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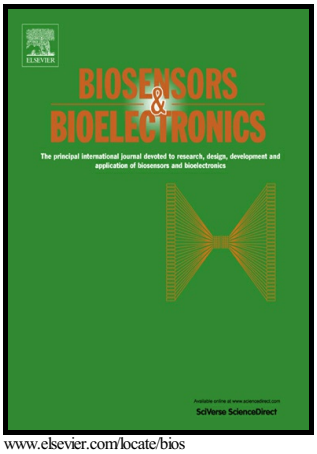

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An innovative paradigm of methods in microRNAs detection: highlighting DNAzymes, the illuminators

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Contents

Abstract

miRNAs are short regulatory sequences that monitor gene expression at post-transcriptional level. miRNAs play outstanding roles in cellular processes and their altered expression or aberrant function has been reported in diseases such as cancer. Furthermore, some of them serve as biomarkers for prognosis, diagnosis, and prediction of drug effectiveness as well as probable utility in the field of personalized medicine. As a result, proper and sensitive detection of miRNAs is a crucial matter. Traditional approaches including Northern blotting, Microarray, and Quantitative reverse transcription polymerase chain reaction have been extensively employed by means of miRNA detection for decades. However, these methods lack sufficient sensitivity, specificity and are labor intensive. In the recent years, DNAzyme-based assays have become strikingly attractive. The principal element in these techniques is a G-rich sequence, called the G-quadruplex, which turns into a DNAzyme with catalytic activity by addition of hemin, and subsequently allows simple and rapid detection of the target. In this review, different methods of miRNA detection, along with related advantages and disadvantages are discussed, with a special focus on DNAzymes and DNAzyme-derived methods.

Keywords: DNAzyme; Biosensor; miRNA; Detection

1. Introduction

microRNAs (miRNAs) are a type of small noncoding RNAs primarily found in *Caenorhabditiselegans* (lin-4 and let-7) in the early 1990s (Wang et al. 2016a). Genes encoding these ~22 nucleotide RNAs (Ha and Kim 2014) comprise about 1-3% of the genome in mammals (Wang et al. 2016a). In fact, there are more than 1000 miRNA encoding genes identified (Calin and Croce 2006; Lin and Gregory 2015; Wang et al. 2016a) which can be found on every chromosome except Y chromosome (de Planell-Saguer and Rodicio 2011). They serve a variety of roles in biological processes (Deng et al. 2009; Tie et al. 2009), including cell proliferation, differentiation, apoptosis and development (Calin and Croce 2006). Considering their vital role in regulation of gene expression, miRNAs could be associated with pathological conditions including psychiatric (Miller and Wahlestedt 2010) and neurodevelopmental disorders (Xu et al. 2010), cardiovascular diseases (Edwards et al. 2010) as well as development of tumors through genome instability, invasion, angiogenesis (Lin and Gregory 2015) and oncogenesis (Calin and Croce 2006). For example, altered expression of miRNA was first identified in B-cell chronic lymphocytic leukemia (CLL) (Calin and Croce 2006; Lee et al. 2003). Furthermore, some miRNAs are found to have oncogenic or tumor suppressive effects (Esquela-Kerscher and Slack 2006): miR15a and miR16-1 act as tumor suppressors while miR155 and miR-17-92 cluster act as oncogenes (Calin and Croce 2006; Zhang and Cui 2013).

These diverse and significant roles of miRNAs have turned them into valuable elements (biomarkers) in prognostic (de Planell-Saguer and Rodicio 2011; Segura et al. 2010), predictive (de Planell-Saguer and Rodicio 2011), diagnostic and therapeutic approaches. With regard to the wide applicability of miRNA as biomarkers, it sounds possible to employ them in personalized medicine(Wang et al. 2016a). Recently, tumor-derived cell-free miRNAs have attracted so much attention as biomarkers; these miRNAs are found in body fluids such as urine (Iorio and Croce 2012), saliva and plasma (Kosaka et al. 2010) and own valuable characters including remarkable stability (Auvinen 2017a), presence of tissue-specific sources, profusion (Kosaka et al. 2010; Laterza et al. 2009), resistance to degrading RNase enzymes (Blanco-Calvo et al. 2012; Zhu et al. 2014b) and non-invasiveness. They can also impact cancer at different levels, either at initiation or progression (Gilad et al. 2008; Swarup and Rajeswari 2007; Zhao et al. 2011). A list of miRNAs involved in diseases and cancers have been shown in Table 1.

<Please insert Table 1 here>

Table 1 miRNAs involved in diseases

Diseases	Up-regulated miRNAs	Down-regulated miRNAs	References
Glioblastoma	miR-21, -132, -140, -155, -182	miR-422, -129-1, -7, -15b, 23b, -32, -95, -101	(Liang et al. 2016; Mobarra et al. 2017; et al. 2016; Yang et al. 2014)
Breast cancer	miR-21, -155, -182, -10b, -9	miR-145, miR-200, -205, -19a, -335	(Banno et al. 2014b; Iorio et al. 2005; V and Luo 2015)
Ovarian cancer	miR-200a, -200b, -200c	miR-15, miR-16, -140, -145	(Banno et al. 2014b)
Colorectal cancer	miR-21, -92a, -221a, -18a	miR-140, -195, -613, -128	(Kim 2017; Ren et al. 2015)
Lung cancer	miR-21, -17~92 cluster, -155, -92a-2	miR-200 family, miR-34 family	(Inamura and Ishikawa 2016; Zhang an 2016)
Gastric cancer	miR-21, -223, -421, -200c, -370, -34a	miR-218, -375, -195-5p, let-7a	(Zhu et al. 2014a)
Pancreatic cancer	miR-21, -155, -221-, -210, -145	miR-217, -141, -29c, -26a	(Li and Sarkar 2016; Yonemori et al. 2
Hepatocellular cancer	miR-101, -221, -222, -223, -224, -122	miR-650, -16, -199a, -29b, -223-3p	(Li et al. 2015a)
Prostate cancer	miR-21, -93, -25, -141-, 148a, -103	miR-221, -222, -205, -145, -125b, -15, -16,	(Bonci et al. 2016; Fabris et al. 2016; K and Lupold 2016)
Esophageal cancer	miR-224, -21, -192, -194, -10b, -151	miR-375, -203, -205, -145, -100, let-7c, -377	(Gu et al. 2013; He et al. 2015; Li et 2017a)
Cervical cancer	miR-21, -16, -155, -183, -146, -215	miR-143, -149, -145, -126, -424, -1, -7	(Banno et al. 2014a; Sharma et al. 20
Skin cancer	miR-184, -205	miR-203, -204	(Ruland and Florea 2014)
Melanoma	miR-150, -425, -210	miR-206, -16	(Armand-Labit and Pradines 2017)
Bladder cancer	miR-183, 17-5p, -20a, -96	miR-199, -195, -145, -195, -125b, -199a, -143	(Enokida et al. 2016; Ruland and Florea Yoshino et al. 2013)
Head and neck cancer	miR-181, -31, -10b, -24	miR-125a, miR-200a	(Lan et al. 2015)
B cell lymphoma	miR-210, -16-1, -155	miR-34a	(Lan et al. 2015)
Oral cancer (OSCC)	miR-31, -223, -29b, -203, -144	miR-125a, -200a, -375, -16	(Manikandan et al. 2016; Siow et al. 2 Yoshizawa and Wong 2013)
Leukemia	miR-181b-5p, 129-5p, 155-5p, 320d	miR-233, -29c	(Lan et al. 2015)
Autism spectrum disorder	miR-142-5p, -451a, -21-5p, has-miR-21-3p	hsa_can_1002-m	(Mor et al. 2015; Wu et al. 2016c)
Hepatitis B	miR-21, -801, -192	miR-223, -122, -27a, -26a	(Auvinen 2017b)
Cardiovascular Diseases	miR-21, -125, -195, -208, -24, -212	miR-150, -30, -1, -29	(Ono et al. 2011)
Myeloproliferative disease	miR-451, 29a, -125	miR-146a, -150, -155,	(Christopher et al. 2016; Palanichamy an 2014; Zhan et al. 2013)
Diabetes	miR-152, -181a, -24, -210, -27a, -25, -9, -510	miR-191, -200, -24, -720, -151-3p, -1275	(Assmann et al. 2017; McClelland a Kantharidis 2014; Wang et al. 2016a

1.1. The importance of miRNA detection

Considering the prominent roles of miRNAs in the regulation of gene expression and cellular processes, their potential use as biomarkers as well as the disease-causing impact resulted by their deregulation, it is necessary to adopt proper detection methods. However, regardless of the method in use, some characteristics of miRNAs emerge as obstacles of proper detection: miRNAs are only about 22 nucleotides long and therefore, with such a small size, it is quite difficult to design appropriate hybridization probes (Zhang and Cui 2013). In addition, sequence similarity among the members of the same family and successful detection of low-abundant miRNAs requires sensitive methods (Shen et al. 2015). miRNAs do not normally share a common sequence. As a result, Selective purification of miRNAs based on a general sequence is not practical. Moreover, normalization of microarray is a serious issue due to the fact that miRNAs from different families are not equally expressed nor have they the same GC proportion (Benes and Castoldi 2010; Zhang and Cui 2013). Besides, it is hard to differentiate pre-miRNAs from the mature ones (Catuogno et al. 2011). To be more specific, it is laborious to comprehend if the generated detection signal is attributed to the mature miRNA, the pri-/pre- miRNA or even other small noncoding RNAs (Zhang and Cui 2013). Finally, various and distinct expression levels of miRNAs in different cells highlights the need for the development of assays applicable to a wide extent as well as simultaneous detection, respectively (Zhang et al. 2015b). It is also noteworthy to mention the impact of sampling and RNA extraction on the final result (Zhang et al. 2015b); the expression data obtained from miRNAs extracted by different methods are fairly similar. However, it is likely that extraction affects the expression profile, especially in case of microarray assays (Ach et al. 2008).

1.2. An overview of miRNA detection methods

Up to the present moment, several detection methods have been employed, including Northern blotting (Koscianska et al. 2011; Varallyay et al. 2008), Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) (Mocellin et al. 2003), Microarray (Shingara et al. 2005), Sequencing (Iorio and Croce 2012), Cloning (Mattie et al. 2006), in situ Hybridization (Nelson and Wilfred 2009; Soe et al. 2011), Surface-enhanced Raman Scattering (SERS) (Muniz-Miranda et al. 2010), Surface Plasmon Resonance (SPR) (Nasheri et al. 2011; Sipova et al. 2010), Bioluminescence-based(Bray et al. 2011), Fluorescence-based(Zhang et al. 2010) and Electrochemical methods (Hamidi-Asl et al. 2013) as well as Capillary Electrophoresis (CE) (Ban and Song 2014). Each method has pros and cons and as a result, different factors such as sample quantity, expenses, accuracy and the goal of detection should be taken into account in the employment of the proper approach. Recently, new biosensor-based methods in terms of miRNA detection, which employ DNAzymes, have attracted enormous attention. The horse-radish-mimicking DNAzymes simply allow target detection through catalyzing a color-producing reaction. These methods

have been attractive to researchers due to various reasons: they exclude the need for amplification prior to detection. Furthermore, these methods do not require complex equipment and reduce on expenses remarkably. Besides, they allow sensitive, simple and high-speed detection (Shi et al. 2014; Zhu et al. 2010). According to various references and to our knowledge, "in situ hybridization" and "capillary electrophoresis" were not classified under "conventional" or "emerging" methods. On the other hand, some methods might have evolved long ago, yet recently established and adapted for miRNA detection. Therefore, in this review, we present a new classification border for detection methods in the text as well as in Table 2, with emphasis on DNAzyme-based strategies as they are the main focus of this article.

<Please insert Figure 1 here>

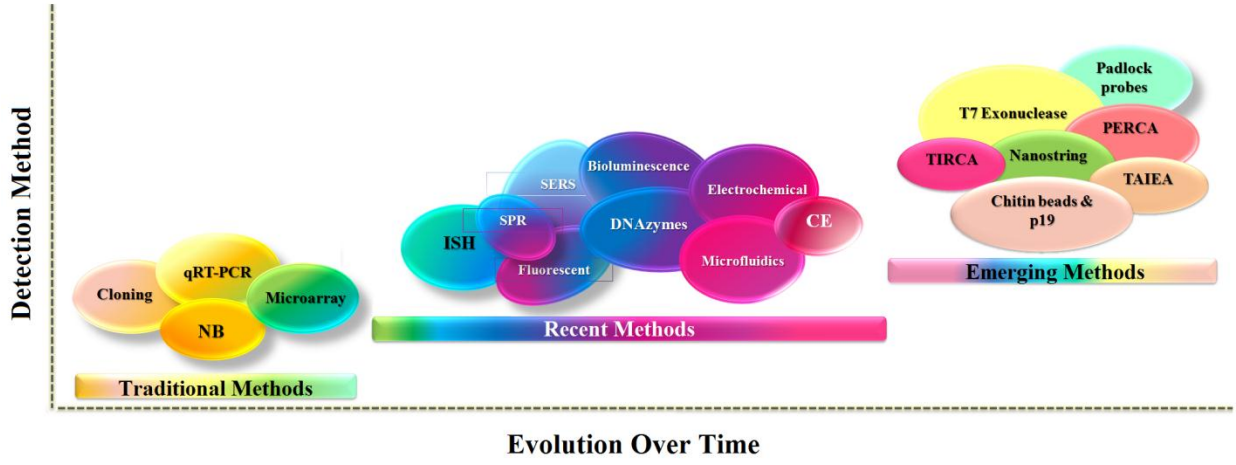

Fig. 1 An overview of miRNA detection methods

2. Traditional Methods

2.1. Hybridization-based Methods

2.1.1. Northern Blotting

Northern blotting is the most common primary method in profiling miRNA expression. This technique is employed for the determination of miRNA size as well as confirmation of expression data obtained from other methods (de Planell-Saguer and Rodicio 2011). Northern blotting procedure involves separation of RNA molecules based on their size and their subsequent transfer onto a membrane, followed by hybridization-based detection via labeled probes (Hamidi-Asl et al. 2013; Zhang et al. 2015b). Various systems have been developed for probe labeling; from the incorporation of radioisotopes, which are unsafe to both the creatures' health and the environment, to the utilization of non-radioisotope labeling by digoxigenin (a type of steroid from foxglove plant, detected by its specific antibody) or biotin (a type of vitamin B, detected by streptavidin with highly affinity)(P. Clark and Pazdernik 2013). In comparison to

radioisotope-labeled probes, digoxigenin-labeled ones are safer and offer the same amount of sensitivity as the radioisotope ^{32}P-incorporated probes. As a solution to low sensitivity, one can perform RNA cross-linking to the membrane, using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), which leads to an increase in efficiency of detection up to 50 times higher (Pall et al. 2007; Yang et al. 2009). It is also practical to implement improved hybridization strategies [e.g. Locked nucleic acids (LNA)] (Stenvang et al. 2008; Valoczi et al. 2004). It is noteworthy that in probe-based detection techniques such as northern blotting, microarrays and in situ hybridization, probe designing is a dilemma. With regard to this matter, utilization of LNA and Peptide nucleic acids (PNA) probes has brought great specificity into probe-based methods. LNAs are stable and nontoxic in biological systems and they also provide sensitive detection of miRNAs which differ by only one nucleotide with no selection bias for nucleotides. Moreover, LNA monomers can combine with other monomers to form new structures. LNAs are successfully used in miRNA detection via in situ hybridization (Stenvang et al. 2008).

