CHEMICAL REVIEWS

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# MicroRNA: Function, Detection, and Bioanalysis

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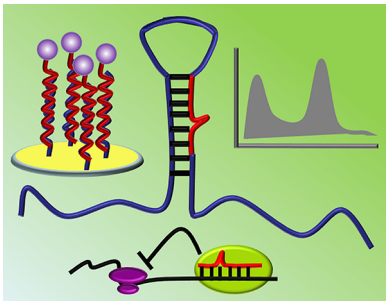

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## 1. INTRODUCTION

Mature microRNAs (miRNAs) are a class of evolutionally conserved, single-stranded, small (approximately 19−23 nucleotides), endogenously expressed, and non-protein-coding RNAs that act as post-transcriptional regulators of gene expression in a broad range of animals, plants, and viruses.[1,2] The biogenesis of miRNAs is a multiple step process. As shown in Figure 1, miRNAs are initially transcribed in the cell nucleus from intragenic or intergenic regions by RNA polymerase II to form primary miRNAs with length of 1−3 kb.[3] These primary miRNAs are cleaved in the nucleus by the RNase III enzyme Drosha and a double-stranded RNA-binding protein Pasha (DiGeorge Syndrome Critical Region 8 Protein) into approximately 70−100 nucleotide-long stem-loop structures, termed pre-miRNAs.[4,5] The pre-miRNAs are then transported from the nucleus to the cytoplasm by Exportin-5,[6] where the pre-miRNAs are further cleaved into the 18−24 double-stranded oligonucleotides by the RNase-III enzyme Dicer into mature double-stranded miRNA:miRNA*.[7]

After strand separation, one of the double strands becomes a mature miRNA molecule incorporated into RNA-induced silencing complex (RISC), while another "passenger" miRNA strand is often degraded or plays a functional role in the regulation of miRNA homeostasis as well as downstream regulation effect.[8,9] The RISC complex functions by perfectly or imperfectly matching with its complementary target mRNA, and induces target mRNA degradation or translational inhibition or sequestration of mRNA from translational machinery (Figure 2).[10,11] Most animal miRNAs pair imperfectly with their targets; thus, the translational inhibition

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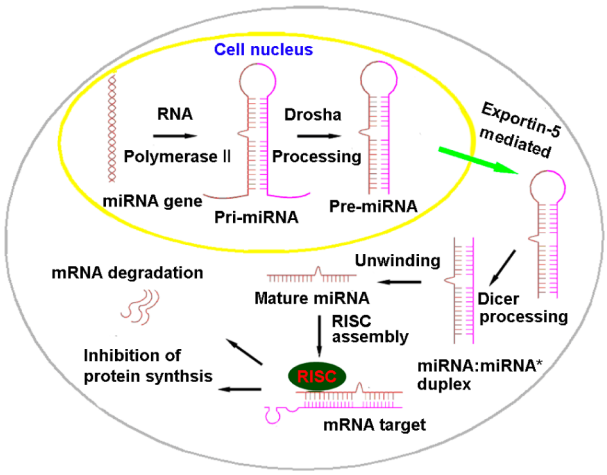


**Figure 1.** MiRNA biogenesis and function. Pri-miRNAs generated by RNA polymerase II are processed to pre-miRNAs by endonuclease Drosha in the nucleus. The pre-miRNAs are subsequently exported to the cytoplasm and cleaved to mature miRNAs by endonuclease Dicer, which are associated with the RISC complexes and bound to complementary sequences of their target mRNAs to repress their translation or induce their degradation.

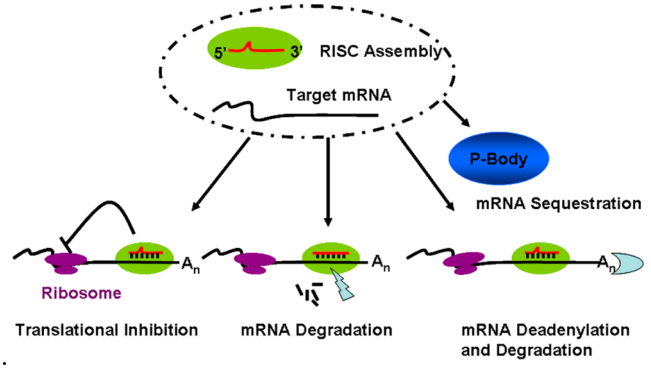


**Figure 2.** Gene regulation mechanisms of miRNAs: repression of mRNA translation, degradation of mRNA transcripts through cleavage, deadenylation, and localization in the processing body (P-body), where the miRNA-targeted mRNA can be sequestered from the translational machinery.

mechanism holds true for many miRNAs. The mechanism ultimately leads to reduced protein level and profound consequences on cellular homeostasis. This is an important topic for understanding miRNAs as tumor suppressors and biomarkers.

To date, over 1000 miRNAs have been identified in humans (http://www.sanger.ac.uk/Software/Rfam/miRNA/), which can target >30% of the human genome.[12] Importantly, a single gene can be regulated by multiple miRNAs, and likewise, a single miRNA might have more than one target due to the imperfectly matching between the miRNA and its target. The increasing evidence has indicated that miRNAs play critical regulatory roles in a vast range of biological processes including early development,[13] cellular differentiation,[14] proliferation,[15] apoptosis,[16] developmental timing,[17] and hematopoiesis,[18] etc. Thus, alterative expression of miRNAs has been associated with a number of diseases. Tremendous observations have been made in linking the aberrant expression levels of miRNAs to the initiation and development of human diseases, genetic disorders, and altered immune system function.[19−26] MiRNAs can act as both oncogenes and tumor suppressors, highlighting their importance in human cancer.[27,28] Therefore, miRNA expression profiles can be used as biomarkers for the onset of disease states,[29] and it is possible to use miRNAs in gene therapy for genetic disorders[30] as well as potential drug targets.[31] Intensive efforts have been made to develop miRNA-based therapy by restoring normal miRNA programs in the cancer cell to rewire the cell connectivity map and reverse cancer phenotypes.[32]

As the miRNA field continues to evolve, it is an essential step to develop efficient and reliable detection strategies for miRNAs toward understanding the functions of miRNAs in diverse regulatory pathways, which eventually influence the development of miRNA-based therapies in diagnostic tests at molecular level and new targets in drug discovery. In recent years miRNA detection and analysis have been quickly developed (Figure 3).[33] However, the analysis of miRNAs

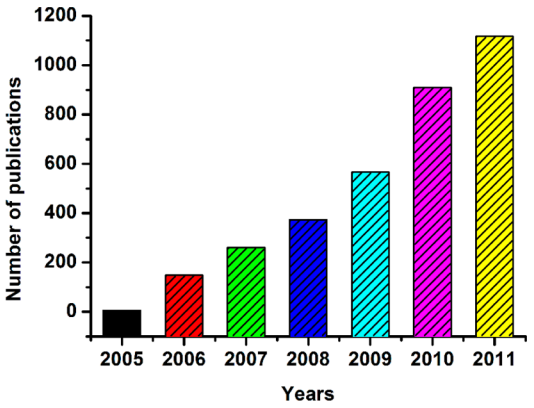


**Figure 3.** Annual trends in the number of publications for miRNA detection and bioanalysis. The terms "microRNA detection" and "microRNA bioanalysis" have been considered as subjects using ISI's Web of Science.[33].

introduces many demands due to unique characteristics of miRNA including small size, low abundance, and sequence similarity among family members.

First, the small size of miRNAs greatly complicates the vast majority of miRNA assays based on polymerase chain reaction (PCR) or hybridization, because the primers used in most conventional PCR are similar in length to miRNAs, which means that very short primers would be required for assay design, which affects the PCR efficiency due to a very low melting temperature.[34,35] As for hybridization-based detection, it is difficult to label the short probe for selective detection of miRNAs. Meanwhile, the duplex melting temperature between the probe and its target is low, which sharply decreases the stringency of hybridization and increases the risk of cross-hybridization. Mismatch sequence can easily produce a false positive signal. Thus, new strategies focusing on improving the specificity of miRNA profiling measurements are significant and urgent.

Second, the miRNAs represent only a small fraction (ca. 0.01%) of the mass in total RNA sample; miRNA concentration in cellular can be as low as a few of molecules per cell.[36] The low abundance requires a highly sensitive assay, while the high sequence similarity among family members makes the specific detection more difficult.

Third, two other important considerations include dynamic range and multiplexing capability. The expression level of


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miRNAs varies by as much as 4 orders of magnitude from a few copies to over 50 000 copies per cell;[1] thus, a wide dynamic range of detection is required for miRNA assay. Meanwhile, a single gene can be simultaneously regulated by multiple miRNAs, which requires methods to detect multiple miRNA to fully understand the important and complex function of these tiny regulators. Considerable effort is continuously devoted to explore high-throughput analysis strategies of multiplexed miRNA gene expression. Northern blotting is widely used as a standard method for multiplexed miRNA analysis. The progress in primer design with high specificity led to the successful application of reverse transcriptase PCR (RT-PCR) in multiplexed miRNA analysis. Various oligonucleotide microarray-based detection platforms have been developed and used as the most-efficient approaches for miRNA high-throughput profiling.

Importantly, another challenge is intracellular or in situ detection. A noninvasive monitoring approach is needed to provide detailed spatial expression patterns for specific miRNAs in vivo with high capability to discriminate the pre- and mature miRNA to elucidate miRNA function and facilitate the translation of miRNA detection methods into clinical practice. Taken together, rapid and highly sensitive and selective miRNA analysis methods that can effectively profile miRNA in minimal amounts of sample and feasibly be applied to in situ detection are an urgent need.

This review summarizes the miRNA biology in a variety of physiological and pathological processes, with emphasis on the significant potential application of miRNA in diagnostics and prognostics as well as drug targets. Some of the conventional techniques used for miRNA detection including standard PCR, Northern blotting, and microarray methods are discussed. Recent emerging strategies in miRNA detection and quantification with superior flexibility and adaptability such as novel molecular biological techniques and lock nucleic acid (LNA) modified probe as well as nanotechnology-based approaches are also included. Importantly, the latest development of detection methods including in situ hybridization (ISH) and molecular imaging techniques for monitoring miRNAs in living cell, circulating blood, and tissue is highlighted, which can assist the elucidation of the biogenesis and biological function of miRNAs in vivo. Additionally, the advantages and disadvantages of various experimental techniques in this fast moving field along with the challenge and new directions are also presented.

## 2. BIOLOGICAL FUNCTIONS OF MIRNA

The degree of complementarity between miRNA and the target is a major determinant factor distinguishing the miRNA−mRNA interaction mechanism. In case of near-perfect complementarity to the miRNA, target mRNA can be cleaved (sliced) and degraded. Otherwise, their translation is repressed.[37−39] For mRNA degradation by a miRNA,[40,41] the mechanism can be explained by mRNA-processing bodies.[11,42−44] However, interestingly, a recent report has shown that miRNA-369-3p can upregulate the expression of tumor necrosisfactor-$\alpha$.[45] Although less explored, miRNAs can also bind to the 5′-UTR[46] and coding sequences.[47−49]

Genetic deletion of miRNAs in organisms has shown that few developmental processes are absolutely dependent on single miRNA.[50,51] The significant redundancy within miRNA families is considered to serve as a buffer against deleterious variations in gene-expression programs.[52,53] The human genome is estimated to encode up to 1000 miRNAs,[1] whereas the number of mRNAs is typically estimated at ∼30 000. Thus, one miRNA may regulate hundreds of mRNAs and, as a result, may substantially affect gene expression networks.[54] The multiplicity of miRNA targets may also promote combinatorial regulation by miRNAs that individually target various mRNAs whose protein products contribute to one particular regulatory axis.[55] Clearly, miRNA biology is a complex and highly orchestrated mode of gene regulation network.

MiRNAs play important roles in almost every biological process, including cell fate determination, proliferation, and cell death. In addition to these vital processes, miRNAs are implicated in diverse cellular activities, such as immune response,[56−58] insulin secretion,[59] neurotransmitter synthesis,[60] circadian rhythm,[61] and viral replication.[46] Recent genome-wide analyses have also identified deregulated miRNA expression in human malignancies.[44] MiRNAs can modulate oncogenic or tumor suppressor pathways, whereas miRNAs themselves can be regulated by oncogenes or tumor suppressors.[43,44] A more complete list of individual miRNAs that function in specific tissues or at specific times can be found in recent published reviews.[62,63]

### 2.1. MiRNA Regulation in Various Physiological Processes

**2.1.1. MiRNA in Cell Development.** The function of miRNAs has been manifested by the earliest discovery of two miRNAs, *lin-4* and *let-7*, both of which control the timing of larva development in *C. elegans*.[64,65] Animals that are unable to produce mature miRNAs do not survive or reproduce.[66] Abundant experiments have demonstrated the importance of miRNAs in neuronal, muscle, and germline development. In *C. elegans*, *dcr-1* mutants display defects in germline development and embryonic morphogenesis.[67] In *Drosophila*, depletion of Loquacious can cause female sterility,[66] and dicer-1 mutant germ-line stem cells display cell division defects.[68] The phenotypes of zebrafish mutants that lack both maternal and zygotic Dicer activity show defects in germ layer formation, morphogenesis, and organogenesis.[69−71] These discoveries underline the importance of miRNA homeostasis on proper development.

