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MicroRNA Detection and Pathological Functions

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Preface

MicroRNAs (miRNAs) are a kind of short noncoding RNAs that regulate gene expression by translational repression or degradation of messenger RNA. They play important roles in many cellular processes, including development, proliferation, differentiation and apoptosis. Particularly, aberrant miRNAs expressions have been proposed to be related to various pathological processes such as cancer, diabetes, cardiovascular disease and other illnesses. Therefore, establishment of an accurate and reliable miRNA expression profile is significant for the investigation of biological processes in health and disease and for exploring their potential as novel diagnostic and prognostic biomarkers and drug targets.

In recent years, due to tremendous expansion of analytical techniques and capability, the scope of miRNA detection has also been widened. A detailed description of covering all detection strategies is beyond the scopes of this book. The book presented is an attempt to describe the significant and emerging research effort being done in the miRNA detection, focusing on the potential clinical application. The miRNA biogenesis, function, regulation and mechanism of action, as well as the challenges for miRNA detection, are briefly described first in Chap. 1. In Chap. 2, special attention is given to the miRNAs pathological function and potential clinic diagnostics indicating their advantages as well as their shortcomings. We then present an overview of various techniques and methods currently used to detect miRNAs. The conventional techniques used for miRNA detection, including standard PCR, Northern blotting and microarray, are critically addressed in Chap. 3.

Subsequently, the most innovative techniques in miRNA detection and quantification with superior flexibility and adaptability were presented in Chaps. 4–6 according to different detection techniques based on electrochemical, optical or other signal detection. These chapters are focusing on nanotechnology techniques, novel molecular biological techniques, enzyme-assisted approaches, capillary electrophoresis methods, etc.

The latest development of clinical-related miRNA detection methods in living cell, circulating blood and tissue including in situ hybridization (ISH) and molecular imaging techniques are reported in Chap. 7. The advantages and deficiency of

various detection techniques in this fast moving field along with the challenge and new directions are proposed in Chap. 8.

The book summarizes most of the significant miRNAs-related detection techniques in a single resource. It is our hope the book will be useful for readers to understand miRNA biogenesis, function and mechanism of action, assisting and enriching readers to understand the various types of miRNA detection available or under development, as well as their potential application in future clinical study. It should be of broad interest for academic and scientific communication worldwide.

We would like to express our sincere appreciation to all the authors who have taken part in this project and written wonderful chapters adding depth and value to the book, and express our sincere gratitude to our editor June Tang who helped us get through the project successfully. Special thanks to the support from National Natural Science Foundation of China, China Postdoctoral Special Foundation and Ph.D. Programs Foundation of Ministry of Education of China and the Fundamental Research Funds for the Central Universities.

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Chapter 1
Introduction

Abstract microRNAs (miRNAs) are a class of nonprotein coding RNAs, which play a significant regulated role in a diverse groups of animals, plants, and viruses. The formation of miRNAs is a multiple-step biogenesis process. There have been identified more than 1800 precursors and more than 2500 mature miRNA sequences for the human species, regulating more than two-thirds of genes that code functional proteins. Therefore, miRNAs are promising biomarkers for diagnosis and prognosis of the onset of disease states, providing an attractive pathway in gene therapy for genetic disorders and potential drug targets. However, the detection of miRNA involves great changes due to the characteristics of miRNA including small size, low abundance, and sequence similarity among family members. Besides traditional miRNA detection strategies such as Northern blotting, microarrays, and quantitative RT-PCR (qRT-PCR), various emerging methods including nanomaterials, molecular biological techniques, and surface plasmon resonance (SPR) novel probe design techniques-based miRNA detection are continuously explored for miRNA detection.

Keywords miRNA biogenesis · miRNAs biological functions · Challenges of detection · Conventional detection strategies · Emerging detection strategies

1.1 miRNA Biogenesis

Mature microRNAs (miRNAs) are a class of single-stranded short (approximately 19–23 nucleotides), evolutionally conserved and nonprotein coding RNAs. They can act as posttranscriptional negative regulators of gene expression by binding to messenger RNAs (mRNAs) in a diverse group of animals, plants, and viruses [1, 2]. The formation of miRNAs is a multiple-step biogenesis process. Typically, miRNAs are initially transcribed by RNA polymerase II with some miRNAs being used by RNA polymerase III to generate 1–3 kb long primary miRNAs (Pri-miRNA) with one or more hairpin structures (Fig. 1.1) in the cell nucleus from

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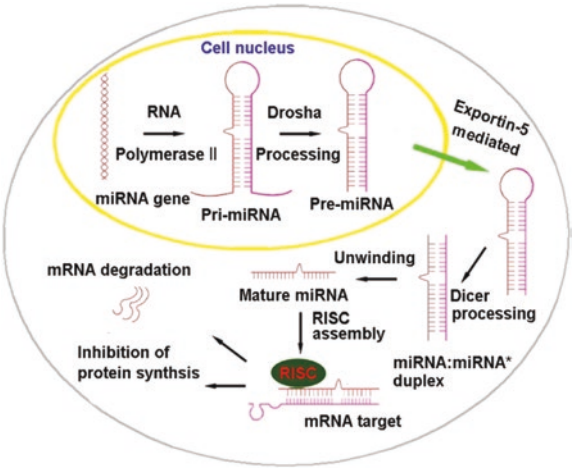

Fig. 1.1 miRNA biogenesis and function (see Footnote 1). Reprinted with permission from Ref. [1]. Copyright 2013 American Chemical Society

intragenic or intergenic regions [3]. Subsequently, these Pri-miRNA are capped, polyadenylated, and then cleaved in the nucleus by the RNase III enzyme Drosha and a double-stranded RNA-binding protein Pasha (DiGeorge Syndrome Critical Region 8 Protein), in order to generate a 70–100 nucleotides precursor miRNA (pre-miRNA) with stem-loop structures [4, 5]. The pre-miRNA can also be alternatively generated from very short introns as a result of splicing [6]. The pre-miRNAs are then translocated into the cytoplasm by the nuclear receptor Exportin-5 and RanGTP, where they are further processed into a 19–24 nucleotides short miRNA duplex by the RNase-III enzyme Dicer [7]. The dissociation of the duplex gives rise to a mature miRNA molecule incorporated into an Argonaute protein, forming an RNA-induced silencing complex (RISC) to regulate gene expression The 'passenger' miRNA strand is often degraded or plays a regulated role in miRNA homeostasis or downstream transcription [8, 9]. The RISC complex can modulate gene expression via perfect or imperfect base pairing to the 3'-untranslated region of the target mRNAs to induce target mRNA degradation or translational repression [10, 11].[1]

1.2 Biological Functions of miRNAs

The importance of miRNA can be demonstrated by the fact that the miRBase database (miRBase 20: http://www.mirbase.org/) identified more than 1800 precursors and more than 2500 mature miRNA sequences for the human species to date, which regulates more than two-thirds of genes that code functional proteins [12].

[1]Xueji Zhang, Haifeng Dong and Yaping Tian contributed together to this chapter.

Notably, due to imperfect matching between the miRNA and its target, one miRNA may regulate hundreds of mRNAs and, as a result, may substantially affect gene expression networks [13]. The miRNA biology is a complex and appears as a highly orchestrated mode of gene regulation network. Thus, miRNAs that individually target various mRNAs whose protein products contribute to one particular regulatory axis can be used for combinatorial regulation due to the multiplicity of miRNA targets [14].

miRNAs play important roles in various biological processes including early development [15], cellular differentiation [16], proliferation [17], apoptosis [18], developmental timing [19], and hematopoiesis [20]. They are also associated with diverse cellular activities such as immune response [21], insulin secretion [22], neurotransmitter synthesis [23], circadian rhythm [24], and viral replication [25]. Excellent observations demonstrate that aberrant expression of miRNAs are implicated to a variety of human diseases and disorders, such as neurodegenerative diseases [26], heart diseases [27], diabetes [28], kidney diseases [29], liver diseases [30], and altered immune system function. [21] Numerous studies have reported that miRNAs are aberrant expression markers in cancer cells. It was discovered that miRNAs could act as both tumor suppressors or oncogenes in human cancer [31, 32]. Thus, miRNA expression profiles are emerging as promising biomarkers for diagnosis and prognosis of the onset of disease states [33] and provide an attractive pathway in gene therapy for genetic disorders [34] and potential drug targets [35].

1.3 Challenges of miRNA Detection

In order to understand the functions of miRNAs and enable the use of miRNAs or miRNA in clinical practice for diagnosis or prognosis, an essential step is to develop efficient and reliable detection strategies. However, the unique characteristics of miRNA such as small size, low abundance, and sequence similarity among family members make miRNA profiling a challenging endeavor. Briefly, the problems are the small size of miRNA and sensitivity of the assay. The small size of miRNAs makes the polymerase chain reaction (PCR) or hybridization inefficient in miRNA analysis. The lengths of primers in conventional PCR are similar to miRNAs, which require very short primers in assay design, leading to very low oligonucleotide annealing temperature and inefficiency of PCR [36, 37]. The low melting temperature decreases the hybridization stringency and sharply increases the cross-hybridization risk. The cellular miRNAs concentration can be as low as a few molecules per cell, representing only a small fraction (ca. 0.01 %) of the mass in the total RNA sample [38], which requires an assay with high sensitivity. The high sequence similarity among homogeneous members makes the specific and sensitive detection more difficult. The different expression level of miRNAs among cells require miRNA assay with wide dynamic range, while multiplicity of miRNA needs an miRNA assay detecting multiple miRNA simultaneously. For intracellular or in situ detection, when sample is a mixture of pre-and mature

miRNA, the nonspecific hybridization to pre-miRNA can lead to a false positive signal for expression levels of mature miRNA. Efficient noninvasive monitoring approaches are of urgent need to provide detailed spatial expression patterns for specific miRNAs in vivo. Taken together, facile, high selective and sensitive miRNA analysis strategies that can effectively implement miRNA expression profile and feasibly be applied to in situ detection are still in demand.

1.4 miRNA Detection Methods

Currently, a wide range of approaches are explored for miRNA profiling. The traditional strategies including Northern blotting, microarrays, and quantitative RT-PCR (qRT-PCR) as well as emerging methods such as nanomaterials, molecular biological techniques, and surface plasmon resonance (SPR) novel probe design techniques-based miRNA detection are briefly discussed in the section. It is important to point out that sample processing and RNA extraction methods have a substantial impact on the results of miRNA profiling regardless of different methods. The preparation and miRNA extraction methods are of critical importance in miRNA detection.

Generally, when Northern blotting, microarrays, and quantitative qRT-PCR are employed for miRNA profiling, numerous technical variants are needed to improve the performance due to the unique characteristics of miRNA. Northern blotting is used to identify and quantify specific miRNAs in profiling studies [13]. However, this method generally needs hundreds of micrograms of total RNA as starting material, and often fails to detect low abundant miRNAs [39]. miRNA microarrays are often used as screening tools rather than as quantitative assay platforms due to low sensitivity and relatively narrow dynamic range. To date, qRT-PCR is the gold-standard of miRNA detection. It possesses the wide dynamic range, high accuracy, and can easily provide absolute miRNA quantification. However, it suffers from expensive instrument and throughput issues [40, 41].

Besides the conventional quantification strategies reported above, a number of emerging assays have been recently reported. The progress of nanotechnology and nanoscience holds great promise in detection of miRNA with high sensitivity and selectivity. The unique optical, electronic, and catalytic properties of nanomaterials facilitate translating of the biorecognition events into an electrochemical or spectroscopic response. They possess high surface areas for improved mass transport and high loading of receptor molecules for synergistic amplification of the target response. In electrochemical miRNA detection, the properties of nanomaterials lead to less amount of sample, shorter detection time, and less labor-cost while increasing sensitivity, specificity, and simplicity [42, 43]. The outstanding optical properties of nanomaterials are valuable for the development of sensing optical strategies. For instance, gold nanoparticles (AuNPs) and carbon-containing nanoparticles have outstanding fluorescence quenching efficiency, allowing their implementation in fluorescence recovery strategies. Silver nanoclusters (AgNCs)

and quantum dots (QDs) can be used to develop fluorescence resonance energy transfer (FRET) strategies, due to their bright and stable fluorescence. Various nanomaterials have been used as carriers or tracers, catalysts, electronic conductors, and optical emitters, to amplify detection signal and stabilize recognition probes in the detection of miRNA.

Molecular-biology-based methods involve enzymatic reactions for signal generation and amplification including target amplification, probe amplification, or signal amplification strategies. Target amplification, such as thermal cycled PCR and isothermal target amplification methods include helicase-dependent amplification (HDA), strand displacement amplification, loop-mediated isothermal amplification (LAMP), and recombinase polymerase amplification (RPA). They provide a 10^8–10^9-fold amplification of a nucleic acids fragment to achieve high target concentration for detection using conventional approaches. The probe amplification strategies are a replication of the probe sequence while keeping the amount of the remains the same, such as rolling circle amplification (RCA). Another technique that improves LOD is signal amplification using a target rather than the sequence to trigger amplifications by chemical approaches.

The expansions of emerging analytical technologies lead to their broad applications in miRNA detection. Examples such as SPR, surface enhanced Raman scattering (SERS), capillary electrophoresis (CP), and inductively coupled plasma mass spectrometry (ICP-MS) are continuously explored in miRNA detection. Each approach has its relative strengths and deficiency, thus researchers should figure out a balance of cost, precision, accuracy, and sample quantity to make their choice based on the given application.