Another type of probe that provides enhanced hybridization detection is PNA. PNAs are DNA analogs with an electrically neutral backbone (instead of negatively-charged phosphate) that prevent repulsion between the probe and the target. Therefore, they own higher specificity and affinity for target hybridization compared to DNA probes (Kim et al. 2012). They are also more stable and since they augment melting temperature, PNAs are capable of target discrimination by a single nucleotide (Fabani et al. 2010; Kim et al. 2012).

No matter the enhanced sensitivity, northern blotting is still behind the standards of a clinically practical technique. The reason is attributable to deficiencies such as the requirement of the sample in high abundance (Chamnongpol et al. 2010; Dangwal et al. 2012). Besides, this technique is time-consuming (Hunt et al. 2009) and involves multiple experimental steps (de Planell-Saguer and Rodicio 2011). Nonetheless, northern blotting also has issues that constrain its applicability in diagnostic practice; due to the small size of miRNAs and their low abundance in a total extract of RNAs, this method is not sensitive enough for miRNA detection (Pall et al. 2007). It should also be noticed that northern blotting is much of a low throughput method (Pall and Hamilton 2008).

2.1.2. Microarray

Among various methods in miRNA detection, microarray has been used to a great extent for many reasons; it allows multiple high-throughput detection of miRNA(Wegman and Krylov 2013) and it can be applied to the identification of mature and precursor miRNA. This method is especially effective for miRNA expression profiling for numerous targets. Basically, all oligonucleotide-based arrays require well-designed and properly labeled probes and for throughput enhancement, signal detection should be carried out with caution (de Planell-Saguer and Rodicio 2013). There are generally two common

approaches for labeling; enzymatic and chemical. The latter comprises direct incorporation of labels, which is beneficial for small miRNAs, as well as fluorescent dyes (mostly common in the parallel analysis) and radioisotopes. One popular enzymatic approach involves utilization of T4 RNA ligase in two ways; the addition of a short oligonucleotide or a single nucleotide labeled fluorescently (Zhang et al. 2015b). Regarding difficulties with fluorescent probes, label-free probes are suggested as proper alternatives [For more information, please refer to (Duan et al. 2011)].

Labeling can be exerted by addition of chemical groups (Pritchard et al. 2012). Even so, it is important to bear in mind that in this labeling method, some of the sequences with 3' end modifications do not affect chemical modification. Besides, there might be nucleotides of preference as targets of labeling (Wark et al. 2008).

A modification in the labeling procedure is post-hybridization labeling of miRNAs; to be more specific, miRNAs are first added to the microarray chip and are labeled afterward. One of these methods is a new enzyme-based method termed RAKE ASSAY (RNA-primed, array-based Klenow enzyme) provides high-throughput detection of miRNA. In brief, this technique involves hybridization of a capture probe connected to the surface of a slide, with miRNA population. Afterwards, the slide is treated with DNA exonuclease I in order to digest unbound probes. Subsequently, the Klenow fragment and dATP nucleotides are added, so the miRNA acts as a primer, followed by extension via Klenow activity. Upon approaching triple thymidines, biotin-dATPs are incorporated as complementary nucleotides for thymidines. As the final step, detection of hybridized pairs is carried out through fluorophores conjugated with streptavidin. Fluorescent signaling allows quantification of the miRNA of interest. The RAKE assay bypasses signaling bias by incorporating two sequence-independent enzymes, DNA exonuclease I and Klenow. Moreover, it is possible to analyze samples containing low concentrations of the target miRNA. Nevertheless, cross-hybridization still remains a setback in this assay (Nelson et al. 2004; Qavi et al. 2010).

In order to overcome labeling issues, implementation of label-free approaches has been proposed. Some of these methods rely on labeling a probe instead of the miRNA(Lee et al. 2010; Qavi and Bailey 2010) and some of them take advantage of stacking hybridization (Shen et al. 2012).

As an alternative to the two-dimensional microarray, magnetic bead-based platforms and one type of universal tagged probe can be implemented for direct detection of miRNAs. A valuable advantage of this method is the elimination of amplification or labeling as well as dependence on stacking hybridization, which provides us with fast and yet specific and sensitive discriminative detection of pre-miRNA from mature miRNAs [more info;(Jiang et al. 2012)]. Compared to conventional microarrays, bead-based

hybridization offers a rapid, more specific and less expensive tool for miRNA detection (validation) (Chaudhuri and Chatterjee 2007); a brilliant example is the method introduced by Lu et al. (2005), according to whom, flow cytometry technique can be adopted for miRNA expression profiling, especially in cancer. The procedure, in brief, involves hybridization of target miRNAs to capture probes linked to beads coated with carboxylated 5-micron polystyrene. These beads are saturated with fluorescent dyes in such a way that implementation of only two dyes results in the production of a diversity of possible combinations so that up to 100 colors can be generated, each of which is indicative of an individual miRNA. Upon linking adaptors the 5' and the 3' ends of miRNAs, amplification with RT-PCR using a biotinylated primer and attachment of PCR product to beads and applying streptavidin-phycoerythrin as a dye, the analysis is performed via flow cytometry (Lu et al. 2005).

Microarray allows high-throughput and simultaneous detection of the target (Yan et al. 2013). However, the considerable efficiency of this technique does not override its limitations. Data obtained from microarray require further confirmation by other techniques. Furthermore, two characteristics of miRNAs (shortness and low concentration) bring about problems (Lee and Jung 2011). In addition, microarrays are incapable of determining the copy number or quantity of the miRNA. Also, false-positive results might be demonstrated as a consequence of cross-hybridization. As the last point, Microarray too faces sequence bias and reproducibility issues, similar to RT-PCR (Qavi et al. 2010).

2.2. Amplification-based Methods

2.2.1. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

RT-PCR is an amplification-based method utilized in the assessment of RNA expression as well as miRNA detection. The RNA sequence of interest is converted to complementary DNA (cDNA) and is further amplified by PCR in real time. Ever since the establishment of this method for miRNA detection, several RT-PCR-based modified approaches have been introduced, a few of which are the following:

2.2.1.1. Resolving the short nature of miRNAs; using longer PCR substrates

Schmittgen et al. reported a sensitive and high-throughput method to analyze the expression profile of pre-miRNAs instead of mature miRNAs in different cell lines. This way, because pre-miRNAs were longer compared to the mature ones, the need for designing short primers was surmounted. The results were the same as those obtained via northern blotting (Schmittgen et al. 2004). However, it is not certain if the number of pre-miRNAs would equally correspond to that of mature miRNAs (Qavi et al. 2010).

Raymond et al. introduced a simple and cost-effective assay to detect mature miRNA in which the primers used for cDNA synthesis contain 5' overhangs. The generated product comprises the fusion of cDNA and the tail, which is subsequently amplified by an LNA-incorporated primer in the first cycle of

real-time PCR. During subsequent cycles, synthesis is progressed using universal and LNA-R primers. Finally, detection takes places using SYBR Green. The major advantage of this method reverts to its ability in the differentiation of miRNAs that belong to the same family (Raymond et al. 2005).

2.2.1.2. Resolving the short nature of miRNAs; enzymatic approaches

Some enzymatic approaches involve the addition of adaptors to the termini of miRNAs using T4 ligase, in order to extend the length and simplify detection (Barad et al. 2004; Miska et al. 2004). Nonetheless, the preferable enzymatic activity of T4 towards some targets over the rest is actually a drawback which leads to error-prone measurement (Qavi et al. 2010).

A proper replacement for adaptors is poly (A) tails located at the 3' end of the target using poly (A) polymerase. The substrates are further amplified using primers with poly (T) tails (Gu et al. 2012; Qavi et al. 2010). This method was later developed by incorporation of LNA primers, which rendered a more specific and sensitive approach for miRNA detection (Andreasen et al. 2010).

2.2.1.3. Resolving the short nature of miRNAs; stem-loop primers

Utilization of stem-loop primers excludes the need for enzymatic reaction. These primers contain complementary sequence to the 3' of the miRNA. The antisense half of the stem-loop is composed of a sequence in the pre-miRNA and comprises the 5' end of the primer. Stem-loop primers offer more sensitivity and specificity compared to linear primers, which is due to base stacking forces as well as a spatial hindrance (Varkonyi-Gasic et al. 2007). Further entailment of the primer would also enhance the melting temperature, which improves detection by eliminating signal bias (Qavi et al. 2010).

A combination of stem-loop and enzymatic reaction was adopted by Li et al., in which two separate stem-loop probes were utilized; in case they both hybridize to the target miRNA, T4 ligase will subsequently proceed extension step. The prominent advantage of this method, which enhances specificity, is the fact that the enzymatic reaction takes place only when the target miRNA is available to probes (Li et al. 2009).

The need to assay multiple miRNAs simultaneously further paved the way towards the establishment of multiplex approaches. However, there were obstacles including the requirement of several sets of primers as well as the necessity of implementing an approach in order to identify each sequence specifically. Nevertheless, these obstacles were bypassed to some extent [for more information, please refer to (Lao et al. 2006)].

RT-PCR is advantageous in terms of sensitivity (Gaur et al. 2007), reasonable expenses and accuracy (Jinek and Doudna 2009; Shen et al. 2015). In spite of this, there are challenges that hinder the efficiency of this method; delicate and laborious primer designing as a result of the short length of miRNAs,

erroneous results, low efficiency and difficult data analysis (Dijkstra et al. 2012; Qavi et al. 2010). At best, the primers have the same length as the target. However, if shorter primers are employed, the low melting temperature of the primer-miRNA duplex renders false positive results (Qavi et al. 2010). Another challenge is the sequence homology among members of miRNA family (Shen et al. 2015). Taking these pitfalls into account, it is necessary to be cautious in cDNA synthesis, acquire proper controls, design proper primers and perform normalization of the results correctly (Zhang et al. 2015b). Further improvement of qRT-PCR has been provided in terms of quantification [please refer to "spiked-in"(Hastings et al. 2012)] and cDNA synthesis [please refer to "miRNA-ID"(Ro et al. 2006)].

3. Recent and Contemporary Methods

3.1. Hybridization-based Methods

3.1.1. Deep/ Next Generation Sequencing (NGS)

Sequencing can be implemented for miRNA profiling in both disease cases including FSHD Myogenesis (Colangelo et al. 2014) and in normal humans [in order to define all circulating miRNAs] (Tonge and Gant 2016). In brief, this technique involves preparation of a cDNA library followed by parallel sequencing of cDNAs (Zhang et al. 2015e). Data analysis, preparation of expression profiling, recognition of isoforms and new miRNAs (Motameny et al. 2010). The huge amount of sequences needs to be aligned. Since there are a massive number of them, some software is acquired in order to perform the alignment task. Some of them include Bowtie (http://bowtie.cbcb.umd.edu/) [provides rapid alignment of short sequences], BWA (http://maq.sourceforge.net/) [includes indels in alignment], MAQ (http://maq.sourceforge.net/) [exclusively performs uninterrupted alignment] , SeqMap (http://www.stanford.edu/group/wonglab/jiangh/seqmap/) [allows up to 5 mismatches and offers manual settings for the final format], SOAP (http://soap.genomics.org.cn/) [high-speed alignment and allows up to 3 gaps] and TopHat (http://tophat.cbcb.umd.edu/) [pinpoints splice junctions]. There is a certain need for miRNA profiling, which relies on using different databases such as miRNAMap, deepBase, miRGen 2.0 (Liu et al. 2011) and miRBase(de Planell-Saguer and Rodicio 2013; Eminaga et al. 2013; Liu et al. 2011). The priority of NGS over microarray includes the absence of background noise, the feasibility of discovering novel miRNA detection (Creighton et al. 2009; Hunt et al. 2009) and elimination of cross-hybridization. Furthermore, NGS can be applied to the paraffin-embedded tissue. However, there are several disadvantages to the application of this technique; NGS requires expensive equipment and analysis of newly identified data is laborious (Creighton et al. 2009; Koshiol et al. 2010). Besides, during the preparation of cDNA library restriction cleavage might demonstrate a preferential selection of some miRNAs over the rest. In spite of these drawbacks, NGS is employed for differential detection of

miRNAs in various diseases (Wyman et al. 2009). A list of NGS platforms used in miRNA detection is shown in Table 2.

