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Novel Biosensing Strategies for the *in Vivo* Detection of microRNA

Junling Zhang, Shanshan Zhao and Jikui Wu

Abstract

As a regulatory molecule of post-transcriptional gene expression, microRNA (miRNA) is a class of endogenous, non-coding small molecule RNAs. MiRNA detection is essential for biochemical research and clinical diagnostics but challenging due to its low abundance, small size, and sequence similarities. In this chapter, traditional methods of detecting miRNA like polymerase chain reaction (PCR), DNA microarray, and northern blotting are introduced briefly. These approaches are usually used to detect miRNA *in vitro*. Some novel strategies for sensing miRNAs *in vivo*, including hybridization probe assays, strand-displacement reaction (SDR), entropy-driven DNA catalysis (EDC), catalytic hairpin assembly (CHA), hybridization chain reaction (HCR), DNAzyme-mediated assays, and CRISPR-mediated assays are elaborated in detail. This chapter describes the principles and designs of these detection technologies and discusses their advantages as well as their shortcomings, providing guidelines for the further development of more sensitive and selective miRNA sensing strategies *in vivo*.

Keywords: microRNA, hybridization probe assays, strand-displacement reaction (SDR), entropy-driven DNA catalysis (EDC), catalytic hairpin assembly (CHA), hybridization chain reaction (HCR), CRISPR

1. Introduction

MicroRNA (miRNA) is a kind of endogenous non-coding RNA with a length of 18–25 nucleic acid sequences. It is usually integrated into the RNA-induced silencing complex (RISC) to execute its biological function of degrading mRNA or inhibiting transcription. MicroRNA is highly conservative and has strict temporal and spatial specificity. It plays a key regulatory role in the development of animals and plants, cell proliferation, differentiation and apoptosis, immunity and metabolism, angiogenesis, tumor invasion, and metastasis. Mature miRNA has the disadvantages of small fragment, no poly (A), high similarity among family members, and low expression level. As a result, it is difficult to sensitively and accurately detect miRNA. Therefore, it is very important to establish fast and simple methods with high sensitivity and specificity for miRNA detection [1].

Many miRNA analysis methods, including polymerase chain reaction (PCR), DNA Microarray, and Northern blotting have been developed. Although these traditional strategies are the gold standard methods for miRNA identification, detection, and analysis *in vitro*, it is very difficult for them to achieve accurate and sensitive spatio-temporal information of miRNA in living organisms. Therefore, in this chapter, we summarize the novel strategies for biosensing miRNAs *in vivo*

including hybridization probe assays, strand-displacement reaction (SDR), entropy-driven DNA catalysis (EDC), catalytic hairpin assembly (CHA), hybridization chain reaction (HCR), DNAzyme-mediated assays and CRISPR-mediated assays. These contents involve their principles and methods, including their advantages and shortcomings, in order to provide important help for the further study of related detection technology.

2. Overview of traditional miRNA detection methods

2.1 Northern blotting

Northern blotting, invented by Alwine in 1979, is the first established method to identify and detect miRNA. It is widely used to detect the expression of miRNAs of various sizes from long primitive miRNAs to mature miRNAs. In the process, the miRNA was separated by polyacrylamide gel electrophoresis in total RNA, then transferred to the imprinted membrane, hybridized with the radionuclide labeled probe. The RNA molecule of interest is detected by the signal of labeled probe. This method can detect both the quantity and the length of miRNA, but it has some defects such as cumbersome operation, low sensitivity, time-consuming, and large sample consumption, which limits its application in clinical diagnosis.

To improve the detection sensitivity, Válczi *et al.* [2] employed locked nucleic acid (LNA) probe instead of traditional oligonucleotide probe to enhance the affinity and stability of the nucleotide double strand. Pall *et al.* [3] utilized 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride crosslinking method instead of the traditional UV crosslinking method to improve the binding efficiency of miRNA and nylon membrane. The method of biotin labeling instead of isotope labeling was used to conduct chemiluminescence detection and reduce radioactive pollution [4].