References

1. Dong H, Lei J, Ding L, Wen Y, Ju H, Zhang X (2013) Chem Rev 113:6207–6233. doi:10.1021/cr300362f
2. He L, Hannon GJ (2004) Nature Rev Genet 5:522–531. doi:10.1038/nrg1415
3. Lee Y, Kim M, Han JJ, Yeom KH, Lee S, Baek SH, Kim VN (2004) EMBO J 23:4051–4060. doi:10.1038/sj.emboj.7600385
4. Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, Shiekhattar R (2004) Nature 432:235–240. doi:10.1038/nature03120
5. Lee Y, Ahn C, Han JJ, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, Kim VN (2003) Nature 425:415–419. doi:10.1038/nature01957
6. de Planell-Saguer M, Celina Rodicio M (2013) Clin Biochem 46:869–878. doi:10.1016/j.clinbiochem.2013.02.017
7. Bohnsack MT, Czaplinski K, Gorlich D (2004) Rna-a Publ of the Rna Soc 10:185–191. doi:10.1261/rna.5167604
8. Bartel DP (2009) Cell 136:215–233. doi:10.1016/j.cell.2009.01.002
9. Suzuki HI, Miyazono K (2011) J Biochem 149:15–25. doi:10.1093/jb/mvq113
10. Liu X, Fortin K, Mourelatos Z (2008) Brain Pathol 18:113–121. doi:10.1111/j.1750-3639.2007.00121.x
11. Krol J, Loedige I, Filipowicz W (2010) Nat Rev Genet 11:597–610. doi:10.1038/nrg2843
12. Friedman RC, Farh KK-H, Burge CB, Bartel DP (2009) Genome Res 19:92–105. doi:10.1101/gr.082701.108

13. Pritchard CC, Cheng HH, Tewari M (2012) Nat Rev Genet 13:358–369. doi:10.1038/nrg3198
14. Small EM, Olson EN (2011) Nature 469:336–342. doi:10.1038/nature09783
15. Brennecke J, Hipfner DR, Stark A, Russell RB, Cohen SM (2003) Cell 113:25–36. doi:10.1016/s0092-8674(03)00231-9
16. Dostie J, Mourelatos Z, Yang M, Sharma A, Dreyfuss G (2003) Rna-a Publ of the Rna Soc 9:180–186. doi:10.1261/rna.2141503
17. Wang Y, Keys DN, Au-Young JK, Chen C (2009) J Cell Physiol 218:251–255. doi:10.1002/jcp.21607
18. Xu PZ, Vernooy SY, Guo M, Hay BA (2003) Curr Biol 13:790–795. doi:10.1016/s0960-9822(03)00250-1
19. Ambros V (2003) Cell 113:673–676. doi:10.1016/s0092-8674(03)00428-8
20. Chen CZ, Li L, Lodish HF, Bartel DP (2004) Science 303:83–86. doi:10.1126/science.1091903
21. O'Connell RM, Rao DS, Chaudhuri AA, Baltimore D (2010) Nat Rev Immunol 10:111–122. doi:10.1038/nri2708
22. Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma XS, MacDonald PE, Pfeffer S, Tuschl T, Rajewsky N, Rorsman P, Stoffel M (2004) Nature 432:226–230. doi:10.1038/nature03076
23. Greco SJ, Rameshwar P (2007) Proc Natl Acad Sci USA 104:15484–15489. doi:10.1073/pnas.0703037104
24. Cheng H-YM, Papp JW, Varlamova O, Dziema H, Russell B, Curfman JP, Nakazawa T, Shimizu K, Okamura H, Impey S, Obrietan K (2007) Neuron 54:813–829. doi:10.1016/j.neuron.2007.05.017
25. Jopling CL, Yi MK, Lancaster AM, Lemon SM, Sarnow P (2005) Science 309:1577–1581. doi:10.1126/science.1113329
26. Eacker SM, Dawson TM, Dawson VL (2009) Nat Rev Neurosci 10:837–841. doi:10.1038/nrn2726
27. Cai BZ, Pan ZW, Lu YJ (2010) Curr Med Chem 17:407–411
28. Pandey AK, Agarwal P, Kaur K, Datta M (2009) Cell Physiol Biochem 23:221–232. doi:10.1159/000218169
29. Saal S, Harvey SJ (2009) Curr Opin Nephrol Hypertens 18:317–323. doi:10.1097/MNH.0b013e32832c9da2
30. Chen X-M (2009) World J Gastroenterol 15:1665–1672. doi:10.3748/wjg.15.1665
31. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D, Slack FJ (2005) Cell 120:635–647. doi:10.1016/j.cell.2005.01.014
32. Stahlhut Espinosa CE, Slack FJ (2006) The Yale J Biol Med 79:131–140
33. Kosaka N, Iguchi H, Ochiya T (2010) Cancer Sci 101:2087–2092. doi:10.1111/j.1349-7006.2010.01650.x
34. Grosshans H, Filipowicz W (2008) Nature 451:414–416. doi:10.1038/451414a
35. Gupta A, Gartner JJ, Sethupathy P, Hatzigeorgiou AG, Fraser NW (2006) Nature 442:82–85. doi:10.1038/nature04836
36. Cissell KA, Shrestha S, Deo SK (2007) Anal Chem 79:4754–4761
37. Qavi AJ, Kindt JT, Bailey RC (2010) Anal Bioanal Chem 398:2535–2549. doi:10.1007/s00216-010-4018-8
38. Lim LP, Lau NC, Weinstein EG, Abdelhakim A, Yekta S, Rhoades MW, Burge CB, Bartel DP (2003) Genes Dev 17:991–1008. doi:10.1101/gad.1074403
39. Yin JQ, Zhao RC, Morris KV (2008) Trends Biotechnol 26:70–76. doi:10.1016/i.tibtech.2007.11.007
40. Chapin SC, Appleyard DC, Pregibon DC, Doyle PS (2011) Angewandte Chemie-International Edition 50:2289–2293. doi:10.1002/anie.201006523
41. Baker M (2010) Nat Methods 7:687–692. doi:10.1038/nmeth0910-687
42. Gao ZQ, Yang ZC (2006) Anal Chem 78:1470–1477. doi:10.1021/ac051726m
43. Peng Y, Yi G, Gao Z (2010) Chem Commun 46:9131–9133. doi:10.1039/c0cc01990a

Chapter 2
miRNA Biology in Pathological Processes

Abstract miRNAs are small (~22 nucleotides) noncoding RNAs, highly conserved in both plants and animals, which mainly function in posttranscriptional regulation through directly degrading target mRNAs or inhibiting the translation. In other words, the final roles of miRNAs are mediated by the regulation of their target genes, which are involved in a series of important pathophysiological events, such as embryonic development, metabolism, cell proliferation and differentiation, tumorigenesis, immune defense, etc. According to their roles as a disease driving force or accompanying feature, miRNAs could be used as molecular therapeutic targets or potential diagnostic/prognostic biomarkers, respectively. Here, we review the latest discoveries of miRNAs alteration involved in common human diseases and discuss their roles in diseases initiation and progression. On the basis of the increasing knowledge on miRNAs, it could be inferred that we might be able to precisely modulate the tissue or cell-specific miRNA levels and this would lead to a new revolution in medical treatment in the future.

Keywords Small noncoding RNAs · miRNAs biogenesis · Pathological processes · Inherited disease · Cancer initiation and progression · Cardiovascular disease · Diabetes mellitus

miRNA genes, located in intergenic regions as independent units or in introns of host genes, are generously transcribed by RNA polymerase II (Pol II). The resulting transcripts, named primary miRNAs (pri-miRNAs), are cleaved by "Microprocessor complex," which is mainly formed by the DiGeorge syndrome critical region 8 (DGCR8) and the enzyme Drosha. The corresponding products, often termed as precursor miRNAs (pre-miRNAs), are then exported out of the nucleus by the nucleocytoplasmic shuttler Exportin-5. In the cytoplasm, pre-miRNAs are further cleaved by the RNase III enzyme Dicer to form an imperfect miRNA: miRNA* duplex, which are about 22 nucleotides in length. Although

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either strand of the duplex might be functional miRNA, it must be incorporated into the RNA-induced silencing complex (RISC) to fulfill its function.

Due to the common features shared by miRNA genes and traditional DNA genes, it could be inferred that the regulating miRNAs might be regulated as well. Indeed, many miRNA genes could be transcriptionally regulated or posttranscriptionally edited, just like traditional genes. This expands the diversity and scope of miRNA function beyond that implicated from just the genome. Therefore, miRNA genes could be expressed in a time and tissue/cell-specific way under fine-tunings. It presents a new regulation layer of gene expression, which ensures that the body could make a rapid and accurate response to various external stimuli. Given that the expression of each individual miRNA is under strict regulation, the altered expression of certain miRNAs plays an important role in human diseases. Specifically, the expression changes could be induced by gene abnormalities, transcriptional or posttranscriptional alteration. Due to the limited space of this book, we mainly discuss the relationship of miRNAs with common diseases, such as inherited diseases, cancer, vascular diseases, diabetes, and so on.

2.1 miRNAs in Inherited Diseases

The first miRNA, named Lin-4, was discovered by Ambrosin *Caenorhabditis elegans* in 1993 [1]. He found that Lin-4 is essential for the normal temporal control of diverse postembryonic developmental events in *C. elegans*. Specifically, adult Lin-4 loss-of function mutants lack many adult structures and they cannot lay eggs because of a failure to develop a vulva, so the eggs accumulate within their bodies [2]. Similarly, it could be inferred that some miRNA mutations might be involved in human inherited diseases due to the dysregulation of their targets. Until now, many hereditary diseases have been found to be caused by germline abnormalities in miRNA or miRNA-related genes [3].

2.1.1 Hereditary Deafness Caused by Mutations of miRNA-96 Gene

Growing evidence has demonstrated that both the mutations in certain miRNA genes and the duplication or deletion of miRNA genes could cause human inherited diseases. Although rare, several human single-gene disorders, which are associated closely with point mutations in miRNA genes have been reported till date. These mutations alter the subsequent processing and target recognition of the miRNAs. The first study of a Mendelian disorder that is associated with mutations in an miRNA gene is reported by Mencía et al. [4]. They found that point mutations in the seed region of miR-96, an miRNA expressed in hair cells of the inner ear, are responsible for nonsyndromic progressive hearing loss. According

to the literature, miRNA-96 is a member of the miRNA-183 family (miRNA-183, miRNA-96, and miRNA-182), which is expressed abundantly in specific types of sensory cells and is required for the physiological development of sensory hair cells [5–7]. The identified mutations are located in the seed region of miRNA-96, which indicate that the mutations result in either the loss of regulation of genes targeted by wild-type miRNA-96 or acquired repression of genes targeted by the mutant miRNA-96, or both. Interestingly, a recent study has found a novel mutation in the seed region of miRNA-96* in an Italian family with autosomal dominant hearing loss [8]. These studies have shown that point mutations in the miRNA-96 gene might play pivotal roles in severe hereditary deafness through affecting the physiological development of sensory hair cells.

2.1.2 Familial Keratoconus with Cataract Due to Mutations of the miRNA-184 Gene

The second example of a human disease that is closely related to mutations in the seed region of an miRNA gene is familial keratoconus with cataract. Hughes and colleagues identified a heterozygous C-to-T transition within the seed region of miRNA-184 by deep sequencing of a linkage region known to contain the mutation [9]. The same point mutation was reported subsequently by another group using bidirectional sequencing [10]. The report suggested that the single-base-pair substitution in the seed region of miRNA-184 is responsible for the corresponding disease phenotype. Recently, two novel heterozygous substitution mutations in miRNA-184 were identified in two patients with isolated keratoconus: miRNA-184(+8C>A) and miRNA-184(+3A>G) [11]. Computational modeling predicted that these mutations would be involved in pathogenesis by altering the miRNA-184 stem-loop stability and secondary structure.

2.1.3 Congenital Malformations Induced by Deletion of the miRNA-17~92 Cluster

In addition to miRNA gene mutations, the duplication, deletion, or inversion of miRNA genes could cause also human inherited diseases. The gene regions that are altered in individuals with chromosomal abnormalities sometimes harbor both protein-coding genes and miRNA genes [12, 13]. Although previous studies mainly focused on the roles of protein-coding gene changes in diseases, the aberrant expression of miRNAs might cause some of the diseases. Here, one example of disease with chromosomal abnormalities that is associated with the dysregulation of miRNAs is introduced as the representative. In 2011, de Pontual and colleagues reported the identification of germline hemizygous deletions of miRNA17HG; encoding the miRNA-17~92 polycistronic miRNA cluster,

in individuals with microcephaly, short stature, and digital abnormalities [14]. Through the establishment of a mouse model, they demonstrated that insufficiency of miRNA-17~92 is responsible for these developmental abnormalities. It is the first example of an miRNA gene responsible for a syndromic developmental defect in humans.

2.1.4 Other Inherited Diseases Closely Related to miRNA Alterations

Besides, many other miRNAs have been reported to be associated with inherited diseases, such as Down's Syndrome, Duchenne muscular dystrophy, methylmalonic acidemia, familial non-medullary thyroid cancer, and others [15–18]. Among these, although the microRNA genes are normal, their expression levels could change significantly during pathogenesis and progression.

Interestingly, the various mutations of miRNA processing enzymes or cofactors, including Drosha, DGCR8, Exportin-5, Dicer, TRBP, and AGO2, result in the reduced efficiency of miRNA processing, which could also lead to human diseases. For example, haploinsufficiency of the DGCR8 gene, induced by hemizygous microdeletions of the 22q11.2 locus, could contribute to the behavioral and cognitive deficits observed in DiGeorge syndrome [19]. Given that mutations in miRNA genes could induce human inherited diseases, it can be inferred that the variants with different miRNA-binding sites in the 3'UTRs of target mRNAs are also associated with diseases. In this regard, Simon and colleagues have found that a mutation in the miRNA-433 binding site of the HDAC6 gene is associated with X-linked dominant chondrodysplasia [20].