In vertebrates, miRNA-124 is considered as a central regulator of neural development. In vitro, miRNA-124 overexpression triggers neural development, while miRNA-124 knockdown prevents neural development.[72] MiRNA-124 is also found to be abundantly expressed in neural progenitors and mature neurons, and enhances neural development.[71,73]

The roles of miRNA in the development of heart, vascular tissue, and blood have been popularly studied. MiRNAs are implicated in various cardiovascular pathologies as diverse as arrhythmias (miRNA-1[74] and miRNA-133[75]), fibrosis (miRNA-21[76] and miRNA-29[77]), pressure overload-induced remodeling (miRNA-208[78] and miRNA-133[79]), and metabolic disorders (miRNA-33[80]). MiRNA-1 is the most abundant miRNA in cardiac myocytes, and is also the first miRNA implicated in heart development.[81] MiRNA-126, miRNA-218, miRNA-143, and miRNA-145 have been reported to be responsible for modulating vascular and blood development.[55] The development of blood cells is also dependent on miRNA activity.[82]

**2.1.2. MiRNA in Cell Differentiation.** Embryonic stem (ES) cells have distinct miRNA signatures. MiRNAs play a significant role in ES cell differentiation. For example, miRNA-290∼295 cluster and miRNA-296 levels decrease during stem cell differentiation, while miRNA-21 and miRNA-22 increase in

this process.[83] Studies on mice bearing deletions of genes in miRNA biogenesis pathway provide early evidence on the importance of miRNAs on stem cell generation and regulation.[71,84]

Individual miRNA may have specific roles in controlling stem cell function.[83] The proliferation defects of Dgcr8 mutant mouse ES cells are partially rescued by expression of miRNA-290∼295/302 family, which are called ES cell-specific cell cycle-regulating miRNAs.[71] Whereas ES cell-specific miRNAs enable Dgcr8 mutant ES cells to proliferate, mature miRNA *let-7* rescues their differentiation defects partially.[85] *Let-7* represses several key pathways that are crucial for ES cell identity, including the promotion of cell cycle progression[86] and stem cell identity.[85,87] The opposing roles of ES cell-specific miRNAs and *let-7* in ES cell illustrate the combined action of miRNA sets to determine cell fates.[71] In addition to *let-7*, several other miRNAs have been implicated in repressing pluripotency in differentiating ES cells.[15] For example, human miRNA-145 downregulates OCT4, SOX2, and KLF4 upon differentiation.[88] In addition, miRNAs are also involved in modulating cancer stem cells. *Let-7* is one of the examples that miRNAs play functional roles in both normal and cancer stem cells.[83] *Let-7* can regulate multiple stem cell-like properties in breast tumor-initiating cells by silencing multiple targets.[89]

**2.1.3. MiRNA in Immune System.** MiRNA control has emerged as a critical regulatory principle in the mammalian immune system. Genetic ablation of the miRNA machinery severely compromises immune development and response, and can lead to immune disorders like autoimmunity and cancer.[83,90] MiRNAs are differentially expressed, both spatially and temporally, in many types of immune cells, and also have distinct expression profiles in resting or activated immune cells.[58,91−93] For example, in monocytes, macrophages, and myeloid dendritic cells, miRNA-155 increases substantially upon a variety of inflammatory stimuli.[92−94]

MiRNAs have crucial roles in regulating the development and function of innate immune cells.[57] Overexpression of miRNA-21 and miRNA-196 in lineage-depleted bone marrow cells blocks the granulopoiesis in vitro. Sustained expression of miRNA-155 can increase immature granulocyte numbers in vivo.[95] The expression levels of miRNA-17-5p, miRNA-20a, and miRNA-106a, and members of the miRNA-17-92 and related miRNA-106a-92 families, have been shown to decrease during the differentiation of human hematopoietic progenitor cells into monocytes.[56] MiRNA-146a, miRNA-132, and miRNA-155 have been found to be regulated in response to immune-cell stimulation by endotoxins.[93] The macrophage inflammatory response to infection involves the upregulation of several miRNAs including miRNA-155, miRNA-146, miRNA-147, miRNA-21, and miRNA-9.[56]

In addition to regulating hematopoietic-cell lineage differentiation, miRNAs are also relative with modulating adaptive immune responses.[96,97] MiRNA-155 can be upregulated following B cell activation in the germinal center. MiRNA-155-deficient B cells display an impaired humoral response to T cell-dependent antigenic stimulation.[96,97] The miRNA-150 has a dynamic expression profile during lymphocyte development. It is highly expressed in mature B cells and T cells but not in their progenitors and then extinguishes after further differentiation into the effector T helper type 1 and T helper type 2 subsets.[98] In another study, miRNA-181a is verified to modulate the strength and threshold of T-cell receptor signaling, thereby influencing T-cell sensitivity to antigens.[99] In addition, the dynamic regulation of miRNA-181a during T-cell development and maturation seems to correlate with changes in T-cell sensitivity to antigens.

## 2.2. MiRNA Regulation in Various Pathological Processes

**2.2.1. MiRNA Aberrant Expression in Cancer.** Since miRNA functions are involved in regulating crucial biological processes, it is now considered that miRNAs have been linked to the etiology, progression, and prognosis of cancer.[49] The alteration of miRNA expression profile is a common characteristic of all human tumors.[43,44] For instance, miRNA-126, miRNA-143, and miRNA-145 are expressed at significantly lower levels in >80% of the tumor samples compared with associated normal tissues, whereas miRNA-21 is found to be overexpressed in 80% of the tumor samples.[100] Thus, miRNAs hold promise as biomarkers for cancer diagnostics and prognostics and for classification, staging, progression, and response to treatment.[101]

Altered miRNA expression in cancer was first observed in miRNA-15a and miRNA-16-1 within locus *13q14*, both of which are deleted or downregulated in the majority of chronic lymphocytic leukemia (CLL) cases.[102] Later, these two miRNAs were shown to negatively regulate antiapoptotic B cell lymphoma 2 protein,[103] suggesting a possible molecular mechanism by which losing miRNA-15a and miRNA-16-1 can cause CLL. Since then, many efforts have been devoted to characterization of miRNA expression levels and their biological correlates in a variety of cancers.[104,105] The first comprehensive profiling of hundreds of tissue and cancer samples was documented by Lu et al. in 2005.[104] There were 217 mammalian miRNAs from 334 samples including multiple human cancers analyzed. There were 129 of the 217 miRNAs globally reduced in tumors compared with normal tissues.

Deregulation of miRNA expression in cancer can arise from four different mechanisms, including chromosomal abnormalities, genomic mutations and polymorphism, epigenetic changes, and alterations in miRNA biogenesis.[43,83] Different tumors show some common characteristics of miRNA deregulation.[83] First of all, miRNA profiles of tumor cells are significantly different from normal cells from the same tissue,[104,106] underlining the biological significance of miRNA function during the cancer progression.[83] Despite some miRNAs increase, most are repressed in cancers relative to normal tissue counterparts.[104,105] Moreover, the difference between the global profiles is not located at a single miRNA, but from many miRNAs, suggesting that the tumorigenesis and progression are relevant with the entire miRNANome.[43] Second, miRNA expression profiles in tumors from similar developmental origins appear to have similar alterations, providing a tool for cancer diagnosis and prognosis.[107] Third, some miRNAs appear to be frequently deregulated in many cancers, suggesting that these miRNAs may participate in fundamental signaling pathways altered in many types of malignancies.

The global depletion of miRNAs by knockdown of the miRNA-processing machinery stimulates cell transformation and tumorigenesis in vivo,[108] which implies that the miRNA alteration may contribute to cancer development. So far, expression profiling studies have identified many miRNAs related to tumor development. A large microarray analysis of 540 samples, including 363 solid tumors from the six most frequent malignancies (breast, prostate, lung, stomach, pancreas, and thyroid) and 177 normal tissues, demonstrates


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a more complex role of miRNAs in cancer by identifying a specific miRNA "miRNANoma" expression signature.[106] The miRNANoma consists of 36 overexpressed and 21 downregulated miRNAs. This study supports the view that miRNA alterations in cancer consist of both downregulated and overexpressed miRNAs with putative tumor suppressive and oncogenic functions.[44]

**2.2.2. MiRNA as Oncogene.** The gain or loss of specific miRNAs can function as an oncogene or tumor suppressor,[49] through miRNA-155, miRNA-17∼92, miRNA-21, miRNA-372, miRNA-373, and *let-7*, miRNA-15a, miRNA-16-1, miRNA-34s, respectively.

miRNA-155 represents the only example that a single miRNA is sufficient to induce tumorigenesis so far. Transgenic mice with miRNA-155 overexpression exhibit a preleukemic pre-B cell proliferation which progresses to B cell leukemia and high-grade lymphoma.[109] High expression of miRNA-155 has been reported in various B cell malignancies, including diffuse large B-cell lymphomas, Hodgkin lymphomas, aggressive CLL, and subsets of Burkitt lymphomas,[110−112] and found to indicate poor prognosis.

One of the first identified oncogenic miRNAs is the cluster miRNA-17-92 (also known as oncomiRNA-1). The cluster is located at chromosome *13q31.3* in humans, which is processed from a polycistronic transcript into seven miRNAs (miRNA-17-5p, miRNA-17-3p, miRNA-18a, miRNA-19a, miRNA-20a, miRNA-19b-1, and miRNA-92-1).[44] The miRNA-17-92 cluster is upregulated in a variety of cancers including lymphomas, lung cancers, and others.[43] The oncogenic activity of miRNA-17-92 cluster is revealed in the E$\mu$−Myc transgenic mouse model of B cell lymphoma.[113] Another example of oncogenic miRNAs is miRNA-21, which is upregulated in almost all kinds of cancers.[43] Transgenic mouse models with loss and gain of function of miRNA-21 combined with a model of lung cancer confirm the role of miRNA-21 as oncogene.[114]

**2.2.3. MiRNA as Tumor Suppressor.** In contrast to oncogenic miRNAs, miRNAs whose expression is decreased in malignant cells may function as tumor suppressor by negatively inhibiting oncogenes and/or genes that inhibit cell differentiation or apoptosis. MiRNAs acting as tumor suppressor genes include the *let-7*,[27] who negatively regulates Ras and HMGA2; miRNA-15a and miRNA-16-1,[102] which negatively regulate B cell lymphoma 2 protein; as well as the miRNA-34,[115] that is induced by DNA damage and oncogenic stress in a p53-dependent manner which leads to apoptosis or cellular senescence.[83]

*Let-7* is one of the earliest-discovered miRNAs. In human, there are 12 paralogous *let-7s*. *Let-7* is probably more abundant than any other miRNA.[43] The role of *let-7* in cancer was first demonstrated by the Slack group when they found that the *let-7* family negatively regulated let-60/RAS in *C. elegans*.[27] Moreover, *let-7* expression was found lower in lung tumors than in normal lung tissue, forced expression of *let-7* was able to suppress cancer cell growth both *in vitro*[86] and *in vivo*.[116] In addition, the reduced expression of *let-7* has been associated with shortened postoperative survival in various types of cancer.[83] Hence, the loss of *let-7* might contribute to the pathogenesis of several types of human tumors.

These studies described above highlight the magnitude of miRNA genes in the pathogenesis and progression of human cancer and provided insight into new opportunities for cancer treatment by modulating miRNA pathways and activities. The more indepth reviews of miRNAs in cancer have been reported by Farazi[117] and Lee.[43]

**2.2.4. Other Pathological Processes.** Since miRNAs are implicated in various cellular activities, including immune response,[56−58] insulin secretion,[59] neurotransmitter synthesis,[60] and circadian rhythm,[61] they play significantly regulated roles in the relating pathological processes.[83] For example, miRNA-133 is involved in secondary complications related to diabetes.[118] MiRNAs have been reported to associate with a number of pathological conditions of the central nervous system, such as Alzheimer's and Parkinson's.[119] In addition, the roles of miRNAs in the pathological processes of heart, vascular tissue, and blood have been popularly studied. For example, miRNAs are associated with various cardiovascular pathologies such as arrhythmias,[74,75] fibrosis,[76,77] pressure overload-induced remodeling,[78,79] and metabolic disorders.[80]

## 3. MIRNA IN VITRO DETECTION

### 3.1. Challenges of MiRNA Expression Analysis

Detection of miRNA expression can help to identify miRNAs that regulate a range of vital processes, and discover miRNA-based biomarkers for diverse molecular diagnostic applications in cancer, cardiovascular and autoimmune diseases, psychiatric and neurological disorders, and forensics.[54] It can also be combined with mRNA profiling and other genome-scale data for integrative analyses of miRNAs-involved interactions and systems-level study of gene regulation.

Currently, a wide range of approaches are available for miRNA profiling. This section highlights the traditional strategies for detection of miRNA, including Northern blotting, microarrays, and quantitative RT-PCR (qRT-PCR). Each platform has its relative strengths and weaknesses; thus, researchers should make their choice based on the given application, and try to figure out a balance of cost, precision, accuracy, and sample quantity.[120]

Novel and previously unidentified miRNAs are often identified by Northern blotting analysis. However, this method requires a large amount of total RNA (hundreds of micrograms) as starting material, and often fails to detect miRNAs with low abundance.[100] MiRNA microarrays are less expensive but tend to have a lower sensitivity and dynamic range; therefore, they are often used as screening tools rather than as quantitative assay platforms. qRT-PCR has the widest dynamic range and highest accuracy and is the only method that can easily provide absolute miRNA quantification. However, it suffers from throughput issues.[121] This section will discuss these methods in detail, and the general consideration of miRNA detection will be introduced first.

### 3.2. General Considerations in MiRNA Detection

MiRNAs represent a small fraction (∼0.01%) of the total RNA mass. The average copy number of an individual miRNA species has been roughly estimated at ∼500 per cell.[122] However, different miRNA species in cells vary widely in concentration over a dynamic range.[122] Sample processing and RNA extraction methods have a substantial impact on the results of miRNA profiling, particularly for samples that are prone to miRNA degradation.[123]

**3.2.1. Specimen Storage and Processing.** A wide range of cell and tissue sources, including cell lines, fresh tissues, formalin-fixed paraffin-embedded tissues, plasma, serum, urine, and other body fluids, can yield high-quality miRNA that is suitable for detection.[54] Unlike mRNA, miRNA is surprisingly


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stable and intact in formalin-fixed paraffin-embedded tissues, from which the data are consistent with those from frozen samples.[124] For some specimen types, such as human blood plasma with distinct miRNA physical states[125] and high levels of endogenous RNase activity,[107] the preparation as well as miRNA extraction methods need to be specially optimized. In addition, the centrifugation conditions, white blood cell counts, and red blood cell hemolysis also affect miRNA measurements.[126]

**3.2.2. MiRNA Extraction.** The principles for isolating miRNA are similar with those for isolation of total RNA, except that miRNA isolation protocols often focus on retaining the small RNA fraction. Chemical extraction using concentrated chaotropic salts, such as guanidinium thiocyanate (for example, Trizol and QIAzol reagents), is widely used in commercially available kits, followed by a solid-phase extraction procedure on silica-based columns.[54] Owing to the miRNA features of low molecular weight and low abundance, miRNA enrichment is an important step for expression analysis.[127] The miRNAVana miRNA Isolation Kit and flashPAGE Fractionator from Ambion Inc. and the PureLink miRNA Isolation Kit from Invitrogen are examples of established methods that applied to miRNA enrichment.[100]

Assessment of the quality and quantity of extracted RNA is important for reproducibility and accuracy in miRNA-profiling studies. It is routine to assess the yield and degree of overall RNA integrity using spectrophotometry and automated capillary electrophoresis instruments.[128] For variation of RNA extraction efficiency and/or inhibitors of reverse transcription or PCR, a known amount of control miRNAs can be "spiked-in" at an early step in RNA isolation to normalize data.[129] Analysis on an RNA gel or Agilent Bioanalyzer can assess whether RNA is degraded to be used for a particular experiment.