2.2 Microarray

The microarray, developed in the early 1990s, enables the high-throughput miRNA detection in a parallel fashion. In this method, the target miRNA is incubated and hybridized with multiple probes (complementary to the target miRNA sequence) on a chip. After removing the non-hybridized part, the signal can be detected and analyzed by fluorescence scanning or northern blotting [5]. DNA probe-based microarray usually consumes a large amount of samples, and has disadvantages such as low sensitivity and specificity and false positives caused by cross-reactions. However, LNA probe can reduce the consumption of starting materials and improve the sensitivity and accuracy of microarray [6]. Furthermore, liquid suspension microsphere hybridization can effectively avoid cross-reaction in the solid chip to decrease the occurrence of false positives.

2.3 qRT-PCR

Quantitative real-time polymerase chain reaction (qRT-PCR) is one of the main methods to detect low abundance miRNA with high sensitivity and accuracy [7]. The principle of this method is to reversely transcribe miRNA into the corresponding cDNA that is used as a template to initiate real-time PCR, and then indirectly analyze miRNA by detecting the signal of amplified products [8].

Because the miRNA sequence is short (18 ~ 25 nt) and similar to the length of PCR primers, researchers overcome this shortcoming by introducing stem-ring

primers for reverse transcription [9] or adding poly (A) into RNA to initiate reverse transcription and dyeing with SYBR Green. Besides, pri-miRNA and pre-miRNA can be introduced into qRT-PCR, causing inaccurate quantification [10]. qRT-PCR usually requires complex primer design and precise reaction temperature control, thereby greatly increasing the cost and complexity of the experiment.

Although the traditional miRNA detection methods are widely used in miRNA detection, there are still some shortcomings such as complex operation, low sensitivity, poor specificity, and large sample consumption. These shortcomings greatly limited the application of these methods in clinical diagnosis and treatment. Importantly, these approaches are only applied to the *in vitro* measurement of miRNA.

3. Emerging sensing techniques in microRNA detection *in Vivo*

3.1 Hybridization probe assay

Hybridization probe assay is a simple and direct detection method without amplification of target miRNA. The principle of this method is as follows: firstly, miRNA is fixed in the tissue or cell. Secondly, signal-labeled nucleic acid probes are added and hybridized with the miRNA based on the principle of complementary pairing. Finally, the position of target miRNA to be detected in the tissue or cell is displayed by certain detection means.

With the principle of hybridization, Wang *et al.* [11] combined the excellent fluorescence-quenching ability of Ti_3C_2 nanosheets with a double-labeled DNA fluorescent probes to design a chimeric DNA-functionalized Ti_3C_2 nanoprobe. When this probe crosses the cell membrane, TAMRA-labeled plasma membrane glycoprotein MUC1 aptamer interacted with MUC1 and peeled off from Ti_3C_2 , lighting up red fluorescence. In the cytoplasm, FAM-tagged hairpin DNA hybridized with miR-21, leading to green fluorescence recovery. This strategy can perform *in vitro* measurement of miR-21 and MUC1 with nanomolar sensitivity and *in situ* simultaneous imaging of dual biomarkers in living cells. Although Ti_3C_2 greatly reduces the fluorescence background, the sensitivity of the probe is still limited because one target miRNA only restore the fluorescence of one fluorescent molecule.

To further improve the detection and imaging sensitivity, fluorescence double-labeling and double-quenching strategy is undoubtedly a good design. Molecular beacon (MB) is hairpin-structure DNA probe. Its two ends are labeled with two identical fluorescent molecules. When MB is in a close state, self-quenching effect between two fluorescent molecules occurs. Graphene oxide (GO) is a good fluorescence quencher. The electrostatic interaction between DNA probe and GO also quenches effectively the fluorescence of DNA probe. Based on the aforementioned properties of MB and GO, Yang *et al.* [12] designed a MB-GO fluorescent sensor for sensitive imaging of intracellular miRNA (**Figure 1**). Two Cy5 fluorophores were tagged at the both ends of MB. MB was electrostatically adsorbed on the surface of GO through the π - π stacking effect. Two Cy5 fluorophores was quenched through their self-quenching effect and cy5-GO resonance energy transfer, which greatly reduced the fluorescence background of sensing system. Once target miRNA hybridize with MB probes, hairpin-structure MBs were unfolded to separate two Cy5 molecules and the MB-miRNA complex with rigid structure was released from the surface of GO, recovering fluorescence signal and significantly enhancing the detection sensitivity (Limit of detection, LOD = 30 pM). This method also visualized miRNA-21 in cancer cells and three xenograft tumor models.

