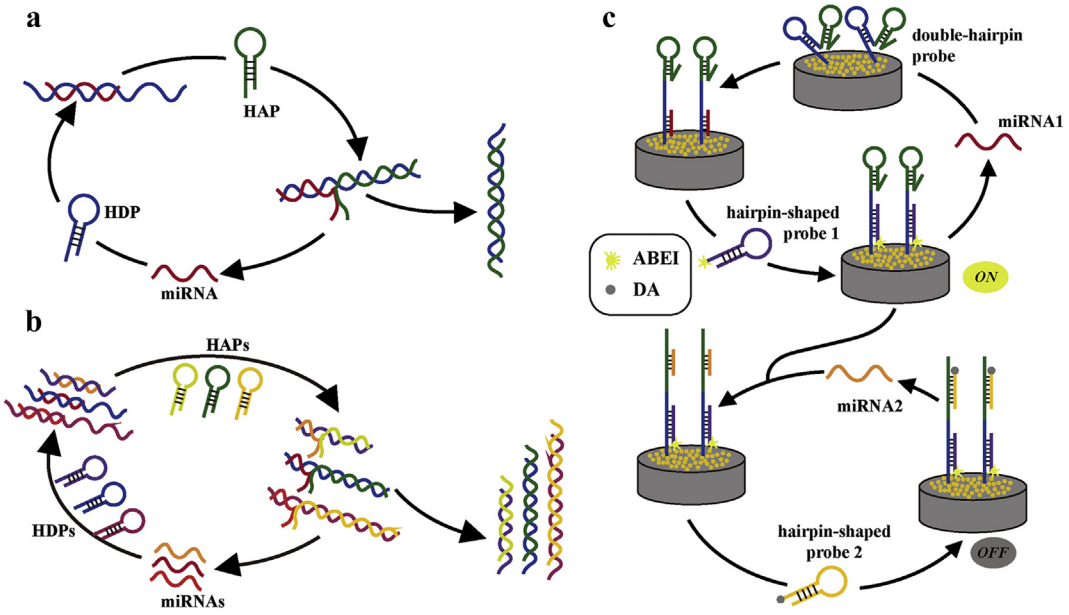

Fig. 5. Schematic illustration of the *in vitro* miRNA detection methods based on CHA. (a) The principle of CHA. Upon interaction with the target miRNA, the HDP will unwind and then form an HDP-miRNA heteroduplex with the exposure of a concealed domain. Subsequently, the corresponding HAP replaces the target miRNA to form a specific HDP/HAP heteroduplex and releases miRNA to trigger another strand displacement cycle, thus finally producing numerous corresponding HDP/HAP heteroduplexes. (b) CHA gel assay for multiple miRNA detection based on different lengths of the designed HDP/HAP pairs. (c) Double-hairpin DNA inducing dual circuit CHA strategy for multiple miRNA detection. One target miRNA could be detected by the significantly enhanced electrochemiluminescence signal (signal-on), while another target miRNA can also achieve sensitive detection by a distinct decline of the electrochemiluminescence signal (signal-off) due to the double quenching effect.

which has shown great potential in nucleic acid detection, especially in complex biosamples [65]. An HCR amplifier consists of two species of DNA hairpin-shaped probes, which can coexist stably in a solution until the introduction of target molecules initiates a cascade of hybridization events to "polymerize" into a nicked double helices analogous to alternating copolymers [64–67]. Yang et al. first reported that miRNAs can initiate HCR and developed a novel miRNA detection method coupled with a GO-surface-anchored fluorescence signal readout pathway [65] (Fig. 6a). Subsequently, to improve the sensitivity and achieve the highly efficient detection of miRNA in human serum, a novel two-layered concatenated HCR-1/HCR-2 circuit was constructed, in which the amplicon product of the upstream layer acts as a transmission trigger to activate the downstream layer [68] (Fig. 6b). Besides, non-linear HCR, which has evolved the conventional HCR from linear probe hybridization to complicated branched probe hybridization, has also been applied in miRNA quantification coupling with Y-shaped DNA as they could achieve higher amplification ratios and molecular weights than the conventional HCR [69].