3.1.2. in situ Hybridization (ISH)

Detection of miRNA is not always limited to quantitative assessment. In fact, it might be necessary to be informed of the localization characteristics of the target in some cases as well (e.g. figuring the role of miRNA under biologic or pathologic conditions). In addition, ISH is able to maintain RNA unity (de Planell-Saguer and Rodicio 2013) and can be further used for multiplex assays (Hanna et al. 2012). As a proper approach, ISH can provide us with miRNA spatial expression profile in histologic sections (Nielsen 2012). There are two platforms for ISH, spatial separation-based (exploits techniques such as electrophoresis in order to separate probe-target duplexes) and non-spatial separation-based (detection is based on alteration of fluorescent signals or other characteristics) (Wegman and Krylov 2013). In order to perform ISH effectively, it is essential to make use of a properly labeled probe [using radioisotopes (Chong et al. 2008), fluorophores(Schneider et al. 2011) or enzymes (Raap et al. 1995)] as well as choosing a suitably stringent environment for hybridization to take place (Kloosterman et al. 2006; Nuovo et al. 2009). Consequently, a combination of LNA probes and imaging was adopted by Pederson et al. (Politz et al. 2006) in order to follow the spatial expression of miRNA-206 in mice. This combination can overcome the low through-put issue of ISH. The so-called LNA-ISH strategy takes advantage of LNAs, RNA analogs that bind their target (DNA or RNA) with high affinity and in a more specific way (de Planell-Saguer et al. 2010) which increases its efficiency (Jorgensen et al. 2010). LNA is also applicable to paraffin-embedded or frozen tissue samples (de Planell-Saguer et al. 2010). Nonetheless, it is not compatible with low concentrations of miRNA. For the more sensitive application of LNA-ISH, two probes can be used in order to detect miRNA, hence, increase detection specificity. These probes are incorporated by fluorophore dyes, which in subsequence, will be detected through laser scanning process, and are able to detect as low as 500 femtomolar amounts of the target miRNA(Guo et al. 2017b). There are also other approaches which detect both pre-miRNAs as well as mature miRNAs benefiting from fluorescently labeled molecular beacon (MB) probes, which can also provide quantitation of the miRNA [for more information, please refer to (Baker et al. 2012)]. Taken together, ISH has the potential to be a diagnostic tool in clinical applications (Wiktor et al. 2006).

3.2. Optical Detection

3.2.1. Surface Plasmon Resonance (SPR)

SPR was first introduced in the 1990s (Nguyen et al. 2015) and is recognized as a surface-sensitive, label-free method used for effective analysis of interplays between biomolecules (Grasso et al. 2015). The procedure of SPR biosensing involves preparation of a surface covered with fixed entrapping tools

(or capture probes) such as enzymes, DNAs, antibodies, etc. Next, the sample is spread across the surface and is followed by molecular interactions between the target and the fixed agents, which leads to alteration of refractive indexes that take place in close proximity to layers made of silver, gold and other metals, changing the SPR angle and providing optical detection of the target (Nguyen et al. 2015). Lately, there has been a wide range of SPR-based methods developed in the field of miRNA detection. Some of which are referred here. Zhang et al. have implemented a sensitive and specific biosensor taking advantage of DNA probes for detection of miR-122 (Zhang et al. 2013). SPR can be used in conjunction with imaging techniques to allow detection by generating signal amplification [termed as surface plasmon resonance imaging (SPRi)] which has been innovated and exploited in the detection of miR-15a by Hu et al. (Hu et al. 2017). Another SPR-based platform was introduced by Šípová et al., according to whom the mentioned method grants quick detection with high sensitivity. This assay puts SPR together with a diffraction grating, hence termed "SPRCD" and brings antibodies into service in order to detect DNA-RNA hybrid duplexes (Šípová et al. 2010). Other strategies on the basis of SPR include: deploying SPR in combination with gold nanoparticles and DNA super sandwich (Wang et al. 2016b), gold nanoparticles-decorated molybdenum sulfide (AuNPs-MoS2) (Nie et al. 2017) and graphene oxide-gold nanoparticles (GO-AuNPs) composites (Li et al. 2017b). SPR biosensor is considered as the gold standard with the ability to screen the interactions of biomolecules as they occur (real-time) (Abadian et al. 2014; Patching 2014). SPR is a high-throughput method, applicable to a wide range of the experimental measurement such as revealing new interactions between molecules (Madeira et al. 2009) as well as facilitating inquiry of the cascade of phenomena in various signaling pathways (e.g. G protein-coupled receptor-mediated cellular response) (Chen et al. 2010). Applicability of SPR to quantitative analysis of samples from body fluids is another remarkable advantage that allows employment of this technique for investigation of clinical cases such as heart diseases, immunity abnormalities, miRNA detection, genetic disorders caused by mutations or single-nucleotide polymorphism (SNP) (Mariani and Minunni 2014), cancer (Uludag and Tothill 2012), Alzheimer's disease (Liu et al. 2012a) as well as viral infections (Jahanshahi et al. 2014). Aside from the sensitive, rapid, wide applicability and the reusable chips, SPR is hindered by a number of issues: data interpretation, non-specific binding, immobilization of the probes or entrapping agents, highly priced chips and the need to employ appropriate controls.

3.3. Other Methods

There are some methods which do not fall under any of the categories mentioned so far. Therefore, a separate title has been dedicated to their introduction.

3.3.1. Capillary electrophoresis

Capillary electrophoresis is one beneficial method in terms of miRNA detection, through which, it is possible to analyze small portions of miRNA samples in order to assess the quantity, interactions, and size of the target with high sensitivity within a short amount of time (Li et al. 2008). This method, in brief, involves the following steps: (i) incubation of probes with miRNAs, (ii) the incubated mix, termed hybridization mixture (HM) is added to a capillary filled with buffer and single-stranded binding protein (SSB). SSBs can only bind ssDNA (unligated) probes, therefore, the probe-target duplexes remain unbound. SSBs accelerate the movement of the probes and as a result, under an electric flow, SSB-bound probes tend to relocate faster. Another utility called "drag-tag" also improves the pace of movement; however, the extent of alteration in the velocity of movement for each duplex varies with each drag-tag. (iii) A detector produces signals in response to hybrid duplexes and unligated probes (signals produced for duplexes differ from those for unligated probes). (iv) Signals are interpreted into graphics in the shape of peaks (Wegman et al. 2015). This method can also be combined with Isotachophoresis (ITP), an assay that allows substrate separation based on the movement of ions under various conditions [for more information on the procedure of ITP-coupled CE, please refer to (Bahga and Santiago 2013)]. Other types of CE including capillary gel electrophoresis (CGE) and capillary zone electrophoresis (CZE) were used in the detection of miRNA and its methylated products (Li et al. 2008) as well as common CE in nasopharyngeal carcinoma (Chang et al. 2008). Moreover, other CE-based approaches have been established: protein-facilitated affinity capillary electrophoresis (ProFACE) (Berezovski and Khan 2013), CE combined with dual-laser-induced fluorescence (CE-dLIF) (Yang et al. 2012), combination of Isothermal exponential amplification reaction (EXPAR) with capillary electrophoresis-based single-strand conformation polymorphism (CE-SSCP) (Na et al. 2017) and capillary electrophoresis – electrospray ionization – mass spectrometry (CE-ESI-MS) (Khan et al. 2016).

CE is used for RNA analysis after synthesis as well as quantitation of miRNA in a mixture of total RNA. It is also possible to perform simultaneous detection of multiple miRNAs using CE-based approaches (Ban et al. 2013; Wegman et al. 2013).In spite of advantages of CE, there are still difficulties to consider, including sensitivity, specificity, and ability to analyze multiple samples simultaneously. CE methods offer reliability, simplicity as well as the ability to detect small amounts of samples. They, however, lack sufficient specificity and sensitivity and simultaneous detection of miRNA (multiple miRNAs or one type of miRNA in multiple samples) is not applicable (Ban and Song 2014).

3.3.2. Microfluidics

Microfluidics-based strategies are among emerging techniques for miRNA detection. The main and meanwhile outstanding priority of these techniques over many others is that they are satisfactorily

consistent with the criteria of point-of-care (Arata et al. 2012), which refers to diagnostic testing of patients without the requirement of complicated facilities (e.g. it can be performed in an ambulance, at home, etc). The microfluidic device offers less time-consuming experiments applicable to small concentrations of samples (Arata et al. 2012). Regarding these advantages, there have been various strategies developed. Among them are the laminar flow-assisted dendritic amplification (LFDA), which involves a 10-minute immunoassay with the LFDA (the overall time of the assay equals ~23 minutes) with a detection limit of 0.15 pM (Hosokawa et al. 2007). It is also made possible to detect the target faster and at femtomolar levels, which provides a sensitive and also portable tool for miRNA detection (Ishihara et al. 2015). Another method termed "microfluidic primer-extension assay (MPEA)" is also based on microfluidics; in fact, MPEA is the combination product of hybridization and enzymatic primer extension on a microfluidic microarray (Beier and Boisguérin 2012), which is capable of detecting various small noncoding RNAs such as miRNAs and piRNAs with noticeable sensitivity. Besides, it does not require labeling prior to hybridization nor amplification (Vorwerk et al. 2008). Microfluidics has also been combined with qPCR in order to achieve the expression profile of the miRNA. One of these methods- "Fluidigm Microfluidics Dynamic Arrays"- introduced by Jen et al. is a sensitive and inexpensive assay which is more effective in terms of throughput as well (Jang et al. 2011). Roy et al. have also reported a sensitive detection method based on microfluidics and microarrays to detect miRNAs using an optical microscope; the considerable resolution and sensitivity of this method make detection possible with only 300 copies of target miRNAs present in nanogram concentrations of RNA samples. On the other hand, independence of this method on labeling amplification processes reveals another attractive dimension of this method (Roy et al. 2011).

3.3.3. *An amplification-based approach in miRNA detection* was introduced by Jonstrup et al., who exploited a specific type of probe called "*the padlock*". Through their terminal sequence, these linear DNA probes can bind two sequences which are in close proximity. In case of accurate hybridization, the enzyme DNA ligase joins the termini of the probe together, which subsequently provides discrimination of the correctly matched duplexes. This process is followed by quantitative amplification of the sequence of interest via rolling circle mechanism using miRNA as the primer. This method is applicable to small amounts of RNA samples [down to a few nanograms] (Jonstrup et al. 2006).

3.3.4. *NanoString* is a probe-based miRNA detection system that implements reporter probes linked to a series of dyes for obtaining both qualitative and quantitative data on the number of miRNAs. This approach requires very-high-resolution microscopy. This method was applied to formalin-fixed paraffin-embedded samples by means of mRNA expression profiling using low amounts of total RNA (Reis et al. 2011).

3.3.5. In a new bead-based approach, detection of known and unknown miRNAs can be carried out using *chitin beads coated with p19*, a protein obtained from a plant virus. This protein has the ability to selectively bind dsRNAs (miRNA-probe duplex). In fact, p19 preferably targets dsRNA 19–21-bp long but not single-stranded RNAs (probe or target). In the end, the target is tracked through nanopores [in case the probe is not labeled] (Wanunu et al. 2010) or radioactivity [probes labeled with radioisotopes] (Jin et al. 2010).

3.3.6. In the category of *cyclic enzymatic amplification* method, Wang et al. have introduced a novel electrochemical biosensor for miRNA detection. To perform this approach, Au nanoparticles (AuNPs) are loaded on the surface of an Au electrode through amperometry technique. Then, thiolated probes are placed onto the surface of the AuNP-deposited gold electrode with nanoparticle by the Au-S bonds. In presence of the target miRNA, probes can bind them and form hybrid RNA/DNA duplexes. Addition of T7 exonuclease promoted DNA digestion [in the RNA/DNA duplex] and the released miRNAs flow back to the solution to hybridize with other DNA probes on the electrode. The positive point about T7 is its ability to recognize RNA/DNA duplexes but not RNA/RNA duplexes or RNA single strands (Wang et al. 2014b).

3.3.7. Detection of miRNAs is provided by the "*padlock probe-based exponential rolling circle amplification (PERCA)*" strategy. After the target miRNA binds the padlock probe, Phi29 DNA polymerase initiates rolling circle amplification to produce a copy of the probe. The products bind other padlock probes and are further cleaved by an endonuclease at the nicking sites present within the probes. The released sequences known as "triggers" start the same cycle over (Liu et al. 2013).

3.3.8. The *toehold-initiated rolling circle amplification (TIRCA)* method is a method of choice for imaging and in situ visualization of miRNAs. This strategy benefits from dually functioned seal probes; they serve as probes for toehold-mediated strand displacement (TMSD) and they promote rolling circle amplification as well. Briefly, in this method, the toe-hold region in the structurally stable seal probe binds the target miRNA and renders its activity, followed by rolling circle amplification. The detection of the target is provided by a probe labeled with 6-carboxyfluorescein (Deng et al. 2014b).

3.3.9. Detection of miRNAs is also possible by isothermal amplification-based methods; Liu et al. reported one such method which brings into view the *combination of fluorescent silver nanoclusters with target-assisted isothermal exponential amplification (TAIEA)*. In this label-free assay, isothermal

amplification promotes a “miRNA-to- reporter oligonucleotide” reaction, the products of which can further initiate the formation of nanoclusters (which allow detection) (Liu et al. 2012b).

3.3.10. There are also methods that take advantage of the enzyme “*T7 exonuclease*” for label-free detection of miRNA either through cyclic enzymatic amplification (Wang et al. 2014b) or based on a dual-amplification method (uses both enzymatic and rolling circle amplification) (Cui et al. 2014).