### 3.3. Conventional MiRNA Detection Strategies

**3.3.1. Northern Blotting.** Northern blotting is the earliest attempt at systematically profiling miRNA expression.[130−132] It is widely used for visualizing miRNA expression of all sizes ranging from the long primal miRNA to the mature form.[133,134] Although Northern blotting is low-throughput, low-sensitivity, and relatively time- and sample-consuming,[135] it continues to be widely used as a gold-standard approach for validating data from newer, more-sensitive detection techniques.[136] For example, Tang et al. introduced an additional array-data-adjustment step-Northern blot analysis of a ratio of a given miRNA and U6 or tRNA to validate results from ambiguous array data and to enable accurate data interpretation.[137]

Numerous reports have shown various improvements of the Northern blotting technique.[138−145] These methods primarily differ in the labeling and design of the probes used to detect miRNA. The most popular probe-labeling protocol is based on incorporation of radio isotopes ($^{32}$P).[145] However, isotope labeling is often inconvenient and hazardous and is restricted by many institutions. As a safer alternative, digoxigenin (DIG)-labeling system has several advantages: high sensitivity, short exposure time, longer shelf life, and increased safety. Ramkissoon et al. reported the use of DIG-labeled RNA oligos for the detection of small RNA molecules (~22 nucleotides), and demonstrated that the DIG-labeled RNA probe was equally sensitive compared to $^{32}$P-labeled probes in detecting miRNA.[138]

Probe-design strategies have also been significantly improved in the recent years. The traditional DNA oligonucleotide probes have been increasingly replaced by LNAs oligonucleotide probes that considerably improve the sensitivity in detecting small RNAs.[143,144] LNAs comprise a class of bicyclic high-affinity RNA analogues in which the furanose ring of LNA monomers is conformationally locked in an RNA-mimicking C3′ *endo*/*N*-type conformation.[101] LNA-modified oligonucleotide probes have been shown to detect miRNAs by at least a 10-fold higher efficiency than traditional DNA probes in Northern blotting of miRNA.[145]

Cross-linking of the RNA to the membrane frequently improves the sensitivity of Northern blots. However, conventional methods such as UV-cross-linking are generally not optimal for detection of small RNAs.[142] Thus, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) has been employed to cross-link RNAs to the nylon membrane, which provided a 25−50-fold increase in the sensitivity of detection of miRNA.[140,141]

Kim et al. reported a new Northern-blot-based protocol for miRNA detection using DIG-labeled oligonucleotide probes containing LNA and EDC for cross-linking the RNA to the membrane.[142] The assays generated clearly visible signals for RNA amounts as low as 0.05 fmol and reduced the exposure time by ~1000-fold.

**3.3.2. MiRNA Microarrays.** miRNA expression patterns provide essential information for determination of their biological function and regulation.[101] Among the most common techniques that are used for miRNA profiling, microarray-based detection is particularly effective for profiling large numbers of miRNAs (Figure 4).[146] However, it requires further validation to quantify the expression more accurately.[135] As a result of the small size and low abundance of miRNAs,

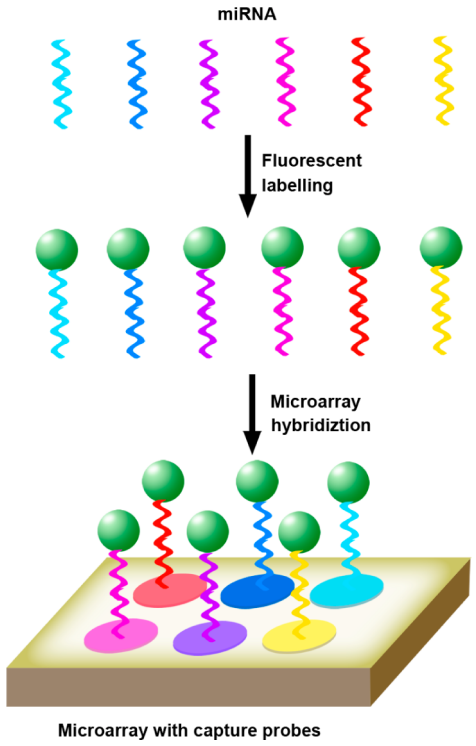


**Figure 4.** Schematic representation of microarray-based miRNA expression profiling.


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current microarray-based strategies have been developed focusing on improvement of the probe design and miRNA labeling.[147]

Detection of miRNAs by DNA microarrays has been described in several early reports.[148,149] However, the major drawback of all DNA-based oligonucleotide array platforms is the difficulty to obtain $T_m$-normalized probe sets for genome-wide expression profiling. In order to solve the issue, LNA-modified capture probe has been introduced in miRNA microarray to allow $T_m$ normalization of array capture probes.[150] Importantly, LNA incorporation improves mismatch discrimination, alleviates the need for purification and amplification of miRNAs,[150] and displays high stability and low toxicity in biological systems.[101] Application of 2′-*O*-(2-methoxyethyl)-modified oligoribonucleotides[127] and adjusting the length of probes according to physicochemical traits of the target miRNA[151] have also been used to balance melting temperatures.[100] The incorporation of hairpin structure onto the 5′ end of the probe confers the ability to distinguish the targeted miRNA from larger RNAs, such as miRNA precursors.[151] In addition, it is important to introduce exogenous and endogenous positive controls and negative control probes to assist normalization and to provide absolute reference points for quality control and quantitative comparison of different microarrays.[100]

In 2003, Krichevsky et al. designed an oligonucleotide array that could detect miRNAs by labeling low molecular weight RNA with radioactive isotopes,[152] which was quickly followed by other labeling technologies.[153,154] Fluorescent labeling of the miRNA in a biological sample for subsequent hybridization to capture probes on the array is the most common strategy for parallel analysis of large numbers of miRNAs.[54] Owing to the extremely small size of miRNAs, direct labeling of miRNA molecules might be advantageous.

Enzymatic labeling mainly includes two approaches. One uses T4 RNA ligase to catalyze the ligation of a fluorophore-conjugated nucleotide or short oligonucleotide to the 3′ end of miRNA. However, this method may lead to circularization owing to an intramolecular ligation reaction. Another approach involves two steps: a fluorophore-conjugated oligonucleotide is ligated by a splinted ligation after 3′ tailing of miRNA. This method avoids the problem of circularization but may add a large and variable number of nucleotides during tailing.[54] In both of the cases, the enzyme favors certain sequences over others, and so creates different artifacts. Worse, such biases tend to be more serious in degraded samples.[120,155] To minimize the interference of structure and sequence differences among miRNAs, Wang et al. introduced dimethylsulfoxide, an effective RNA denaturant, into the reaction solution and found that up to 20% dimethylsulfoxide stimulated T4 RNA ligase activity.[151] Another shortcoming of enzyme-based labeling methods is that they are unable to detect 5′ end mismatches due to the 3′ activity of the enzymes. Besides, for miRNAs with natural modifications at 3′ ends such as plant miRNAs, enzymatic labeling is also problematic.[156]

Alternative chemical approaches of miRNA labeling include chemical alkylation-based labeling along the miRNA (for example, MiRNA Bio Label IT) and approaches based on platinum coordination chemistry with nucleic acids (for example, Kreatech ULS).[54] However, chemical labeling methods are insensitive to the 3′ end modifications, and are suspicious of introducing bias by selectively labeling certain nucleotides with higher efficiency than others.[136]

In both enzymatic and chemical approaches, coexisting pre-miRNAs may be labeled, leading to background signal as well as cross-hybridization.[156] Initial size fractionation of small RNA using column- or gel-purification-based methods may be helpful to solve the problem.[54] In addition, background correction and normalization must be performed to remove dye labeling bias and differences in hybridization and scanning.[65]

Current mainstream commercial miRNA microarrays are all label-based,[157] and have been reported to have poor performances in terms of interplatform concordance[155] owing to the drawbacks associated with labeling procedure.[146] Although some problems have been partially solved in the RAKE assay devised by Nelson et al. via posthybridization labeling,[153] remaining difficulties accompany the usage of two different enzymes.

MiRNA array detection method free from labeling and amplification reactions can clearly simplify the process and greatly bolster the credibility of miRNA-profiling studies, particularly for diagnostic purposes. Novel efforts have been continuously made to develop nonlabeling methods for direct miRNA detection in the past decade.[158,159] These works mainly focus on two aspects: first, labeling secondary probe rather than miRNA target, and second, making use of special equipment.

"Stacking hybridization" has also been introduced into label-free miRNA microarray to combine target capturing and fluorescent signaling in a single step, but without labeling the targets. Here, the total RNA is directly applied to the microarray with a short fluorophore-linked oligonucleotide universal tag which can be selectively captured by the target-bound probes via base-stacking effects (Figure 5). This

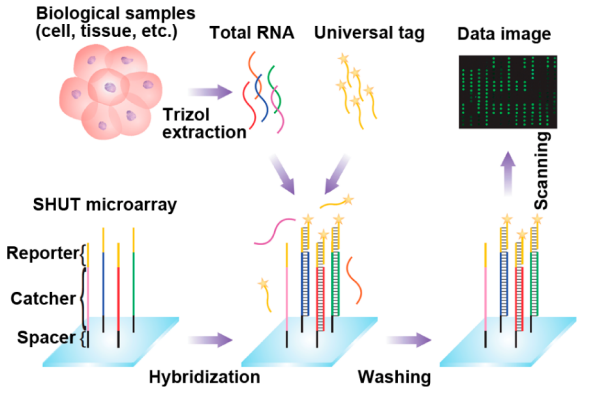


**Figure 5.** Schematic diagram of the stacking-hybridized universal tag (SHUT) assay. Reprinted with permission from ref 156. Copyright 2011 Oxford University Press.

stacking-hybridized universal tag assay has been successfully used to analyze as little as 100 ng total RNA, and found to be highly specific to homogeneous miRNAs.[156] Stacking-hybridized universal tag microarray assay with high capability of discriminating single-base difference has been found to be a useful way for unbiased profiling of both normal and methylated small RNA species.[160]

To further eliminate cross-hybridization between genome-wide miRNAs and short probes, a two-temperature hybridization procedure, 42 °C for the capture step and 64 °C for the detection step, has been developed for miRNA profiling (Figure 6). In this assay format, label-free miRNAs are trapped between two short probes (approximately 10 nt). This method can achieve detection at attomolar concentrations, and can be


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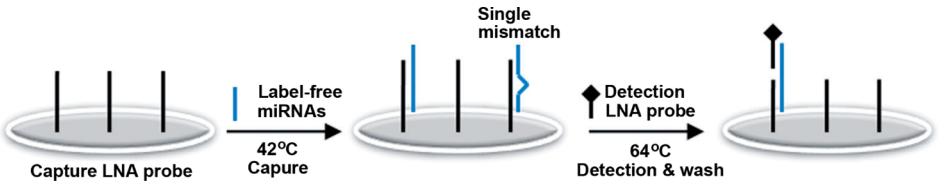


**Figure 6.** Representation of miRNA array detection by two short, LNA-modified probes in a two-temperature hybridization procedure. Reprinted with permission from ref 147. Copyright 2011 John Wiley and Sons Ltd.

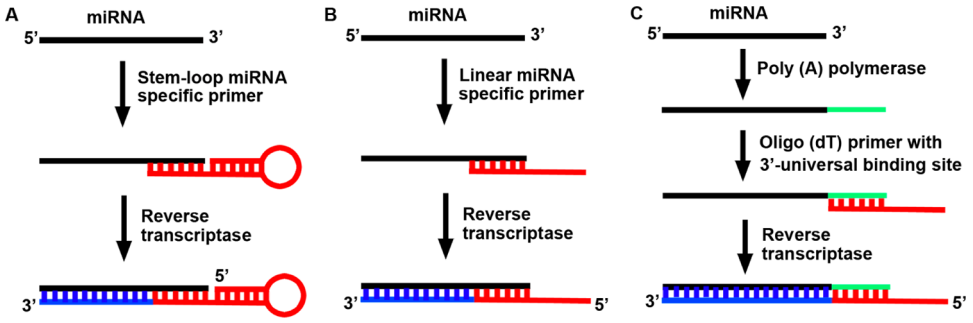


**Figure 7.** Schematic representation of alternative reverse transcription methodologies to generate cDNA. Reverse transcription of individual miRNAs using (A) stem-loop or (B) linear primers, and (C) enzymatic addition of a poly(A) tail to miRNAs followed by reverse transcription using oligo(dT) primers.

improved by combination with bead-based or microfluidic platforms.[147]

Besides adjusting hybridization format, novel recognition elements for miRNA have also been introduced in label-free miRNA detection. Lee et al. described the first example using antibody-like protein-based array for detection of miRNA through linking dsRBD motif with the PAZ domain.[161] PAZ, an RNA-binding module, can specifically recognize dsRNA with a 2 nt 3′ overhang. The dsRNA-binding domain (dsRBD) can further stabilize the RNA−PAZ complex. The resulting RNA binder, PAZ−dsRBD, acting like an antibody, enabled sensitive detection of miRNA in the concentration range from 10 to 500 pM without the need for enzymatic amplification or labeling reactions. Further engineering of the PAZ−dsRBD construct by addition of signal-amplifying enzymes for ELISA-like detection may lead to improved sensitivity.[158]

In conclusion, miRNA microarrays have the advantage of generally being less expensive and yet they allow large numbers of parallel measurements. At present, many companies offer microarray platforms for miRNA profiling.[120] Thus, miRNA microarrays have been successfully used to explore the biogenesis of miRNAs, tissue distribution, differential miRNA expression between normal and abnormal states, disease characterization, stem cell development, pathway mapping, mechanisms of action, and tumorigenesis. Limitations include a restricted linear range of quantification, imperfect specificity in some cases for miRNAs that are closely related in sequence, inability to compare results between different techniques, and lack of ability to perform absolute quantification of miRNA abundance easily.

**3.3.3. qRT-PCR.** Generally, qRT-PCR covers a large dynamic range and is regularly deemed as the "gold standard" for gene expression.[156] qRT-PCR-based miRNA profiling relies on reverse transcription of miRNA to cDNA, followed by qPCR with real-time monitoring of reaction product accumulation. This technology represents a balance of cost, precision, and sample size, and is often used for expression profiling of miRNAs and for validating results obtained by other methods such as microarray and Northern blot assays.[162]

The development of qRT-PCR methods has improved the sensitivity of miRNA detection down to a few nanograms of total RNA.[163] Several parameters such as RNA integrity control, cDNA synthesis, primer design, amplicon detection, and normalization must be taken into account to obtain meaningful and reproducible results.

The reverse transcription of miRNA to cDNA includes two common strategies and poly(A) polymerase method (Figure 7).[54,164] In the latter method, polyadenylation of all miRNAs by *Escherichia coli* poly(A) polymerase is followed by reverse transcription using universal primers consisting an oligo (dT) sequence on its 5′ end,[165,166] whereas the former reverse transcribes only particular miRNAs using miRNAs-specific reverse transcription primers.

The poly(A) polymerase method is more suitable for detecting several miRNAs from a very small amount of starting material, such as plasma.[135] However, it can neither distinguish pre-miRNAs from mature miRNAs, nor detect small RNAs carrying a 2′-oxymethyl modification at their 3′-ends (2′-OMe RNAs; e.g., plant miRNAs).[167]

In the former approach, the 3′-end of the primer has to be complementary to the miRNA.[168,169] The stem-loop at 5′-end can help to reduce annealing of the primer to pre- and primary miRNAs. A particular disadvantage of a stem-loop primer is the reduced ability to achieve reverse transcription of isomiRNA sequences.[170] The TaqMan miRNA assay, which uses stem−loop RT primers together with miRNA-specific TaqMan probes, is often considered as the "gold standard" for miRNA detection.[167] Modified versions of the TaqMan assay[171,172] and SYBR Green-based qRT-PCR miRNA assays[173,174] have also been described recently.