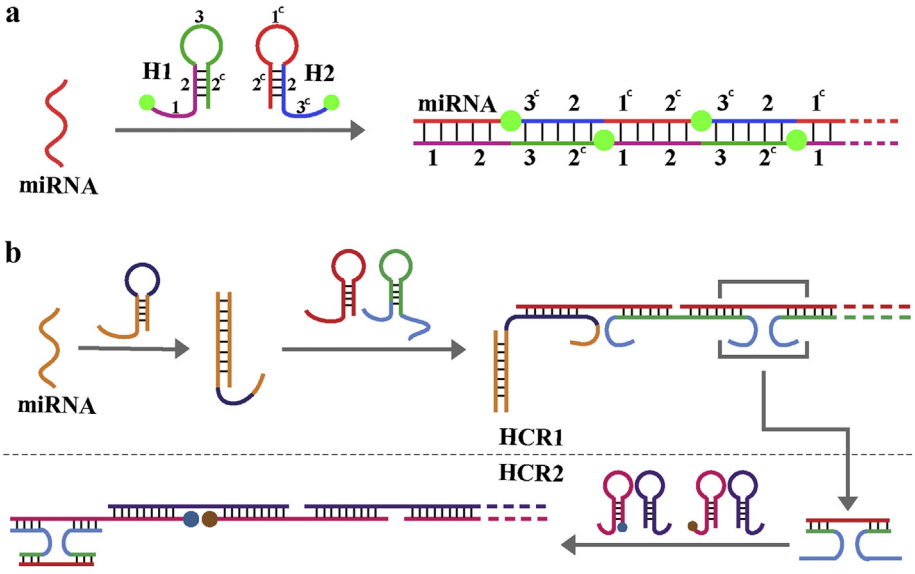

Fig. 6. Schematic illustration of the *in vitro* miRNA detection methods based on HCR. (a) GO-assisted HCR method. With the design of a pair of hairpin-shaped probes bearing fluorophores, miRNA could initially bind to the unfolded H1 probe through SDR. The newly exposed sticky end of H1 probe could subsequently hybridize with a H2 probe, generating another sticky end which could repeatedly overlap with a new H1 probe. Hence, each copy of miRNA can propagate a chain reaction of hybridization and yield a long nicked double-stranded DNA with accumulated fluorescence signals. The sequences of 1^c, 2^c and 3^c are complementary to the sequences of 1, 2, 3 respectively. (b) Two-layered concatenated HCR-1/HCR-2 circuit. The execution of HCR-2 is dependent on the formation of the assembled HCR-1 nanowire, a particular DNA nanostructure.

2.3.3. Duplex-Specific Nuclease Signal Amplification (DSNSA)

DSNSA is a novel signal-amplifying mechanism based on the duplex-specific nuclease (DSN), which displays a specific cleavage preference for double-stranded DNA or DNA in DNA–RNA hybrid duplexes [70,71]. In DSNSA design, a probe (usually with special 5′-modifications and/or 3′-modifications) and target miRNA are annealed to create a restriction site that is recognized by DSN. DSN cleaves the probe in a DNA–RNA heteroduplex into pieces, releasing the intact miRNA to bind a fresh probe and initiate another round of the reaction as a template. Hybridization, cleavage, and dissociation were repeated, resulting in signal amplification and release of signaling molecules, such as the fluorescent signal with fluorophore-labeled Taqman probe [70], the release of magnetic nanoparticle with magnetic microparticle-DNA probe-magnetic nanoparticle conjugates [71], and the bareness of the 3′-OH terminal after the digestion of the 3′-PO_4 terminated capture probe [72] (Fig. 7a). These methodologies show great sensitivity and specificity to capture and identify miRNA owing to the recycling of target and DSN's one-base discrimination ability. Recently, ingeniously designed hairpin-shaped probes showed significant potential to replace the ssDNA probes as the miRNA capturer due to their high sensitivity and selectivity to differentiate single-base mismatched targets [13,31,73–75] (Fig. 7b). The hairpin-shaped probe is modified with 2-OMe-RNA on its stem to avoid the digestion by DSN [73,74]. Besides, the Zhang group proved that only by controlling the length of the stem, hairpin-shaped probe can be prevented from digestion by DSN without special modification [75]. In addition, T7 exonuclease [76,77] and nuclease-mimicking DNAzyme [78], with the function similar to DSN, have also been utilized in miRNA detection to achieve cyclic enzymatic amplification.

3. Additional Micro- and Nano-Materials

3.1. Magnetic Bead

Magnetic beads have drawn great concern because of their convenience, quick magnetic responsiveness, easy automation and good liquid-phase kinetics [79], which have been increasingly used in the miRNA sensing field. Generally, they are utilized as the first step in the detection procedure to achieve additional signal amplification. For instance, miRNA targets are firstly hybridized with a magnetic-bead-functionalized capture probe, and the hybridized targets are subsequently isolated, purified, and heat-released to homogeneous solution to achieve enrichment [80–83]. Magnetic beads can also provide an attractive solid support to carry out miRNA assay in homogeneous solution [84]. A highly efficient chemiluminescence imaging array for the simultaneous detection of three different miRNAs was developed in which capture probes-modified magnetic beads would specially recognize their respective miRNAs and then initiate DNA machines, thus achieving exponential amplification of the targets [85]. Moreover, an immobilization-free electrochemical impedance biosensor was constructed based on the capture probes-modified magnetic beads and DSNSA. Capture probes in different forms (intact or hydrolyzed) could be attached to the surface of magnetic glass carbon electrode in the absence or presence of miR-21, resulting in different electrochemical signal [86]. In addition, magnetic beads are able to provide a way to concentrate signal. For example, anti-miRNA-modified AuNP-coated magnetic microbeads were constructed in this assay and combined with p19 protein-conjugated fluorescent dyes-loaded Alb nanoparticles to achieve the high usability and sensitivity of miRNA sensing, in which magnet was utilized to enrich the magnetic microbead complex [87].