Figure 1.

Illustration of 2Cy5-MB-GO complex sensing miRNA in living cells. Reprinted with permission from ref 12. Copyright 2018 American Chemical Society.

Another approach to enhance the sensitivity of hybridization probe assay is to use the plasmon coupling effect of assembled nanostructures, especially, dissymmetric nanostructures. Xu *et al.* [13] developed a plasmonic nanorod probe by miR-21-directed *in situ* self-assembly of DNA-modified Gold nanorods (AuNR) in living cells. The formation of NR@PS-PAA dimers gives rise to intense chiroplasmonic signal and surface-enhanced Raman scattering (SERS). The LOD were 0.081 fmol/10 μ g $\cdot$ RNA (CD signal) and 1.12 fmol/10 μ g $\cdot$ RNA (SERS signal), respectively. Highly sensitive quantitative analysis and *in situ* imaging of microRNA in cells are achieved.

Although the introduction of nanomaterials (gold nanoparticles, graphene oxide, upconversion nanoparticles, MnO₂ nanosheets, quantum dots, silver nanoparticles, noble metal nanoclusters, and silica nanoparticles) remarkably enhanced the sensitivity of hybridization probe assay, the lack of signal amplification limits its practical application in the sensitive detection of intracellular miRNA.

3.2 Strand displacement reaction (SDR)

Strand displacement reaction (SDR) is a dynamic process of hybridization-driven DNA strand exchange accompanied by branch migration [14]. In this process, a single-stranded reactant (input, target miRNA) reacts with multi-stranded DNA complex and releases another single-stranded product (output signal) and a new DNA complex. This process operates autonomously through a series of reversible DNA hybridization and dissociation steps to produce numerous output strands, thereby generating cascaded signal amplification [15, 16].

Using SDR, Wang *et al.* [17] constructed a double-stranded fuel catalyzed DNA molecular machine. The machine was divided into two parts: the first part was composed of the Cy5-labeled signal strand and BHQ-2 labeled complementary quenching strand. The second part was double-strand fuel (DSF), consisting of fuel strand and protected fuel. The target miRNA bound to the quenched strand and triggered the first SDR to obtain the miRNA-quenching double-strand, releasing the signal strand to recover the fluorescence. At this time, DSF initiated the second SDR, the dominated fuel hybridized with quencher strand, replacing miRNA to produce double-stranded waste and intermediated miRNA protected fuel. The

(MCF-7) membrane (DMSN-DP@CM) and simultaneously modified with DNA photoacoustic probe and fuel strand. The target miRNA triggered the release of the near-infrared fluorescence strand from the quenched strand and the fuel strand further replaced the target miRNA. The alternate replacement of target miRNA and DNA fuel strand generated an amplified photoacoustic signal ratio to realize the miRNA detection.

Surface-enhanced Raman spectroscopy (SERS) refers to that when molecules approach the metal surface of nanostructures, the Raman signal of molecules is enhanced. Gold nanoparticles with the core-satellite structure are important SERS substrate structures, which are composed of a single Au (or Ag) inside and multiple Au (or Ag) linked outside. The core-satellite structure is combined with SDR to detect miRNA and produce the SERS signal.

Li *et al.* [21] designed the core-satellite plasma rulers (PRs) for the quantitative determination of miRNA-21 in living cells. Several small gold nanoparticles were assembled on large gold nanoparticles with dsDNA to form a core-satellite. MiRNA-21 initiated a single hybridization event, leading to the destruction of core-satellite structural components. The plasma displacement between the core and the satellite initiated the wavelength changing of scattering intensity. The expression and the amount of miRNA-21 could be detected via statistical analysis of the wavelength signal. This method enabled highly sensitive detection of the intracellular miRNA without side effect.