A hurdle in performing highly parallel qRT-PCR is that optimal reaction conditions may vary substantially among miRNAs owing to sequence-specific differences in primer annealing. An effective strategy to solve this problem is the incorporation of LNAs into primers to standardize optimal

miRNA primer hybridization conditions.[54] Several manufacturers, including Applied Biosystems, Exiqon, Fluidigm, and SA Biosystems, offer qRT-PCR kits that can assess hundreds of miRNAs in parallel, and some offer customizable assays.[120] Quantification of circulating miRNAs in body fluids, such as plasma, requires a "spike in" internal standard for array/qRT-PCR data normalization.[135] More problematic for qRT-PCR is the technique used to normalize miRNA expression. Most normalization curves rely on genes for small RNAs that might not be transcribed by the same polymerases and are less representative of general miRNA regulation.[120] An alternative normalization technique uses the mean miRNA present in each sample.[175] However, whether the selected reference holds constant across samples is unknown.

Current development of the qRT-PCR technique for miRNA detection mainly focuses on the innovation of the synthesis of complementary DNA (cDNA). Kumar et al. reported a circularization-based platform called "miRNA-ID" for detection of miRNAs.[166] The miRNA-ID was featured by circularization of the miRNA by a ligase and reverse transcription of the circularized miRNA. The circular RNA and multimeric cDNA templates provided unmatched flexibility in the positioning of primers, which straddled the boundaries between these repetitive miRNA sequences.

T4 DNA ligase, which can repair nicks in the DNA strand of a DNA:RNA hybrid, has been combined with size-coded DNA probes (Figure 8) to detect multiple miRNAs in total RNA

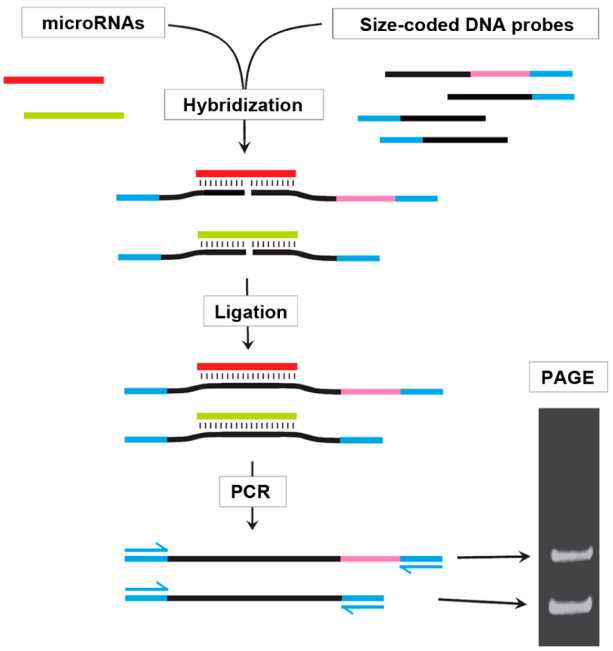


**Figure 8.** Illustration of the size-coded ligation-mediated PCR method. Reprinted with permission from ref 176. Copyright 2011 Oxford University Press.

with the detection limit down to 1 pM (~$10^5$ copies per microliter).[176] A reverse transcription-free real-time approach has also been proposed for rapid quantification of miRNAs ranging from 5 amol to 500 fmol.[177] An exponential amplification process is only carried out efficiently in the presence of target miRNA which provides additional stability for the heterodimer through contiguous stacking hybridization of miRNA and bridging sequence.

To further increase the sensitivity, Zhang et al.[178] demonstrated that the DNA probe modified with ribonucleotides can be efficiently ligated by using miRNA as the template with the catalysis of T4 RNA ligase 2. The modification of the DNA probe with two ribonucleotides at its 3′-terminus can greatly improve the ligation efficiency, and target miRNAs at the level as low as 0.2 fM (e.g., 4 zmol) can be detected. The dynamic range spans over 7 orders of magnitude, which is comparable to the sensitivity of RT-PCR assays. High specificity to well discriminate a single-nucleotide difference among miRNA sequences and specific detection of mature miRNAs against their precursors can also be achieved.

### 3.4. Detection Strategies

**3.4.1. Nanoparticle-Based Detection.** With the achievements of nanotechnology and nanoscience, nanomaterial-based signal amplification holds great promise in realizing high sensitivity and selectivity for in situ or online detection of miRNA. Over bulk materials-biosensors, nanoparticles (NPs)-based biosensors exhibit the significant advantages as follows: (i) The small sizes of NPs break through the limitation of structure miniaturization, leading to the possibility for designing the low-cost and minimized equipment in point-of care diagnostics. (ii) NPs are in direct contact with the environment, which can accelerate the signal transduction, enhance the ability of rapid analysis, and lower the detection limit. (iii) The utilization of nanomaterials also brings out new concepts such as biomimetic, reagent-less biosensing, and in vivo detection with less cytotoxicity and long-term stability. A lot of nanomaterials have been introduced as carriers or tracers, catalysts, electronic conductors, and optical emitters, to obtain the amplified detection signal and the stabilized recognition probes in the detection of miRNA.

Due to large surface area, good conductivity, and biocompatibility, NPs, especially carbon and metal-based NPs, are the excellent candidate as the carrier to enhance the signal transduction. Typically, gold nanoparticles (AuNPs) can be employed as carriers of miRNAs to exploit their unique optical properties, and low cytotoxicity in biosensing or cell transfection.[179] On the basis of a biotin-labeled bridge DNA−AuNPs−biobarcode as detection probe, an "off−on" signaling genosensor platform for miRNA-21 detection has been developed with a linear range of 0.01−700 pM and detection limit of 6 fM.[180]

Magnetic NPs easily achieve concentration and purification of analysts to dramatically improve the sensitivity in biosensing. Drndić and co-workers[181] developed a protocol for nanopore-based miRNA detection. The probe:miRNA duplex is first enriched by binding to p19-functionalized magnetic beads. The hybridized probe:miRNA duplex is then eluted from the magnetic beads, and the eluted probe:miRNA duplex is electronically detected using a nanopore. This approach can achieve over 100 000-fold enrichment of the probe:miRNA duplex from total RNA with picogram level for detection of a liver-specific miRNA (miRNA-122a) from rat liver RNA.

Nanomaterials show excellent conductivity to promote the direct electron transfer between the biomolecules and electrode surface. On the basis of the formation of an electrically conducting polyaniline nanowire network in the gaps, a simplified and sensitive electrical biosensor is proposed to directly detect target miRNA in a range from 10 fM to 20 pM (Figure 9).[182] To further enhance the sensitivity and lower the detection limit, the chemical ligation procedure must be


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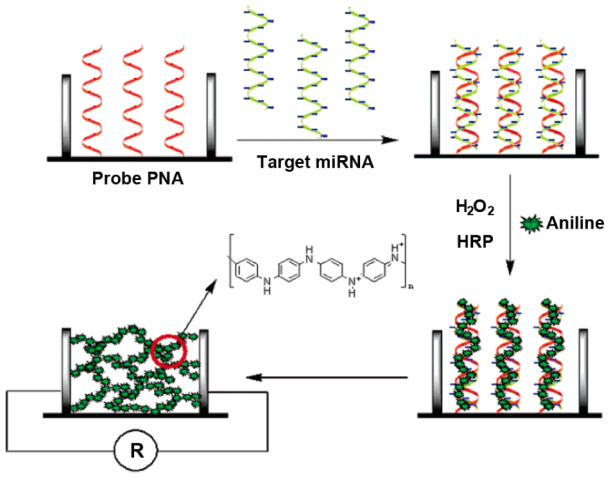


**Figure 9.** Schematic illustration of the miRNA sensing mechanism using target-guided formation of conducting polymer nanowires in nanogaps. Adapted with permission from ref 182. Copyright 2007 American Chemical Society.

coupled to a chemical or biological amplification scheme in the assay. The electrocatalytic behavior of NPs or functionalized NPs is an alternative way for highly sensitive miRNA expression analysis. An ultrasensitive miRNA assay has been designed by employing isoniazid-capped $OsO_2$ nanoparticle as electrocatalytic nanoparticle tags. The resulting electrode exhibits electrocatalytic activity toward the oxidation of hydrazine at −0.10 V, reducing the oxidation overpotential by 900 mV, and thereby lowering the detection limit to femtomolar levels.[183] Ruthenium oxide nanoparticle is employed to initiate polymerization of 3,3′-dimethoxybenzidine and deposit an insulating poly(3,3′-dimethoxybenzidine) film on the electrode for construction of a highly sensitive miRNA biosensor by electrochemical impedance spectroscopy.[184]

Optical detection is advantageous in biosensor design because of its high sensitivity, wide dynamic range, and multiplexing capabilities. A colorimetric assay for convenient detection of miRNA has been established in a homogeneous and label-free manner based on conformational and colorimetric changes of a polythiophene derivative (PMNT) in the duplex of DNA/PMNT and triplex of DNA/miRNA/PMNT.[185] Moreover, NP probes provide significant advantages in signal brightness, photostability, and multicolor-light emission. Mirkin and co-workers[186] designed a scanometric miRNA platform for the detection of relatively low abundance miRNAs with high specificity and reproducibility. As shown in Figure 10, isolated miRNAs are enzymatically ligated to a universal linker followed by hybridization onto miRNA microarray. After washing away unbound miRNA species, universal spherical nucleic acid-functionalized AuNP conjugates are subsequently hybridized to detect captured miRNA targets. Next, signal intensity is amplified by depositing gold with gold enhancing solution (1:1 (v/v) mixture of 1 mM $HAuCl_4$ and 10 mM $NH_2OH$) for 5 min and imaged with a scanner. The scanometric miRNA system was able to detect miRNA of 1 fM in serum with single-nucleotide mismatch specificity.

The small silver clusters as new, bright, and photostable labels have received significant attention in detection of miRNA. Using the fluorescence properties of DNA-nanosilver clusters (DNA/AgNC), a DNA/AgNC probe has been designed to detect the presence of target miRNA without pre- or postmodification, addition of extra enhancer molecules, or labeling.[187] Coupled with target assisted isothermal exponential amplification, attomolar sensitivity has been achieved to quantify miRNA expression levels by fluorescent DNA-scaffolded AgNCs.[188] Interestingly, a label-free approach for detecting low abundant miRNAs with high specificity and reproducibility has also constructed by using plasmonic coupling effect from a nanonetwork of silver nanoparticles, leading to a useful diagnostic tool for biomedical diagnostics and biosensing applications.[189]

**3.4.2. Molecular Biology-Based Detection.** Many molecular biological techniques such as target RNA cycling, ligase chain reaction (LCR), rolling-circle amplification (RCA) isothermal strand-displacement polymerase reaction, and pyrophosphate detection can be applied in identifying and detecting miRNA targets.[190−194] Target miRNA recycling using various nucleases, e.g., endonuclease, polymerase, and exonuclease, has attracted considerable attention for producing strong detectable signal in the analysis of trace target miRNA. For example, on the basis of DNase I-assisted signal amplification and quenching nature of graphene oxide for dye-labeled single-stranded DNA (ssDNA) probe, a cyclic enzymatic amplification method was reported for sensitive miRNA detection within the range from 20 pM to 1 nM in complex biological samples.[195] Further, a one-step, direct method to quantitatively detect multiple miRNAs was developed on the basis of duplex specific nuclease (DSN) signal amplification.[196] As shown in Figure 11, Taqman probe, as a signal output, hybridizes to a target miRNA to form DNA:RNA heteroduplex. Duplex-specific nuclease enzyme is employed to recycle the process of target-assisted digestion of Taqman probes, thus, resulting in a significant fluorescence signal amplification through which one target molecule cleaves thousands of probe molecules within 30 min. DSN signal amplification method allows the direct detection of

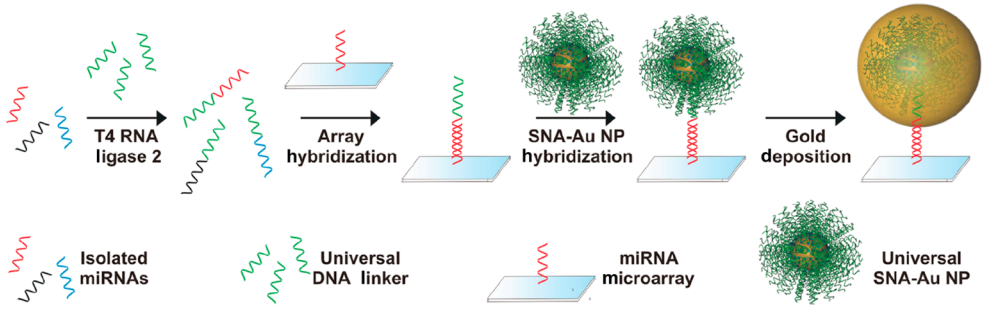


**Figure 10.** Scheme for the scanometric array-based multiplexed detection of miRNA species. Reprinted with permission from ref 186. Copyright 2012 American Chemical Society.


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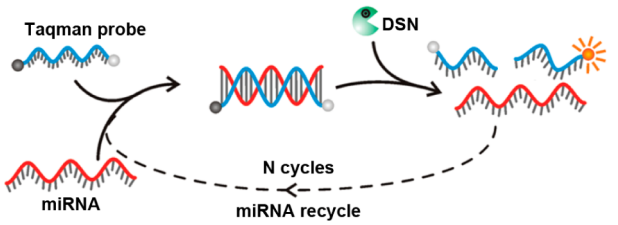


**Figure 11.** Schematic representation of miRNA direct detection based on DSNSA. Reprinted with permission from ref 196. Copyright 2012 American Chemical Society.

miRNAs in the femtomolar range. Moreover, it can be easily applied to all miRNAs, because DSN enzyme has no requirement for specific recognition sequence.