3.2. Nanomaterials for Immobilization

Immobilization of target miRNA or reporter on the transduction surfaces is one of the critical requirements for designing biosensors [88]. Recently, engineered nanomaterials have been integrated on electrode surfaces to improve the electrode reaction efficiency and further the sensitivity of biosensor due to their high stability, surface-to-volume ratio, conductivity, and biocompatibility [17,89]. Their high surface areas greatly enhance the enzyme-loading quantity, and their good conductivities partly facilitate the electron transfer from the enzyme redox center to the electrode [25]. For example, hairpin-modified AuNPs were used as the sensing unit to initiate CHA reaction, because they can generate several electroactive molecules and induce a great change in current [89,90]. Well-dispersed nitrogen-doped hollow carbon nanospheres with large pores (a carbon-based 3D material) were fabricated to improve the performance of a self-powered biosensor [25], and GO–AuNP hybrids were used to construct a novel SPR biosensor, because they can increase the available surface area for analyte binding and improve their electrical conductivity and electron mobility [91]. GO has some distinctive properties because its surface possesses abundant carboxylic acid and hydroxyl groups, making it more water-soluble and suitable for miRNA analysis in homogeneous solution [17,92]. Lu et al. proposed a new concept of Faraday cage-type model, which greatly improved the electrode reaction efficiency and further the sensitivity of the biosensor [92].

3.3. Nanomaterials for Catalytic Signal Amplification

An increasing number of reports have indicated that functional nanomaterials can be an effective alternative strategy for enhancing the sensitivity and specificity of biosensors due to their electrocatalytic activity [93], such as metal oxide nanomaterials [94], platinum nanoparticle (PtNPs) [95] and magnetic nanoparticles [96]. For instance, a

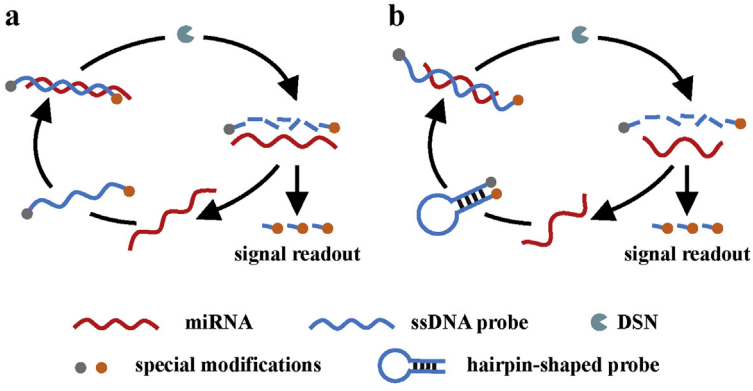

Fig. 7. Schematic illustration of the *in vitro* miRNA detection methods based on DSNSA. (a) DSNSA coupling with ssDNA probes. The DSN could specifically cleave the ssDNA probe in the DNA-RNA heteroduplexes and then release both the signaling molecules from the ssDNA probe and the intact miRNA. The intact miRNA can participate in another round of the reaction to generate amplified readout signals. (b) Improvement of the detection specificity of DSNSA through coupling with hairpin-shaped probes.

miRNA sensing scheme was developed based on the electrocatalytic properties of PtNPs and DSNSA, in which PtNPs are modified with complementary ssDNA probes at first, then with the progress of DSNSA, some of the PtNPs surfaces are exposed, reactivating the PtNPs-based electrocatalytic amplification [95]. Moreover, a dual-mode electrochemical platform was proposed by using CeO_2 nanospheres codoped with Cu and Co (CuCo−CeO_2 NSs) as an efficient electrocatalyst [94]. In this assay, Cu and Co are doped into the CeO_2 lattice, to generate large amounts of extra oxygen vacancies, and then remarkably enhance the redox and electrocatalytic properties of the CeO_2 material [94]. In more recent years, increasing interest in the nanomaterial-based signal amplification strategies has led to the development of many novel composite nanomaterials, which have more superior characteristics than the monometallic frameworks counterparts. A new class of gold-loaded nanoporous superparamagnetic iron oxide nanocubes (Au@NPFe_2O_3NC) was synthesized, which possesses both of the merits of Fe_2O_3 with high surface area, conductivity, thermal/chemical stability and superparamagnetism and the AuNPs with bio-favorable physicochemical properties, leading to its high eletrocatalytic activity and surface loading capacity [96]. Through the gold−DNA/RNA/proteins affinity interactions, this advanced material is highly suitable for the development of simple, inexpensive and rapid miRNA biosensors for a wide range of molecular biomarkers including miRNA [80,96].

3.4. Nanomaterials in Fluorescent Assays

Due to the advantages of unique optical properties, large surface area and good biocompatibility, several nanomaterials, such as carbon nitride nanosheet [97], GO [98] and MoS_2 nanosheets [99], can be used as efficient fluorescence quenchers for the fluorophore-labeled probe based miRNA detection. Intriguingly, fluorescent metal nanoclusters (<1 nm), collected from two to tens atoms of gold or silver [100], have been developed as a new class of fluorophores and widely applied in the area of miRNA bioassays owing to their facile synthesis and tunable fluorescence emission [101]. In particular, DNA-scaffolded silver nanoclusters (DNA-AgNCs) using DNA as scaffolds have attracted more attention. A label-free and environmental-friendly miRNA detection method was developed based on EXPAR and DNA-AgNCs in which the product of EXPAR can act as scaffolds for the synthesis of fluorescent AgNCs [101]. Furthermore, the optical properties of DNA-AgNCs are influenced by the length, sequences and intrinsic secondary structures of the DNA templates. Thus, multiple miRNAs can be detected in one step by designing various fluorescent DNA-AgNCs probes [102].