<Please insert Table 2 here>

Table 2 miRNA detection methods; other approaches

Method	Description	Features	LOD	References
Electrocatalytic amplification (ECA)	A single-stranded DNA oligonucleotide is connected to Pt nanoparticles via a linker, these oligonucleotides bind their target miRNA and current transients generated by the electrode surface allow detection.	Colloidal stability A "signal on" assay Applicable to a variety of nucleic acids Utilization of DSN enzyme for cleavage which targets only DNA/RNA duplexes	NS	(Castañeda et al. 2017)
Signal-on fluorescence biosensor (SDA and DNAzyme-based)	In presence of the target, DNA polymerase promotes strand displacement amplification and the products form DNAzymes by addition of Mg^{2+}.	High sensitivity and specificity Dual signal detection Possible application to clinical samples Utilization of organic fluorescent labels Signal intensity indicates target quantity	NS	(Yin et al. 2017)
izMiR framework	13 approaches for miRNA detection were put together into different predictors. izMiR framework allows comparison, addition, and combination of various approaches.	Computational tools facilitate detection	NS	(Saçar Demirci et al. 2017)
Electropolymerization based doping	A hybridization-based assay that allows detection through electrochemical impedance spectroscopy.	Excellent selectivity Detection range of 0.17 nM Potential accommodation to chip technology Target detection in total RNA	nM	(Kaplan et al. 2017)
miRNAs detection via intercalation of hemin	Binding of the target to the probe and addition of hemin allow electrochemical detection.	Easy Label-free	µM	(Yi and Lu 2017)
Optomagnetic Detection	The target-probe duplex is cleaved by DSN, leading to release of magnetic nanoparticle and miRNAs.	Reasonable expenses Possibility of multiplex detection Accurate and rapid quantification Single-base mismatch recognition	fM	(Tian et al. 2017)
Enzymatically engineered primer extension poly-thymine (EPEPT)	Poly-thymine sequences are added to short miRNAs and allow fluorescence detection.	Quick Simple Reasonable expenses	fM	(Chi et al. 2017)

Electrochemical detection via sulfonamide-bound antisense hybridization	A glassy carbon electrode with naphthalene sulfonic acid deposits provides miRNA detection	Quick, simple and cost-effective Detection of urinary miRNA	fM	(Smith et al. 2017)
Combination of RCA and nicking endonuclease signal amplification (NESA)	The products of quadratic RCA form tandem repeats which hybridize with a molecular beacon. Upon cleavage of the molecular beacon, detection is provided through fluorescence.	Highly sensitive and specific	aM	(Xu et al. 2017a)
Metal-enhanced fluorescence/visual bimodal platform	Employs fluorophore-functionalized DNA1 and flower-like silver (FLS), hybridized with the quencher-containing strand. In presence of the target miRNA and H_2O_2, the fluorescent signal is amplified to allow detection.	Excellent sensitivity Provides multiple detection Follows the basics of multi-channel microfluidic paper-based analytical devices (μPADs)	fM	(Liang et al. 2017)
Padlock exponential rolling circle amplification (P-ERCA) with $CoFe_2O_4$ magnetic nanoparticles	One capture probe is located on an electrode coated with $HAuCL_4$. Meanwhile another capture probe is fixed on Au@CoFe2O4/Gra-Tb composites to form an assembly, which is exposed to the electrode by the assistance of RCA amplification products.	Highly sensitive and specific Detection does not require substrate Based on nano electrocatalysis	NS	(Yu et al. 2017)
Dual microRNAs-fueled DNA nanogears	Each of the two nanogears is set to operate upon binding with a distinct type of miRNA. Next, they start to roll against each other until being situated in the proper mode for detection.	Sensitive Multiplex assessment Non-enzymatic detection Electrochemiluminescence detection Single luminophore for dual detection	fM	(Zhang et al. 2017)
Etching (top-down approach)	After preparation of luminescent copper nanoclusters, single strand DNAs are used as templates for miRNA detection based on the formation of RNA-DNA duplex, which alters the fluorescence signal.	Sensitive Label-free Non-enzymatic No chemical treatment required	$2.2 * 10^{-12}$ M	(Borghei et al. 2017)
Fluorescent dyes-loaded albumin nanoparticles around functionalized magnetic beads	The probe-miRNA duplex is tracked by p19, tagged on fluorescent albumin nanoparticles. Detection takes place after signal amplification by albumin nanoparticles.	Quick and sensitive Used for detection in cell lysate and serum	fM	(Wei et al. 2017)
Morpholino-Functionalized Nanochannel Biosensor	After the target miRNA has bound the probes (Phosphorodiamidatemorpholinooligos), alteration of charge density allows target detection.	Label-free Reduced background noise Potential application in clinics Considerable sensitive and specificity	NS	(Liao et al. 2017)
Theranostic One-Step RNA Detector (TORNADO)	Platinum nanoparticles with thiolated probe strand miRNA on the surface, as well as the target miRNA, are placed within a microfluidic disc. The thiol group of capture miRNAs can	Independent of PCR Remarkably selective Fulfills fast point of care Useful for biomarker detection	pM	(McArdle et al. 2017)

	interact with a gold electrode. Upon spinning, the electrode allows electrochemical detection.	Used for detection of miR-134 in plasma and cerebrospinal fluid		
Combination of gold nanoparticles, hollow molybdenum disulfide microcubes, and DSN	The biotinilated probe-target duplex is cleaved by DSN, the biotin tag is released and streptavidin-conjugated alkaline phosphatase can be loaded onto the electrode. By the addition of ascorbic acid, detection is allowed by electrochemical–chemical–chemical redox cycling.	Detection limit at 0.086 fM Used for detection of miR-21	fM	(Shuai et al. 2017)
3D DNA Walking Machine Based Target Conversion and Distance-Controllable Signal Quenching and Enhancing	miRNA is used as a template to produce an intermediate DNA for remarkable signal amplification. These DNAs bind to gold nanoparticles, increase the distance between the nanoparticles and CdS:Mn quantum dots and intensify the electrochemiluminescence signal resulted by SPR.	Ultrasensitive Used for detection of miR-141 Reduction of background noise Detection in human prostate cancer cells	fM	(Xu et al. 2017b)
HCR on silica coated Qbeads	Probes bind the beads via their 5' and form a loop in absence of the target. However, in presence of the miRNA, the loop splits open and the 3'-initiator sequence promotes HCR and the products were stained with GelRed.	Multiplex detection Sensitive and specific Easy detection based on colorimetry	NS	(Guo et al. 2017a)
Flow cytometric bead assay (FCBA)	The probe-miRNA duplex is cleaved by DSN. Then, terminal deoxynucleotidyl transferase (TdT) will add dTTPs to the 3'-OH of the cleavage site, which will finally bind poly (A) sequences tagged with FAM.	Highly sensitive Allows multiplex detection Facilitated probe designing process	fM	(Qiu et al. 2017)
Loop-mediated isothermal amplification (LAMP)	A cDNA copy of the miRNA is generated, and RNase H eliminates the miRNA from the duplex and the remaining cDNA hybridizes with two probes. Finally, DNA ligase promotes ligation of the probes.	Easy Quick Highly specific Highly sensitive	fM	(Du et al. 2016)
Particle-based DNA circuit system	Continual and multiple displacements of the DNA from magnetic beads in presence of the target.	Sensitive Enzyme-free Easy detection (colorimetric) No complicated tools required	NS	(Oishi and Sugiyama 2016)
Quantitative PCR SplintR ligation assay	This assay exploits two DNA oligonucleotides to bind the target miRNA. The products of PCR are detected by a double-quenched DNA probe labeled with FAM.	Rapid action of SplintR ligase Considerable specificity and sensitivity Multiplex detection in combination with NGS	NS	(Jin et al. 2016)

The miR-Test case	Establishes a way to boost the efficiency of detection in terms of operation and consistency.	Potential clinical application Detection of circulating miRNAs	NS	(Marzi et al. 2016)
Fe_3O_4@Ag magnetic nanoparticles	miRNAs bound to the probes placed on Fe_3O_4@Ag magnetic nanoparticles are cleaved by DNS and provide detection.	Reasonable costs Remarkable sensitivity miRNA detection in total RNA Adjusts to point of care clinical diagnosis	NS	(Fiammengo 2017; Pang et al. 2016)
Square wave voltammetry	DNA probes are fixed on the nanoparticles and then exposed to target miRNA. A biotinylated DNA sequence participates in hybridization to be captured by streptavidin on the nanospheres coated with and Cd^{2+}.	Easy and highly sensitive Detection limit at 0.76 aM Used for detection of miR-21	aM	(Cheng et al. 2015; Fiammengo 2017)
ICP-MS	A series of DNA probes are labeled with lanthanide and then hybridize to miRNAs blotted on a membrane, followed by washing, laser ablation, and detection.	High specificity Provides multiplex detection	NS	(Zhang et al. 2015e)
Carbon nanomaterials	Detection relies on alteration of the electrical activity of a nanoscale polymer film in presence of the target miRNA.	Label-free detection A "signal on" method	NS	(Zhang et al. 2015c)
Enzyme-based electrochemical signal amplification	Enzymes including horseradish peroxidase and alkaline phosphatase act as biosensors.	Enzymes promote signal amplification Utilization of enzymes enhances sensitivity	NS	(Zhang et al. 2015c)
Silver nanoclusters (AgNCs)	Involves fabrication of DNA-AgNC probes that target miRNAs.	Rapid and simple Cost-effective Used in imaging assays Allows multiplex detection in some assays	NS	(Zhang et al. 2015d)
Cascade Hybridization Reaction (CHR)	This method benefits from two programmable nucleic acid sequences which are labeled with fluorophore dyes; detection occurs by FRET signaling.	Single-step and simple Real-time detection in live cells Detection of short and low-abundant miRNAs	NS	(Cheglakov et al. 2015)
Isothermal EXPonential Amplification Reaction (EXPAR)	Short sequences are amplified by DNA polymerase and the cleaved by a nicking enzyme. This method has been combined with the arched probe and time-resolved fluorescence.	High-speed Cost-effective Highly sensitive and specific Possible application in diagnosis Differentiates miRNAs of one family Requires low amounts of the substrate	NS	(Wang et al. 2014a; Yu et al. 2014; Zhang et al. 2015d; Zhang and Zhang 2012)

Enzymatic repairing amplification (ERA)	This assay implements DNA replication by DNA polymerase and is then cleaved and repaired by various enzymes. The target miRNA has the role of the primer.	Highly sensitive Real-time analysis	fM	(Zhou et al. 2014)
Dual signal amplification; catalyzed hairpin assembly reaction (CHA) and HCR	Binding of the target miRNA to a hairpin attached to a surface leads to its opening and the second probe is added to form a duplex while the miRNA is released. Detection is provided by using methylene blue.	Highly sensitive	nM-fM	(Wu et al. 2014)
Nanopore ionic flow	Polyethylene glycol (PEG)-bound DNA probes capture the target and the ion current throughout the nanopore is modulated, leading to the production of a signal.	Allows simultaneous detection Applicable in clinics	NS	(Zhang et al. 2014b)
Deposition of polyaniline (PAn) via hybridized miRNAs	Deposition of polyaniline by hybridized miRNAs on the surface of the biosensor allows miRNA detection.	Remarkable recognition of mismatches miRNA detection in total RNA from blood and cancer cells	fM	(Deng et al. 2014a)
Ligase chain reaction (LCR)	This method exploits two probes with total complementation to the target, followed by ligation, amplification, and detection.	Highly sensitive Does not require thermal cycler Offers simultaneous and multiplex detection	fM	(Yuan et al. 2014; Zhang et al. 2014a; Zhang et al. 2015d)
Carbon nanotubes field-effect transistor along p19 protein	Upon binding of the probe to the target miRNA, an RNA duplex is formed and subsequently captured by p19. This attachment results in alteration of the resistance of the biosensor.	Wide dynamic range Simple and early detection of the target	aM	(Ramnani et al. 2013)
Nanopore single-molecule detection	Based on the alteration of the ion flow as the target blocks the pore. The number of pulses is indicative of the target quantity.	High sensitivity Diverse synthesis approaches Determination of the target's location and conformation Simple quantification based on pulse estimation	NS	(Gu et al. 2012)
DNA nanostructure-based interfacial engineering	miRNA detection takes place by an electrochemical sensor (EMRS) and signal transduction of enzymes.	Label-free Highly reproducible	aM	(Wen et al. 2012)
Duplex-specific nuclease signal amplification (DSNSA)	After formation of the probe-miRNA duplex and its cleavage by DSN, the probes generate a signal for detection.	Sequence-specific function Multiplex and easy detection Excellent specificity and sensitivity Applicable in research and clinics	fM	(Yin et al. 2012)
miRNA imaging	Luciferase reporter-based imaging	Practical in clinics	NS	(Wang et al. 2011)

	Fluorescence protein-based imaging Fluorescence beacon-based imaging	Applicable to living systems Background noise may be observed Allows non-invasive evaluation of miRNAs Tracking biogenesis and functions of miRNAs Bioluminescent and fluorescent methods are signal off approaches, but molecular beacon imaging is a signal on approach		
Quantum dots	They offer various applications in terms of detection such as DNA biosensors and barcode assays.	High-speed analysis Provides multiplex detection Significant signal amplification Effective detection of molecules and nucleic acids (barcode assays)	NS	(Dolatabadi et al. 2011; Dong et al. 2012)
NGS	Different platforms including SOLiD, Illumina and Roche 454; a cDNA library must be prepared and massively parallel sequencing is carried out subsequently followed by data analysis. SOLiD; ligation-dependent Illumina; sequencing-by-synthesis Roche 454; based on Pyrosequencing	Highly specific and sensitive High-throughput No limitation for precursor size Discovery of novel miRNAs Investigation of known and unknown miRNAs Bridge PCR, read length: 50 or 75 bp Emulsion PCR, read length: 150-300 bp Emulsion PCR, read length 400 bp	NS	(Liu et al. 2011; Mardis 2017; Tang et al. 2012)
Molecular beacon (MB) assays	In presence of the target, the loop pops open and takes the fluorophore away from the quencher and provides signal detection.	Rapid, Specific and Sensitive Label-free Applicable in Diagnostics Amplification-independent Does not require reverse transcription	NS	(Baker et al. 2013; Dolatabadi et al. 2011)
Combination of capture probes and gold nanoparticles	Capture probes bind the target miRNA through their 3' end. Detection probes (GOx-DP) hybridize to the 5’ end of the capture probes. The number of GOx molecules correlates with the miRNA concentration.	PCR-independent Ligation-independent Cross-hybridization may occur miRNA detection in total RNA obtained from cell lines	fM	(Gao and Peng 2011)
Protein facilitated affinity capillary electrophoresis (ProFACE) assay	Target miRNA is captured by an RNA probe, accumulated by a magnetic bead covered in p19 protein and finally added into a capillary for further separation by electrophoresis.	PCR amplification-independent Considerable sensitivity Multiplex detection is possible	fM	(Khan et al. 2011)
Metal oxide nanoparticles	Exploitation of ruthenium oxide nanoparticles	High sensitive	NS	(Peng et al. 2010)