The direct detection of RNAs can be achieved with ribozymes by taking advantage of their ability to perform RNA-cleavage reactions. Hairpin ribozymes cleave a short RNA substrate labeled with a fluorophor at the 3′- and a quencher at the 5′-end, as a function of the presence or absence of a miRNA effector. This assay can achieve detection at miRNA concentration as low as 5 nM, corresponding to a detection limit of 50 fmol miRNA in the reaction mixture.[197]

LCR reaction can be designed to exponentially amplify and sensitively detect miRNAs by using T4 RNA ligase 2 to initiate the ligation of DNA probes templated by target miRNAs. Due to the high specificity of the LCR reaction, the LCR miRNA assay can clearly discriminate one base difference between miRNA sequences.[198] On the basis of enzymatic ligation of DNA stem-loop probes, a real-time PCR miRNA detection method is presented to reduce nonspecific ligation at least 100-fold with wide dynamic range, and single-base mismatch discrimination among miRNA sequences.[199]

As a result of simplicity, robustness, specificity, and high sensitivity, RCA has become increasingly popular in the detection of DNA, RNA, and proteins. RCA offers an exquisite strategy for detecting miRNAs because the short miRNAs are suitable to be used as templates for ligation of the padlock probes and can subsequently prime the isothermal RCA reaction.[200−202] T4 RNA ligase 2 can greatly improve the specificity for the ligation of padlock probes by using miRNA as the template so that the RCA-based assay can clearly discriminate one-nucleotide differences between miRNAs. Furthermore, by introduction of a second primer complementary to the RCA products, which leads to a branched rolling-circle amplification (BRCA) reaction, the RCA products can be sensitively determined in a homogeneous manner by using SYBR Green 1 (SG) as the fluorescence dye (Figure 12).[203] Therefore, the proposed assay is practical for highly sensitive detection of miRNAs with a simple process. On the basis of the detection of pyrophosphate molecules released during BRCA, a bioluminescence assay for small RNA quantification is capable of detecting at least 20 amol of target RNA molecules.[204] On encoded hydrogel microparticles, RCA is used to achieve subfemtomolar sensitivity and single-molecule reporting resolution for multiplexed quantification of miRNAs.[205]

On the basis of isothermal strand-displacement polymerase reaction and graphene oxide fluorescence quenching, a multiple miRNA detection can be realized in the same solution with a detection limit down to 2.1 fM and a linear range of 4 orders of magnitude.[206] Li and co-workers[207] devised an exponential amplification reaction (EXPAR) for miRNA by a combination of polymerase strand extension and single-strand nicking. By means of the real-time fluorescence detection of EXPAR products, miRNAs target can be detected in an amount as low as 0.1 zmol with the dynamic range more than 10 orders of magnitude. An exponential amplification strategy for ultrasensitive detection of miRNA is also developed by a cross-catalyst strand displacement reaction.[208] The generated signals can be sensitively read out in the form of chemiluminescence resonance energy transfer through flow injection measurement, achieving a detection limit of *let-7a* miRNA as low as 0.68 fM.

Pyrophosphate (PPi) detection is often used for monitoring of DNA polymerase activity and DNA sequencing. On the basis of the release of PPi, a rapid quantification method for miRNA is developed for the detection of miRNA in SiHa cells.[209] Coupling the exponential amplification reaction of DNA polymerase and a single-quantum-dot-based nanosensor, a miRNA assay can even discriminate single-nucleotide differences between miRNA family members with a detection limit of 0.1 aM, and potentially become a promising miRNA quantification method in biomedical research and clinical diagnosis.[210]

**3.4.3. LNA-Based Detection.** For specific detection of miRNA, the use of the LNA has shown great advantages to enable specific identification of highly similar sequences such as miRNA family members and single mutations.[211] The LNA-based probe has been widely employed in miRNA analysis and miRNA-based cancer diagnostics and therapeutics due to the remarkable affinity and specificity of LNA to miRNA.[212]

Sulfydryl functionalized LNA integrated MB capture probe can be directly immobilized on the dendritic gold nanostructure modified electrode. After being hybridized with miRNA-21 and biotin multifunctionalized bio-barcodes on AuNPs, streptavidin−HRP is brought to the electrode through the specific interaction with biotin to catalyze the chemical oxidation of hydroquinone by $H_2O_2$ to form benzoquinone affording the electrochemical reduction signal. The biosensor shows excellent selectivity and high sensitivity with low detection limit of 0.06 pM.[213] By using chemiluminescence imaging, a new method for

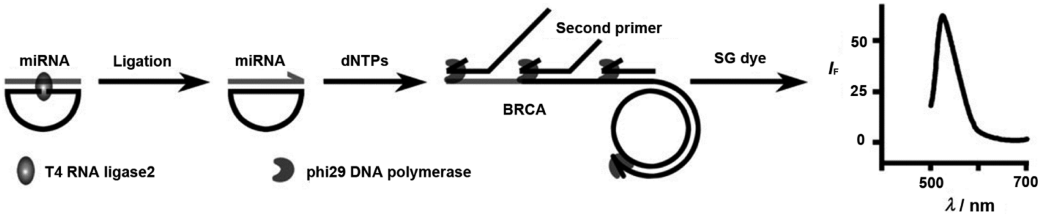


**Figure 12.** Illustration of the target-primed BRCA reaction and fluorescence detection of miRNA. Reprinted with permission from ref 203. Copyright 2009 John Wiley and Sons Ltd.


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miRNA assay is achieved with a 50 fM detection limit on the LNA probe immobilized coverslip.[214]

The use of LNA−DNA chimeric probes for in situ hybridization analysis has evolved a series of detection strategies in chromogenic and fluorescence detections. For example, a single-molecule two-color coincident detection strategy has been designed by the direct hybridization of two spectrally distinguishable fluorescent LNA−DNA oligonucleotide probes to the target miRNA, and the count of the tagged molecules in a microfluidic, multicolor confocal laser system for single-molecule detection. The assay is sensitive to femtomolar concentration of miRNA (500 fM) with a three-log linear dynamic range.[154] On the basis of the fluorescence recovery, MBs combined with LNA−DNA backbones can readily distinguish mature- and pre-miRNAs, and reliably quantify miRNA expression with detection limit low 1 nM.[215]

Although the use of DNA-LNA probe has enabled the specific detection of miRNAs by in situ hybridization, detection of low-copy number miRNAs is still not always possible. A novel probe consisting of 2′-*O*-methyl RNAs and LNA at every third base can provide superior performance in detection of miRNA targets due to the higher sensitivity and signal-to-noise ratio compared to DNA-LNA probes. Furthermore, the hybridization can be performed in buffers of 4 M urea instead of 50% formamide, thereby yielding an equally specific but nontoxic assay.[216]

**3.4.4. Analytical Chemistry-Based Sensing.** Surface plasmon resonance (SPR), with a rapid, sensitive, and on-site analysis, can offer an attractive alternative to conventional techniques in the detection of miRNA.[217] By using SPR technology and a DNA*RNA antibody-based assay, an approach, which allows detection of miRNA in less than 30 min at concentration down to 2 pM with an absolute amount at attomole level, has been developed.[218] Further, the technique of SPR imaging has also been designed for the detection of miRNAs down to 10 fM on LNA microarrays. Figure 13 shows the proposed three-step scheme for the detection and identification of miRNAs.[219] The target miRNAs are first adsorbed from solution onto a single-stranded LNA microarray (step i). In step ii, poly(A) tails are added to the surface bound miRNAs via the poly(A) polymerase surface reaction. Finally, in step iii the poly(A) tails are hybridized with T30 DNA-coated AuNPs for signal amplification and subsequently detected with SPR imaging. This ultrasensitive nanoparticle-amplified SPR imaging methodology can be applied to determination of miRNA concentrations in a total RNA sample from mouse liver tissue. Another method can detect subpicomolar concentrations of multiple short ssRNA by using enzymatic silica nanoparticles combined with SPR imaging measurement.[220]

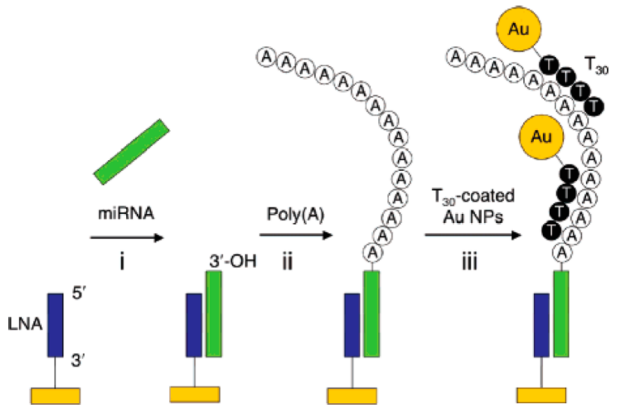


**Figure 13.** Schematic showing the detection of miRNAs using a combination of surface polyadenylation chemistry and nanoparticle amplified SPRI detection: (i) hybridization adsorption of miRNA onto a complementary LNA array element; (ii) addition of poly(A) tails to the surface bound miRNAs using poly(A) polymerase; and (iii) hybridization adsorption of T30-coated Au nanoparticles to poly(A) tails detected by SPRI measurements. Reprinted with permission from ref 219. Copyright 2006 American Chemical Society.

The strength of surface enhanced Raman scattering (SERS)-based sensor is its sensitivity to detect extremely low levels of analyte and specificity to provide the molecular fingerprint of the analyte. By using silver nanorod substrates, the SERS spectra of miRNAs can be realized in near-real time with high accuracy.[221] A silver nanorod array has been used for the intrinsic SERS detection of miRNAs based on different binding affinities of ssRNA, thiolated ssDNA, and RNA:DNA duplex, eliminating the need for a labeling step.[222]

On the basis of the strong resonance Rayleigh scattering of single-Ag NPs, an ultrahighly sensitive homogeneous detection of miRNA has been achieved with a good linearity in 2−5 orders of magnitude and detection limit of 1 fM by using a single-silver-nanoparticle counting technique with a highly focused laser beam.[223]

Capillary electrophoresis with laser-induced fluorescence as an instrumental platform can be used in direct quantitative analysis of multiple miRNAs.[224] Coupling a tandem adenosine-tailed DNA bridge-assisted splinted ligation, this capillary electrophoresis platform allows for the detection of multiple miRNAs within a single capillary with linear range covering 3 orders of magnitude (1.0 nM to 1.0 pM).[225] A protein facilitated affinity capillary electrophoresis assay has been designed for rapid quantification of miRNA levels in blood serum using ssDNA binding protein and double-stranded RNA binding protein (p19) as separation enhancers. The detection limit is 0.5 fM or 30 000 miRNA molecules in 1 mL of serum as a potential source of miRNAs.[226] Further, through confocal time-resolved fluorescence detection on an embedded capillary interface, a 300-fold improvement is achieved below the 1000-copy detection limit in breast cancer for miRNAs 125b and 145. This sensitive method may facilitate the analysis of miRNA even in a single cell without enrichment or modification of miRNA.[227] In addition, isotachophoresis is an electrophoretic preconcentration technique, which allows for the measurement of the total miRNA content in a sample and its comparison between different cell types and tissues.[228,229]

To enhance the sensitivity and selectivity, and lower the cost, electrochemical biosensors have been investigated to detect miRNAs. The first electrochemical method for miRNA analysis was developed by Gao's group.[230−233] On the basis of electrocatalytic oxidation of isoniazid-substituted osmium complex toward ascorbic acid, an increase in sensitivity of 2000-fold over direct voltammetry is obtained by amperometry.[230] Similarly, the detection of target miRNAs can be easily realized at femtomolar levels by using the activated GOx tags as the catalyst toward glucose oxidation.[231]

Through competitive hybridization between the biotinylated short-stranded RNA and the miRNA target for the preimmobilization of oligonucleotide probe onto an electrode, a voltammetric quantification of the miRNA target is accomplished by using ferrocene-capped AuNPs/streptavidin con-


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jugate as signal probe with the low detection levels (10 fM or 0.1 attomoles of miRNA in a 10 $\mu$L solution).[234] On the basis of four component DNA/RNA hybridization and electrochemical detection using esterase 2-oligodeoxynucleotide conjugates, a rapid, selective, and sensitive gap hybridization assay is established for detection of mature miRNAs with a detection limit of 2 pM or 2 amol of miRNA-16.[235]

Microring resonators as a promising class of refractive index-sensitive devices have recently been applied to monitoring chemical reactions and biomolecular binding events. Given a defined microring structure, the resonance wavelength is sensitive to changes in the local refractive index. As illustrated in Figure 14, the scanning electron micrograph shows six

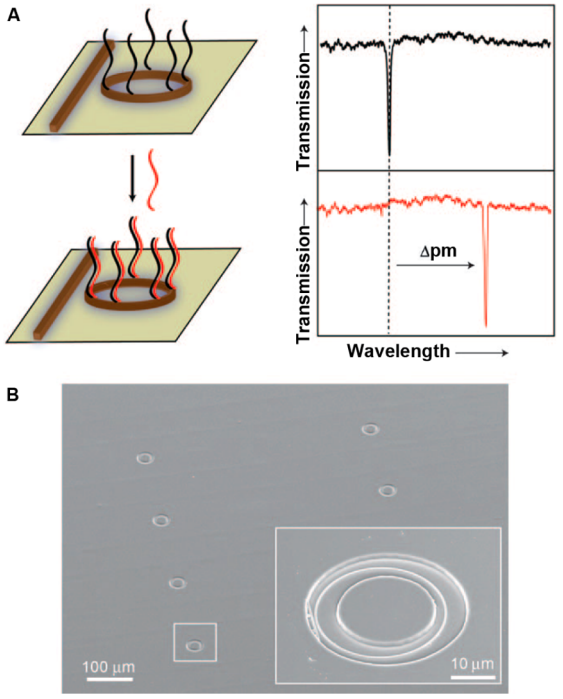


**Figure 14.** (A) Schematic of microring sensor and detection of miRNA target. (B) Scanning electron micrograph of array chip. The inset shows a single microring and its corresponding linear access waveguide revealed within an annular opening in the fluoropolymer cladding layer. Reprinted with permission from ref 159. Copyright 2010 John Wiley and Sons Ltd.

microrings on a sensor array chip, and each microring sensor is functionalized with a capture sequence of DNA (black).[159] The sequence-specific hybridization of the target miRNA (red) causes a shift in the wavelength. These silicon photonic microring resonators are able to rapidly (10 min) quantitate down to approximately 150 fmol of miRNA and discriminate between single-nucleotide polymorphisms within the biologically important *let-7* family of miRNAs. Utilizing an antibody that specifically recognizes DNA:RNA heteroduplexes, a silicon photonic microring resonator array allows for the detection of miRNAs at concentrations as low as 10 pM (350 amol).[236]

Silicon nanowire field effect transistors offer advantages of low cost, label-free detection, and potential for massive parallelization. Using peptide nucleic acid as a receptor to recognize miRNA, a silicon nanowire (SiNW) device has been developed for sensitive detection of miRNA with detection limit of 1 fM.[237] Further, for point-of-care medical diagnostics, a stable $H_2O_2$ dielectric-based SiNW is constructed to detect the miRNA cousins of miRNA-10b and miRNA-21.[238]

Interestingly, an autonomous DNA machine recycling the output triggered by target miRNA as the input has been designed for isothermal, sensitive, and specific detection of miRNAs. This machine shows considerably high signal amplification efficiency (~1000-fold) with a low detection limit (~20 amol), and also can discriminate 50 amol of synthetic miRNA from 100-fold larger amounts of its family member. Therefore, it is available for practically detecting natural miRNAs in total RNAs.[239]