4. Signal Transduction and Readout Elements

4.1. Electrochemical Sensing

Electrochemical sensing is one of the most prospective analysis methods to meet the needs of miRNA detection because of its high sensitivity and specificity, quick response, convenient operation, strong controllability, and relatively low cost, and they have gained wide attention in the field of miRNA detection [51,72,76,92,103,104]. Unfortunately, the accuracy and sensitivity of electrochemical sensors alone are insufficient to detect miRNA in biological samples, and the low electrode reaction efficiency mainly limit the enhancement of sensitivity of the traditional electrochemical biosensor [92]. Accordingly, various kinds of amplification methods have been devoted to collaborate with the electrochemical biosensors, such as T7-exonuclease-assisted cascade signal amplification [76], strand displacement reaction (SDR) [103], CHA [51,105] and the cyclic cleavage reaction of metal-ion-dependent DNAzyme [106,107]. In addition, nanomaterials with high surface-to-volume ratio and conductivity have been widely applied in electrochemical biosensors as electrode materials to improve the electrode reaction efficiency, such as GO [92], graphitic-phase carbon nitride nanosheet (g-C_3N_4) [108] and 2D metal–organic framework (MOF) nanosheets [105,108]. Photoelectrochemical (PEC) sensing, as a vibrant electrochemical analytical technology, exhibits striking advantages such as instinctive mechanism and prominent anti-interference for chemical analysis [109]. Notably, PEC analysis involves the conversion of photoactive materials from photo to electricity in which the photo-induced formation of electron-hole pairs in a photoactive material is utilized to initiate oxidation–reduction reactions [110,111]. Therefore, for PEC biosensors, eminent photoactive materials with appropriate band gap, high carrier mobilities, good corrosion resistance, and relatively low cost are critical to performance [109]. Based on that, several novel species with distinctive properties were constructed and applied in PEC biosensor as photoactive materials, such as a MoS_2/g-C_3N_4/black TiO_2 heterojunction [112], a novel iridium(III)-complex-based photoactive material [111], and a TiO_2-α-Fe_2O_3 heterojunction [109], to greatly improve the PEC performance of electrode.

4.2. Optical Biosensors

SPR sensor, which monitors the change in refractive index near the surface during complex formation or dissociation, is one of the most widely used optical sensors [91,113]. Due to its excellent specificity, reproducibility and precision, SPR sensor has been widely applied in miRNA detection [114]. Such method, however, suffers from a major drawback of relative low sensitivity. Considering that the participation of any protein enzymes may induce irreversible damage to the architecture of SPR biosensors [115], enzyme-free amplification strategies, such as CHA [116,117] and HCR [117], have been developed and applied to SPR sensing platform urgently. Various nanomaterials have also been used in SPR analysis owing to their distinctive properties, such as carbon nanotube [118], GO [119], Au@Ag nanorods [117], and GO-AuNPs [91], and show great potential to improve the sensitivity.

SERS has been developed as another powerful miRNA detection method through highly specific and sensitive biological imaging and sensing due to its distinct advantages of high stability, good specificity, and low background signal. This surface-sensitive technology is based on a phenomenon that Raman scattering is enhanced by molecules adsorbed on the plasmonic metal surface or by plasmonic metal nanostructures due to the strong electromagnetic coupling generated nearby the metal nanoparticles [120,121]. However, relatively low sensitivity and extremely rare electromagnetic hot spots in substrates limit its wide applications [55]. Fortunately, the introduction of engineered metallic nanoparticles such as target-triggered CHA-induced core-satellite [55], superwettable nanodendritic gold substrates [121], and nanodendritic gold/graphene [122] addressed these problems in principle due to their abilities to aggregate with strong electromagnetic hot spots and finally improve the detection sensitivity for trace amount of miRNA targets.

4.3. Fluorescent Assays

Fluorescent assays are the most frequently used strategies among these signal readout methods and can be generally divided into three categories, assays based on fluorescence dye, fluorescence-labeled probe, and fluorescence resonance energy transfer (FRET). Fluorescence-dye-based fluorescent assay, usually refers to SYBR Green I, has been frequently used in miRNA detection due to its simplicity and real-time detection [39,42]. However, SYBR Green I suffers from defects such as high background, unstable fluorescent output, and the preferential binding to GC-rich sequences [123], which limits further applications. Fluorescence-labelled probes can be divided into fluorescence-labelled ssDNA/ssRNA probes and fluorescence-labelled MBs. The former ones are typically used in nanomaterial-based biosensors in which their fluorescence is quenched when immobilized and restored upon capturing the target miRNA [27,97–99]. The latter ones rely on the distance-dependent fluorescence quenching property,

because they can be disrupted to linear forms in the presence of target miRNA, thus restoring their fluorescence [13,31,32].

FRET is a mechanism describing the electronic excitation energy transfer from an energy donor chromophore to an energy acceptor [124]. Recently, FRET-based sensing platforms have gained extensive attention due to their sensitivity and simplicity, which could advance many homogeneous and sensitive miRNA detections [124]. Increasing number of FRET probes with favorable photophysical properties are synthesized and applied in the biological imaging of miRNA [125]. However, traditional FRET probes are exposed to analytes, and single fluorescent signal turn-on is limited [126]. Accordingly, an oriented gold nanocross (AuNC)-decorated gold nanorod (AuNR) probe with "OFF-enhanced ON" fluorescence switching was developed to improve the detection sensitivity and expand the sensing range [124]. A novel FRET-based platform was constructed for the dual-color simultaneous detection of multiple tumor-related miRNAs, in which polydopamine nanospheres were used as energy acceptors, and dual colored AuNCs were utilized as energy donors [127].