	(RuO_2NPs) as the label for miRNA, followed by hybridization to probes on the gold-film electrode. In presence of aniline and H_2O_2, probe/miRNA duplexes generate a peak.	Allows direct detection Remarkable catalytic activity Successful function in tough environments		
Hybridization chain reactions (HCR)	Upon binding of RNA probes to the target, fluorescent RNA hairpins turn into amplification polymers.	Programmable amplification cycles Sequence-specific function HCR amplifiers can be implemented simultaneously and in the same sample	NS	(Choi et al. 2010)
Rybozome method	Hybridization of the target miRNA to the inactive ribozyme leads to its activation, resulting in separation of the fluorophore from the quencher.	Real-time analysis Utilization of FRET Provides direct detection More sensitive than molecular beacons	NS	(Wang and Yang 2010e)
Electrocatalytic Nanoparticle Tags (ENT)	Placement of nanoparticle tags leads to signal amplification.	Label-free Amplification-free	fM	(Catuogno et al. 2011; Wang and Yang 2010b)
Surface-enhanced Raman spectroscopy (SERS)	a nanostructured surface can link the target. The generated spectra are evaluated and analyzed by partial least squares discriminate analysis (PLS-DA).	Rapid and highly sensitive Labeling is optional Offers multiplex detection Determination of miRNA pattern Provides qualitative & quantitative data Laborious analysis & elucidation of data	fM	(Wang and Yang 2010f; Zhang et al. 2015d)
Silicon Nanowire	PNA probes linked to a silicon nanowire hybridize to their complementary unlabeled target miRNA. Detection is allowed by evaluation of the resistance alteration.	Quick Label-free Highly sensitive Direct hybridization	fM	(Zhang et al. 2009)
Rolling circle amplification (RCA)	It is based on the designation of a padlock probe complementary to the target miRNA. Next, the two termini of the probe are brought together and the miRNA acts as a primer for amplification.	Simple Specific Sensitive	fM	(Neubacher and Arenz 2009)
Enzyme-linked oligonucleotide assay (ELONA)	Exploits aptamers with fluorescent tags in order to detect miRNA	Rapid Requires small amounts of miRNA	NS	(Catuogno et al. 2011; Mok and Yingfu 2008)
Stem-loop qRT-PCR	Requires a stem-loop probe, forward and reverse primers as well as a hydrolysis probe.	Expensive Highly specific Highly sensitive Low-throughput	NS	(Catuogno et al. 2011; Kramer 2011; Mestdagh et al. 2008)
miR-Q	cDNA is synthesized using hexamers with 5' overhangs. Next, quantitative PCR is carried out	Specifically detects mature miRNAs Highly specific	fM	(Catuogno et al. 2011; Sharbati-Tehrani et al. 2008;

	using three DNA-oligonucleotides.	A rapid and simple assay A qRT-PCR-derived assay Applicable to clinical cases Detection of mature miRNAs Highly sensitive (down to 0.2 fM)		Wang and Yang 2010c)
Electrocatalytic Moiety Labeling	Direct labeling of miRNAs in the total RNA solution without taking advantage of enzymes.	Highly sensitive Enzyme-independent labeling	pM	(Gao and Yu 2007; Wang and Yang 2010a)
Polyaniline nanowire	Different experiments take advantage of this nanomaterials; target-guided formation of conducting polymer nanowires in nanogaps, magnetic bead-based hybridization assay and electrochemical detection using DNA concatamers.	Simple and sensitive	fM	(Bartosik et al. 2014; Fan et al. 2007; Hong et al. 2013)
Splinted ligation	A labeled oligonucleotide is ligated to the target miRNA by DNA ligase. After separation on a denaturing gel, detection takes place via autoradiography or phosphor-imaging.	Amplification-free Rapid and simple No specialized instrument required More sensitive than northern blotting Applicable to evaluation of other small RNAs	Ng-mg	(Maroney et al. 2007; Maroney et al. 2008)
miRAGE	Similar to the procedure of cloning, except that it benefits from SAGE which has taken the place of the steps of amplification through sequencing	Identification of one tag Multiple tags in one experiment	fM	(Cummins et al. 2006; Wang and Yang 2010d)
Single-molecule method	Fluorescent probes hybridize the target and allow single-molecule detection and quantification of miRNA.	Direct assessment Single-molecule-based Discriminates miRNAs of one family	fM	(Neely et al. 2006)
Invader assay	This technique employs two types of oligonucleotides: the “invader” and the labeled probe, each complementary to a specific segment of miRNA. Detection is allowed through FRET approach.	High throughput Sensitive and specific Short incubation time Allows parallel experiment Target detection in a few cells Differentiation of pre-miRNA from mature miRNA	NS	(Allawi et al. 2004; Hunt et al. 2009; Nicolas et al. 2009)
Metal nanoparticles	Implementation of gold nanoparticles (AuNPs) in different assays such as sandwich model.	Compatible with nucleic acids Considerable stability Palladium nanoparticles label-free detection	fM	(Barad et al. 2004; Wu et al. 2013; Zhang et al. 2015c; Zhou et al. 2012)

LOD: limite of detection; aM: attomolar; pM: picomolar;
fM: femtomolar; µM: micromolar; ng:nanogram; mg: milligram

4. DNAzymes-based approaches

The methods discussed so far each is powerful in their own way (e.g. high throughput, multiplex detection, sensitivity and specificity, providing local information, interactions of biomolecules, etc). In spite of this, development of approaches which allow all/most of these options altogether, with lower expenses and less time consumption, or better, simpler detection without demanding special instruments or analyses is highly crucial and attractive. In this regard, it is worthwhile to consider DNAzymes and DNAzyme-derived approaches. As their public figure shows, DNAzymes are catalytic enzymes based on DNA. These single-stranded structures were first mentioned over 20 years ago and ever since they started to get popular (Breaker and Joyce 1994; Kosman and Juskowiak 2011; Tram et al. 2012). DNAzymes are less costly to prepare and they are remarkably stable (Kosman and Juskowiak 2011). Besides, they take part in a variety of fields and applications. For instance, they serve as the molecular recognition component in biosensors and those with peroxidase-like activity have therapeutic applications and are also able to detect heavy metals [$Mg^{2+,}$ Cu^{2+}, Pb^{2+}, Hg^{2+}, etc] (Deng et al. 2013b) and biomolecules such as proteins. They can also analyze enzymatic activity (methyltransferase, telomerase, etc). One of the most interesting implementations of this type of DNAzymes is in detection of different sequences; in this case, detection relies on proper designation of a G-rich sequence (known as the G-quadruplex; hydrogen bonds are formed between guanines), which will form an active DNAzyme in presence of hemin (cofactor; required for peroxidase activity), H_2O_2 (boosts peroxidase activity), metal ion (e.g. Pb2+, NH4+ or K+) and the substrate (such as luminol or 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid [ABTS]; indicates the catalytic activity of DNAzymes) that produce an observable color, hence termed as colorimetric detection (Deng et al. 2013b; Du and Dong 2017; Tram et al. 2012). In this section, some of the DNAzyme-based methods used for detection of miRNAs have been reviewed.

4.1 One DNAzyme-based approach relies on *dual amplification for miRNA detection*: (i) hybridization amplification by duplex-specific nuclease (DSN), (ii) signal amplification (chemiluminescence) by DNAzyme. DSN is an endonuclease which can only cleave DNA–RNA hybrids and DNA-DNA duplexes but not single-stranded RNA/DNA and RNA–RNA duplexes. For performing the assay, capture probes (CPs) are designed to contain a region for miRNA capture and a DNAzyme sequence placed at their termini. Subsequently, magnetic beads can bind the terminal amino group of capture probes through their carboxyl group and upon folding of probes followed by addition of hemin, the enzymatic activity of DNAzymeinitiates. In presence of the target miRNA, the CPs bind them and then hybridized segments of the probes are cleaved by DSN, which results in detachment of DNAzyme segments and the release of miRNAs into the sample. This procedure takes place repeatedly and a great

number of DNAzyme sequences are released from the beads. Finally, intact probes and beads are separated via a magnet, and presence of the target miRNA is reported through chemiluminescence [Please see Fig. 2] (Deng et al. 2013a).

<Please insert Figure 2 here>

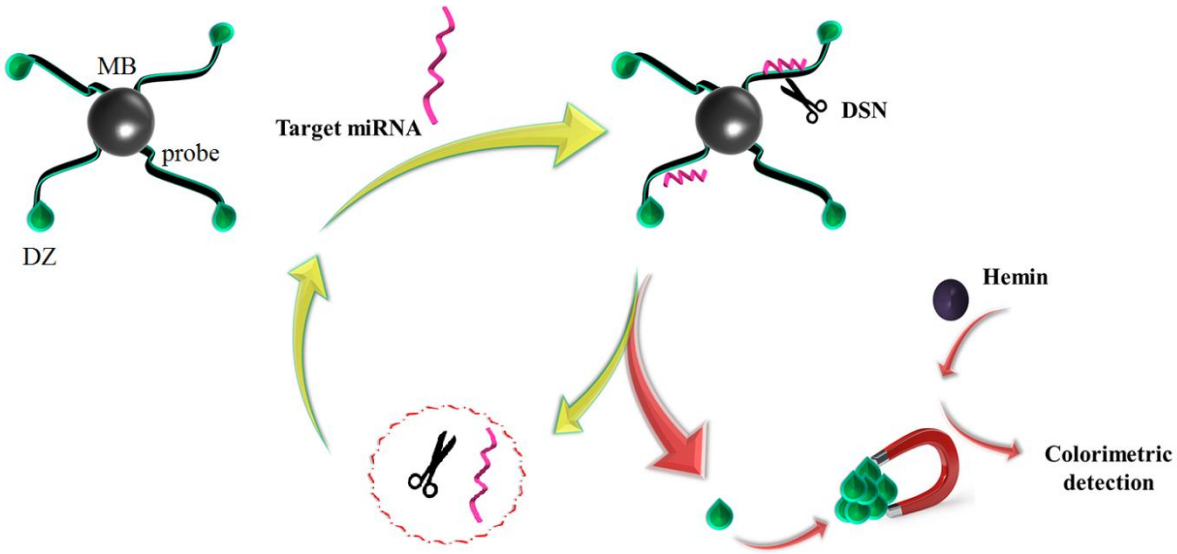

Fig. 2 Chemiluminescent assay for miRNA detection (MB: molecular beacon; DZ: DNAzyme; DSN: duplex-specific nuclease)

4.2 "*DNAzymeferris wheel*" nanostructures are novel biosensors used in the detection of miRNAs via a colorimetric reaction. These structures are label-independent and were developed by Zhou et al. in order to detect miR-141 in prostate cancer cell lines. This method involves the employment of three hairpin loops, a G-quadruplex sequence, hemin, H_2O_2, H_2SO_4 and 3,3′,5,5′-tetramethylbenzidine sulfate (TMB). Each hairpin has two segments linked to their ends (5' and 3' respectively). By the addition of the G-quadruplex to hairpins, these sequences form a nanostructure with horseradish peroxidase-like activity. Upon addition of hemin, the cofactor for the enzyme, the target miRNA binds to its complementary region in the first hairpin and unwinds it. Next, it binds to the complementary sequence in the second hairpin and frees a sequence in this hairpin. The same mechanism is applied to the third hairpin sequence,

which unfolds the first hairpin and the whole cycle of self-assembly starts over. In the end, in presence of H_2O_2, colorless TMB substrate produces a yellowish color, which provides simple detection of the target miRNA. H_2SO_4 is the turn-off key for this cyclic reaction. It is clear that if the target miRNA is absent, the self-assembly process does not take place and therefore is not followed by the rest of detection procedure [Please see Fig. 3] (Zhou et al. 2015).

<Please insert Figure 3 here>

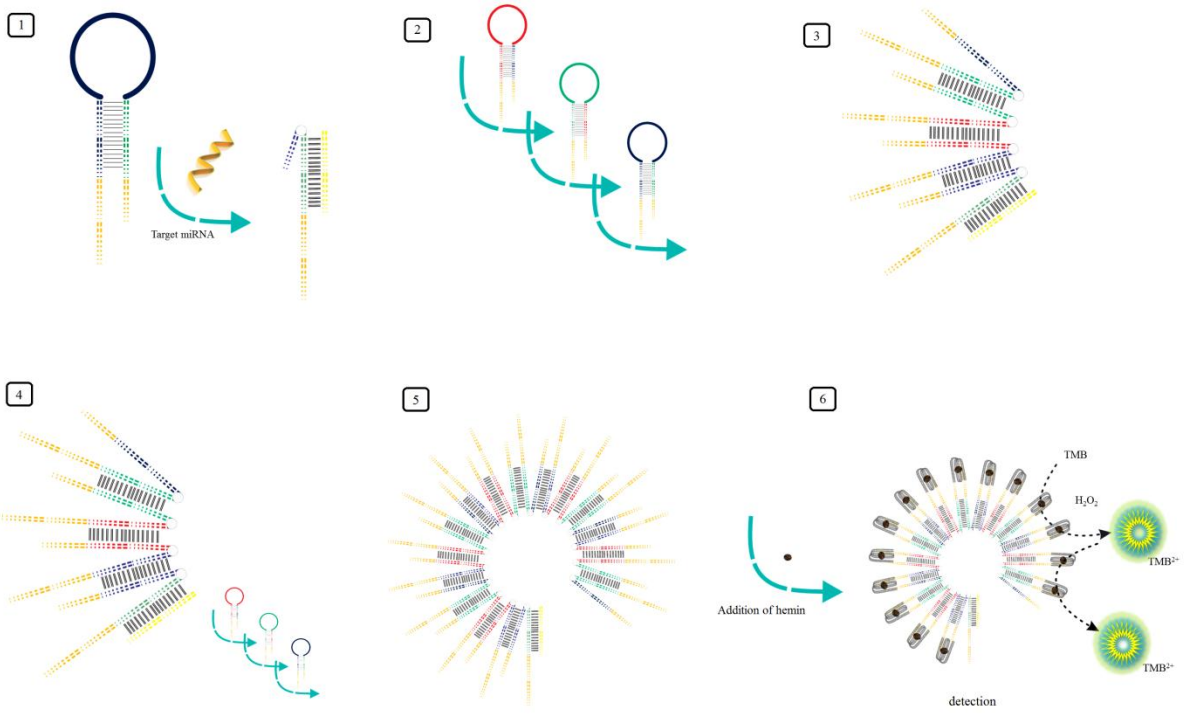

Fig. 3 DNAzymeferris-wheel assay: (1) recognition of target miRNA; (2) subsequent addition of hairpins; each hairpin binds to the complementary sequence in within the previously added hairpin; (3) construction of a hairpin network; (4,5) the same sequenc

4.3 *2'-O-methyl modified DNAzymes* are among recent approaches in biochemical miRNA detection using biosensors. In this method, a 5'-thiolated signal probe, consisting of a recognition sequence and a 2'-O-methyl modified G-rich sequence, hybridizes with the target miRNA to form a hybrid duplex. Next, a duplex-specific nuclease cleaves the generated duplex at the recognition site, so that only the 2'-O-methyl modified G-rich sequence remains on the gold electrode. After performing the washing step, the target miRNA is released from the electrode surface to hybridize with a new probe. This means that a

single miRNA is capable of creating a positive loop (the cycle in which signal probe turns into 2'-O-methyl modified sequence). In the end, all the signal probes turn into 2'-O-methyl modified sequences and followed by addition of hemin and TBM, the active DNAzyme provides colorimetric detection of the target in presence of H_2O_2 [Please see Fig. 4] (Zhang et al. 2014c).