Gold nanorods have strong and controllable plasma resonance properties, which can be widely used in photothermal therapy and amplification detection of miRNA. Qu *et al.* [22] used the assembly of Ag₂S on the surface of platinum (Pt)-modified gold nanorods (AuNR-Pt) to form a nuclear-satellite structure (AuNR-Pt@Ag₂S). The probe can complete the near-infrared imaging of miRNA *in vivo*. Target miRNA bound to the complementary DNA sequence and released the strand modified with Ag₂S, thereby destroying the core-satellite structure and recovering the fluorescence. The LOD was 0.0082 amol ng_{RNA}⁻¹, providing a multifunctional nanoplatform for tumor diagnosis and treatment *in vivo*.

Inspired by Qu's work, Yan *et al.* [23] utilized the electrostatic interaction between polyethylenimine (PEI)-modified gold nanorod and nucleic acid probe to construct a Fuel Improved miRNA Explorer (FIRE) sensing platform. The detection system included a double-strand DNA labeled with BHQ-2, Cy5 fluorescence-quenching group and a fuel strand that realizing the cycling of miRNA for signal amplification. The target miRNA triggered the release of dsDNA to recover fluorescence. In this design, the preparation of FIRE is simple via electrostatic interactions. Moreover, AuNRs enhanced the photoacoustic imaging and real-time monitoring capabilities of FIRE, resulting in sensitive detection of miRNA and effective tumor treatment effect.

In addition to the strand displacement reaction catalyzed by fuel strand, toehold-mediated strand displacement is another commonly used amplification strategy. A novel catalytic self-assembly nanosensor based on quantum dots was constructed [24] to detect miRNA *in vivo*. As an efficient catalyst, miRNA triggered the toehold-mediated strand displacement cascade reaction to produce multiple Cy5/biotin double-labeled dsDNA, which were assembled on a 605QD surface, resulting in significant FRET signal. The nanosensor was simple to operate and has high specificity and sensitivity. Also, the synergistic effect between telomerase and the catalytic strand was helpful to distinguish cancer cells from normal cells, which provided a valuable method for clinical diagnosis.

Enzyme-mediated strand displacement reaction enables the exponential accumulation of DNA products through the continuous polymerization-nicking-displacement cycle process catalyzed by polymerases. Based on the amplification

methods, Yang *et al.* [25] proposed a novel strategy by utilizing branched DNA ligation enhanced isothermal strand displacement polymerization (B-ICSDP) for *in vitro* quantitation and intracellular miRNA imaging. This internal Y-shaped DNA structure consisted of a circular DNA scaffold and three repeatable molecular beacons (MB). MiRNA triggered the conformation conversion of MB and recovered the fluorescence. Additionally, polymerase-based cyclic SDR produced a large number of extended Y-type DNA structures (one DNA scaffold could hybridize with three MBs), thus generating amplified signals. This method had the advantages of high sensitivity, high specificity, and simple design. Besides, it could be used to detect single or multiple target miRNAs in living cells.

Peng's team [26] developed a telomerase-catalyzed FRET ratio probe for accurate miRNA detection. AuNPs were modified with capture probe containing recognition sequence and telomerase primer located at the 5' of capture probe strand. The detection probe (a molecular beacon labeled with donor FAM and acceptor TAMRA) hybridized with the capture probe, separating the fluorescent donor and acceptor and causing low FRET signal. Once miRNA specifically recognized and hybridized the capture probe. The detection probe was then replaced by miRNA to form a stem-ring structure. Thus, the FAM and TAMRA were brought in close proximity to produce high FRET signal. In addition, the capture probe was extended with telomerase primers and hybridized with the catalytic strand to displace target miRNA. The released miRNA also triggered the above-mentioned detection system. This method had low background signal and can detect low abundance miRNA molecules in living cells.