2.2 miRNAs in Cancer

Both miRNAs and cancer are very hot and ever-changing fields of biomedical research. Growing evidence has shown that miRNAs play pivotal roles in the whole process of cancer development, including tumor initiation, progression, metastasis, and even the final outcome. Generously, miRNAs repress the expression of target genes through reducing the mRNA stability or inhibiting the following translation. It is estimated that about two-thirds of all protein-coding genes could be targeted and then regulated by miRNAs. These targets include genes that mediate different stages of tumorigenesis, such as inflammation, cell cycle regulation, stress response, differentiation, apoptosis, invasion, metastasis, and so on. On the other hand, it is not unexpected that miRNAs are involved in various aspects of cancer through the regulation of oncogenes and tumor suppressor genes. In this regard, cancer-related miRNAs could be divided into two kinds depending on their functions, namely the onco-miRNAs which inhibit tumor suppressor genes

and tumor suppressor miRNAs which inhibit oncogenes. Therefore, the mutation or dysregulation of miRNAs could play important roles in tumor initiation and development.

2.2.1 Genetic Changes of miRNAs in Cancer

Single nucleotide polymorphisms (SNPs) have been identified in many protein-coding genes and some of these variants have been found to be associated with cancer risk [21, 22]. It is not unexpected that the SNPs could be closely related to cancer risk or even prognosis. One of the most recent examples of miRNAs SNPs in cancer is the miRNA-196a2 SNP rs11614913. Du and colleagues found that the CC genotype was associated with the significantly decreased expression of miRNA-196a-5p in some renal cell cancer (RCC) tissues. Moreover, luciferase reporter assays revealed that this SNP could potentially affect the binding efficiency of miRNA-196a-3p to its targets. It showed that this SNP may contribute to the genetic susceptibility and prognosis for RCC, which may act as a biomarker for RCC occurrence and prognosis [23]. Similarly, other SNPs within miRNA genes could be associated with cancer risk as well. For instance, a single SNP in the miRNA-499 gene is closely related to the risk of various cancers including breast cancer, squamous cell carcinoma of the head and neck, and hepatocellular carcinoma [24–27]. However, further study is needed to elucidate the detailed mechanisms.

In addition, point mutations could also be associated with cancer risk. Using miRNA-16-1 as an example, in 2005, a germline point mutation in the miRNA-16-1-miRNA-15a primary precursor, which caused low levels of miRNA expression in vitro and in vivo, was identified in patients with chronic lymphocytic leukemia (CLL) [28]. This suggested that the mutations in miRNA transcripts are common and may have functional importance. Aside from SNPs and point mutations, the most common chromosomal abnormality identified in CLL is deletion of 13q14.3, which could cause both hemizygous and homozygous loss [29]. This was also found in other tumors, including multiple myeloma, diffuse large B-cell lymphoma (DLBCL), and prostate cancer, which suggests that this region harbors tumor suppressor genes [30–32]. Combining with mouse models, Klein and colleagues found that miRNA-15a/16-1-deletion could accelerate the proliferation of both human and mouse B cells by modulating the expression of genes controlling cell-cycle progression [33].

Interestingly, SNPs within miRNA binding sites could be a novel genre of cancer biomarkers as well. Given that miRNA regulation is dependent on sequence complementarity between the target mRNA and miRNA seed region, it can be inferred that even single nucleotide alterations have significant effects. In recent years, many examples of such functional SNPs within the miRNA binding site have been identified as cancer biomarkers [34]. For instance, Dzikiewicz and colleagues found that variant alleles of TLX1_rs2742038 and ETV6_rs1573613 were

associated with increased risk of childhood ALL, while PML_rs9479 was associated with decreased ALL risk. Using luciferase reporter assays, it was revealed that SNPs within an microRNA-binding site could modulate leukemia risk by interfering with the miRNA-mediated regulation [35].

2.2.2 Alterations of miRNAs Expression in Cancer

As reported by many studies, miRNAs play more and more important roles in the whole process of tumor development, such as cancer initiation, progression, metastasis, and so on. Here, we will take some typical examples to illustrate the specific mechanisms involved in various steps of cancer development: For the first step, miRNAs expression might be associated with carcinogenesis. Through qPCR detection, Wang and colleagues found that miRNA-185 expression decreased in human breast cancer tissues compared with healthy tissue controls. In addition, upregulation of miRNA-185 could inhibit breast cancer cell proliferation and invasion and vice versa. Using bioinformatics techniques and a dual luciferase reporter system, they found that miRNA-185 was shown to bind to the 3′-untranslated region (UTR) of vascular endothelial growth factor a (Vegfa), which was found to be high in human breast cancer tissues [36]. Coincidentally, Zhang and colleagues revealed that miRNA-125b is abundantly expressed in both human and mouse, particularly at early stages of malignant progression to squamous cell carcinoma (SCC). Through further molecular and genetic analysis of miRNA-125b targets, they uncovered new insights underlying miRNA-125b's oncogenic function. On the one hand, miRNA-125b directly represses stress-responsive MAP kinase genes and associated signaling. On the other hand, it indirectly prolongs activated (phosphorylated) EGFR signaling [37]. These findings suggested that miRNAs could be associated with cancer initiation by targeting key molecules of cell proliferation and differentiation pathways.

For the second step, accumulating evidence has indicated that miRNAs act as critical regulators in tumor progression. Recently, Wang and colleagues reported that miRNA-199a-3p was significantly upregulated in gastric cancer (GC) cell lines and tissues. Functional studies demonstrated that miRNA-199a-3p dramatically increased cell proliferation and suppressed cell apoptosis both in vitro and in vivo by targeting the transcriptional regulator zinc fingers and homeoboxes 1 (ZHX1) [38]. Similarly, Zhang and colleagues reported that miRNA-214 was overexpressed in nasopharyngeal carcinoma (NPC) cell lines and tissues. Silencing of miRNA-214 by LNA-antimiRNA-214 in NPC cells resulted in promoting apoptosis and suppressing cell proliferation in vitro, while it suppressed tumor growth in nude mice in vivo. In addition, Bim was identified as a direct target of miRNA-214 by luciferase reporter assay [39]. This suggested that miRNAs could be associated with cancer progression by regulating key molecules of cell proliferation and apoptosis pathways.

For the third step, angiogenesis is a fundamental characteristic of cancer and is necessary in its multi-step progression. Although evidence for arsenite-induced lung cancer in humans is strong, the molecular mechanisms by which arsenite causes cancer remain to be established in practice. During a recent investigation, Zhao and colleagues evaluated the mechanism for arsenite-induced angiogenesis. They found that the knockdown of miRNA-21 could prevent tumors, which were formed from human bronchial epithelial (HBE) cells transformed by arsenite, from developing new blood vessels. Furthermore, downregulation of miRNA-21 in human umbilical vein endothelial cells (HUVEC) might inhibit the arsenite-induced increases of VEGF levels, which promotes angiogenesis. Thus, it is concluded that miRNA-21 could mediate tumor angiogenesis induced by arsenite [40]. Additionally, Kumar and colleagues demonstrated that ectopic expression of miRNA-34a in head and neck squamous cell carcinoma (HNSCC) cell lines significantly inhibited tumor cell proliferation, colony formation, and migration. Through an SCID mouse xenograft model, they found that ectopic expression of miRNA-34a also significantly inhibited tumor growth and tumor angiogenesis, which is mediated by blocking VEGF production as well as directly inhibiting endothelial cell functions [41]. This suggested that miRNAs could be associated with cancer initiation and progression by targeting key molecules in angiogenesis.

Finally metastasis, which can be regulated by miRNAs, causes most cancer deaths. Comparing the expression of miRNAs in metastatic and nonmetastatic primary mouse sarcomas, Sachdeva and colleagues found that miRNA-182 was markedly overexpressed in some tumors that metastasized to the lungs. By utilizing genetically engineered mice, they discovered that deletion of miRNA-182 substantially decreased, while overexpression of miRNA-182 considerably increased the rate of lung metastasis. Moreover, overexpression of miRNA-182 increased circulating tumor cells (CTCs), while deletion of miRNA-182 decreased CTCs, suggesting that miRNA-182 regulates invasion of cancer cells into the circulation. They identified four miRNA-182 targets that inhibit either the migration of tumor cells or the degradation of the extracellular matrix [42]. In addition, Wang and colleagues demonstrated that miRNA-133a expression negatively correlates with cell invasiveness in both transformed normal bronchial epithelial cells and lung cancer cell lines. miRNA-133a can inhibit cell invasiveness and cell growth through suppressing the expressions of three oncogenic receptors, including insulin-like growth factor 1 receptor (IGF-1R), TGF-beta receptor type-1 (TGFBR1), and epidermal growth factor receptor (EGFR) [43]. These results demonstrate that a single miRNA can regulate metastasis by coordinated regulation of multiple genes.

2.3 miRNAs in Cardiovascular Development and Disease

Cardiovascular disease is the leading cause of morbidity and mortality in developed countries and its incidence has increased gradually in developing countries. The implications of miRNAs in the pathological mechanism of cardiovascular

disease have widely been recognized, and research on their relationship has now become one of the most rapidly evolving fields. Many studies have demonstrated that miRNAs are abnormally expressed in the cardiovascular system under some pathological conditions. Using in vitro and in vivo models, both gain- and loss-of-function studies have revealed various roles for specific miRNAs in cardiovascular development, and physiological processes. Here, we review the latest studies that show the association of miRNAs with different aspects of cardiovascular disease.

2.3.1 miRNAs in Cardiovascular Development

Previous studies indicated that miRNAs play pivotal roles in proper cardiac development. However, their specific temporal and spatial functions during organogenesis are largely unknown. The results of inhibition of miRNA expression have been tested by deletion of Dicer, which is the essential RNase for miRNA biosynthesis. Interestingly, using Cre recombinase under control of cardiac regulatory DNA sequences to achieve cardiac deletion of Dicer could result in lethality at different developmental stages; which depends on the temporal expression pattern of the Cre transgene [44, 45]. Similarly, using a tamoxifen-inducible Cre recombinase to delete Dicer in the adult heart could cause heart failure and death [46]. Although these reports verify the importance of miRNAs in heart development and function, it is still unclear which miRNAs play decisive roles in the process.

miRNA-1, which is highly conserved from fruit flies to humans, was the first miRNA to be implicated in heart development. Interestingly, miRNA-1 and miRNA-133 are generated from a common bicistronic transcript in vertebrates, whereas these miRNAs are transcribed separately in invertebrates. Research on embryonic stem (ES) cells has revealed roles for miRNA-1 and miRNA-133 in the specification of mesodermal cell fates. However, miRNA-1 and miRNA-133 have opposite effects on the differentiation of muscle lineages: miRNA-1 promotes differentiation of ES cells toward a cardiac fate, whereas miRNA-133 inhibits the process. It is has been suggested that miRNA-1 exerts its effects by targeting the Notch ligand Delta-like (Dll-1) [47]. Both miRNA-1 and miRNA-133a could regulate the fundamental aspects of cardiac growth and development. Furthermore, miRNA-1 could control cardiac rhythm and remodeling through modulating numerous ion channels involved in cardiac conduction.

Additionally, many other miRNAs have been found to be involved in cardiac development. For example, miRNA-138 is specifically expressed in the ventricular chamber of the zebrafish heart and plays pivotal roles in the control of cardiac patterning. Some research has shown that miRNA-138 plays similar roles in patterning of the mammalian four-chambered heart because it is conserved from zebrafish to human [48]. This will be interesting to investigate in future studies. In addition, although miRNA-143/145 has different sequences, they are transcribed as a bicistronic unit. These miRNAs are cardiovascular-specific miRNAs and play key roles in modulating vascular smooth muscle cell (VSMC) phenotypes between

differentiated, proliferative, or migratory states in response to vascular injury or growth factor signaling [49]. Specifically, both miRNA-143 and 145 could target various genes involved in the regulation of SRF activity and actin dynamics to promote differentiation, repress proliferation of VSMCs, and modulate cytoskeletal assembly and dynamics [50, 51].

Despite current progress in the roles of miRNAs in cardiovascular development, our understanding of the specific mechanism is far from complete and numerous conceptual and experimental questions remain to be solved in future studies. Till now, only a small part of the miRNAs expressed in the cardiovascular system has been functionally determined by study. Identification and verification of additional miRNAs and further analysis of the functions of their targets might provide innovative insights into mechanisms of cardiovascular development, function, and dysfunction [52].

2.3.2 miRNAs in Cardiovascular Diseases

Expression profiles of miRNAs have been identified and verified in a variety of cardiovascular disorders, such as hypertrophy, heart failure, ischemic cardiomyopathy, myocardial infarction, and so on. According to the multifactorial nature of cardiovascular disease, it could be inferred that miRNAs might orchestrate many aspects of disease progression, from regulating metabolic risk factors (e.g., cholesterol and hormones) to controlling the response to acute cardiovascular events (e.g., inflammation and hypoxia) [53].

At the very beginning, miRNAs regulate lipid metabolism, lipoprotein clearance, and the pro- or anti-atherogenic effects in multiple organs. As we know, low-density lipoprotein (LDL) delivers cholesterol and phospholipids from the liver to tissues in need, whereas high-density lipoprotein (HDL) carries redundant lipids away from peripheral tissues back to the liver for excretion. Both high levels of LDL cholesterol and low levels of HDL cholesterol are independent risk factors for the development of atherosclerosis and its downstream disorders. Based on knockdown experiments using antisense technology, Najafi-Shoushtari and colleagues found that miRNA-33 acts in concert with the SREBP host genes to control cholesterol homeostasis and suggested that miRNA-33 may represent a therapeutic target for ameliorating cardiometabolic diseases [54]. Work by Ramírez and colleagues suggest that miRNA-144 regulates cholesterol metabolism via suppressing ABCA1 expression and modulation of miRNAs may represent a potential therapeutic intervention for treating dyslipidemia and atherosclerotic vascular disease [55]. Unlike that of HDL, the miRNA-mediated regulation of LDL cholesterol has been less documented. miRNA-122, which is the most plentiful miRNA expressed in liver, controls both LDL and HDL cholesterol levels, mainly by indirect modulation of cholesterol biosynthesis [56].