<Please insert Figure 4 here>

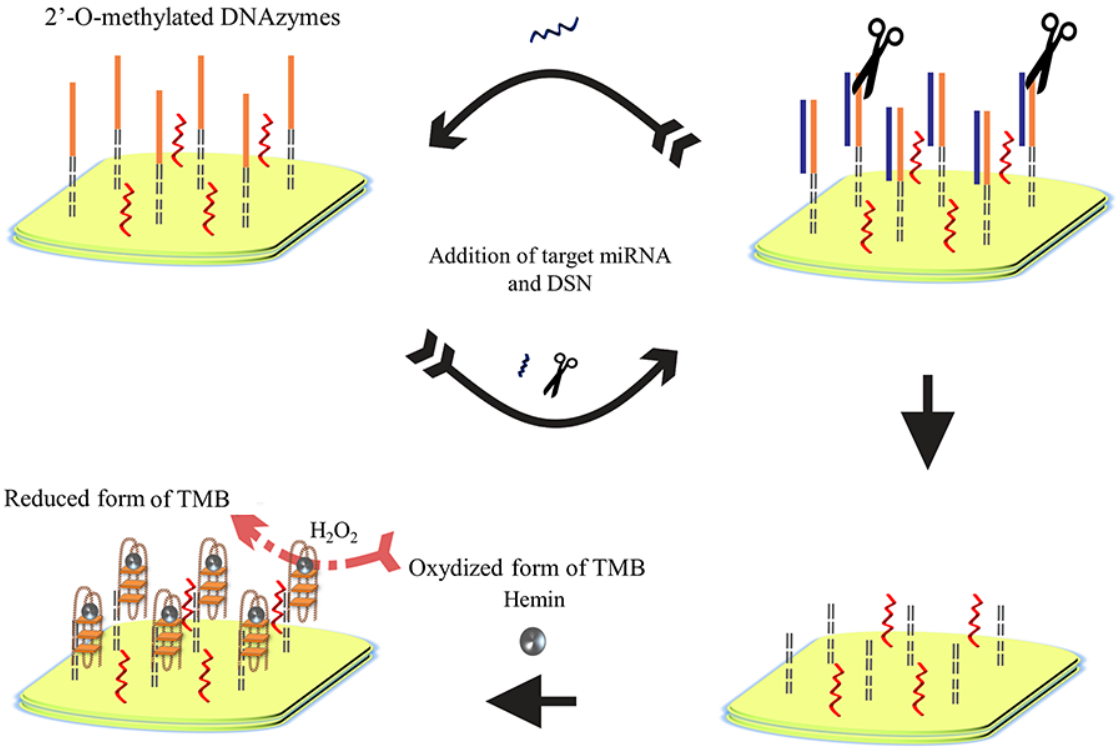

Fig. 4 2'-O-methyl-modified DNAzymes (DSN: duplex-specific nuclease)

4.4 Recently, an *electrical method* was developed for assessment of *cell-free miRNA in low concentrations*. This method exploits capture probes (to capture miRNAs from the blood), detection probes (targets the miRNAs captured), alkaline phosphatase (ALP; dephosphorylation of a substrate), Au nanoclusters (AuNC; promotes silver deposition). The first step is to create gold nanoclusters, followed by their incorporation into ALP to generate ALP-AuNCs, which have catalytic activities of both ALP and AuNCs (e.g. dephosphorylation as well as silver deposition). In fact, the addition of AuNCs to ALP results in augment of enzymatic activity; therefore, ALP-AuNCs act as catalytic probes. After the capture probes have found target miRNAs, the detection probes hybridize with them and then, ligation takes place by DNA ligase. Finally, signal amplification of silver depositions provides detection of the target. This

technique is advantageous for it can carry out detection of miRNA amounts as little as 21.5 attomolar [Please see Fig. 5] (Si et al. 2014).

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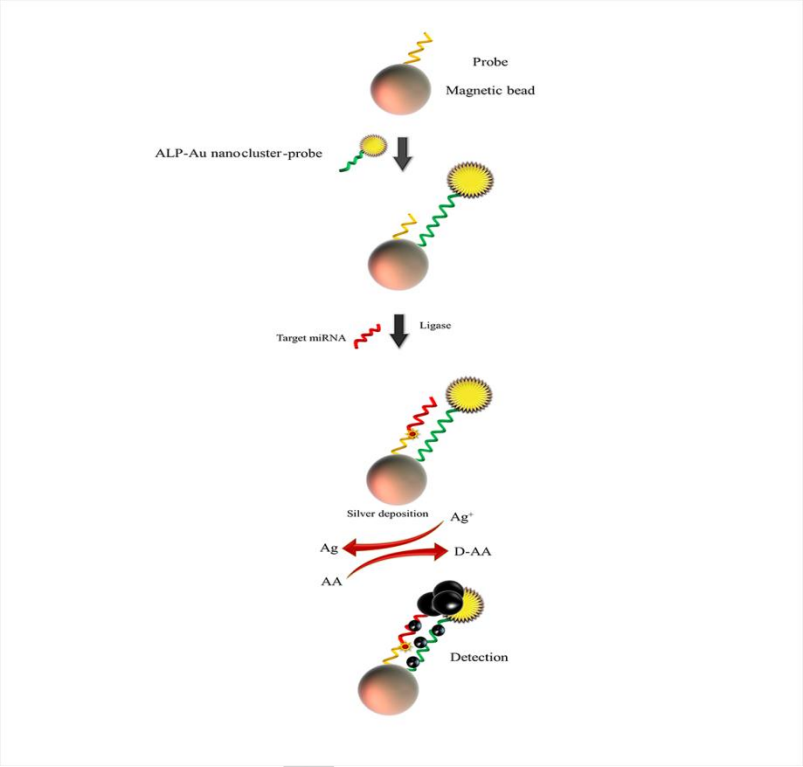

Fig. 5 Electrical detection of cell-free miRNA (ALP: alkalyn phosphatase)

4.5 Another colorimetric-based detection method of miRNAs relies on *molecular beacons (MBs) to induce strand displacement amplification coupled with catalytic hairpin assembly with the formation of DNAzyme*. The molecular beacon template comprises three different domains including (1) recognition domain, (2) nicking domain (target of Nb.BbvCI) and (3) amplification domain (stand displacement-type). When the target miRNA hybridizes with the recognition domain of the MB, it spreads over the other two domains (2, 3) and the Klenow fragment extends the formed duplex. Subsequently, cleavage of nicking sites results in production/release of "nicking triggers", which in turn promote catalytic hairpin (HPI and HPII) assembly (CHA). The generated CHAs further increase the release of triggers and therefore, aid in hairpin recycling assembly. Addition of hemin in presence of H_2O_2 turns on DNAzyme activity placed at the 5' end of the H1 hairpin and leads into colorimetric detection of the target miRNA. It is noteworthy that detection of various miRNAs is made possible through changing the recognition domain [Please see Fig. 6] (Yan et al. 2015).

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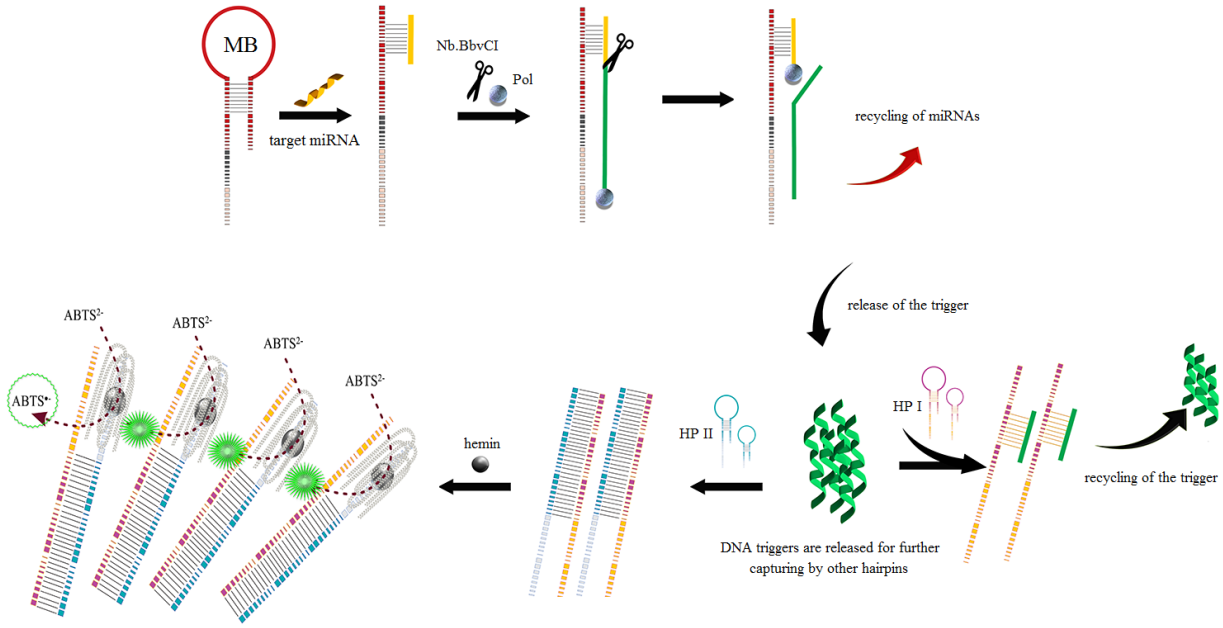

Fig. 6 Combination of molecular beacon and strand displacement amplification

4.6 One of the methods based on biosensor detection uses a *glassy carbon electrode covered in* $HAuCl_4$ as a platform onto which hairpin probes can link. In order to avoid nonspecific hybridization, the electrode is incubated with hexanethiol. Followed by this, the first hybridizing strand is added (dopamine-bound probe). This sequence carries a dopamine molecule and opens the hairpin loop upon binding the hairpin. Dopamine acts as a quencher for ECL signal to turn the system off. Afterwards, another sequence (probe II) is added to displace the dopamine-bound probe. It should be mentioned that miRNA forms a Y-shaped structure by binding the primer-probe and the attachment probe. This structure can be cleaved by Pb^{2+} to form S_2 and release the miRNA. In fact, S_2 acts as a primer to promote rolling circle amplification which results in the production of numerous repeated sequences. Addition of hemin to these sequences leads to the formation of hemin/G-quadruplex and switches on the system (detection of the target) [Please see Fig. 7] (Zhang et al. 2015a).

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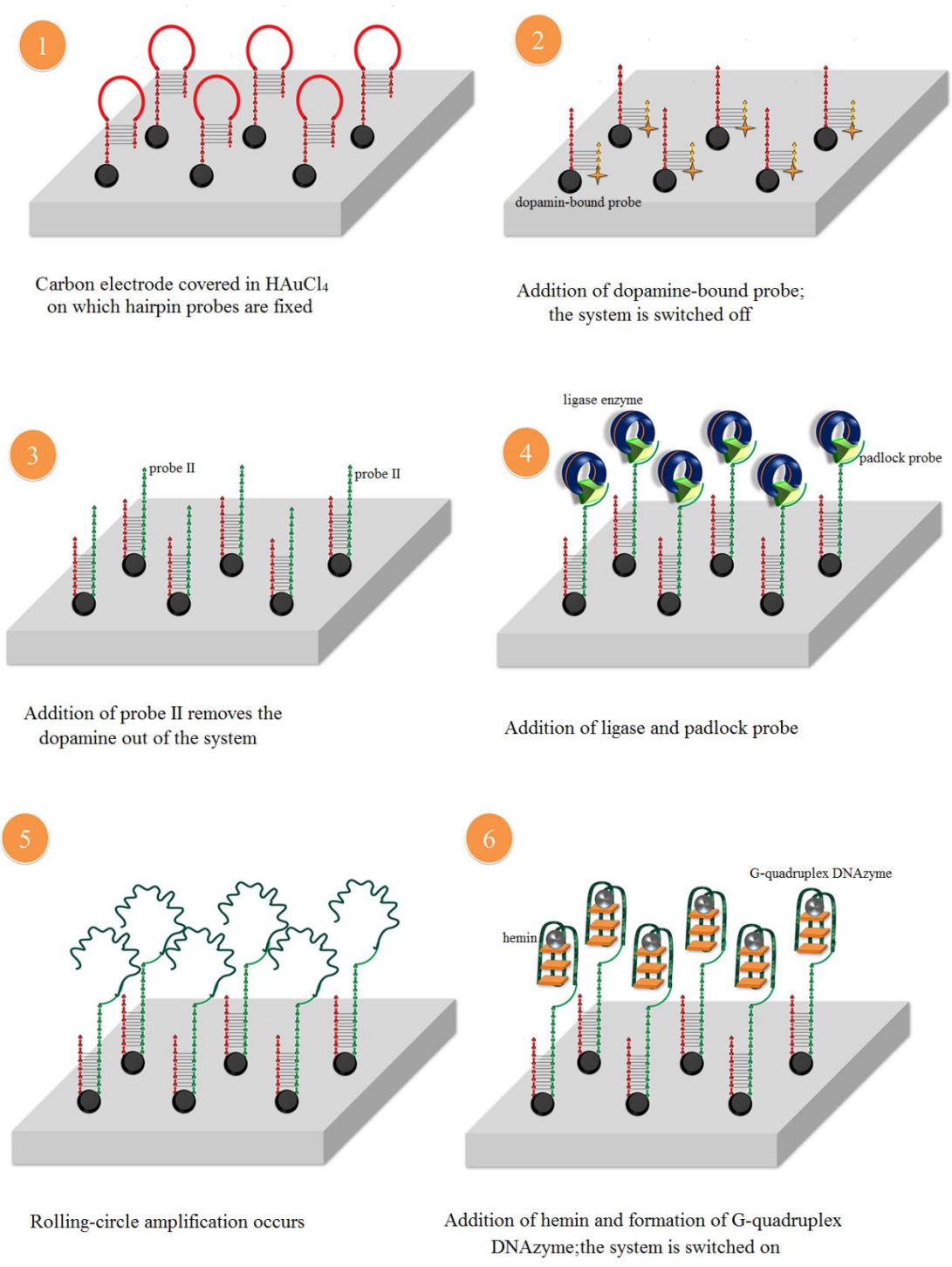

Fig. 7 "Off-on" electrochemiluminescent biosensor

4.7 Zhuang et al. carried out an experiment for miRNA detection based on *isothermal amplification relying on rolling circle mechanism*. They utilized a padlock probe (with a hybridization sequence for miRNA at their 5' and 3' ends as well as a catalyst sequence) to bind the miRNA of interest. In subsequence, two ends are ligated by T4 ligase and form a circular structure, which will replicate via Phi29 and through the rolling circle amplification. This results in production of padlock probe concatamers (includes catalysts). The catalysts lead the formation of hairpin duplex and are released afterwards. This sequence is able to go through the same cycle continually. After addition of hemin, the formation of DNAzyme takes place which catalyzes an H_2O_2-dependent reaction on TMB to provide colorimetric detection [Please see Fig. 8] (Zhuang et al. 2014).