4.4. Colorimetric Assays

Colorimetric assays significantly reduce the cost and complexity of signal readout as they performed to quantify and describe physically the human color perception, which allows rapid and accurate miRNA detection at the point-of-care (POC) [128]. Unfortunately, traditional colorimetric assays are not sensitive enough to detect miRNA with ultra-low concentration. Consequently, various kinds of amplification strategies are utilized [128]. Among these methods, DNAzyme-based colorimetric assays without using any protein enzymes offer a cost-effective, rapid, and efficient option for miRNA detection. Typically, isothermal amplification methods are coupled to DNAzyme to further enhance the sensitivity. Wen et al. integrated RCA, EXPAR, and a horseradish peroxidase (HRP)-mimicking DNAzyme to achieve ultrasensitive detection of target miRNA [129]. Different from the former miRNA assay, non-enzymatic isothermal amplification (CHA and HCR) were combined with G-quadruplex/hemin DNAzyme in this work to develop an enzyme-free colorimetric biosensing system [130]. Another intriguing design is the AuNP-based colorimetric assay [131]. AuNPs possesses some unique properties such as strong SPR absorptions and high extinction coefficients, resulting in a color change of the AuNP solution from red to purple when aggregating, providing its wide application in analytical methods. Subsequently, this ultrasensitive colorimetric method was developed coupled with EXPAR. In summary, colorimetric assays provide an approach with decreased cost and simpler equipment for detecting miRNAs, while extra amplification methods are also urgently needed to achieve remarkable efficacy.

4.5. Lateral Flow Assay (LFA)

Due to the demand for biomedical and clinical applications, considerable effort has been devoted for exploring feasible and reliable POC testing (POCT) methods [132]. LFA-based POC devices are rapidly growing for qualitative and quantitative analysis in recent years [133]. LFA is performed over a strip adopted from the well-developed immunochromatography strip technology [134]. However, the traditional nucleic acid sandwich methods in which the tested oligonucleotide matches with the detection and capture probes seemed to be unavailable for miRNA detection because of its small size [134]. To address this issue, AuNP labeled with thiol-DNA and biotin-ssDNA were introduced as detection probe and capture probe respectively, thus the target miRNA and the detection probe were perfectly matched with the capture probe [134]. To address the pivotal problem of low sensitivity of the LFA method, signal amplification enzyme like HRP was immobilized on the AuNPs surface, and the sensitivity was significantly increased by applying the 3,3,5,5-tetramethylbenzidine enzymatic substrate (TMB/H_2O_2 enzymatic substrate) onto the test zone [135]. Another notable concern is about the paper-based POCT. An interesting work presented a pH-responsive miRNA amplification method, which allows the detection of miRNA by using a pH test paper. In their design, an improved netlike RCA technique was utilized to amplify the miRNA target, thus producing large amounts of H^+ as a by-product and subsequently induced substantial changes in pH [136]. Although the specificity and the false negative/positive signals of the paper-based work remains to be improved and tested in future studies, we could envision that the paper-based detection techniques will obtain prosperous development considering the projected cost, reaction time, ease of use, and no requirement for laboratory infrastructure.

4.6. CRISPR-Cas System

CRISPR–Cas systems, which were first found as RNA-mediated adaptive immune systems in bacteria and archaea against invading nucleic acid components, have become a prominent tool in biosensing applications. Various CRISPR effectors, including Cas12a [137,138], Cas13a [139], and Cas14a [140], have been recently applied to CRISPR-based nucleic acids diagnostics (CRISPR-Dx) owing to their "collateral" cleavage activities, wherein RNA-guided target DNA/RNA binding would unleash indiscriminate ssDNA cleavage activity. However, all CRISRP-Dx approaches reported so far were not capable of miRNA detection owing to their short size. Our group recently built a novel RCA-CRISPR-split-HRP (RCH) method based on catalytically dead Cas9, TIRCA, which elongate short-length RNAs into considerably regular structures, and split-HRP techniques to produce a colorimetric signal readout [21] (Fig. 8). Circulating let-7a, a reported biomarker of non-small cell lung cancer, was selected as the target miRNA as a proof of concept. Such scheme can effectively detect target miRNAs in human serum samples and significantly distinguish patients from healthy volunteers through the naked eye. As the first to use CRISPR-Cas system in miRNA detection, this scheme shows great application prospect in POCT with the rapid development of paper-based techniques and smartphone-based ELISA detection devices.