3.3 Catalytic hairpin assembly (CHA)

Catalytic hairpin assembly (CHA) is an enzyme-free, hairpin fuel-driven, and autonomous nucleic acid amplification technology. A CHA system needs to design two hairpin structures according to the sequence of target miRNA [27]. One segment of the first hairpin is complementary to the target miRNA sequence [28]. Its hairpin structure can be unfolded by miRNA, and then form a complementary structure with another hairpin probe. The target miRNA will be replaced and dissociated, which can further catalyze CHA between other hairpin probes, forming a cycle to generate amplification signal. The catalytic hairpin assembly has been widely used in nucleic acid detection due to its enzyme-free and target-recyclable advantages.

Like SDR, CHA usually employs nanomaterials as a scaffold and carrier to deliver DNA probes into living cells. As shown in **Figure 3**, Liu *et al.* [29] developed a core-satellite nanoprobe (AuNPs-AuNDs CS). Hairpin1 (H1) was immobilized on the Au nanoparticle surface (AuNPs-H1, satellites) and hairpin2 (H2) was assembled on the surface of the plasmonic gold nanodumbbells (AuNDs-H2, core) via Au – S bonds. MiRNA triggered CHA by targeting AuNPs-H1 (AuNPs-H1-miRNA). AuNDs-H2 then hybridized with AuNPs-H1-miRNA to form a core-satellite nanostructure, whereas target miRNA was displaced from AuNPs-H1-miRNA. The released miRNA originated the next assembly of AuNPs-AuNDs CS, generating an amplified signal. This design engineered metallic nanoparticle aggregates to increase electromagnetic hot spots, thereby realizing the highly-sensitive SERS detection of low abundant miRNA.

Wang *et al.* [30] modified the fluorescence-labeled hairpin H1 to the surface of AuNPs. The fluorescence of H1 was quenched by AuNPs. When the probes delivered into cells, the hybridization between H1 and miRNA unfolded H1. The exposed single-strand domain of H1 induced H2 (hairpin fuel) hybridization and displaced miR-21. The released miR-21 participated in the subsequent reaction cycle. The

Figure 3. Schematic representation of (A) the Assembly process of AuNPs-AuNDs-CS and (B) AuNPs-AuNDs-CS sensing miRNA and imaging in living cells. Reprinted with permission from ref 29. Copyright 2018 American Chemical Society.

sensitivity of hairpin-fuelled catalytic nanobeacons is two orders of magnitude lower than that of noncatalytic nanobeacons.

Aruni *et al.* [31] exploited a genetically encoded means to design a RNA-based CHA circuits for imaging miRNA in living cells. In this design, Broccoli, a specific RNA aptamer for DFHBI-1 T, was used as fluorescence reporter. It was split into two non-fluorescent parts (Broc and Coli). Broc was attached to the 5' end of H1, while Coli was conjugated to the 3' end of H2. The target miRNA initiated CHA and hybridized with H1. The unfolded H1 then hybridized with H2 to displace target miRNA and form dsDNA. The recombinant Broccoli activated the dye DFHBI-1 T to emit fluorescence, and the target miRNA can be recycled to catalyze the fluorescence of ten to hundreds of broccolis, achieving signal amplification. This strategy provided a new opportunity for the application of gene-encoded RNA circuits in cells.

To improve the kinetics and efficiency of CHA in the complex intracellular environment, inspired by spatial-confinement effects of cells, Liu *et al.* [32] constructed a three-dimensional DNA nanocube to implement the localized hairpin-DNA cascade signal amplification. The hairpin H1 and H2 labeled with Cy3 and Cy5 were modified in the cube, respectively. MiRNA could specifically unfold H1 and then unfold H2. The rapid and efficient imaging of miRNA could be monitored

by FRET. This cascade amplifier significantly improved the speed (7 times faster) and efficiency (2.6 times higher) of signal amplification. Furthermore, this probe possesses good cell permeability, good nuclease resistance, and the ability to avoid false-positive signals.