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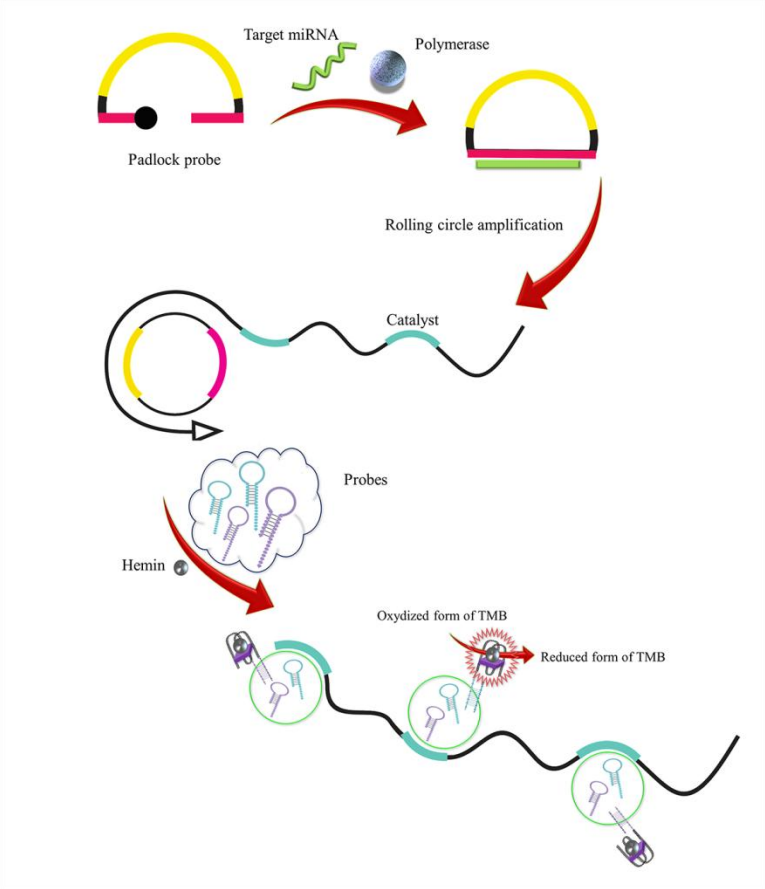

Fig. 8 Rolling-circle amplification-based DNA machine with hairpin assembly

4.8 One of the rolling circle amplification-based techniques for miRNAs detection is the "*functional nucleic acid-based amplification machine*" comprising several components: a MB/GDNA probe (C-rich DNA modified molecular beacon and G-rich DNA), Phi29 polymerase, nicking enzyme and an amplification template. The MB template includes several parts: (i) miRNA-detection domain, (ii) GDNA

hybridization domain, (iii) amplification domain, (iv) nicking domain. After binding of miRNA to (i), the circular structure of MB turns into a linear form and allows miRNA is extended along (i) and down to (ii) by subsequent polymerase activity of Phi29 which will displace the GDNA at (ii). Then, the Nb.BbvCI recognizes the nicking site and cleaves the DNA strand at (iii), which will be released as the nicking trigger. These nicking triggers bind to seal probes and act as primers for rolling circle amplification. To be more specific, these probes are amplification-inactive structures which begin the rolling circle mechanism upon binding with the triggers. This procedure goes on over and over to result in production of numerous GDNA probes. Finally, hemin is added to form DNAzymes which catalyzes the conversion reaction of colorless 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{2-}$) into green $ABTS^{\bullet-}$, which provides simple colorimetric detection of the target miRNA with the naked eye. This method is advantageous for allowing sensitive and specific detection of miRNAs in amounts as little as 10 attomolar to 1 nanomolar [Please see Fig. 9] (Li et al. 2016).

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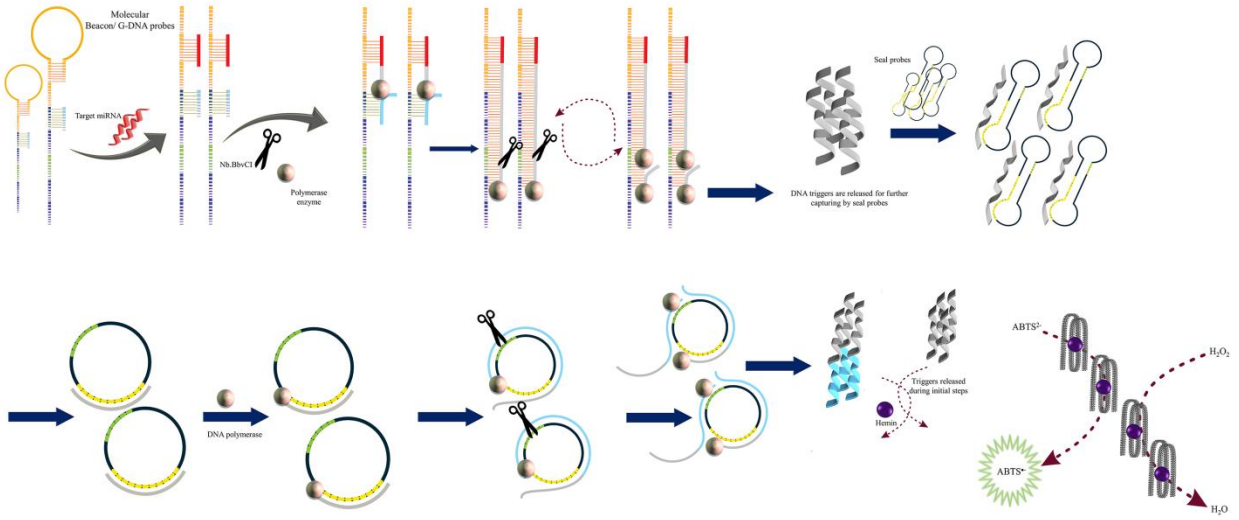

Fig. 9 Functional nucleic acid-based amplification machine

4.9 Meng et al. introduced an approach for *electrochemical detection of miRNA-21 using DNAzymes*. In this method, a gold electrode covered with Au nanoparticles (AuNPs) is prepared to be implemented as a surface for DNA probes to attach. After detection and hybridization to miRNA-21, the whole set is called the "miRNA-21/DNA-Au/probe/AuNPs/Au. Addition of hemin leads to formation of G-quadruplexDNAzyme, which in presence of H_2O_2, oxidizes hydroquinone and alters the response of benzoquinone, hence detects the presence of miRNA-21 [Please see Fig. 10] (Meng et al. 2013).

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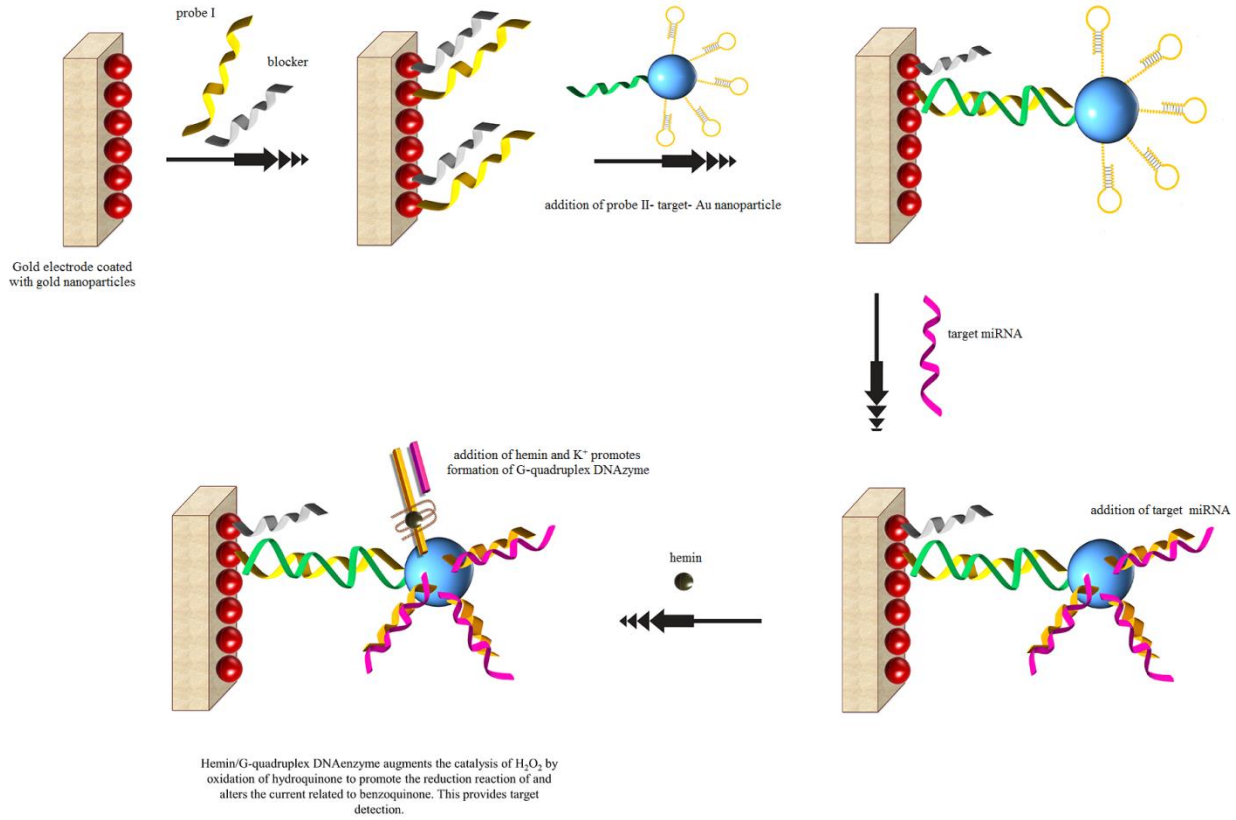

Fig. 10 Electrochemical detection using bio bar code system

4.10 A fluorescence biosensor using *DNAzymes activity on a platform of quantum dots (QDs)*. Alleged Yuan et al., capture probes were linked to QDs, followed by attachment of DNA nanostructures to capture probes. Addition of the target miRNA promotes cyclic hairpin assembly and upon addition of hemin, the whole bunch of G-quadruplexes provides detection in two ways: (a) the enzymatic activity of DNAzyme is triggered by hemin and in presence of H_2O_2, therefore fluorescence detection; (b) excited electrons might transfer to Fe^{III}core of hemin. As a result, the current assay is regarded as dual quenching strategy [Please see Fig. 11] (Yuan et al. 2017).