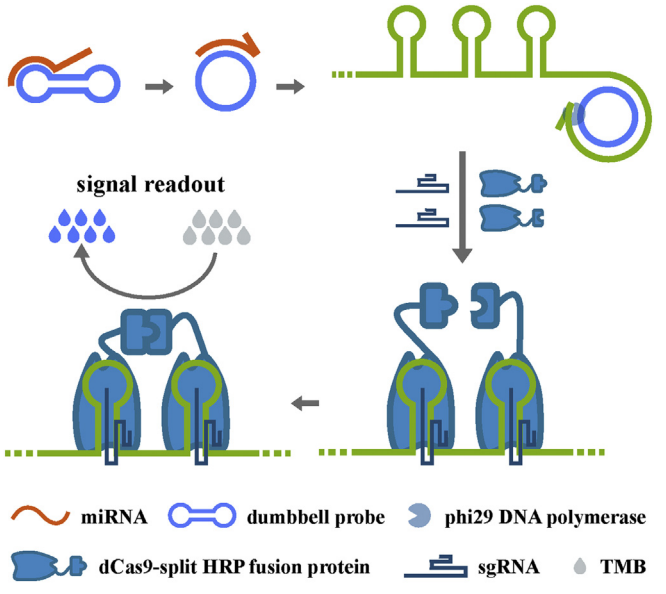

Fig. 8. Schematic representation for the detection workflow of the RCH method for miRNA detection. The CRISPR-dCas9 system conjugated with split-HRP reporters have been introduced to the miRNA detection process. The sgRNAs are designed to target the repeated sequences of the RCA products, thereby recruiting the dCas9-split-HRP fusion proteins and reactivating the HRP activity of TMB-catalytic colorimetric reaction.

Table 1
Typical miRNA *in vitro* detection systems.

Name of miRNA	Biological recognition elements	Additional micro- and nano- materials	Signal transduction and readout elements	Additional information	Limit of detection (LOD)	Quantitative (dynamic range)	Specificity	Multiplex	Required sample volumes	Total assay time	Ref.
let-7a	RCA	–	CRISPR-Cas system	Split-HRP techniques to amplify signal	$3.54 * 10^{-17}$ mol/L	Y	1 nt	N	a little	<4h	[21]
let-7a	CHA	–	colorimetric assay	HCR to promote the form of massive peroxidase-mimicking DNAzymes	$7.4 * 10^{-15}$ mol/L	Y (10^{-14}–10^{-8} mol/L)	1 nt	N	5 μl	≈3 h	[130]
let-7a, miR-21	ssDNA probe	MOFs	Electrochemical sensing	–	$3.6 * 10^{-15}$ mol/L	Y (10^{-14}–10^{-11} mol/L)	2 nt	Y	40 μl	≈1 h	[151]
miR-21	ssDNA probe	AuNPs	Lateral flow assay	Silver enhancement reagents to intensify the signal of AuNPs	$1.0 * 10^{-13}$ mol/L	Y (10^{-13}–$2 * 10^{-12}$ mol/L)	N	N	a lot	70 min	[134]
miR-21	ssRNA probe	AuNPs superlattice	Electrochemical sensing	Toluidine blue as a redox indicator	$7.8 * 10^{-17}$ mol/L	Y (10^{-16}–10^{-9} mol/L)	1 nt	N	a lot	3.5 h	[24]
miR-21	Hairpin-shaped probe	AuNPs, AgNPs, magnetic bead	Optical biosensor	–	$6.0 * 10^{-16}$ mol/L	N	1 nt	N	a lot	≈3.5 h	[115]
miR-21	Hairpin-shaped probe	–	Colorimetric assay	EXPAR to achieve isothermal amplification	$2.91 * 10^{-15}$ mol/L	Y (10^{-14}–10^{-10} mol/L)	1 nt	N	a little	≈2.5 h	[152]
miR-21	EXPAR	–	Fluorescent assays	CHA to induce significant enhancement of fluorescence signals	$3.0 * 10^{-15}$ mol/L	Y (10^{-14}–10^{-10} mol/L)	12 nt	N	1 μl	≈1.5 h	[153]
miR-21	CHA	–	Optical biosensor	Programmable streptavidin aptamer	$1.0 * 10^{-12}$ mol/L	Y ($5 * 10^{-12}$–10^{-7} mol/L)	1 nt	N	a little	≈1.5 h	[116]
miR-21, miR-141	Hairpin-shaped probe	g-C_3N_4@AuNPs, Ru-MOF, Fe_3O_4@AuNPs	Electrochemical sensing	–	$3.0 * 10^{-16}$ mol/L	Y (10^{-15}–10^{-11} mol/L)	10 nt	Y	a lot	4 h	[108]
miR-141	ssDNA probe	GO-AuNPs hybrids	Optical biosensor	–	$1.0 * 10^{-15}$ mol/L	N	2 nt	N	a lot	≈2 h	[91]
miR-141	EXPAR	–	Colorimetric assay	generation of catalytic G-quadruplex DNAzyme	$1.0 * 10^{-15}$ mol/L	Y (10^{-15}–10^{-7} mol/L)	4 nt	N	a little	≈4.5 h	[44]
miR-141	HCR	GO, Fe_3O_4@SiO_2@AuNPs	Electrochemical sensing	faraday cage-type strategy	$3.0 * 10^{-17}$ mol/L	Y (10^{-16}–10^{-12} mol/L)	1 nt	N	5 μl	2 h	[92]
miR-122	CHA	PdNPs@Fe-MOFs AuNPs@N-G	Electrochemical sensing	–	$3.0 * 10^{-18}$ mol/L	Y (10^{-17}–10^{-11} mol/L)	N	N	10 μl	≈1.5 h	[105]
miR-133a	CHA	Hollow Ag/Au nanospheres	Optical biosensor	–	$3.06 * 10^{-16}$ mol/L	Y (10^{-15}–10^{-8} mol/L)	14 nt	N	a little	≈5 h	[54]
miR-146a	RCA	AuNPs	Fluorescent assays	a CTG repeat-based hairpin structure	$1.4 * 10^{-17}$ mol/L	Y (10^{-15}–10^{-10} mol/L)	1 nt	N	a little	>21 h	[154]
miR-148	DSNSA	Quantum dots	Fluorescent assays	–	$4.2 * 10^{-14}$ mol/L	Y (10^{-15}–10^{-8} mol/L)	3 nt	N	a little	≈3 h	[155]
miR-185	ssDNA probe	Ruthenium(II) complexes	Fluorescent assays	–	$2.8 * 10^{-10}$ mol/L	Y	1 nt	N	a lot	1 min	[27]
miR-196a	DSNSA	–	Electrochemical sensing	Template-free DNA extension reaction, methylene blue	$1.5 * 10^{-17}$ mol/L	Y ($5 * 10^{-17}$–$5 * 10^{-11}$ mol/L)	1 nt	N	a little	≈3.5 h	[72]
miR-221	EXPAR	AuNPs	Colorimetric assay	–	$4.6 * 10^{-14}$ mol/L	Y ($5 * 10^{-14}$–10^{-8} mol/L)	1 nt	N	a little	<1 h	[131]
miR-224	ssDNA probe	AuNPs	Lateral flow assay	Signal amplification enzyme (HRP) to improve sensitivity	$7.5 * 10^{-12}$ mol/L	Y ($7.5 * 10^{-12}$–$7.5 * 10^{-8}$ mol/L)	N	N	100 μl	30 min	[135]
let-7d	RCA	–	Colorimetric assay	Nicking enzyme signal amplification, DNAzyme signal amplification	$2.0 * 10^{-18}$ mol/L	Y (10^{-17}–10^{-11} mol/L)	2 nt	N	a little	≈6 h	[129]
miR-1178, let-7b, miR-1248	DSNSA	Magnetic bead	Colorimetric assay	DNAzyme moieties to further enhance the sensitivity	$1.0 * 10^{-16}$ mol/L	Y ($2 * 10^{-16}$–$5 * 10^{-14}$ mol/L)	1 nt	N	25 μl	2 h	[128]