Coronary atherosclerosis is one of the most common cardiovascular diseases. Till now, various studies have identified many risk factors for it, including

hypertension, dyslipidemia, overweight/obesity, high blood sugar/diabetes, unhealthy lifestyle, smoking, unreasonable diet, excessive drinking, and so on. Interestingly, cardiovascular inflammation or injury is common in early molecular events of coronary atherosclerosis. Inflammation in the vessel wall is regulated by multiple miRNAs, including miRNA-126, miRNA-143/145, miRNA-155, miRNA-342-5p, and so on [53]. For example, miRNA-126 in endothelial cells (ECs) directly targets the 3'untranslated region of VCAM-1 mRNA to repress its expression, and inhibition of miRNA-126 could upregulate VCAM-1 expression and leukocyte adherence to ECs [57]. In addition, EC apoptotic bodies shed microvesicles containing miRNA-126, which represses RGS16 (regulator of G protein signaling 16) in arteries, inhibits the expression of inflammatory chemokine CXCL12 (chemokine (C-X-C motif) ligand 12), and then decreases the recruitment of inflammatory cells. This further reduces inflammation and overall stabilizes the atherosclerotic plaque [58]. miRNAs also play pivotal roles in cardiac fibrosis, hypertrophy, and remodeling and repair post-ischemic injury or myocardial infarction (MI). Although miRNA-21 is the most well studied miRNA in the post-MI environment, its role in cardiac remodeling remains controversial. Thum and colleagues found that blocking of miRNA-21 inhibits ERK-MAPK kinase pathways in cardiac fibroblasts through miRNA-21's specific target, Spry1, resulting in the attenuation of interstitial fibrosis and cardiac hypertrophy and dysfunction [59]. In contrast, another group used locked nucleic acid modified anti-miRNA oligonucleotides to inhibit miRNA-21 and verified that no difference was found in pathological cardiac remodeling in response to cardiac stress, which was further confirmed in an miRNA-21–knockout model [60].

In summary, the understanding that miRNAs play pivotal roles in modulation of inherited and acquired diseases of a cardiovascular system provides a new perspective on these disorders and has shown innovative cellular mechanisms of disease and potential new therapeutic targets. Therefore, the results that abnormal miRNA expression patterns contribute at various levels to the pathogenesis of cardiovascular disease has brought about considerable optimism for their use as therapeutic targets.

2.4 miRNAs in Diabetes Mellitus

According to the high prevalence and associated complications, diabetes mellitus (DM) is a major socioeconomic health problem worldwide. Diabetes is currently the most common metabolic disorder and its prevalence is rapidly increasing in both developed and developing countries. Specifically, diabetes affected 285 million adults in 2010, and it is expected to ascend to 7.7 % or 439 million adults by 2030 [61]. Diabetes is a complex metabolic disorder with an etiology that includes genetic, environmental, and lifestyle factors that lead to several different defects of glucose homeostasis. Depending on the distinct pathogenesis, diabetes could be classified into two forms: type I and type II. Between these, type I mostly occurs

in children and adolescents. This type is associated with absolute insulin deficiency due to a destruction of pancreatic β cells, often subsequent to autoimmune β-cell destruction. Type II, often seen in older people, is caused by peripheral insulin resistance and β-cell dysfunction. It can clearly be seen that the absolute deficiency or relative lack of insulin is the root cause of diabetes. Till now, many studies suggest that miRNAs regulate multiple biological processes, including the pathogenesis of diabetes.

2.4.1 miRNAs Involved in β Cell Development and Function

Pancreatic β cells, located in the islet of Langerhans, secrete insulin in response to increased blood glucose concentration, thereby maintaining glucose homeostasis within the body. These cells play key roles in the pathogenesis and progression of diabetes. Obviously, a variety of genes in them are distinctly regulated in response to alterations in blood glucose. Many researchers examined the contribution of miRNAs to this process.

In order to assess the overall contribution of miRNAs to pancreatic development, Dicer1, an essential enzyme for miRNA processing, was conditionally deleted from an embryonic developing mouse pancreas. The results showed that the expression of a unique profile of miRNAs is required during pancreas development and is necessary for β cell formation [62]. A recent study by Wang and colleagues shows that Dicer is essential for maintenance of acinar cell identity. Specifically, acinar cells lacking Dicer showed increased plasticity (as evidenced by loss of polarity), initiation of epithelial-to-mesenchymal transition (EMT), and acinar-to-ductal metaplasia (ADM) [63]. Poy and colleagues cloned and identified a novel, evolutionarily conserved and islet-specific miRNA (miRNA-375) and found that it is a regulator of insulin secretion and may thereby constitute a novel pharmacological target for treatment of diabetes [64]. miRNA-375, which is highly expressed in pancreatic β cells, is one of the most widely studied miRNAs involved in β cell functions. Soon after this discovery, the same group found that miRNA-375 is essential for normal glucose homeostasis, alpha- and beta-cell turnover, and adaptive beta-cell expansion in response to increasing insulin demand in insulin resistance [65]. In addition, increasing evidence has suggested that many other miRNAs are involved in insulin secretion, such as miRNA-124a, miRNA-9, miRNA-96, and so on [66].

2.4.2 miRNA Alterations During Pre-diabetic Metabolism

As discussed in the part “miRNAs in cardiovascular diseases,” miRNAs play pivotal roles in glucose and lipid metabolism, which is very important in the pathogenesis of both cardiovascular diseases and diabetes. On one hand, several studies

have revealed the role of miRNAs in glucose-induced vascular dysfunction. For example, Caporali and colleagues found that the expression of miRNA-503 was shown to be increased in human umbilical vein endothelial cells (HUVECs) and human microvascular endothelial cells (HMVECs) in culture conditions mimicking diabetes mellitus (high D-glucose) and ischemia-associated starvation (low growth factors) [67]. On the other hand, under diabetes conditions the vascular smooth muscle cell (VSMC) changes its phenotype from a contractile to a synthetic and proliferative state, which is an early event in the pathogenesis of atherosclerosis and is also accompanied by increased inflammation. Interestingly, Villeneuve and coworkers demonstrated a novel upstream role for miRNA-125b in the epigenetic regulation of inflammatory genes in VSMC of db/db mice through downregulation of Suv39h1 [68].

2.4.3 miRNAs in the Manifestation of Long-Term Complications

Due to consistently high blood glucose levels, diabetics have an elevated risk of developing a number of serious health problems, which could affect the heart and blood vessels, eyes, kidneys, nerves, and teeth. Additionally, these patients also have a higher risk of suffering from infectious diseases. In almost all developed countries, diabetes is a leading cause of cardiovascular disease, blindness, kidney failure, and lower limb amputation. According to statistics by the WHO, diabetes could lead to the largest number of complications in the world. Until now, it is widely reported that miRNAs play important roles in these processes.

Diabetic cardiomyopathy is one of the diabetes-induced organ complications. Using a microarray analysis of myocardial tissue, Shen et al. [69] identified changes in expression in 19 miRNAs, of which 16 miRNAs were further validated by qPCR. Specifically, overexpression of miRNA-373 decreased the cell size, and also reduced the level of its target gene MEF2C; this allows edp38 to regulate miRNA-373 expression. Another important complication of diabetes is represented by diabetic nephropathy (DN), which is the major cause of end-stage renal disease in developed countries. Krupa [70] found that loss of miRNA-192 expression associates with increased fibrosis and decreased estimated glomerular filtration rate (eGFR) in diabetic nephropathy in vivo, perhaps by enhancing TGF-beta-mediated downregulation of E-cadherin in proximal tubular cells (PTCs). Furthermore, diabetic neuropathy is a major debilitating complication of diabetes, which leads to high psychological strain. Recently, combining a mouse model of diabetic peripheral neuropathy with cultured dorsal root ganglion (DRG) neurons, Wang and colleagues showed that hyperglycemia downregulated miRNA-146a expression and elevated interleukin-1 receptor-activated kinase (IRAK1) and tumor necrosis factor receptor-associated factor 6 (TRAF6) levels in DRG neurons. Their data provide the first evidence showing that miRNA-146a plays an important role in mediating DRG neuron apoptosis under hyperglycemic conditions [71].

2.5 Conclusions

It is well known that one miRNA could target up to hundreds of mRNAs and one mRNA could be regulated by multiple miRNAs at the same time. Therefore, various miRNAs collaboratively work together to form a comprehensive regulatory net, which provides a new layer of gene regulation, mainly at the transcriptional level. Unexpectedly, it could be inferred that miRNAs participate in nearly all of the physiological and pathological processes of the body, including embryonic and tissue development, stem cell differentiation, apoptosis, inflammation, and so on. Up to now, miRNAs are widely reported to be involved in many kinds of diseases. Apart from the above-mentioned diseases, many other diseases are closely related to aberrantly expressed miRNAs, such as infertility, infectious diseases, autoimmune diseases, neurodegenerative diseases, and so on. Therefore, modulation of miRNA levels by administration of specific miRNA mimics or antisense oligonucleotides has recently come into focus as an attractive and promising alternative therapeutic option to halt or attenuate disease progression. These findings give us hope that one day we might be able to precisely modulate the tissue or cell-specific miRNA levels by targeting technology and this would lead to a new revolution in medical treatment.

References

1. Lee RC, Feinbaum RL, Ambros V (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75(5):843–854
2. Ambros V (2008) The evolution of our thinking about microRNAs. Nat Med 14(10):1036–1040
3. Kawahara Y (2014) Human diseases caused by germline and somatic abnormalities in microRNA and microRNA-related genes. Congenit Anom (Kyoto). 54(1):12–21
4. Mencía A, Modamio-Høybjør S, Redshaw N et al (2009) Mutations in the seed region of human miRNA-96 are responsible for nonsyndromic progressive hearing loss. Nat Genet 41(5):609–613
5. Gu C, Li X, Tan Q, Wang Z, Chen L, Liu Y (2013) MiRNA-183 family regulates chloride intracellular channel 5 expression in inner ear hair cells. Toxicol In Vitro 27(1):486–491
6. Li H, Kloosterman W, Fekete DM (2010) MicroRNA-183 family members regulate sensorineural fates in the inner ear. J Neurosci 30(9):3254–3263
7. Weston MD, Pierce ML, Jensen-Smith HC et al (2011) MicroRNA-183 family expression in hair cell development and requirement of microRNAs for hair cell maintenance and survival. Dev Dyn 240(4):808–819
8. Soldà G1, Robusto M, Primignani P et al (2012) A novel mutation within the MIRNA96 gene causes non-syndromic inherited hearing loss in an Italian family by altering pre-miRNA processing. Hum Mol Genet 21(3):577–585
9. Hughes AE, Bradley DT, Campbell M et al (2011) Mutation altering the miRNA-184 seed region causes familial keratoconus with cataract. Am J Hum Genet 89(5):628–633
10. Iliff BW, Riazuddin SA, Gottsch JD (2012) A single-base substitution in the seed region of miRNA-184 causes EDICT syndrome. Invest Ophthalmol Vis Sci 53(1):348–353
11. Lechner J1, Bae HA, Guduric-Fuchs J et al (2013) Mutational analysis of MIRNA184 in sporadic keratoconus and myopia. Invest Ophthalmol Vis Sci 54(8):5266–5272