3.4 Hybrid chain reaction (HCR)

The hybridized chain reaction proposed by Dirks and Pierce in 2004 is an isothermal signal amplification technology based on DNA strand displacement reaction [33]. Single strand promoter DNA (target miRNA) binds to the stem-loop nucleic acid probe and causes conformation changing of hairpin DNA. The unfolded hairpin structure can unfold a new DNA hairpin. Two kinds of stem-loop probes were alternately hybridized to form double-stranded DNA containing a large number of repeat units [34]. This method has the advantages of constant temperature, efficient signal amplification, and without the requirement of enzyme. It has been applied to the detection of DNA or RNA.

Wang *et al.* [35] combined CHA with HCR to design a sensing system with six DNA hairpins (**Figure 4**). Target miRNA first catalyzed CHA and formed numerous double-stranded products ($H_1 \cdot H_2$) containing initiator sequences to initiate downstream HCR circuit. The resultant dsDNA products then triggered subsequently autonomous cross-hybridization reactions to form HCR copolymer ($H_3 \cdot H_4 \cdot H_5 \cdot H_6$). The resultant HCR copolymer carries many donor-acceptor pairs that can generate FRET signal. The synergistic amplification effect of CHA-HCR system significantly increased the selectivity and sensitivity of miRNA detection.

Exploiting the signal amplification function of protein with multiple binding sites, Huang *et al.* [36] used streptavidin (SA) as a protein scaffold and four biotinylated hairpin DNA probes to construct a DNA tetrads probe. When miRNA was

Figure 4. Schematic of the isothermal CHA-HCR cascaded circuit for miRNAs assay. Adapted with permission from ref 35. Copyright 2018 Royal Society of Chemistry.

present, miRNA unfolded the Cy3-labeled hairpin H1, triggered HCR, and hybridized with Cy5-labeled H2 to form a cross-linked hydrogel network, generating the signal. It is proved that the DNA tetrad was a highly robust delivery agent and could realize the sensitive imaging of miRNA.

To improved the stability of miRNA sensing system, Wu and coworkers [37] designed a DNA probe composed of tripartite Y-shaped DNA structures, folate probe FAP and hairpin probe H1, H2. MiRNA triggered H1 and H2 hairpin probes to assemble HCR, separating Cy5 and BHQ-2 labeled on H1 to recover fluorescence signal. This method was proved to have high sensitivity with a sub-picomolar limit *in vitro*, and the probe had high stability *in vivo*.

3.5 Entropy-driven DNA catalysis (EDC)

Entropy-driven DNA catalysis (EDC) exponentially amplifies DNA signal by target-induced entropy change of pre-design sensing system [38]. EDC is a simple, rapid, and enzyme-free isothermal signal amplification technology based on toehold exchange mechanism and adaptable to different low-abundance targets due to its modular design and tunability.

A EDC system usually is composed of a three-strand substrate complex (output strand and signal strand are complementary with link strand) and a fuel strand [39]. In the absence of targets, the sensing system does not work because the toehold domain in substrate that binds to the fuel strand has been protected. As a catalyst, miRNA can combine with the substrate link strand to replace the signal strand, and then the fuel strand replaced miRNA to be recycled. The production of liberated molecules leads to the increase of entropy, repeating the abovementioned strand displacement reaction to generate amplified signals.

Liang *et al.* [40] constructed an entropy-driven DNA nanomachine. A three-stranded substrate complex (A/B/C) and an affinity ligand (L) were modified on the AuNP surface, respectively. Target miRNA hybridized with L to replace B from C. A fuel strand (F) bound to C and the C-F complex departed from the AuNP surface, restoring the fluorescence of the FAM-labeled C strand. Thus, such entropy-driven catalytic DNA nanomachine operated automatically and progressively to realize signal amplification. The assay had superior sensitivity (LOD = 8 pM) due to the accelerated intramolecular reaction.

To avoiding the addition of external enzyme or fuel transfection, Lu *et al.* [41] developed a NIR-controlled DNA sensing system based on entropy-driven catalysis to detect intracellular miRNA. Hollow copper sulfide nanoparticle (HCuSNPs) served as the photothermal conversion agent and a carrier. An entropy-driven DNA probe and DNA fuel were conjugated to HCuSNPs. Under the irradiation of the near-infrared laser, target miRNA-155 recognized toe1 and combined with the probe, replaced Cy3-DNA and exposed toe2 that initiates toehold-mediated strand displacement reactions. Cy3-DNA was released and its fluorescence was recovered. This method possessed facile design and its sensitivity is two orders of magnitude higher than that of molecular beacons (MBs).