In addition, the cation exchange-based fluorescence amplification method, in which the $Cd^{2+}$ from the labeled CdSe turns on the fluorescence of thousands of fluorogenic Rhod-5N molecules, has been designed for the detection of miRNAs. The assay achieves a detection limit of 35 fM with analyte concentrations spanning over 7 orders of magnitude.[240]

## 4. INTRACELLULAR MIRNA DETECTION

### 4.1. In Situ Hybridization

Generally, the conventional routine for indirect measurement of the expression levels of miRNAs in cells is always conducted as follows: the cells are first lysised, and the extracted RNA are then detected by qRT-PCR,[241−245] Northern blotting,[246] or microarray hybridization.[247−252] The insight into the physiologic function of miRNA requires quantifying miRNA expression at the single-cell level expression. To obtain a more complete spatial profile of gene expression in single cell, a promising alternative option is ISH. A typical practice for ISH is to link probes to a fluorophores maintaining spatial resolution[253−255] or enzymes catalyzing fluorogenic reactions[256,257] or radioactively labeled probes detected by autoradiography.[5] Recent advances in ISH have allowed it to be a powerful technique for visualization of the spatial localization of RNA at the tissue, cellular, and even subcellular level.[258−261]

The small size of the mature miRNAs makes it difficult to visualize and target specifically miRNA in cell by conventional ISH methods. An alternative approach is LNA-based ISH system, which derives a high degree of sequence specificity from the base-pairing properties of LNA probes.[262,263] Pederson et al. determined intracellular localization of miRNA-206 during differentiation in single rat myogenic cell by coupling LNA-based ISH system with high-resolution imaging microscopy.[261] From the higher spatial resolution imaging, it was found that a substantial miRNA-206 fraction colocalizing with 28S rRNA occupied a cytoplasmic location and concentrated in nucleoli. These results indicated that miRNA-206 may associate with both functional ribosomes in the cytoplasm and nascent ribosomes in the nucleolus, respectively.[261] Some previous reports have reviewed the detailed procedures about the LNA-ISH system for miRNA detection in tissue or cells.[264,265]

It is well documented that the target copy number, probe modification, and conditions of stringency affect the signal of in situ hybridization.[266] In contrast to the LNA-ISH system concentrated on the probe modification, Turner et al. developed a modified ISH system for miRNA detection by using high-stringency wash conditions based on tetramethylammonium chloride (TMAC) in combination with RNase A.[258,267] The expression patterns for several miRNAs expressed in the development of adult nervous system cells such as


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miRNA-124a, miRNA-9, miRNA-92, and miRNA-204 could be determined by in vivo imaging.[267] Furthermore, the same group summarized the TMAC-based ISH for analysis of miRNA expression in tissue sections or cultured cells by using nonradioactive hapten-conjugated probes detected by enzyme-coupled antibodies, or radioactively labeled probes detected by autoradiography.[258]

The LNA-ISH system allows a qualitative assessment of miRNA localization patterns and tissue distribution due to a remarkable affinity and specificity of the probes against RNA targets. Unfortunately, quantitative detection of low-copy number miRNAs is less amenable by LNA-ISH system. The introduction of amino-allyl modified bases[268] permits the chemical synthesis of multiply labeled fluorescent oligomer hybridization probes.[269,270] Recently, heavily labeled probes or a larger number of singly labeled probes have been employed to visualize single mRNA in cell.[255,260]

Unfortunately, it is not simply possible to extend the approach of heavily labeled probes to miRNA quantitative detection due to their short length. An alternative strategy is to develop more promising fluorescent label agent with adequate sensitivity. For example, a method for detection of individual miRNA molecule in cancer cells has been described by using fluorescence LNA-based ISH system with enzyme-labeled fluorescence, which exhibits 40 times brighter signal than those from probes directly labeled with fluorophores (Figure 15).[271] Using this approach, miRNA-15a and miRNA-155 were successfully quantified in HeLa cells and MCF-7 cells, respectively, which displayed good correction with the measurements acquired by qRT-PCR. An ISH system for detecting single miRNA-486 molecules in the lung cells was developed by using a fluorescent metal nanoshell label composed of silica spheres with encapsulated $Ru(bpy)_3^{2+}$ complexes as cores and thin silver layers as shells as label.[272] It is worth mentioning that ISH with radioactively labeled probes provides promising potential in accurate semiquantitative or quantitative miRNA expression levels due to the high sensitivity.[258]

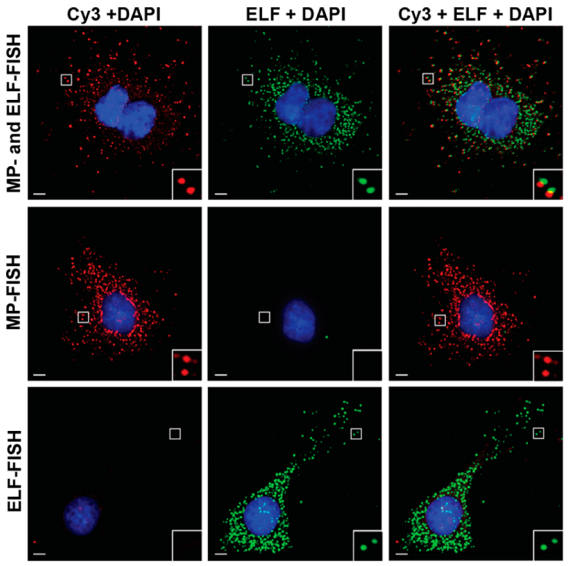


**Figure 15.** Simultaneous detection of individual mRNA molecules using MP-FISH and LNA-ELF-FISH. HeLa cells were engineered to constitutively express luciferase mRNA with 24 MS2 binding repeats in the 3′-untranslated region. Each MS2 site was hybridized by an oligonucleotide probe labeled at its 5′- and 3′-end with Cy3. In addition, the coding region of the luciferase RNA was labeled with a single dig-labeled LNA probe. The LNA probes were subsequently labeled with antidig-alkaline phosphatase conjugates, and ELF signal amplification was performed. Two-dimensional, deconvolved images of the Cy3 fluorescence, ELF signal, and a merged image are shown (top panel). Analogous studies were performed using just Cy3 probes (middle panel) or just LNA probes + ELF amplification (bottom panel). Scale bar, 5 $\mu$m. Reprinted with permission from ref 271. Copyright 2009 Oxford University Press.

## 4.2. Imaging Analysis

Conventional detection methods including Northern blot,[246] qRT-PCR,[241] or microarray[247] are capable of evaluating miRNA expression, and the in situ hybridization analysis provides a strategy for directly identifying the location of the miRNA in cells.[254] However, these techniques are labor-intensive and time-consuming, and cannot be carried out on the same subject repeatedly. Meanwhile, the lysis or fixation process included in these methods makes the study of the dynamic function of miRNAs in living cells impossible. A noninvasive manner for repetitive monitoring and real-time imaging of the miRNA-production is needed for the detection of miRNAs in clinic research.

Recent significant progress in optical imaging techniques using multimodal reporter systems has offered the opportunities of noninvasive and repeated real-time analysis of molecular agent expression in living cell. Various reporter-based optical imaging systems with luciferase or fluorescent probes, and radionuclide imaging using sodium iodide symporter and herpes simplex virus 1-thymidine kinase, have been intensely employed to analyze the location of implanted stem cells and investigate the endogenous gene expression.[273−276] These miRNA imaging strategies provide a better understanding of the biological process and product of miRNAs in vivo as well miRNAs expression profile in human diseases. In this section, a brief discussion related to recently developed miRNA imaging strategies such as the various bioluminescence systems, fluorescent imaging approaches, as well as magnetic resonance imaging is presented. In addition, both the advantages and inherent inefficiencies of various imaging system are also included.

**4.2.1. Luciferase-Based MiRNA Imaging.** Bioluminescence imaging via the detection of light emitted from an enzymatic reaction has been widely employed to visualize endogenous gene expression and molecular distribution in living animals. The typical luciferase optical reporter proteins include firefly luciferase (Fluc), Renilla luciferase (Rluc), and Gaussia luciferase (Gluc). Fluc displays a maximum emission peak at about 562 nm through oxidizing its substrate beetle D-luciferin to oxyluciferin (benzothiazole),[277,278] while Rluc and Gluc catalyze the oxidation of coelenterazine to emit bioluminescence light at about 480 nm.[279] The emission light from Gluc is up to 1000-fold more intense than that from native Rluc or Fluc. Importantly, Gluc is the smallest luciferase and stays stable at elevated temperatures, which facilitates it to construct clones and cell transfection.[279−281] On the other hand, the better tissue penetration of photons with longer wavelengths makes Fluc better than Rluc and Gluc for in vivo imaging in tissue.

The available luciferase reporter-based imaging strategies have been utilized to assess not only the miRNA biogenesis including the pri-miRNA to mature miRNA but also the


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functional action of miRNA. As shown in Figure 16, to monitor the production patterns of miRNAs involved in miRNA

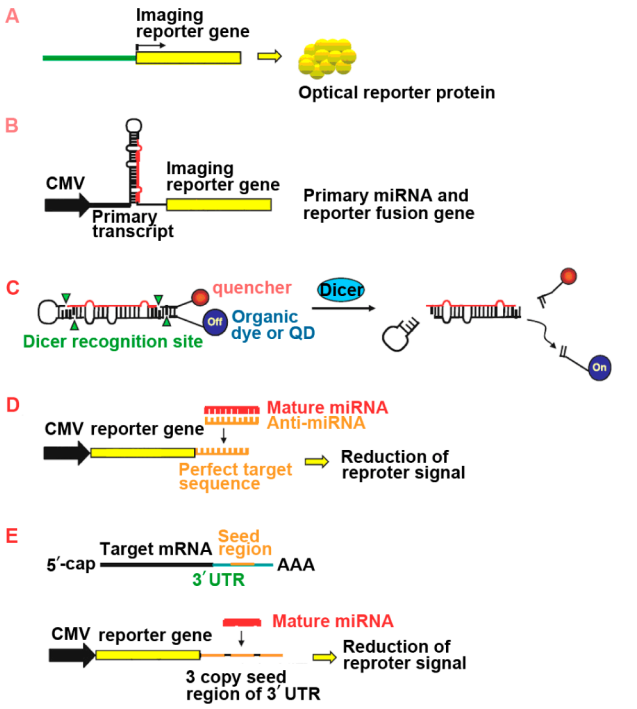


**Figure 16.** (A) Design for imaging pri-miRNAs. (B) Imaging strategy for pre-miRNAs. (C) Molecular beacon for imaging a partially double-stranded miRNA complex (miRNA−miRNA*), which is released from pre-miRNA by Dicer. (D) Schematic strategy for the reporter gene imaging of mature miRNAs. (E) Reporter-gene frame imaging of miRNA targets. Reprinted with permission from ref 282. Copyright 2009 John Wiley and Sons.

generation, various imaging systems based on luciferase reporters have been developed.[282] It is well-known that intergenic or intragenic miRNAs are transcribed into primal miRNA by RNA polymerase II, and 5′-U upstream region of genomic miRNA controls the long primary transcripts of miRNA.[283] The optical reporter gene system monitoring primal miRNA generation can be obtained by fusing the 5′-U upstream region of miRNA into the cassette of a promoterless optical reporter gene vector. For example, Lee et al. used miRNA-23a specific reporter vector fusing the 5′-upstream promoter region of primal miRNA-23a to the cassette of pGL3 containing a promoterless Fluc gene to acquire the images of endogenous expression of primal miRNA23a in HeLa, 293, and P19 cells.[284] Using the same miRNA-specific reporter vector, the highly expressed pattern of a brain-specific primal miRNA-9 during neurogenesis was also observed.[285]

By annealing and cloning sense and antisense oligonucleotides of the primal miRNA-23a between cytomegalovirus promoter and the start codon of the Gluc gene, an optical reporter gene vector can be obtained. The cleavage of primal miRNA-23a by Drosha and Pasha causes an increase in luciferase activity of the pre-miRNA imaging system.[284] Using an analogous imaging system, the image of the cleavage of pre-miRNA by the Dicer enzyme is acquired.[286] The assay is based on the displacement of the fluorescence dye from the quenching molecule by the Dicer enzyme, and endogenous or exogenous Dicer enzyme can be detected by the intensity of fluorescence signals.

To investigate the expression of mature miRNA, an imaging system based on luciferase reporter has been constructed by fusing complementary sequence of mature miRNAs to the downstream of the reporter system under the control of the cytomegalovirus promoter.[287] Three perfectly matched complementary sequences of miRNA-221 are cloned immediately after the stop codon of the Gluc reporter vector to develop a Gluc imaging system, and the binding of endogenous miRNA-221 with the imaging system causes destabilization of reporter gene, and thus decreases the activity of reporter gene.

Pervious reports indicate that different levels of primal miRNA and mature miRNA occur simultaneously in living cells due to the unknown regulatory maturation.[288] To simultaneously monitor the production of both primal miRNA and mature miRNA, a noninvasive imaging system containing two different bioluminescent proteins, Fluc and Gluc, has been constructed.[284] This dual-luciferase reporter system provides a promising avenue to evaluate the spatial and temporal expression of miRNAs.

The widely used 3′-untranslated region (UTR)-based method for miRNA target detection is designed on the basis of the functional mechanism of miRNA that partially or completely complements the 3′-UTR of target to mediate mRNA cleavage or prevent protein synthesis.[289,290] In this way, 3′-UTR of targets is cloned to the downstream of the luciferase reporter gene to construct a reporter system, and the target can be detected by the reduction of the reporter signal in cells. Using this 3′-UTR-based Rluc reporter gene system, nine potential transcripts have been predicted as the targets of miRNA-9 based on the ensemble machine learning algorithm.[291] A 3′-UTR-based Gluc reporter system is obtained by fusing 3′-UTR of the chromosome 14 open reading frame, a target of miRNA-124a. From the in vivo imaging, the miRNA-124a-mediated repression of chromosome 14 open reading frame during neurogenesis is observed.[292] Another reporter system is described by cloning Gluc gene to the 3′-UTR of homeobox B5, one of the miRNA-221 target genes for miRNA-221 imaging. The presence of miRNA-221 during the development of papillary thyroid carcinoma causes significant decrease of Gluc reporter gene activities.[283] In addition, lucirase-based miRNA imaging system is also developed for monitoring the therapeutic potential of miRNAs.[293]

**4.2.2. Fluorescence-Based MiRNA Imaging.** Many fluorescent proteins with various emission wavelengths such as the green-fluorescent protein (GFP) and red-emitting fluorescent proteins have been used for in vivo imaging.[294] GFP from the jellyfish *Aequorea Victoria* provides a permanent and heritable label in living cells due to the high flexibility to almost any protein. It is always encoded with multitargeting sites of miRNA, complementing against the particular miRNA, to construct lentiviral or retroviral vectors for in vivo imaging.[295,296] With this strategy, a lentiviral vector has been developed by fusing several target sequences for miRNA-142-3p into the 3′-UTR of a GFP expression cassette driven by the ubiquitously expressed phosphoglycerate kinase promoter.[297] The in vivo imaging and flow cytometry analysis show that the presence of target miRNA effectively suppresses transgene expression from vectors in intravascular and extravascular hematopoietic lineages, which provides in vivo imaging evidence of miRNA regulation and demonstrates a new model in vector design.[297] To quantify miRNA-mediated suppression, a bidirectional lentiviral vector coordinately expressing two transgenes, miRNA-mediated regulation GFP


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and control gene of low-affinity nerve growth factor receptor, is also designed.