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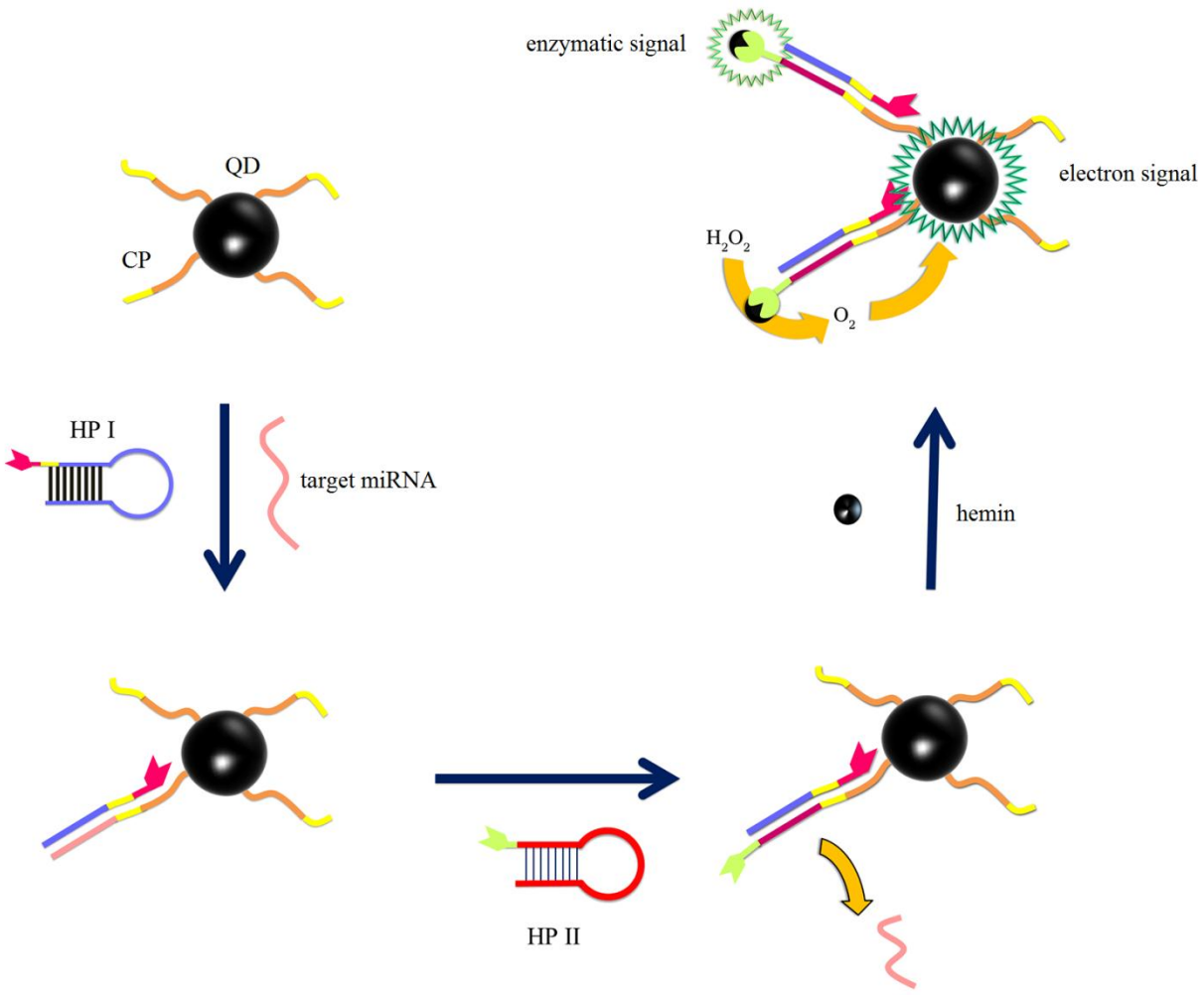

Fig. 11 Implementation of quantum dots in miRNA detection (QD: quantum dot; CP: capture probe; HP I: hairpin I; HP II: hairpin II)

4.11 It has been shown thatDNAzymes allow easy colorimetric detection of miRNAs in a variety of ways. For instance, Tian et al. carried out an experiment on the basis of *cascade amplification of DNAzymes*. They designed an oligomer in the shape of a hairpin, comprising three segments including a sequence to form into G-quadruplex, a loop to target miRNA and a 9-bp spacer (designed to prevent the formation of the G-quadruplex), which is capable of binding the G-rich sequence. When the miRNA is recognized and bound by the oligomer, the loop unwinds and results in formation of a DNA/RNA duplex. The DNA sequence in this duplex is further cleaved by DSN and with hemin, the G-quadruplex gains enzymatic activity. Conversion of $ABTS^{2-}$ to $ABTS^{\bullet-}$ allows colorimetric detection with the naked eye. Another experiment with a similar arrangement was performed: a combination of a DNAzyme and an inhibitory spacer generates a hairpin, which opens up in presence of the target miRNA and the DNA in the formed duplex is targeted and cleaved by DSN, leading to release of DNAzyme, which this time

assembles with a molecular beacon. This interaction separates the quencher from the fluorophore and enables fluorescence detection and quantification of the target [Please see Fig. 12] (Tian et al. 2013).
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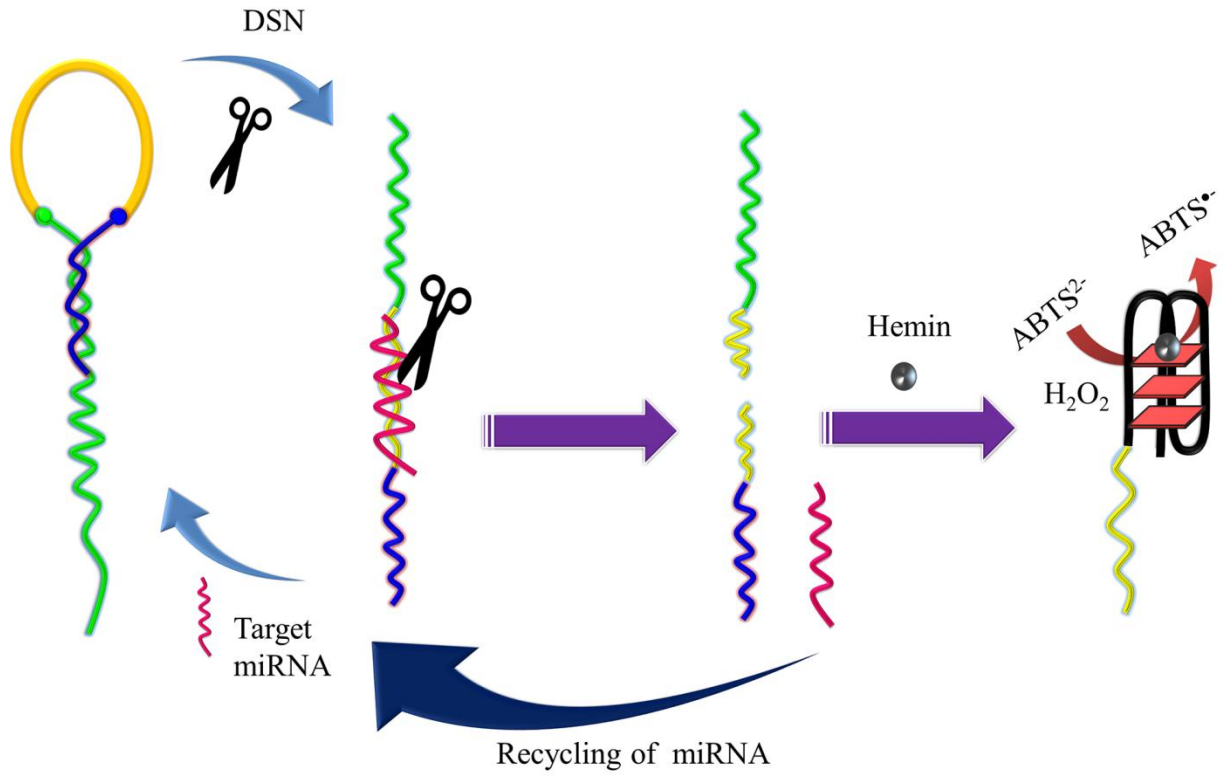

Fig. 12 Cascade amplification for miRNA detection (DSN: duplex-specific nuclease)

4.12 Another novel experiment for colorimetric detection of miRNA profits from a *multi-segment hairpin probe*, with each segment resolving a unique purpose: (i) hybridization region, (ii) the complementary sequence of G-rich DNAzyme, (iii) the stem of the probe to preserve hairpin stability and (iv) the recognition sequences containing two nicking spots for Nb.BbvCI. In brief, the procedure of detection goes as following: (a) Hybridization of the miRNA with the probe which spreads the hairpin into a linear shaped sequence. (b) Extension of the probe using primers and the polymerase enzyme. (c) Cleavage takes place at nicking spots by the nicking enzyme. As amplification of the target is accompanied with strand displacement, therefore, the target goes through the same cycle of probe hybridization, and the growing amount of amplified products enhances signal transduction. Nevertheless, in the absence of the target sequence, the probe does not open and maintains its hairpin structure. Obviously, the rest of the procedure will not be carried out [Please see Fig. 13] (Yan et al. 2013).
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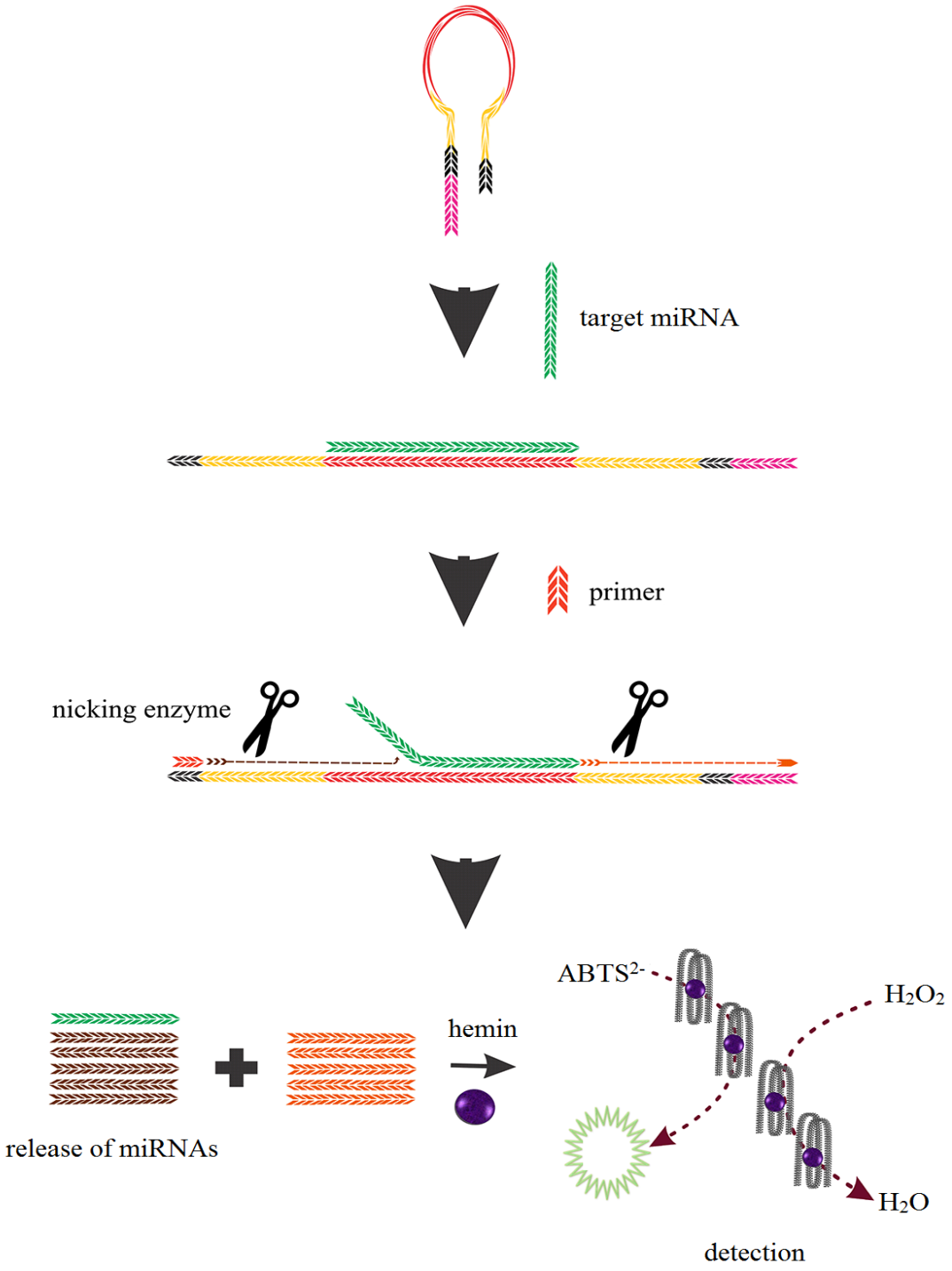

Fig. 13 Colorimetric detection using a multi-segment hairpin probe

4.13 Other DNAzyme based methods include formation of a thin insulating polymer film (Shen et al. 2013), exponential isothermal amplification-based chemiluminescence imaging of microRNAs (Xu et al. 2016) or exponential isothermal amplification-dependent generation of catalytic G-quadruplexDNAzyme(Wang et al. 2013), implementation of T7 enzyme (Sang et al. 2017), electrochemical detection with enzyme spheres (Wu et al. 2016b), self-cleaning functionalized microarray (Li et al. 2015b) and colorimetric detection by catalyzed hairpin assembly and hybridization chain reaction (Wu et al. 2016a).

Regarding the significant pace of emergence of DNAzyme-based methods, the hope is to develop methods with higher levels of sensitivity and specificity, which are also applicable to multiplex profiling in clinical and therapeutic approaches, where the time consumption and expenses are lowered to the least possible amount.

5. Summary and Conclusions

miRNAs are important molecules in terms of post-transcriptional gene expression. They are finely tuned in biological processes and their deregulation in pathologic cases such as cancer is indicative of their importance. For these reasons, development of appropriate methods for miRNA detection is in high demand. Selection of the method depends on different factors (e.g. cost, available techniques, type of sample, amount of miRNA). Besides, the method of choice must be appropriate regarding the goals of the investigation. Compared to traditional methods such as Microarray and qRT-PCR, newly emerged methods, especially those established on the basis of DNAzymes, have been shown to eliminate the labor of the detection process. On the other hand, these techniques are capable of providing more precise detection of the target miRNAs present in small amounts. Despite the fact that DNAzymes were introduced over twenty years ago, their application in different aspects such as miRNA detection is interesting. Methods based on these enzymes allow simple, rapid and sensitive detection by means of color production, which is observable with the naked eye and therefore, does not require complex tools.

6. Future perspectives

Currently, it is not easy to keep count of the numerous techniques emerging at a rising pace every day. Taking this fact into account, it is hard to predict the future application of these techniques. Besides, one cannot simply define the possible usefulness of a specific technique or the extent to which it might be helpful. However, the exertion of data mining or employment of systematic reviews seems to be a bright solution that shines a light on this area of research. It is the hope that in near future, colorimetric or more improved assays on the basis of DNAzymes develop to be utilized in clinical approaches, with regard to their ability in the practical detection of miRNAs with prognostic, diagnostic and predictive application in

diseases. It is hopefully expected that miRNA detection can serve as a promising tool with a major impact in personalized medicine, a wide and rapidly growing field in terms of clinics.

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Conflict of interest

The authors declare no conflict of interest.

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Highlights

- miRNAs serve as prognostic, diagnostic and predictive biomarkers and are candidate tools in personalized medicine.
- Detection of miRNAs is an essential task and can be performed through various methods.
- Some of the traditional methods include northern blotting, qRT-PCR and microarray.
- There are a variety of emerging methods advantaged by high sensitivity and specificity including DNAzyme-based assays.
- DNAzymes comprise a G-rich sequence which folds into a catalytic enzyme in presence of hemin (the cofactor), H_2O_2 and a dye, allowing simple detection of the target.