3.6 DNAzyme-mediated assays

DNAzyme is a kind of DNA with catalytic function and structure recognition ability. It was screened by Breaker and Joyce through the systematic evolution of ligands by exponential enrichment (SELEX) technology in 1994. The single strand, simulating the function of enzymes *in vivo*, can catalyze different chemical reactions, including nucleic acid cleavage, nucleic acid ligation, phosphorylation, porphyrin metallization enzyme activity, and peroxidase activity. It has high

catalytic efficiency, simple modification of fluorescent dyes, and strong chemical stability. Also, compared with the traditional protease, DNAzyme can be denatured repeatedly and renatured without loss of enzyme activity. DNAzyme catalytic amplification technology is a constant temperature amplification technology, which is especially suitable for high sensitivity detection of intracellular targets.

Wu *et al.* [42] constructed a signal-enhanced split DNAzyme probe loaded on gold nanoparticles for miRNA detection in living cells. They split Mg^{2+} -dependent DNAzyme into two nucleic acid strands, which were hybridized with the substrate to form a complex. The fluorescence of the complex was quenched without target miRNA. In the presence of miRNA, two split strands hybridized with target miRNA to form a secondary structure with catalytic activity, cleaving the substrate to separate fluorescence reporter and quenching groups and restoring fluorescence. The released miRNA targeted the next DNAzyme probe and switched on recognition-cleavage-release cycles to produce signal amplification. In this experiment, split DNAzyme serves as both recognition element and signal reporter. As a carrier, gold nanoparticles increase the biological affinity of nanoprobe and avoid the degradation of the nucleic acid probe in the process of transport into cells. This method improved the detection sensitivity and specificity. Additionally, it had low cytotoxicity, high enzymatic degradation resistance which is effective for detection in living cells.

Yang *et al.* [43] integrated DNAzyme, its substrate, and recognition strand into a FAM-labeled hairpin-locked-DNAzyme probe. The probe was immobilized on surface of gold nanoparticles. The catalytic activity of DNAzyme was inhibited and the fluorescence of FAM was quenched by gold nanoparticles. When target miRNA hybridized with the hairpin probe, the change of the probe structure activated the DNAzyme to cleave the substrate strand and made the FAM-labeled substrate strand emit fluorescence. After the miRNA was released, it entered the next cycle and generated amplification signals. This design significantly reduced fluorescent background signal. The detection limit of the target miRNA was 25 pM. It can be applied to the *in vivo* detection of different types of miRNA.

Although AuNP-DNA probes are highly sensitive and selective, they suffer from the aggregation of AuNPs in the complex intracellular environment. To overcome this limitation, there is highly desirable for homogeneous DNA (composed entirely of DNAs) sensing system. Xue *et al.* [44] utilized a Y-shaped backbone-rigidified triangular DNA scaffold (YTDS) to develop a self-powered DNAzyme walker (**Figure 5**). This sensing platform consists of YTDS (carrier), nuclide aptamer (transportation), and a locked M-DNAzyme-substrate complex (recognition and signal reporter). The binding of miRNA trigger DNAzyme walker to perform self-powered stepwise walking and amplify the signal at the same time. The detection platform has the advantages of efficient delivery without any transfection agent and amplification of output signal without any protein enzyme.

To effectively protect the probe from degradation by nuclease and greatly improve its cell permeability, Li *et al.* [45] constructed a DNAzyme probe based on the tetrahedral nanostructure. FAM and Dabcyl were labeled the linker strand and partial complementary strand, respectively. Catalytic activity of DNAzyme was effectively silenced by the locking strand in the absence of target miRNA. The target miRNA hybridized with the locking strand to release DNAzyme. With the assist of Na^+ cofactor, the substrate strand was cleaved and the fluorescence was recovered. Activated DNAzyme could compete with inactive DNAzyme for the next locking strand, starting the next hybridization, and generating amplified signals circularly. The LOD of the DNAzyme probe is 16 pM. It possessed high specificity and distinguished target miRNA from its family members.