Multiple colors system provides a promising platform for monitoring dynamic function of miRNAs and multiple miRNA functions. A novel two-color retroviral vector system for monitoring miRNA-133 in living cells has been constructed by fusing target site for miRNA-133 in the 3′-UTR of a GFP with RFP for normalization.[295] The in vivo imaging results suggest that the miRNA-133 specifically down-regulates its targets during myogenesis. Using this bidirectional lentiviral vector system, adenovirus-derived miRNAs expression in living cells is further monitored.[296] MiRNA expression can be successfully imaged by the fluorescence reduction of GFP due to the interaction of target with the 3′-UTR of GFP. To investigate the functional orthologous relationship of viral and cellular miRNAs, a simple dual-fluorescent protein reporter system containing three separate promoters to imaging miRNA function has been developed.[298]

Both luciferase-based and fluorescent-protein-based miRNA reporter systems for imaging mature miRNAs or miRNA targets have been established. The sensitivity is related to the reporter gene expression level and the pharmacokinetics of probe reporter. It inherently suffers from low sensitivity and the interpretation of cell death. An alterative signal-on system provides promising potential to overcome these limitations for in vivo miRNA. MB is a hairpin-shaped single-stranded oligonucleotide hybridization probe in which the loop contains a probe sequence that is complementary to a target sequence and the self-complementary 5′ and 3′ ends forms a stem.[299] In general, the fluorophore−quencher pair covalently linked at 5′- and 3′- termini forms a quenched approach; upon hybridization with the target, the separation of the donor and acceptor dye results in a bright fluorescence signal for detection.[300,301] The MB-based molecular detection of intracellular targets attracts intensive interest due to its unique thermodynamic and relative low background.[302−304]

Several MB-based signal-on imaging strategies have been developed to image endogenous miRNAs.[305−308] To monitor the biogenesis of miRNA-206 and miRNA-26a in C2C12 cells during myogenic differentiation, two MBs with 6-FAM-BHQ1 pair and Texas Red-BHQ2 pair were designed, respectively.[305] The same group further developed a MB-based imaging system, in which the therangostics probe intergraded an AS1411 aptamer for targeting tumor cell with miRNA-221 MB-conjugated magnetic fluorescence nanoparticle to inhibit miRNA-221 expression and to simultaneously monitor the miRNA-221 function involved carcinogenesis.[306]

The efficient takeup of MB by the cell always requires the use of various vectorization agents. Ju et al. proposed an effective gene vector of polyethylenimine-grafted graphene nanoribbon to deliver LNA modified MB probes into cell for in vivo miRNA recognition.[307] By combining with the remarkable affinity and specificity of LNA to miRNA and high transfection efficiency of polyethylenimine-grafted graphene nanoribbon, mi-RNA 21 in single-cell HeLa cells was effectively detected. The same group further developed a multifunctional $SnO_2$ nanoprobe for target-cell-specific delivery, imaging, and detection of intracellular miRNA (Figure 17).[308] The multifunctional nanoprobe used folic acid for cell-specific delivery and gene probe conjugated to fluorescence $SnO_2$ nanoparticle with a disulfide linkage to detect or inhibit intracellular target miRNAs. It demonstrated that the multifunctional nanoprobe could be used for intracellular miRNA imaging and its suppression expression monitoring.

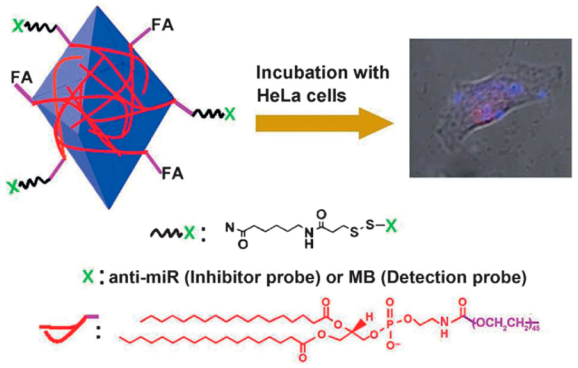


**Figure 17.** Schematic representation of mf-$SnO_2$ nanoprobe for target-specific cell imaging and intracellular detection of miRNA. FA = folic acid, MB = molecular beacon. Reprinted with permission from ref 308. Copyright 2012 John Wiley and Sons.

**4.2.3. Radionuclide Imaging.** Besides the optical system mentioned above, the advance in radionuclide imaging modalities, such as the sodium iodide symporter and the magnetic resonance imaging (MRI) reporter gene, provides a promising tool for clinical diagnosis and biomedicine.[309−311] However, few reports have been dedicated to the radionuclide-based miRNA imaging due to innate limit of spatial resolution in radionuclide imaging (ca. 100 mm), which is difficult to monitor the intracellular miRNA.[312] A new field named radiogenomics linking gene expression profiles with MRI phenotypes has emerged.[313] A comprehensive radiogenomic analysis is conducted by using quantitative MRI volumetrics with large-scale miRNA expression profiling in glioblastoma multiforme. The high periostin and low miRNA-219 expression are significantly correlated with the mesenchymal glioblastoma multiforme subtype.[314]

## 5. CIRCULATING MIRNA DETECTION

Extracellular miRNAs circulating in the blood of both healthy and diseased people showed extreme stability, and resistance to degradation from RNases activity.[315,316] Most of the circulating miRNAs are well protected from RNases because they can reside in membrane structure of microvesicles such as exosomes, microparticles, and apoptotic bodies[317,318] or associate with RNA-binding proteins including Argonaute2 (Ago2) and nucleophosmin (NPM1).[125,319] The circulating miRNAs expressed aberrantly in the blood of cancer patients have great potential to serve as novel diagnostic markers in the clinic due to their remarkable stability.[320,321] This application raises the need of the improved profiling to accurately identify miRNA in complex clinic samples.

### 5.1. Mechanism of Circulating MiRNA Stability

A crucial question surrounding circulating miRNAs is the high stability despite the presence of ubiquitous RNases. The following possible reasons have been proposed. The microvesicles offer protection by shedding of miRNAs from RNase activity or the existence of nonvesicle associated miRNA−RNA-binding proteins complexes in extracellular fluids. Both microvesicle membrane shedding and protein association provide a protected internal microenvironment for miRNAs circulating in the blood without degradation (Figure 18).[322]


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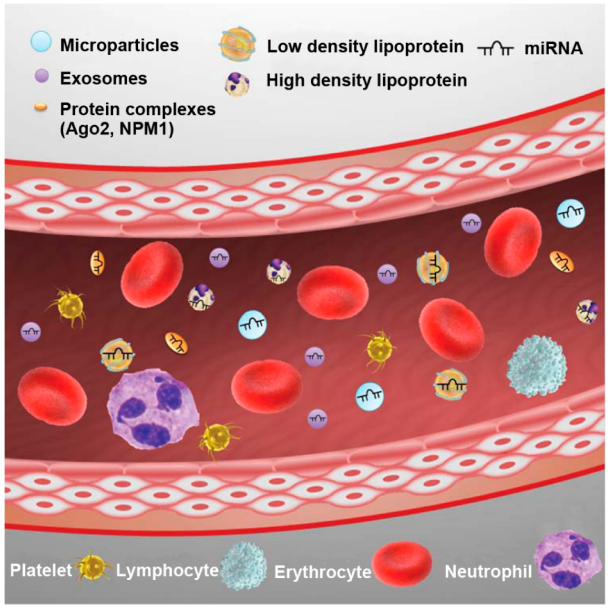


**Figure 18.** Compartmentalization of circulating miRNAs. Circulating miRNAs are contained within vesicles (exosomes, microparticles, apoptotic bodies), in protein complexes (Ago2, NPM1), and in lipoprotein complexes (HDL, LDL complexes). Although various tissues contribute to the circulating miRNA pool, most miRNAs are probably derived from blood cells. In response to injury, such as AMI, cardiac-specific miRNAs, which are otherwise undetectable, are released into the circulation. Reprinted with permission from ref 322. Copyright 2012 Oxford University Press.

**5.1.1. Microvesicle-Based Stability.** The significance difference between these microvesicles depends on the source and mechanism of derivation. Exosomes are small vesicles (50–90 nm) derived from intracellular multivesicular bodies of the late endosome and fused with the plasma membrane.[323,324] Exosomes contain substantial amounts of lipid rafts, RNA, and miRNA, but are devoid of DNA.[325] There were 121 miRNAs identified in exosomes of mast cells, and the package of exosomes with miRNA shows a highly selective pattern.[318,326] The cellular selection mechanism for miRNA release and package is also indicated by the selective release of miRNA-451 and miRNA-1246 produced by mammary epithelial cells.[327] Importantly, exosomes released from human mast cell lines not only contain numerous mRNAs and miRNAs but also can mediate communication between cells to transfer miRNA from one cell to anther.[328]

Microparticles released by exocytosis are typically larger in size than exosomes (100 nm diameter).[329,330] Diverse stimulus can produce microparticles different in their numbers, compositions, and cell origins, and microparticles influence diverse biological functions.[331,332] The miRNAs contained in the microparticles produced from blood can regulate the cellular differentiation of blood cells and metabolic pathways as well as modulate immune function.[329] Similar to the exosomes, the selective package of miRNAs into microparticles has been reported.[329]

Larger in size than microparticles, apoptotic bodies are generated from the surface of blebbing membranes in response to apoptotic stimuli.[333] Analysis of circulating microparticles in plasma has been performed in Orozco's group.[334] The apoptotic bodies can also provide protection for circulating miRNA, regulation of the biological function and transfer of miRNAs between the cells.[335] For example, a panel of miRNAs can be derived from endothelial cell-derived apoptotic bodies, and the most abundant miRNA-126 packaged in apoptotic bodies can induce CXC motif ligand 12-dependent vascular protection.[336] Interestingly, Tim4, a phosphatidylserine receptor for the engulfment of apoptotic cells, is involved in exosome-mediated intercellular signaling.[337]

**5.1.2. RNA-Binding Protein-Based Stability.** High-density lipoprotein (HDL) can readily associate with endogenous miRNAs in plasma and transport them to recipient cells by acting as a carrier with functional targeting capabilities.[338] A specific miRNA signature of HDL–miRNA (hsa–miRNA-22, hsa–miRNA-105, and hsa–miRNA-106a) complexes has been observed in patients with familial hypercholesterolaemia, suggesting that HDL participates in intercellular communication between cells by transporting of miRNAs.

Some studies have shown that the circulating miRNAs in plasma associate with the Ago2 ribonucleoprotein complex rather than with microvesicles.[125,339] Using differential centrifugation and size-exclusion chromatography, the majority of miRNAs except the miRNA-16 and miRNA-92a can be copurified with Ago2 complexes rather than microvesicles.[125] The majority of the nuclease-resistant extracellular miRNA in plasma is bound to Ago2 protein rather than endosomes.[339] Besides Ago2 protein, other Ago proteins including Ago1, Ago3, and Ago4 also associate with circulating miRNA.[339] In addition, NPM1, another RNA-binding protein, is found to bind circulating miRNAs to form NPM1-associated complex and protect miRNAs from degradation.[319]

## 5.2. Circulating MiRNAs as Biomarkers

Aberrant expression of circulating miRNA in cancer patients combing the simplicity of getting a blood sample and remarkable stability of circulating miRNA raises the possibility of employing the miRNA as a class of invasive and promising diagnostic markers. The changes of miRNA expression profiles in plasma and serum miRNA profiles have been reported to be associated with various physiologic and pathologic conditions, which provide promising diagnostic and prognostic values.

The first discovered serum-miRNA biomarker is miRNA-21, which was discovered in diffuse large B cell lymphoma patient by Lawrie and co-workers.[340] The expression levels of miRNA-155, miRNA-210, and miRNA-21 in blood of diffuse large B cell lymphoma patient are higher than control sera, and high serum levels of miRNA-21 is associated with relapse-free survival.

The abberrant expression of miRNA in blood is observed in ovarian cancer patients. The levels of eight specific miRNAs are similar between exosome-derived and tumor cell-derived miRNA from ovarian cancer patients, while these displays are significantly distinct from patients in benign disease.[341] Furthermore, a study has found that the miRNA-21, -92, -93, -126, and -29a display elevated expression in the serum from ovarian cancer patients compared to controls, and suggests that the oncogenes of miRNA-21, -92, -93 have the potential for therapeutics or as biomarker.[342] The ovarian cancer-associated miRNA pattern combining with other markers can improve the screening for ovarian cancer.[343]

The expression levels of miRNA-17-5p, miRNA-21, miRNA-106a, and miRNA-106b in plasma are significantly higher in patients with gastric cancers than controls, while *let-7a* is lower in patients.[344] On the basis of the receiver operating

characteristics curves, a significant correlation between the number of cancer cells and the levels of both miRNA-106a and miRNA-17 is observed, which provides a strategy for monitoring circulating gastric tumor cells.[345]

Chen et al. compared the miRNA expression profiles for lung cancer, colorectal cancer, and diabetes patients to those of healthy subjects, and found the elevated expression of miRNA-25 and miRNA-223 in cancer patients.[320] The circulating miRNA expression profiles in early stage of nonsmall cell lung cancer (NSCLC) by comparing cases and control have been described, and the expression of *let-7b* is associated with prognosis in NSCLC.[346] A test to identify asymptomatic high-risk individuals with early stage NSCLCs with 80% accuracy for serum circulating miRNA detection has been developed.[347] To develop a novel invasive diagnostic tool for detection and risk assessment of lung cancer, four circulating miRNAs including miRNA10b, miRNA34a, miRNA141, and miRNA155 and the caspase activity in serum of patients with benign lung tumors and control healthy individuals have been measured.[348]

Ng et al. described the differential expression of miRNAs in plasma of patients with colorectal cancer. The elevated expression of miRNA-17-3p and miRNA-92 was observed in the patients, while these miRNAs levels in plasma were reduced after surgery. The abnormal expression of circulating miRNA provides a potential marker for colorectal cancer screening.[349] Furthermore, circulating miRNA-141 in plasma has been reported to be a novel biomarker for metastatic colon cancer, and the integration of miRNA-141 and carcinoembryonic antigen can improve the accuracy of detection.[350]

Circulating miRNA-based diagnostics for breast cancer,[351,352] hepatocellular carcinoma (HCC),[353,354] esphageal carcinogenesis,[355] pancreatic cancer,[356] etc. have also been reported. The surgical treatment leads to the decrease of miRNA-195 levels in plasma of patients with breast cancer, which provides a novel minimally invasive biomarker for breast cancer.[351] The different expression levels of serum miRNA-155 between women with hormone-sensitive and women with hormone-insensitive breast cancer have also been reported.[352] The HCC patients show an increased expression of serum miRNA-500, which can return to normal after operation.[353] The expression profiles of circulating miRNA in serum pools from patients with HCC and healthy controls have been investigated. Elevated expression of serum miRNA-885-5p in patients with liver pathologies indicates serum miRNAs can serve as new complementary biomarker for the diagnosis of HCC.[354]

### 5.3. Detection Strategy of Circulating MiRNA

qRT-PCR and microarray are the mainstream approaches for identification and quantification of circulating miRNAs in plasma or serum. The advantages and disadvantages of the qRT-PCR or microarray methodology employed in procedures of extracellular miRNA analysis have been described in a previous report.[357] Microarrays have a major advantage in high throughout analysis over all the other methods used in RNA expression profiles, while qRT-PCR is the better choice for quantifying miRNAs with low levels due to its high sensitivity. Importantly, the measurement of circulating miRNAs is likely to be affected by a various factors, such as specimen collection and processing, RNA extraction efficiency, successful operation of method, and data analysis. These issues generate an urgent need for reproducible RNA isolation methods and advances in qRT-PCR and microarray technology.