5. Summary and Outlook

Since the circulating miRNAs have been generally demonstrated as stable blood-based markers for cancer diagnosis and prognosis, as well as targets for therapy, the biochemical and synthetic biological technologies for miRNA detection *in vitro* has received a lot of attentions and gradually developed into an important research area. Until now, systematical techniques composed with a serials of biological recognition elements, additional micro- and nano-materials, and signal transduction and readout elements have been developed and provided significant supports for the detection of more than ten typical miRNA biomarkers, and more miRNA targets monitoring is still ongoing (Table 1).

Along the development history of miRNA detection methods, we could find that ssDNA/ssRNA probes are the first developed and also the most commonly used way to capture target miRNA among various kinds of biological recognition elements because of their simplicity and universal applicability, typically combining with all sorts of biosensors (Fig. 9). Immediately after that, hairpin-shaped probes are designed as an advanced version of capture probes. With the introduction of a specific SDR, their target sequence specificity is superior to the ssDNA/ssRNA probes. With regard to the detection pipeline, the hairpin-shaped probes were initially combined with fluorescent assays as MBs with high signal-to-background ratios, and then developed to couple with electrochemical sensing [108], optical biosensors [115] or LFA [141] with biotin or thiol groups labeled. In addition, they supply a novel non-radioactive method to detect miRNA in homogenous solutions [31], and show great potential in DNAzyme field due to their special structure [142]. Several years later, with the increased demands of higher sensitivity for the trace amount of specific miRNA detection, amplification methods have been successively developed to capture the target miRNAs, including the molecular amplification methods (RCA and EXPAR) and the signal amplification methods (CHA, HCR and DSNSA). RCA was firstly introduced in miRNA detection and obtained lots of attentions. The updated RCA methods, such as TIRCA [39] and RCA-LAMP [143], helped to reduce the signal background and enhance the amplification efficiency, which could broaden the RCA's further applications in miRNA detection. Another idea to achieve the goal of massive amplification of the target miRNA signal is to reuse the same miRNA as the initiator for repeated rounds of the amplification reaction. The further developed methods of EXPAR and DSNSA are the products of this supposition. Notably, these methods can only combined with fluorescent assays or colorimetric assays instead of biosensors because the participation of any protein enzymes may induce irreversible damage to the architecture of biosensors [115]. This problem could be addressed by the most recent developed CHA and HCR, which are two major non-enzymatic biological recognition elements playing important roles in various kinds of biosensors. However, both of them still experience drawbacks such as background leakage caused by the non-specific hybridization of two hairpin-shaped probes [59–61], which need further improvements.