12. Xu W, Ahmad A, Dagenais S et al (2012) Chromosome 4q deletion syndrome: narrowing the cardiovascular critical region to 4q32.2-q34.3. Am J Med Genet A. 158A(3):635–640
13. Elton TS, Sansom SE, Martin MM (2010) Trisomy-21 gene dosage over-expression of miRNAs results in the haploinsufficiency of specific target proteins. RNA Biol 7(5):540–547
14. de Pontual L1, Yao E, Callier P et al (2011) Germline deletion of the miRNA-17∼92 cluster causes skeletal and growth defects in humans. Nat Genet 43(10):1026–1030
15. Li YY, Alexandrov PN, Pogue AI et al (2012) miRNA-155 upregulation and complement factor H deficits in Down's syndrome. NeuroReport 23(3):168–173
16. Zaharieva IT, Calissano M, Scoto M et al (2013) Dystromirs as serum biomarkers for monitoring the disease severity in Duchenne muscular dystrophy. PLoS ONE 8(11):e80263
17. Li Y, Peng T, Wang X et al (2014) A primary study on down-regulated miRNA-9-1 and its biological significances in methylmalonic acidemia. J Mol Neurosci 53(2):280–286
18. Xiong Y, Zhang L, Holloway AK et al (2011) MiRNA-886-3p regulates cell proliferation and migration, and is dysregulated in familial non-medullary thyroid cancer. PLoS ONE 6(10):e24717
19. Kobrynski LJ, Sullivan KE (2007) Velocardiofacial syndrome, DiGeorge syndrome: the chromosome 22q11.2 deletion syndromes. Lancet 370(9596):1443–1452
20. Simon D, Laloo B, Barillot M et al (2010) A mutation in the 3'-UTR of the HDAC6 gene abolishing the post-transcriptional regulation mediated by hsa-miRNA-433 is linked to a new form of dominant X-linked chondrodysplasia. Hum Mol Genet 19(10):2015–2027
21. Shi H, Shu H, Huang C et al (2014) Association of LEPR K109R polymorphisms with cancer risk: a systematic review and pooled analysis. J BUON 19(3):847–854
22. Zhu J, Hua RX, Jiang J et al (2014) Association studies of ERCC1 polymorphisms with lung cancer susceptibility: a systematic review and meta-analysis. PLoS ONE 9(5):e97616
23. Du M, Lu D, Wang Q et al (2014) Genetic variations in microRNAs and the risk and survival of renal cell cancer. Carcinogenesis 35(7):1629–1635
24. Hu Z, Liang J, Wang Z et al (2009) Common genetic variants in pre-microRNAs were associated with increased risk of breast cancer in Chinese women. Hum Mutat 30(1):79–84
25. Liu Z, Li G, Wei S et al (2010) Genetic variants in selected pre-microRNA genes and the risk of squamous cell carcinoma of the head and neck. Cancer 116(20):4753–4760
26. Xiang Y, Fan S, Cao J et al (2012) Association of the microRNA-499 variants with susceptibility to hepatocellular carcinoma in a Chinese population. Mol Biol Rep 39(6):7019–7023
27. Wang N, Tian ZQ, Li Y et al (2013) An A/G polymorphism rs3746444 in miRNA-499 is associated with increased cancer risk: a meta-analysis. Genet Mol Res 12(3):3955–3964
28. Calin GA, Ferracin M, Cimmino A et al (2005) A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med 353(17):1793–1801
29. Döhner H, Stilgenbauer S, Benner A et al (2000) Genomic aberrations and survival in chronic lymphocytic leukemia. N Engl J Med 343(26):1910–1916
30. Dong JT, Boyd JC, Frierson HF Jr (2001) Loss of heterozygosity at 13q14 and 13q21 in high grade, high stage prostate cancer. Prostate 49(3):166–171
31. Migliazza A, Bosch F, Komatsu H et al (2001) Nucleotide sequence, transcription map, and mutation analysis of the 13q14 chromosomal region deleted in B-cell chronic lymphocytic leukemia. Blood 97(7):2098–2104
32. Mian M, Scandurra M, Chigrinova E et al (2012) Clinical and molecular characterization of diffuse large B-cell lymphomas with 13q14.3 deletion. Ann Oncol 23(3):729–735
33. Klein U, Lia M, Crespo M et al (2010) The DLEU2/miRNA-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia. Cancer Cell 17(1):28–40
34. Preskill C, Weidhaas JB (2013) SNPs in microRNA binding sites as prognostic and predictive cancer biomarkers. Crit Rev Oncog 18(4):327–340
35. Dzikiewicz-Krawczyk A, Macieja A, Mały E et al (2014) Polymorphisms in microRNA target sites modulate risk of lymphoblastic and myeloid leukemias and affect microRNA binding. J Hematol Oncol 7(1):43

36. Wang R, Tian S, Wang HB et al (2014) MiRNA-185 is involved in human breast carcinogenesis by targeting Vegfa. FEBS Lett 588(23):4438–4447
37. Zhang L, Ge Y, Fuchs E (2014) miRNA-125b can enhance skin tumor initiation and promote malignant progression by repressing differentiation and prolonging cell survival. Genes Dev 28(22):2532–2546
38. Wang Z, Ma X, Cai Q et al (2014) MiRNA-199a-3p promotes gastric cancer progression by targeting ZHX1. FEBS Lett 588(23):4504–4512
39. Zhang ZC, Li YY, Wang HY et al (2014) Knockdown of miRNA-214 promotes apoptosis and inhibits cell proliferation in nasopharyngeal carcinoma. PLoS ONE 9(1):e86149
40. Zhao Y, Xu Y, Luo F et al (2013) Angiogenesis, mediated by miRNA-21, is involved arsenite-induced carcinogenesis. Toxicol Lett 223(1):35–41
41. Kumar B, Yadav A, Lang J et al (2012) Dysregulation of microRNA-34a expression in head and neck squamous cell carcinoma promotes tumor growth and tumor angiogenesis. PLoS ONE 7(5):e37601
42. Sachdeva M, Mito JK, Lee CL et al (2014) MicroRNA-182 drives metastasis of primary sarcomas by targeting multiple genes. J Clin Invest 124(10):4305–4319
43. Wang LK, Hsiao TH, Hong TM et al (2014) MicroRNA-133a suppresses multiple oncogenic membrane receptors and cell invasion in non-small cell lung carcinoma. PLoS ONE 9(5):e96765
44. Zhao Y, Ransom JF, Li A et al (2007) Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. Cell 129(2):303–317
45. Chen JF, Murchison EP, Tang R et al (2008) Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure. Proc Natl Acad Sci USA 105(6):2111–2116
46. da Costa Martins PA, Bourajjaj M, Gladka M et al (2008) Conditional dicer gene deletion in the postnatal myocardium provokes spontaneous cardiac remodeling. Circulation 118(15):1567–1576
47. Ivey KN, Muth A, Arnold J et al (2008) MicroRNA regulation of cell lineages in mouse and human embryonic stem cells. Cell Stem Cell 2(3):219–229
48. Morton SU, Scherz PJ, Cordes KR et al (2008) microRNA-138 modulates cardiac patterning during embryonic development. Proc Natl Acad Sci USA 105(46):17830–17835
49. Owens GK, Kumar MS, Wamhoff BR (2004) Molecular regulation of vascular smooth muscle cell differentiation in development and disease. Physiol Rev 84(3):767–801
50. Cordes KR, Sheehy NT, White MP et al (2009) miRNA-145 and miRNA-143 regulate smooth muscle cell fate and plasticity. Nature 460(7256):705–710
51. Xin M, Small EM, Sutherland LB et al (2009) MicroRNAs miRNA-143 and miRNA-145 modulate cytoskeletal dynamics and responsiveness of smooth muscle cells to injury. Genes Dev 23(18):2166–2178
52. Liu N, Olson EN (2010) MicroRNA regulatory networks in cardiovascular development. Dev Cell 18(4):510–525
53. Karunakaran D, Rayner KJ (2013) MicroRNAs in cardiovascular health: from order to disorder. Endocrinology 154(11):4000–4009
54. Najafi-Shoushtari SH, Kristo F, Li Y et al (2010) MicroRNA-33 and the SREBP host genes cooperate to control cholesterol homeostasis. Science 328(5985):1566–1569
55. Ramírez CM, Rotllan N, Vlassov AV et al (2013) Control of cholesterol metabolism and plasma high-density lipoprotein levels by microRNA-144. Circ Res 112(12):1592–1601
56. Lanford RE, Hildebrandt-Eriksen ES, Petri A et al (2010) Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science 327(5962):198–201
57. Harris TA, Yamakuchi M, Ferlito M et al (2008) MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1. Proc Natl Acad Sci USA 105(5):1516–1521
58. Zernecke A, Bidzhekov K, Noels H et al (2009) Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. Sci Signal 2(100):ra81
59. Thum T, Gross C, Fiedler J et al (2008) MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. Nature 456(7224):980–984

60. Patrick DM, Montgomery RL, Qi X et al (2010) Stress-dependent cardiac remodeling occurs in the absence of microRNA-21 in mice. J Clin Invest 120(11):3912–3916
61. Shaw JE, Sicree RA, Zimmet PZ (2010) Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pract 87(1):4–14
62. Lynn FC, Skewes-Cox P, Kosaka Y et al (2007) MicroRNA expression is required for pancreatic islet cell genesis in the mouse. Diabetes 56(12):2938–2945
63. Wang YJ, McAllister F, Bailey JM et al (2014) Dicer is required for maintenance of adult pancreatic acinar cell identity and plays a role in Kras-driven pancreatic neoplasia. PLoS ONE 9(11):e113127
64. Poy MN, Eliasson L, Krutzfeldt J et al (2004) A pancreatic islet-specific microRNA regulates insulin secretion. Nature 432(7014):226–230
65. Poy MN, Hausser J, Trajkovski M et al (2009) miRNA-375 maintains normal pancreatic alpha- and beta-cell mass. Proc Natl Acad Sci USA 106(14):5813–5818
66. Plaisance V, Waeber G, Regazzi R et al (2014) Role of microRNAs in islet beta-cell compensation and failure during diabetes. J Diabetes Res 2014:618652
67. Caporali A, Meloni M, Völlenkle C et al (2011) Deregulation of microRNA-503 contributes to diabetes mellitus-induced impairment of endothelial function and reparative angiogenesis after limb ischemia. Circulation 123(3):282–291
68. Villeneuve LM, Kato M, Reddy MA et al (2010) Enhanced levels of microRNA-125b in vascular smooth muscle cells of diabetic db/db mice lead to increased inflammatory gene expression by targeting the histone methyltransferase Suv39h1. Diabetes 59(11):2904–2915
69. Shen E, Diao X, Wang X et al (2011) MicroRNAs involved in the mitogen-activated protein kinase cascades pathway during glucose-induced cardiomyocyte hypertrophy. Am J Pathol 179(2):639–650
70. Krupa A, Jenkins R, Luo DD et al (2010) Loss of MicroRNA-192 promotes fibrogenesis in diabetic nephropathy. J Am Soc Nephrol 21(3):438–447
71. Wang L, Chopp M, Szalad A et al (2014) The role of miRNA-146a in dorsal root ganglia neurons of experimental diabetic peripheral neuropathy. Neuroscience 14(259):155–163

Chapter 3
Conventional miRNA Detection Strategies

Abstract Conventional detection strategies for miRNA, including Northern blotting, quantitative real-time reverse-transcription, miRNA microarrays and cloning, are basically and widely used technologies. As gold-standard methods, conventional miRNA detection strategies are continuously used for confirming results from newer detection techniques. For improving the sensitivity and reducing the throughput of these conventional strategies, numerous efforts were focused on the probe design and miRNA labeling in the past several years. Although much progress has been made, researchers are devoted to improving miRNA detection strategies with the criteria of high sensitivity and specificity, cost-effectiveness, and minimal sample preparation.

Keywords Conventional strategies · Northern blotting · Qrt-PCR · miRNA microarrays · Cloning

3.1 Northern Blotting

Northern blotting is the earliest attempt at methodically measuring an miRNA expression profile [1–3]. It is broadly employed for visualizing miRNA expression of all sizes ranging from the long primal miRNA to the mature form [4, 5]. In short, the technique includes an initial electrophoretic step separating the extracted RNA in the light of size, followed by transferring to a blotting membrane. Finally, detection/quantification of mature miRNAs is achieved by hybridization with labeled, sequence-specific oligonucleotide probes [6]. Although this technology is low-throughput, low-sensitivity, and relatively time-consuming with a large sample-requirement [7], it continues to be widely used as a gold-standard method for confirming data from newer, more-sensitive detection techniques [8]. As an example, Tang et al. validated results from ambiguous array data and enabled accurate data interpretation by an additional array-data adjustment with step-Northern blot analysis of a ratio of a given miRNA and U6 or tRNA [9].

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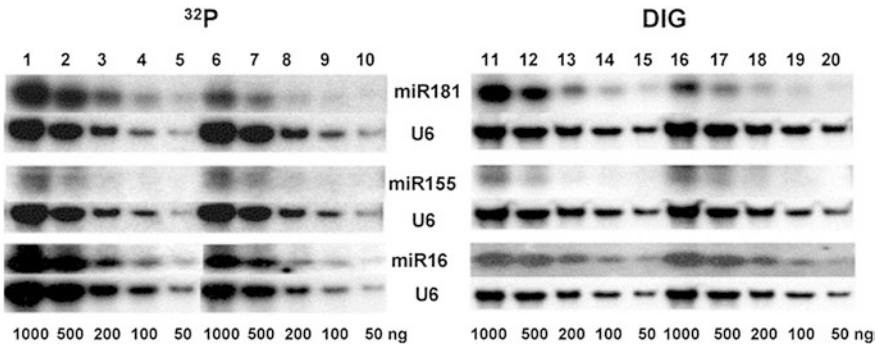

Fig. 3.1 Comparison of ^{32}P- and DIG-labeled RNA probes for the detection of miRNAs. Reprinted from Ref. [10], Copyright 2006, with permission from Elsevier

In the past few years, various improvements of the Northern blotting technique have been reported [5, 10–15], which mainly differ in the labeling and design of the probes used to detect miRNA. The most popular probe-labeling protocol is incorporation of radio isotopes (^{32}P) to the probe [15]. However, the probes are radioactively labeled, leading to health and environmental safety concerns and the requirement of dedicated facilities. As a safer alternative, a digoxigenin (DIG)-labeling system has been successfully applied to Northern-blotting analysis of miRNA with several advantages including high sensitivity, short exposure time, long shelf life, and increased safety. The use of DIG-labeled RNA oligos for detection of small RNA molecules (~22 nucleotides) with equal sensitivity is compared to ^{32}P-labeled probes in detecting miRNA (Fig. 3.1) [10][1].

In order to improve the sensitivity of Northern blots, the RNA was frequently cross-linked to the blotting membrane [13, 16]. However, conventional methods such as UV-cross-linking are generally not first-rank for detection of small RNAs [16]. Therefore, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as an alternative choice was employed to cross-link RNAs to the nylon membrane, giving rise to a 25−50-fold increase in the sensitivity of detection of miRNA [11, 13, 17].

Probe-design strategies have also been significantly improved. Locked-nucleic acids (LNAs) oligonucleotide probes, which considerably enhance the sensitivity of small RNAs detection, have taken the place of traditional DNA oligonucleotide probes [12, 14]. LNAs are a class of bicyclic high-affinity RNA analogues where the furanose ring of LNA monomers is conformationally locked in an RNA-mimicking C3' endo/N-type conformation (Fig. 3.2) [18]. Compared to traditional DNA probes, LNA-modified oligonucleotide probes have shown a 10-fold higher efficiency to detect miRNAs in Northern blotting of miRNA [15]. In 2010, Hu et al. reported the use of a new synthetic locked nucleotide, locMeCytidine-5′-triphosphate (LNA-mCTP), which can be transcribed into a short (~30-nt) RNA

[1]Haifeng Dong and Conghui Liu contributed together to this chapter.