The catalytic activity of DNAzyme depends on the concentration of its cofactor Mg^{2+} . However, the content of Mg^{2+} in the cell is too low to support the long-time

Figure 5. (A) The preparation routes of Ap-YTDS-DzW. (B) Ap-YTDS-DzW imaging of miRNAs in vivo. Reprinted with permission from ref 44. Copyright 2019 American Chemical Society.

catalytic reactions used for the signal amplification. To circumvent this limitation, Wei *et al.* [46] constructed a smart autocatalytic DNAzyme imaging machinery to execute magnetic resonance imaging (MRI) of miRNA *in vivo*. The imaging system composed of honeycomb-like MnO₂ nanosponge (hMNS), HCR, and autocatalytic DNAzyme. In this system, hMNS act as three roles, that is, nanocarrier, DNAzyme cofactors, and MRI agents. The multifunctional hMNS effectively delivered the system into the cells and was degraded into Mn²⁺ by intracellular glutathione (GSH) as a DNAzyme cofactor. MiR-21 activates HCR amplification to produce DNAzyme nanowires, mediating the automatic catalytic accumulation of the new trigger and the retroaction to the original HCR sensor. This robust assay can accurately locate miRNAs *in vivo* and enhance the amplification signal.

3.7 CRISPR-mediated assays

CRISPR/Cas9 system is a new gene-editing technology based on the bacterial adaptive immune defense system. It can insert or delete genes accurately to knock out target genes [47]. CRISPR/Cas9 system consists of CRISPR RNA, transactivating crRNA, and endonuclease Cas9. Cas9 is specific to the protospacer adjacent

motif with the guidance of crRNA and tracrRNA to form RNA–DNA complex. Cas9 can cut double-stranded DNA to complete gene editing.

For the convenience of operation, scientists fused the mature tracrRNA-crRNA dual structure into a sgRNA, its 5' strand sequence is complementary to the target miRNA, and the 3' stranded structure could bind to Cas9. Therefore, only one sgRNA needs to be designed to edit the related genes. This method has some advantages such as simple operation, high efficiency, low cost, and no introduction of foreign genes. So far, it has become the most popular gene-editing technology. Similarly, this method has been applied to the detection of miRNA.

Wang *et al.* [48] constructed a CRISPR–Cas9 sensing platform based on a miRNA-mediated sgRNA releasing strategy. The sensing approach successfully realized miRNA imaging and cell-specific regulation of the CRISPR gene editing system. The sensing system was divided into two parts. One part was composed of Cas9 or Cas9 mutant fusion protein to edit or inhibit gene expression, the other was sgRNA with miRNA binding site without activity, mediating the binding of Cas9 protein in a specific position of the genome. As a result, only specific miRNA could finish cleavage reaction, producing mature sgRNA, and starting CRISPR system. The system could sense two kinds of miRNAs in the same cell and turn on red or green fluorescence respectively. It was proved that the detection platform could accomplish precise regulation of different gene sites by multiple miRNAs, and provided new ideas for other gene therapy technologies.

4. Conclusions

An ideal method for detecting intracellular miRNA should possess high throughput, high specificity, high detection sensitivity, wide detection range, and low detection cost. To achieve this goal, a variety of miRNA detection methods have been developed, but there are many shortcomings, and the technology needs to be improved. The additionally introduced nanomaterials is self-aggregated and enriched in different tissues in a complex living environment. Hybridization probe assay lacks signal amplification capabilities. SDA, HCR, and CHA need to avoid high background signal caused by probe leakage. DNzyme-mediated assays usually require exogenous cofactors to initiate signal amplification. Therefore, EDC and CRISPR-mediated assays are the most promising detection methods of miRNA *in vivo* in the future. The establishment of an ideal miRNA detection technology still needs the efforts of scientists and the continuous progress of related science and technology.

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Conflict of interest

There are no conflicts to declare.

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