A study to standardize and optimize circulating miRNA detection has been presented.[358] The detailed procedure of qRT-PCR-based analysis of circulating miRNAs, from the sample preparation and experimental design to data analysis, provides a successful protocol for carrying out extraction sample and qRT-PCR of miRNAs from clinical plasma and serum specimens.[129] The preanalytical and analytical steps involved in qRT-PCR-based miRNA detection influence the accurate analysis of circulating miRNAs in clinical practice. Therefore, the optimization conditions to decrease variability of replicate miRNA analysis are important.[126] To improve the performance of qRT-PCR in circulating miRNA analysis, several innovative solutions have been processed. A serious of successful qRT-PCR technologies to amplify and quantify both the precursor and mature miRNA have been reported.[359] A sensitive qRT-PCR method for direct detection of circulating miRNAs-21 in 0.625 $\mu$L of serum has been described.[360]

The microarray is usually employed for high-throughout analysis of miRNA expression profiles. There can be 900 human miRNAs screened to detect deregulated miRNAs in blood of melanoma patients using a microarray-based approach, which provides a miRNA expression profiles of blood to be used as melanoma biomarkers.[361] Using a high-density microarray from the fluorescent or electrochemical signals, miRNA expression patterns in human serum for five types of human cancer, prostate, colon, ovarian, breast, and lung cancer, have been investigated.[362] Recently, the different expression of serum miRNAs in samples from surviving to nonsurviving sepsis patients using microarray screens has been observed. The results show that miRNA-297 and miRNA-574-5p display differential expression in sepsis survivors and nonsurvivors, which provides the promising prognostic value of serum miRNA-574-5p for sepsis patients.[363] For more efficient detection of the miRNA, novel assays are progressing to sensitively and selectively detect the miRNA without time-consuming amplification processing and purification steps.

## 6. BIOANALYSIS OF TISSUE OR ORGANISMS MIRNA

Nowadays, the detection methods of miRNA in tissue or organisms fall into two broad categories: indirect detection and direct analysis. The indirect detection strategies involved tissue level analysis including qRT-PCR, Northern blotting, or microarrays, which require more laborious or extraction processes compared with the cellular level miRNA anlysis due to more complex composition. The direct analysis methods involve the ISH or imaging, in which the short emission wavelength of the labeling dye molecules in cell imaging is not appropriate for in vivo imaging, while the cellular analysis miRNA requires higher resolution. Technologies for direct detection of the temporal and spatial expression sequence of specific miRNA in tissues or entire organism patterns are extremely important for elucidating miRNA biology.

ISH is a mainstreamed technology used for direct miRNA detection in tissues or organisms. An ISH strategy for the detection of miRNAs in tissue sections from mouse embryos and adult organs has been developed,[267] which shows high capability of selective detection of the predominantly mature miRNAs. The temporal and spatial expression patterns of 115 conserved vertebrate miRNAs in zebrafish embryos has been obtained by LNA-based ISH.[262] The ability of LNA-modified DNA probes is compared with unmodified DNA probes of identical length and sequence to detect miRNA-206 (muscle-specific miRNA), miRNA-124a (brain-specific miRNA), and

miRNA-122a (liver-specific miRNA). The LNA probes show a much higher affinity for their complementary RNAs than conventional RNA- or DNA-based probes, thus increasing the specificity and sensitivity of miRNA detection. To explore the usefulness of LNA-modified DNA probes for miRNA detection in organisms, the whole mount in situ hybridization on mouse and frog embryos has been further studied.[263] Most miRNAs show a highly tissue-specific expression manner during segmentation and later stages, but not early in development, which indicates that the miRNAs play a key role in differentiation or maintenance of tissue identity rather than fate establishment. An approach for the in situ detection of a mature miRNA in formalin fixed, paraffin-embedded tissues using labeled extension on ultramer templates has been designed, which shows high selectivity for mature miRNA detection, and is much less expensive than the LNA-based method.[364]

Besides ISH technology, novel imaging strategy has also been employed for in vivo miRNA detection in tissue or organisms. By replacing traditional cytomegalovirus promoter with a chicken beta-actin promoter, a reporter system for the long-term monitoring of mouse liver miRNA-122, miRNA-142, and miRNA-34a activities, as well as for exogenous miR-34a activity, has been constructed. Such reporter system allows convenient monitoring of miRNAs in a single animal at different time points, but avoids the killing of test animals. This method is fast and extremely sensitive for monitoring miRNA activity without the accumulation of reporter over time.[365]

Effectively delivering the probe into the desired position is significant for in vivo miRNA detection in tissue. Nanoparticle delivery systems with safe, biodegradable properties (<1 $\mu$m diameter) show promise to support a multitude of enhancing delivery and modifications.[366] Su et al. used a interfering nanoparticles to deliver a chemically stabilized anti-miRNA-122 for effectively silencing the liver specific expressed miRNA-122 in mice. Under optimized conditions, the miRNA-122 in liver can be specifically silenced by 83.2 ± 3.2%, accompanied by regulating gene expression in liver and lowering of plasma cholesterol without inducing an immune response.[367] A functional nanosystem composed of poly(lactic-*co*-glycolic acid) has been developed to deliver antisense peptide nucleic acids probe to liver for miRNA analysis. The oncogenic miRNA-155 overexpressed in numerous cancers is effectively inhibited in the transfected liver, which indicates a promising therapeutic option for lymphoma/leukemia.[368]

## 7. SUMMARY AND PROSPECTS

MiRNAs play important roles in numerous developmental, metabolic, and disease processes of plants and animals. They can be regarded as useful biomarkers for cellular events or early disease diagnosis. Since mature miRNAs are small with only about 19−23 nucleotides, highly similar sequences in their family, and the low expression, the creation of efficient tools for rapid, specific, and sensitive detection of miRNAs becomes significant in complex samples. Conventional miRNA detection techniques including qRT-PCR, Northern blotting, and microarrays have their individual advantages for the screening of miRNAs. Northern blotting is considered as the standard method for miRNA detection since it can locate specific RNA sequences in a solution of mixed RNAs and provide information on the size and expression of predicted miRNAs and precursor miRNAs. qRT-PCR can easily provide absolute miRNA quantification and has the wide dynamic range and high accuracy. MiRNA microarrays with a low sensitivity and dynamic range are less expensive as discovery tools due to their high-throughput-screening capability. However, these techniques require miRNA isolation and/or target amplification, which makes the assays complicated and labor-intensive. Meanwhile, they require the lysis of a population of cells, and thus cannot drive phenotypic diversity and cell-to-cell variations in miRNA expression. Therefore, more sensitive and selective diagnostic assays are in urgent need for detection of the miRNA without time-consuming amplification process and purification step.

Fortunately, physical scientists and engineers have become increasingly interested in exploring the emerging tools in miRNAs detection by incorporating novel materials and reagents, such as metallic nanoparticles, semiconductor quantum dots, and bioluminescent proteins, etc. Especially, the nanoscale sizes of nanoparticles break through the limitation of structure miniaturization, and result in low detection limit. By utilizing the unique electrical or optical properties of nanostructures, a series of emerging approaches such as electrochemical assay, SPR, SERS, SiNWs, and nanomachine devices and so on are presented. Over traditional methods, all of them offer one or more advantages with high sensitivity, good specificity, multiplexing capability, and easy operation, and are supposed to be an alternative technique for miRNA detection. Further, by combining with the molecular biological techniques such as enzymatic reaction, LCR, RCA, and EXPAR, the integrated approaches for miRNA detection exhibit lower and lower detection limit. These methods have applied in the detection of miRNAs in cells, and circular blood and tissue samples.

For point-of-care analysis of miRNAs, there are still great demands for innovating the detection methods. The ideal miRNA profiling technique should be multiplex and possesses high specificity against other family RNAs, minimum sample manipulation, and a wide dynamic range from attomolar to nanomolar concentrations. Aiming at the advantages and disadvantages of the current miRNA analytical methods, several future directions in this field should be developed: (i) The need for ultrasensitive assay of low-abundance miRNA and the trend toward miniaturized devices make nanomaterials significant, since the nanoscale nanomaterials can produce a synergic effect among catalytic activity, conductivity, and biocompatibility to accelerate the signal transduction, leading to the ultrasensitive detection. (ii) The specificity in the detection of miRNA should be further improved. Typically, by using the molecular biological techniques such as target cycling, LAN recognition, ISH, and enzyme catalysis, the specificity of the assay can be efficiently enhanced in the detection of miRNA. (iii) On the basis of the unique optical, electronic, and magnetic properties of nanomaterials, the novel physical biosensors with total internal reflection fluorescence microscopy, SPR, SERS, and SiNWs are supposed to be an alternative technique for miRNA detection, and even permit detection of miRNA in single-molecular level.[369] (iv) Since most of the reported methods require a large amount of miRNAs and relatively pure miRNA samples, an integrated microfluidic chip provides a powerful tool for high-throughput analysis of miRNA in single-cell gene expression measurements with improved performance, reduced cost, and high sensitivity. (v) In point-of-care analysis of miRNAs, the stability and biocompatibility involving the immobilization of the signal probes should be considered in the design of the next generation biosensing methods. The

novel strategies in miRNA detection will provide a powerful tool to not only decode the functions of these miRNA molecules in a variety of biological systems but also improve human health in early disease diagnostics.

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### Notes

The authors declare no competing financial interest.

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Jianping Lei received his B.S. and M.S. degrees from Nanjing University and Ph.D. degree from Kanazawa University in Japan in 2004, and then pursued his postdoc research at Stanford University, in 2005−2006. He became an associate professor in 2006 and full professor in 2010 in Nanjing University. His research interests include functionalization and self-assembly of nanomaterials for signal amplification in biosensing and bioanalysis.

Lin Ding graduated with a B.S. degree in chemistry from Nanjing University in 2004. She then started a project on the electrochemical detection of tumor cells and cell surface glycans under the supervision of Professor Huangxian Ju at Nanjing University, where she obtained her Ph.D. in 2009. She is now an associate professor in Department of Chemistry, Nanjing University. Her research interest focuses on the detection of cellular carbohydrate expression and glycosyltransferase.

Professor Yongqiang Wen received his Ph.D. degree in 2005 at Institute of Chemistry, Chinese Academy of Sciences. After working in this institute for three years, he started his research as a CIFAR (Canadian Institute for Advanced Research) junior fellow at McGill University, Canada. He moved back to the institute in July, 2009. In January 2011, he joined the Research Center for Bioengineering & Sensing Technology, University of Science and Technology Beijing. His research mainly focuses on nanobiotechnology, using organic functional molecules and biomolecules to fabricate nanostructures and nanodevices.

Huangxian Ju received his B.S., M.S., and Ph.D. degrees from Nanjing University during 1982−1992. He was a postdoc in Montreal

University (Canada) from 1996 to 1997 and a guest professor in three universities of Germany and Ireland in 1999−2000. He became an associate and full professor of Nanjing University in 1993 and 1999. He won the National Funds for National Distinguished Young Scholars in 2003 and National Creative Research Groups in 2005, and was selected as a Changjiang Professor by Education Ministry of China in 2007. His research interests focus on analytical biochemistry and molecular diagnosis. He has published 430 papers with an h-index value of 56, authored 24 patents, 2 English books, 6 Chinese books, and 6 Chinese and 8 English chapters.

Xueji Zhang received his B.S. degree in 1989 and Ph.D. degree in 1994 from Wuhan University. He pursued his research as a postdoctoral researcher at National Institute of Chemistry Slovenia from 1995 to 1997, Swiss Federal Institute of Technology (ETH) from 1997 to 1998, and New Mexico State University from 1998 to 1999. He then joined the World Precision Instruments in 1999 and became the senior vice president in 2004. He is the courtesy professor at Univerty of South Florida. He joined University of Science & Technology Beijing via "Thousand Talents Program" of China in 2009. His researches include bioanalysis, electrochemical sensors, and design and application of biomedical instrumentation.

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## ABBREVIATIONS

| | |
|---|---|
| Ago2 | argonaute2 |
| AuNPs | gold nanoparticles |
| BRCA | branched rolling-circle amplification |
| cDNA | complementary DNA |
| CLL | chronic lymphocytic leukemia |
| DIG | digoxigenin |
| DSN | duplex-specific nuclease |
| dsRBD | dsRNA-binding domain |
| EDC | ethyl-3-(3-dimethylaminopropyl) carbodiimide |
| ES | embryonic stem |
| EXPAR | exponential amplification reaction |
| Fluc | firefly luciferase |
| GFP | green-fluorescent protein |
| Gluc | Gaussia luciferase |
| HCC | hepatocellular carcinoma |
| HDL | high-density lipoprotein |
| ISH | in situ hybridization |
| LCR | ligase chain reaction |
| LNA | lock nucleic acid |
| MB | molecular beacon |
| MRI | magnetic resonance imaging |
| miRNAs | microRNAs |
| NPM1 | nucleophosmin |
| NPs | nanoparticles |
| NSCLC | nonsmall cell lung cancer |
| qRT-PCR | quantitative reverse transcription PCR |
| PCR | polymerase chain reaction |
| PMNT | polythiophene derivative |
| PPi | pyrophosphate |
| pre-miRNAs | precursors miRNAs |
| RCA | rolling-circle amplification |
| RISC | RNA-induced silencing complex |
| Rluc | Renilla luciferase |
| RNases | ribonuclease |
| SERS | Raman scattering |
| SG | SYBR Green 1 |
| SiNWs | silicon nanowires |
| SPR | surface Plasmon resonance |
| ssDNA | single-stranded DNA |
| TMAC | tetramethylammonium chloride |
| UTR | untranslated region |

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