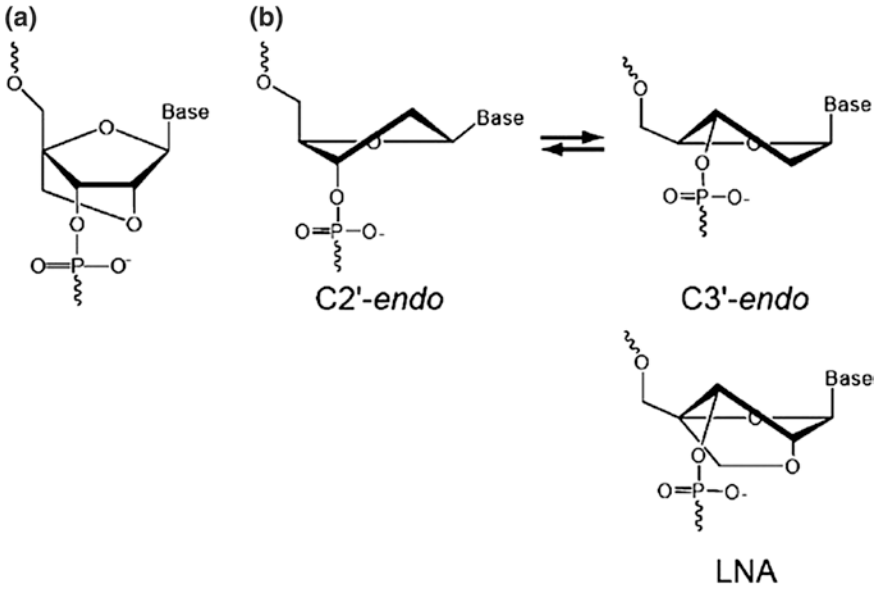

Fig. 3.2 Chemical structure of LNA (locked nucleic acid). Reprinted from Ref. [18], Copyright 2008, with permission from Elsevier

probe hybridized at a high temperature to increase hybridization specificity and reduce background [19]. A Northern-blot-based protocol for miRNA detection using DIG-labeled oligonucleotide probes containing LNA and EDC for cross-linking the RNA to the membrane has been reported [16], which generated clearly visible signals for RNA amounts as low as 0.05 fmol and reduced the exposure time by ~1000-fold.

3.2 Quantitative Real-Time Reverse-Transcription

Generally, quantitative real-time reverse-transcription (qRT-PCR) covers a large dynamic range and is regularly termed as the gold standard for gene expression [20]. qRT-PCR-based miRNA profiling leans on reverse transcription of miRNA to cDNA, followed by real-time monitoring of reaction product accumulation using qPCR. This technology represents a balance of cost, precision, and sample size, and is always employed for profiling of miRNAs expression level and confirming results generated by other strategies including microarray and Northern blot assays. [21, 22].

The development of qRT-PCR methods renders high-sensitivity miRNA detection down to a few nanograms of total RNA [23]. In order to obtain meaningful and reproducible results, RNA integrity control, cDNA synthesis, primer design,

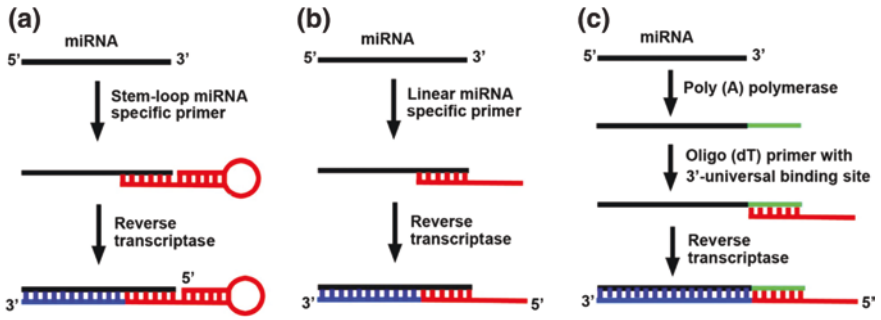

Fig. 3.3 Schematic of several strategies of reverse transcription to generate cDNA. Reverse transcription of individual miRNAs using (**a**) stem-loop or (**b**) linear primers, and (**c**) enzymatic addition of a poly(A) tail to miRNAs followed by reverse transcription using oligo(dT) primers. Reprinted by permission from Macmillan Publishers Ltd: Ref. [24], Copyright 2012

amplicon detection and normalization must be taken into account. The reverse transcription of miRNA to cDNA includes several strategies (Fig. 3.3) [24, 25]. The poly(A) polymerase method is polyadenylation of all miRNAs by Escherichia coli poly(A) polymerase, followed by reverse transcription using universal primers consisting of an oligo (dT) sequence on its 5′end, [26, 27], whereas specific-primers reversely transcribe only particular miRNAs using miRNAs-specific reverse transcription primers. In order to detect several miRNAs from a very small amount of starting material, such as plasma, the poly(A) polymerase method is more suitable [7]. However, it cannot distinguish pre-miRNAs from mature miRNAs, or detect small RNAs carrying a 2′-oxymethyl modification at their 3′-ends either [28]. The 3′-end of the primer needs to be complementary to the miRNA in the stem-loop primers approach [29, 30]. The stem-loop at the 5′-end facilitates the annealing of the primer to pre- and primary miRNAs, but reduces the ability to achieve reverse transcription of iso-miRNA sequences [31]. The TaqMan miRNA assay using stem−loop RT primers along with miRNA-specific TaqMan probes is the ‘‘gold standard’’ for miRNA detection (Fig. 3.4) [28]. Various modified versions of the TaqMan assay [17, 32] and SYBR green-based qRT-PCR miRNA assays [33, 34] have also been developed.

However, it was reported that in stem-loop quantitative PCR, the accurate detection could be severely influenced by total RNA input and DNA contamination, and the carryover DNA contamination could also mislead the detection in a sequence-specific manner. Additionally, it was proved that different 3′ iso-miR species of a particular miRNA could be reversely transcribed and cross-detected, even by specifically targeted assays. Therefore, it was suggested to refine protocol of miRNA detection by stem-loop real-time PCR technology [35].

Owing to sequence-specific differences in primer annealing, optimal reaction conditions in performing highly parallel qRT-PCR may vary substantially among miRNAs. In order to solve this problem, the incorporation of LNAs into primers to standardize the hybridization conditions of optimal miRNA primer is an

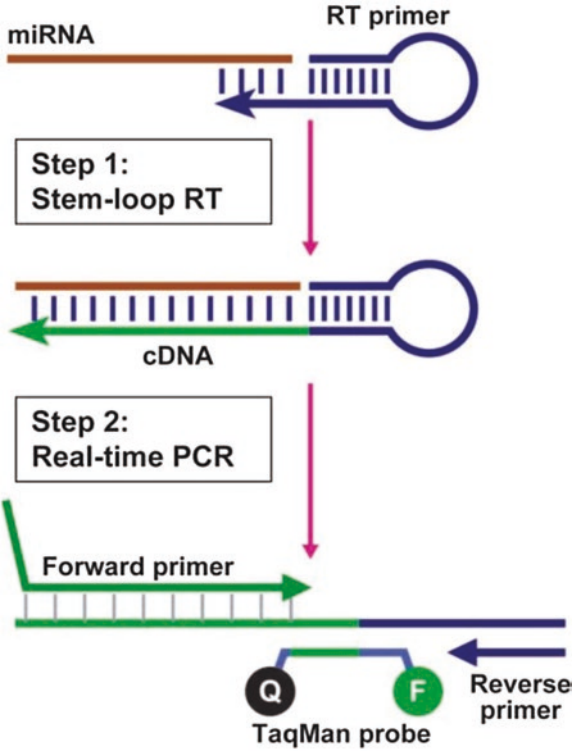

Fig. 3.4 Schematic of TaqMan miRNA assays, TaqMan-based qRT of miRNAs includes two steps, stem–loop RT, and real-time PCR. Reprinted with permission from Ref. [30]. Copyright 2005, Oxford University Press

effective method [24]. Manufacturers like Exiqon, Fluidigm, and SA Biosystems offer qRT-PCR kits that can evaluate hundreds of miRNAs in parallel, and some have customizable assays to sell to customers [36]. These require a "spike in" internal standard for array/qRT-PCR data normalization to quantificate circulating miRNAs in body fluids, such as plasma [7]. Another problem for qRT-PCR is the normalization of miRNA expression. Most normalization curves depend on genes for small RNAs, which might not be transcribed by the same polymerases and are unrepresentative of general miRNA regulation [36]. An alternative normalization method introduces the mean miRNA present in each sample [37], but the selected reference may not stay constant across samples.

Currently, the improvement of the synthesis of complementary DNA (cDNA) is the main development of the qRT-PCR technique for miRNA detection. It has been reported that a circularization-based platform called "miRNA-ID" exists for miRNA detection [38]. Circularization of the miRNA by a ligase and reverse transcription of the circularized miRNA was the main feature of the miRNA-ID. The circular RNA and multimeric cDNA templates were unrivalled as being flexible in the primers positioning, which straddled the boundaries between these repetitive miRNA sequences.

In order to improve reliability of the quantification by qRT-PCR, addition of "spiked-in" exogenous controls (e.g., miRNAs from the nematode C. elegans) at

the beginning of sample preparation can be employed to monitor miRNA recovery efficiency [39]. However, in this case, there are potential pitfalls such as sequence dependence of miRNA recovery [40] and therefore misdirection of normalization to a specific exogenous miRNA.

T4 DNA ligase has the capacity for repairing nicks in the DNA strand of a DNA:RNA hybrid and it has been combined with size-coded DNA probes (Fig. 3.5) to detect multiple miRNAs in all RNA with the detection limit down to 1 pM (~105 copies per microliter) [41]. A reverse transcription-free real-time approach has also been used for rapid quantification of miRNAs ranging from 5 amol to 500 fmol [42]. Only in the presence of target miRNA, which provides additional stability for the heterodimer through contiguous stacking hybridization of miRNA and bridging sequence, is an exponential amplification process carried out efficiently.

In order to identify serum miRNAs that diagnose and correlate with the prognosis of prostate cancer, a multiplex qRT- PCR method, which involved the purification of multiplex PCR products followed by uniplex analysis on a microfluidics chip to evaluate 384 human miRNAs, has been developed [43].

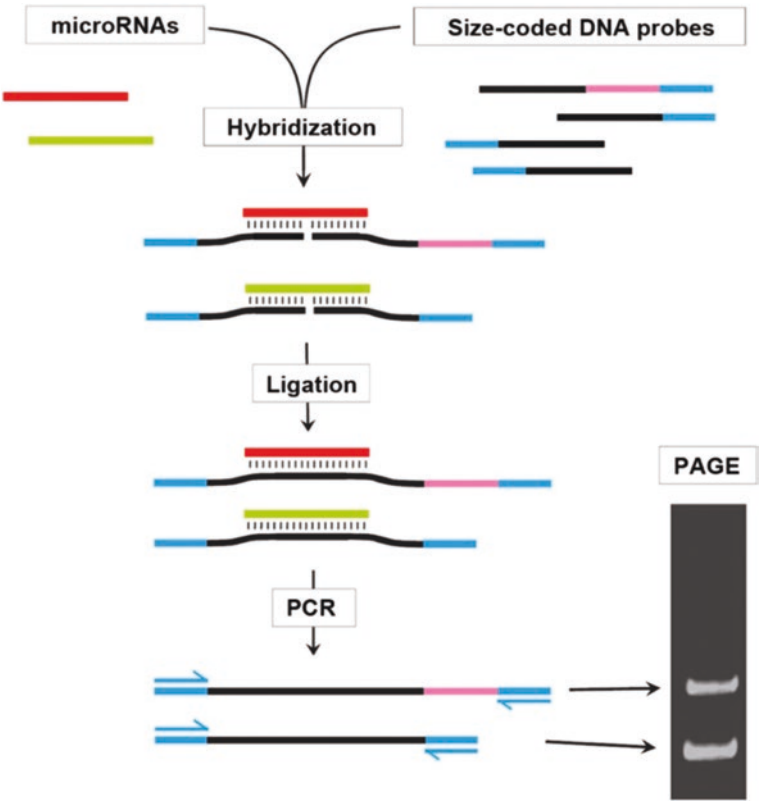

Fig. 3.5 Diagram of the size-coded ligation-mediated PCR method. Reprinted with permission from Ref. [41], Copyright 2011 Oxford University Press

For the sensitivity to further increase, the DNA probe modified with ribonucleotides can be effectively ligated by using miRNA as the template with the catalysis of T4 RNA ligase 2 [44]. The DNA probe modified with two ribonucleotides at its 3′-terminus can make great contribution to ligation efficiency improvement and limit of detection of target miRNAs as low as 0.2 fM (e.g., 4 zmol). The dynamic range spanning over seven orders of magnitude is comparable to the sensitivity of RT-PCR assays. Great discrimination of a single-nucleotide difference among miRNA sequences and high specific detection of mature miRNAs against their precursors can also be achieved.

3.3 miRNA Microarrays

Basic information for determination of miRNA biological function and regulation can be provided by their expression patterns [18]. Among several common techniques for miRNA detection, microarrays are highly efficient for profiling numerous miRNAs [45]. However, it still needs further confirmation to obtain more accurate expression for quantification [7]. Due to the low abundance and small size of miRNAs, current microarray-based strategies are focusing on improvement of the probe design and miRNA labeling [46].