For signal readout elements, traditional fluorescent assays still account for a large proportion of the total. With the in-depth understanding of FRET, some novel platforms with high sensitivity and simplicity have been proposed [124,127]. Biosensors have been developed rapidly and widely applied in miRNA detection in the last decade, especially in electrochemical biosensors. However, biosensors suffer from the same drawbacks such as low sensitivity and poor accuracy and require special materials to transduce signal [92,109]. Hence, various kinds of nanomaterials [103,105] and nonenzymatic amplification methods [105,115] have been applied in miRNA biosensing. Novel biosensors, such as self-powered biosensors, have also been applied due to their excellent biocompatibility and considerable power output [144–148]. Colorimetric assays and optical biosensors have been applied to this field for a relatively long time but have been developed very slowly because of their relatively low sensitivity. With the demand of feasible and reliable POCT, LFA-based portable devices are rapidly developed in recent years, however, they still have a long way to go to address the problem of low sensitivity and collect enough experimental cases to identify the false positive and negative rates.

Collectively, although numerous achievements for miRNA detection *in vitro* have been extensively reported, there are many essential issues remain to be addressed for *in vitro* applications in the clinic, global health, industry, research, and education. Firstly, most of the capture probes rely on the immobilization on corresponding surfaces, which requires strict experimental conditions and complicated experimental process. Furthermore, inappropriate immobilization would result in decreased or even no signal response [149]. Thus, novel homogeneous biosensors are supposed to be used to avoid the immobilization of ssDNA/ssRNA probes. Secondly, the sensitivity still needs further improvement and the precision of quantitative analysis remains insufficient. Thus, novel strategies have to focus more on the increase of the signal/noise

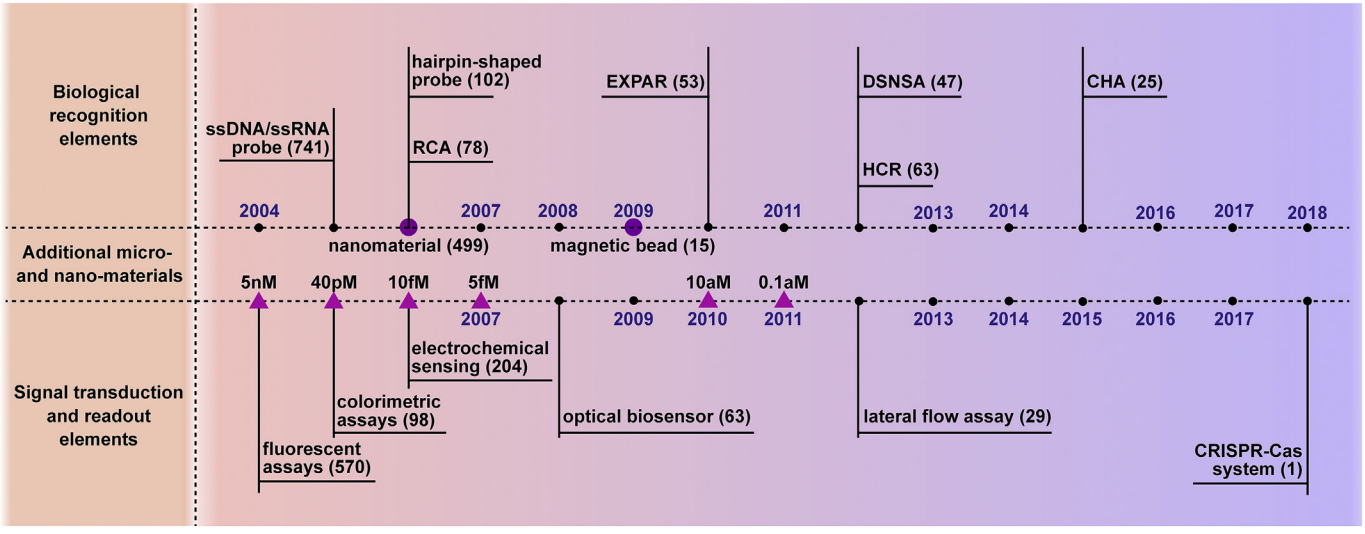

Fig. 9. The historical development of the miRNA detection technologies *in vitro*. The timeline integrates all the three elements of biological recognition, additional micro- and nano-materials, and signal transduction and readout. Each method is indicated at the time when it was first introduced to miRNA detection. The numbers in the brackets represent the number of the papers studying the corresponding methods to date (data are collected from the PubMed database). The numbers above the triangles along the timeline indicate the breakthrough of detection sensitivities.

ratio and the expansion of the dynamic range of quantitative analysis. Thirdly, precise diagnosis or prognosis of diseases always needs a composition of multiple miRNA biomarkers [108]. Thus, more studies are necessary to develop some highly efficient multitask techniques for simultaneous and multiplexed detection of different groups of miRNA combination. Finally, for the application in low-tech conditions and a feasible and reliable POCT, more efficient portable platforms for miRNA detection are essential be exploited. Notably, the newly developed CRISPR-Dx may offer some guidelines. Several portable nucleic acid detection platforms have been constructed, utilizing their "collateral" cleavage activities [137–140] with ultra-high sensitivity and specificity as well as their POCT capability. Moreover, field-deployable viral diagnostics has already been accomplished via HUDSON and SHERLOCK [150]. However, additional strategies, which can convert short-length RNAs into longer oligonucleotides or proper structures that can be targeted by Cas-crRNA (CRISPR RNA)/sgRNA (single-guide RNA) complex, are needed when applying CRISPR-Dx to miRNA detection.

Declarations of competing interest

The authors have declared that they have no conflicts of interest to this work.

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