In several early reports, DNA microarrays detections of miRNAs have been introduced [47, 48]. However, the difficulty to obtain Tm-normalized probe sets for genome-wide expression profiling is the central defect of all DNA-based oligonucleotide array platforms. For the purpose of solving the issue, an LNA modified capture probe has been developed in an miRNA microarray, which allows Tm normalization of array capture probes [49]. In addition, LNA incorporation can not only improve mismatch discrimination, but also moderate the requirement for purification and amplification of miRNAs [49]. Notably, it represents low toxicity and high stability in biological systems [18]. In order to balance melting temperatures, application of 2′-O-(2-methoxyethyl)-modified oligoribonucleotides [50] and adjusting the length of probes according to physicochemical characteristics of the target miRNA [51] have also been used for this process [52]. By incorporating a hairpin structure onto the 5′ end of the probe, distinguishing the targeted miRNA between larger RNAs like miRNA precursors can be achieved [51]. Moreover, introduction of exogenous and endogenous positive controls and negative control probes which can help normalization and establishment of absolute reference points for quality control and quantitative comparison of different microarrays are also important [52].

In 2003, an oligonucleotide array which could detect miRNAs by labeling low molecular weight RNA with radioactive isotopes was designed [53], quickly followed by other labeling technologies [54, 55]. Among them, the most common method for parallel analysis of numerous miRNAs is the fluorescent labeling of miRNA in a biological sample for succeeding hybridization to capture probes on the array [24]. Direct labeling of miRNA molecules might be beneficial due to the extremely small size of miRNAs.

Another labeling technology, enzymatic labeling, can be mainly divided into two approaches. One of them uses T4 RNA ligase to catalyze the ligation of a fluorophore-conjugated nucleotide or short oligonucleotide to the 3′ end of miRNA. By this way, the problem of circularization can be avoided, but a large and variable number of nucleotides may be added during tailing [24]. In both the cases, the enzyme prefers certain sequences to others, so creates different artifacts. Making matters worse, such biases might be more serious in degraded samples [36, 56]. In order to minimize the disturbance of structure and sequence differences among miRNAs, dimethylsulfoxide(as an effective RNA denaturant) was introduced into the reaction solution. It was discovered that up to 20 % dimethylsulfoxide can have impact on the activity of T4 RNA ligase [51]. Failure to detect 5′ end mismatches due to the 3′ activity of the enzymes is another shortcoming of enzyme-based labeling methods. Furthermore, there is also uncertainty for enzymatic labeling to detect miRNAs with natural modifications at 3′ ends such as plant miRNAs [20].

Chemical alkylation-based labeling along the miRNA and approaches based on platinum coordination chemistry with nucleic acids are alternative chemical approaches of miRNA labeling. [24]. However, insensitivity to the 3′ end modifications and the possibility of introducing bias by selectively labeling certain nucleotides with higher efficiency than others are the main disadvantages of chemical labeling approaches [8].

There are some common problems in both enzymatic and chemical approaches where background signal as well as cross-hybridization may be caused if coexisting pre-miRNAs are labeled [20]. This problem may be solved by initial size fractionation of small RNA using column- or gel-purification-based methods [24]. In addition, for removing dye labeling bias and differences in hybridization and scanning, background correction and normalization must be performed [57].

Label-based commercial miRNA microarrays are mainstream [58] and have been reported to have poor performances in interplatform concordance [56], due to the defects associated with labeling procedure [45]. Some problems have been solved by Nelson et al. via posthybridization labeling [55]. However, the usage of two different enzymes is still a problem.

The miRNA array detection method, which is free from labeling and amplification reactions, can clearly make the process simple and greatly bolster the credibility of miRNA-profiling studies, especially for diagnostic purposes. In the past decade, novel efforts have been continuously made to develop nonlabeling methods for direct miRNA detection [59, 60]. These works mainly focused on labeling a secondary probe rather than miRNA target and making use of special equipment.

“Stacking hybridization,” which can combine target capturing and fluorescent signaling in one step without labeling the targets, has also been introduced into a label-free miRNA microarray. With a short fluorophore-linked oligonucleotide universal tag, which can be selectively captured by the target bound probes via base-stacking effects, the total RNA is directly applied to the microarray (Fig. 3.6). It has been found that this assay is a useful way for unbiased profiling of both normal and methylated small RNA species [61] by analyzing as little as 100 ng total RNA and was highly specific to conspecific miRNAs [20].

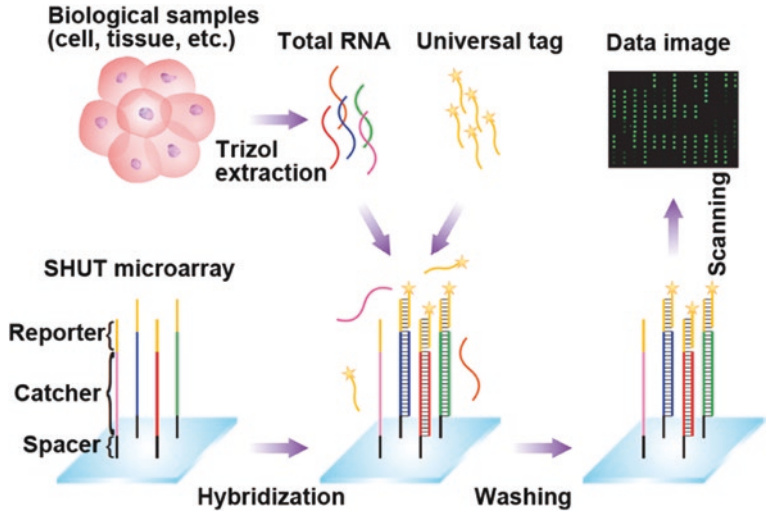

Fig. 3.6 Schematic of the stacking-hybridized universal tag (SHUT) assay. Reprinted with permission from Ref. [61], Copyright 2012 American Chemical Society

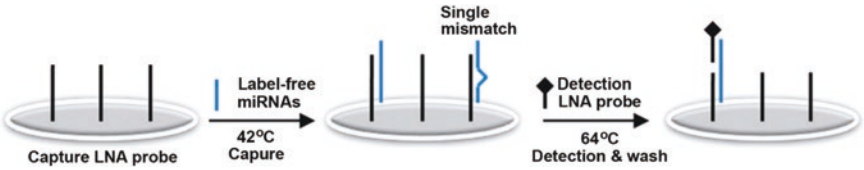

Fig. 3.7 Representation of miRNA array detection by two short, LNA-modified probes in a two-temperature hybridization procedure. Reproduced from Ref. [46] by permission of John Wiley & Sons Ltd

A two-temperature hybridization procedure, 42 °C for the capture step and 64 °C for the detection step, has been applied for miRNA profiling to further eliminate cross-hybridization between genomewide miRNAs and short probes (Fig. 3.7). In this assay, label-free miRNAs are captured between two short probes (~10 nt). This method can not only achieve detection at attomolar concentrations, but also can be improved by combination with bead-based or microfluidic platforms [46].

In summary, low cost and allowance of numerous parallel measurements are the main strengths of miRNA microarrays. For now, many companies offer microarray platforms for miRNA profiling [36]. There are still some limitations existing such as a restricted linear range of quantification, imperfect specificity in some cases for miRNAs that are closely related in sequence, failure to compare results between different techniques, and inability to perform absolute quantification of miRNA abundance easily.

3.4 Cloning

Cloning, one of the techniques first used to detect and discover miRNAs [62, 63], is still occasionally used for miRNA detection. Conventional cloning has its shortcomings. Cloning is extremely labor intensive, requires lots of RNA, and only provides information about the presence or absence of a particular miRNA in a sample. Based on these shortcomings of the technique, cloning is impractical for high throughput miRNA detection and expression profiling [64]. Recently, the discovery of miRAGE(miRNA serial analysis of gene expression) has been realized [65].

Small RNAs are extracted and amplified by RT-PCR into cDNA, which is similar to cloning. In this application, biotinylated primers are used in the PCR step and allow the cDNA products to be purified by affinity chromatography with streptavidin-coated beads. The cDNAs are enzymatically cleaved from the beads and the washed products can be cloned and sequenced. miRAGE is beneficial because it can identify up to 35 tags in a single iteration, compared to five when using conventional cloning [65].

References

1. Ruby JG, Jan C, Player C, Axtell MJ, Lee W, Nusbaum C, Ge H, Bartel DP (2006) Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in C-elegans. Cell 127:1193–1207
2. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T (2001) Identification of novel genes coding for small expressed RNAs. Science 294:853–858
3. Berezikov E, Cuppen E, Plasterk RHA (2006) Approaches to microRNA discovery. Nat Genet 38:S2–S7
4. Wu YQ, Chen DJ, He HB, Chen DS, Chen LL, Chen HC, Liu ZF (2012) Pseudorabies virus infected porcine epithelial cell line generates a diverse set of host MicroRNAs and a special cluster of viral microRNAs. PloS One 7
5. Koscianska E, Starega-Roslan J, Sznajder LJ, Olejniczak M, Galka-Marciniak P, Krzyzosiak WJ (2011) Northern blotting analysis of microRNAs, their precursors and RNA interference triggers. Bmc Mol Biol 12
6. Degliangeli F, Pompa PP, Fiammengo R (2014) Nanotechnology-based strategies for the detection and quantification of microRNA. Chem Eur J 20:9476–9492
7. Dangwal S, Bang C, Thum T (2012) Novel techniques and targets in cardiovascular micro-RNA research. Cardiovasc Res 93:545–554
8. Wark AW, Lee HJ, Corn RM (2008) Multiplexed detection methods for profiling microRNA expression in biological samples. Angew Chem Int Ed 47:644–652
9. Tang X, Gal J, Zhuang X, Wang W, Zhu H, Tang G (2007) A simple array platform for microRNA analysis and its application in mouse tissues. Rna-a Publ Rna Soc 13:1803–1822
10. Rarnkissoon SH, Mainwaring LA, Sloand EM, Young NS, Kajigaya S (2006) Nonisotopic detection of microRNA using digoxigenin labeled RNA probes. Mol Cell Probes 20:1–4
11. Pall GS, Codony-Servat C, Byrne J, Ritchie L, Hamilton A (2007) Carbodiimide-mediated cross-linking of RNA to nylon membranes improves the detection of siRNA, miRNA and piRNA by northern blot. Nucleic Acids Res 35
12. Varallyay E, Burgyan J, Havelda Z (2008) MicroRNA detection by northern blotting using locked nucleic acid probes. Nat Protoc 3:190–196

13. Pall GS, Hamilton AJ (2008) Improved northern blot method for enhanced detection of small RNA. Nat Protoc 3:1077–1084
14. Varallyay E, Burgyan J, Havelda Z (2007) Detection of microRNAs by Northern blot analyses using LNA probes. Methods 43:140–145
15. Valoczi A, Hornyik C, Varga N, Burgyan J, Kauppinen S, Havelda Z (2004) Sensitive and specific detection of microRNAs by northern blot analysis using LNA-modified oligonucleotide probes. Nucleic Acids Res 32:556
16. Kim SW, Li Z, Moore PS, Monaghan AP, Chang Y, Nichols M, John B (2010) A sensitive non-radioactive northern blot method to detect small RNAs. Nucleic Acids Res 38
17. Yang H, Schmuke JJ, Flagg LM, Roberts JK, Allen EM, Ivashuta S, Gilbertson LA, Armstrong TA, Christian AT (2009) A novel real-time polymerase chain reaction method for high throughput quantification of small regulatory RNAs. Plant Biotechnol J 7:621–630
18. Stenvang J, Silahtaroglu AN, Lindow M, Elmen J, Kauppinen S (2008) The utility of LNA in microRNA-based cancer diagnostics and therapeutics. Semin Cancer Biol 18:89–102
19. Kore AR, Hodeib M, Hu Z (2008) Chemical synthesis of LNA-mCTP and its application for microRNA detection. Nucleosides Nucleotides Nucleic Acids 27:1–17
20. Duan D, Zheng KX, Shen Y, Cao R, Jiang L, Lu Z, Yan X, Li J (2011) Label-free high-throughput microRNA expression profiling from total RNA. Nucleic Acids Res 39
21. Jinek M, Doudna JA (2009) A three-dimensional view of the molecular machinery of RNA interference. Nature 457:405–412
22. Murphy J, Bustin SA (2009) Reliability of real-time reverse-transcription PCR in clinical diagnostics: gold standard or substandard? Expert Rev Mol Diagn 9:187–197
23. Gaur A, Jewell DA, Liang Y, Ridzon D, Moore JH, Chen C, Ambros VR, Israel MA (2007) Characterization of microRNA expression levels and their biological correlates in human cancer cell lines. Cancer Res 67:2456–2468
24. Pritchard CC, Cheng HH, Tewari M (2012) MicroRNA profiling: approaches and considerations. Nat Rev Genet 13:358–369
25. van Rooij E (2011) The art of microRNA research. Circ Res 108:219–234
26. Ach RA, Wang H, Curry B (2008) Measuring microRNAs: comparisons of microarray and quantitative PCR measurements, and of different total RNA prep methods. Bmc Biotechnol 8
27. Reichenstein I, Aizenberg N, Goshen M, Bentwich Z, Avni YS (2010) A novel qPCR assay for viral encoded rnicroRNAs. J Virol Methods 163:323–328
28. Kumar P, Johnston BH, Kazakov SA (2011) miR-ID: a novel, circularization-based platform for detection of microRNAs. Rna-a Publ Rna Soc 17:365–380
29. Raymond CK, Roberts BS, Garrett-Engele P, Lim LP, Johnson JM (2005) Simple, quantitative primer-extension PCR assay for direct monitoring of microRNAs and short-interfering RNAs. Rna-a Publ Rna Soc 11:1737–1744
30. Chen CF, Ridzon DA, Broomer AJ, Zhou ZH, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, Lao KQ, Livak KJ, Guegler KJ (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 33
31. Wu ZS, Yang S, Sun Y, Parvez K, Feng X, Mullen K (2012) 3D nitrogen-doped graphene aerogel-supported Fe3O4 nanoparticles as efficient electrocatalysts for the oxygen reduction reaction. J Am Chem Soc 134:9082–9085
32. Wan G, Lim QE, Too H-P (2010) High-performance quantification of mature microRNAs by real-time RT-PCR using deoxyuridine-incorporated oligonucleotides and hemi-nested primers. Rna-a Publ Rna Soc 16:1436–1445
33. Wang X (2009) A PCR-based platform for microRNA expression profiling studies. Rna-a Publ Rna Soc 15:716–723
34. Sharbati-Tehrani S, Kutz-Lohroff B, Bergbauer R, Scholven J, Einspanier R, miR-Q (2008) A novel quantitative RT-PCR approach for the expression profiling of small RNA molecules such as miRNAs in a complex sample. Bmc Mol Biol 9
35. Schamberger A, Orban TI (2014) 3' IsomiR species and DNA contamination influence reliable quantification of microRNAs by stem-loop quantitative PCR. PloS One 9
36. Baker M (2010) MicroRNA profiling: separating signal from noise. Nat Methods 7:687–692

37. Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F, Speleman F, Vandesompele J (2009) A novel and universal method for microRNA RT-qPCR data normalization. Genome Biol 10
38. Ro S, Park C, Jin J, Sanders KM, Yan W (2006) A PCR-based method for detection and quantification of small RNAs. Biochem Biophys Res Commun 351:756–763
39. Hastings ML, Palma J, Duelli DM (2012) Sensitive PCR-based quantitation of cell-free circulating microRNAs. Methods 58:144–150
40. Kim Y-K, Yeo J, Kim B, Ha M, Kim VN (2012) Short structured RNAs with low GC content are selectively lost during extraction from a small number of cells. Mol Cell 46:893–895
41. Arefian E, Kiani J, Soleimani M, Shariati SAM, Aghaee-Bakhtiari SH, Atashi A, Gheisari Y, Ahmadbeigi N, Banaei-Moghaddam AM, Naderi M, Namvarasl N, Good L, Faridani OR (2011) Analysis of microRNA signatures using size-coded ligation-mediated PCR. Nucleic Acids Res 39
42. Lu Z, Duan D, Cao R, Zhang L, Zheng K, Li J (2011) A reverse transcription-free real-time PCR assay for rapid miRNAs quantification based on effects of base stacking. Chem Commun 47:7452–7454
43. Moltzahn F, Olshen AB, Baehner L, Peek A, Fong L, Stoeppler H, Simko J, Hilton JF, Carroll P, Blelloch R (2011) Microfluidic-based multiplex qRT-PCR identifies diagnostic and prognostic microRNA signatures in the sera of prostate cancer patients. Cancer Res 71:550–560
44. Zhang J, Li Z, Wang H, Wang Y, Jia H, Yan J (2011) Ultrasensitive quantification of mature microRNAs by real-time PCR based on ligation of a ribonucleotide-modified DNA probe. Chem Commun 47:9465–9467
45. Li W, Ruan K (2009) MicroRNA detection by microarray. Anal Bioanal Chem 394:1117–1124
46. Lee JM, Jung Y (2011) Two-temperature hybridization for microarray detection of label-free microRNAs with attomole detection and superior specificity. Angew Chem Int Ed 50:12487–12490
47. Liu CG, Calin GA, Meloon B, Gamliel N, Sevignani C, Ferracin M, Dumitru CD, Shimizu M, Zupo S, Dono M, Alder H, Bullrich F, Negrini M, Croce CM (2004) An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. Proc Natl Acad Sci 101:9740–9744
48. Thomson JM, Parker J, Perou CM, Hammond SM (2004) A custom microarray platform for analysis of microRNA gene expression. Nat Methods 1:47–53
49. Castoldi M, Schmidt S, Benes V, Noerholm M, Kulozik AE, Hentze MW, Muckenthaler MU (2006) A sensitive array for microRNA expression profiling (miChip) based on locked nucleic acids (LNA). Rna-a Publ Rna Soc 12:913–920
50. Beuvink I, Kolb FA, Budach W, Garnier A, Lange J, Natt F, Dengler U, Hall J, Filipowicz W, Weiler J (2007) A novel microarray approach reveals new tissue-specific signatures of known and predicted mammalian microRNAs. Nucleic Acids Res 35
51. Wang H, Ach RA, Curry B (2007) Direct and sensitive miRNA profiling from low-input total RNA. Rna-a Publ Rna Soc 13:151–159
52. Yin JQ, Zhao RC, Morris KV (2008) Profiling microRNA expression with microarrays. Trends Biotechnol 26:70–76
53. Krichevsky AM, King KS, Donahue CP, Khrapko K, Kosik KS (2003) A microRNA array reveals extensive regulation of microRNAs during brain development. Rna-a Publ Rna Soc 9:1274–1281
54. Neely LA, Patel S, Garver J, Gallo M, Hackett M, McLaughlin S, Nadel M, Harris J, Gullans S, Rooke J (2006) A single-molecule method for the quantitation of microRNA gene expression. Nat Methods 3:41–46
55. Nelson PT, Baldwin DA, Scearce LM, Oberholtzer JC, Tobias JW, Mourelatos Z (2004) Microarray-based, high-throughput gene expression profiling of microRNAs. Nat Methods 1:155–161

56. Sato F, Tsuchiya S, Terasawa K, Tsujimoto G (2009) Intra-platform repeatability and inter-platform comparability of microRNA microarray technology. PloS One 4
57. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G (2000) The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 403:901–906
58. Git A, Dvinge H, Salmon-Divon M, Osborne M, Kutter C, Hadfield J, Bertone P, Caldas C (2010) Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. Rna-a Publ Rna Soc 16:991–1006
59. Qavi AJ, Bailey RC (2010) Multiplexed detection and label-free quantitation of microRNAs using arrays of silicon photonic microring resonators. Angew Chem Int Ed 49:4608–4611
60. Lee JM, Cho H, Jung Y (2010) Fabrication of a structure-specific rna binder for array detection of label-free microRNA. Angew Chem Int Ed 49:8662–8665
61. Shen Y, Zheng K-X, Duan D, Jiang L, Li J (2012) Label-free microrna profiling not biased by 3 ‘ end 2 ‘-O-Methylation. Anal Chem 84:6361–6365
62. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T (2002) Identification of tissue-specific microRNAs from mouse. Curr Biol 12:735–739
63. Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, Pfeffer S, Rice A, Kamphorst AO, Landthaler M, Lin C, Socci ND, Hermida L, Fulci V, Chiaretti S, Foa R, Schliwka J, Fuchs U, Novosel A, Mueller R-U, Schermer B, Bissels U, Inman J, Phan Q, Chien M, Weir DB, Choksi R, De Vita G, Frezzetti D, Trompeter H-I, Hornung V, Teng G, Hartmann G, Palkovits M, Di Lauro R, Wernet P, Macino G, Rogler CE, Nagle JW, Ju J, Papavasiliou FN, Benzing T, Lichter P, Tam W, Brownstein MJ, Bosio A, Borkhardt A, Russo JJ, Sander C, Zavolan M, Tuschl T (2007) A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 129:1401–1414
64. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O’Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M (2008) Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci 105:10513–10518
65. Cummins JM, He YP, Leary RJ, Pagliarini R, Diaz LA, Sjoblom T, Barad O, Bentwich Z, Szafranska AE, Labourier E, Raymond CK, Roberts BS, Juhl H, Kinzler KW, Vogelstein B, Velculescu VE (2006) The colorectal microRNAome. Proc Natl Acad Sci 103:3687–3692

Chapter 4
miRNA Electrochemical Detection

Abstract Electrochemical genosensors hold great promise for point-of-care diagnostics and multiplexed platforms for fast, simple, and inexpensive miRNA analysis. A typical electrochemical biosensor consists of an electroactive hybridization indicator and a solid electrode with an immobilized short single-stranded nucleotide probe [1]. Hybridization between the probe and the complementary sequence influences the electrochemical response signal, producing a signal for detection. The electrochemical miRNA detection methods are based on hybridization, which require translating the hybridization event into a measurable signal when hybridization occurs in the sequence. Sensitivity is challenging for the development of high performance electrochemical genosensors, due to the unique characterization of miRNA. In order to implement the detection of the specific target gene down to attomolar to femtomolar level presented in the organism genome, [2] various approaches are continuously being explored for signal amplification to improve the sensitivity.

Keywords Electrochemical genosensors · Nanoparticle-based signal amplification · Enzyme-assisted signal amplification

4.1 Nanomaterials-Based Electrochemical miRNA Detection

Nanoparticles have attracted considerable attention for producing a strong detectable signal in the analysis of trace target miRNA. This provides a promising sensing platform in nucleic acid detection. The unique optical, electronic, and catalytic properties of nanomaterials are useful for translation of biorecognition events into an electrochemical or spectroscopic response. The high surface areas can be used to improve mass transport, while high loading of receptor molecules are employed for synergistic amplification of the target response. Nanomaterials show excellent

Xueji Zhang, Fang Xu and Haifeng Dong contributed together to this chapter.

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conductivity to promote direct electron transfer between the biomolecules and electrode surface. Electrochemical miRNA detection employs the unique properties of nanomaterials to decrease the requirement for high amount of sample, time, and number of steps while increasing sensitivity, specificity, and simplicity [3, 4]. The combination of unique properties of nanoparticles with the advantage of an electrochemical method has evolved a series of miRNA detection strategies. According to the nanoscale element, we address the application of nanoparticles in miRNA detection in this part.

4.1.1 Notable Metal Nanoparticles

Gold nanoparticles (AuNPs) have attracted intensive interests in biosensors due to their excellent controllable size distribution, high stability, and compatibility with biomaterials such as nucleic acids and proteins [5]. Yang et al. [6] used AuNPs and silver enhancement to detect miRNA with a sensitive, specific, simple, and colorimetric technique. They used a sandwich model, which was comprised of two probes: an AuNPs probe hybridized to the complementary target miRNA and a biotinylated probe (capture probe). The hybridization complex is immobilized on the streptavidin-coated microplate surface. Then, silver enrichment is applied to amplify the signal of adsorbed AuNPs and is recorded with a microplate reader. Their reported LOD was 10 fM miRNA (2 ng of total RNA), so the method seemed to be applicable for detection of low amounts of the small RNA molecule [7].

In another work, application of AuNPs in a ferrocene capped AuNP/streptavidin complex has been reported for detection of glioma-related miRNA in glioma patients' samples [8]. In their study, they immobilize thiolated-OND probes on the surface of a gold electrode first, then the total miRNA sample was mixed with previously biotinylated competitor miRNA (which had the same sequence of target miRNA) and applied on the electrode. After hybridization of miRNA with immobilized probes, a ferrocene-capped AuNPs/streptavidin complex was added on these hybridized miRNAs. The complex was condensed with biotin molecules of competitive miRNA and ferrocene molecules of the complex produced considerable voltammetric signals. This signal was proportional to the amount of target miRNA molecules, and, with increasing target-miRNA concentration in the sample (due to the competition between target miRNA and competitor miRNA in hybridization to the immobilized probes) the voltammetric signal became weaker. In other words, in the presence of more miRNA targets, the capacity of probes becomes full, and competitor miRNAs cannot hybridize to probes and are removed after washing steps. Therefore, less of the ferrocene-capped AuNP/streptavidin complex is condensed with them, reducing the voltammetric signal [8].

Based on the three-step amplification, another chronoamperometry method was developed to detect the presence of target miRNA. The first step of amplification was achieved by applying a barcode nanostructure probe: comprising of a reporter locked nucleic-acid (LNA) probe and biotin-functionalized reporter DNAs conjugated on AuNPs. The second level of amplification was conducted through

hybridization of this barcode AuNP probe to the target miRNA captured by a hairpin probe, which was pre-immobilized on the surface of a glassy carbon electrode comprising a primary layer of graphene nanosheets and a secondary layer of dendritic gold nanostructure (DenAu). In fact, target miRNA was sandwiched between the hairpin and barcode nanostructure probes. In the final step of amplification, the positive signal was intensified more by reaction of streptavidin-conjugated HRP (streptavidin-HRP) with terminal biotin molecules of barcode DNA. Then, hydroquinone and H_2O_2 were added as substrates for HRP [9]. Finally, the chronoamperometric readout of this method was achieved by electrochemical reduction of the benzoquinone, which was produced in the previous steps.

Zhou et al. [10] designed a strategy based on LNA-modified DNA hairpin probes carrying a 5′-end biotin immobilized on AuNPs and deposited on the electrode surface. Hybridization of miRNA-21 unfolds the hairpins exposing the biotin tag, which can then bind to streptavidin–HRP conjugates. Therefore, it can translate miRNA hybridization into a redox reaction at the electrode (the HRP-catalyzed oxidation of hydroquinone), the extent of which is followed by amperometry and allows miRNA quantification (LoD $\sim$ 0.4 pm).

The same HRP-based transduction mechanism was used by Yin et al. [11]. Compared to the previously described assay, this approach is modified to largely increase the number of biotin tags (and, hence, signal-generating HRP conjugates) from each miRNA hybridization event. This strategy gave improved sensitivity with an estimated LoD of 6 fm miRNA. In the other two sensors, immobilization of HRP–streptavidin conjugates is replaced by target miRNA hybridization-driven unmasking of DNA sequences able to fold into G-quadruplexes. Addition of hemin results in supramolecular complexes with peroxidase-like catalytic activity allowing miRNA quantification. Although similar in design, these two approaches considerably differ in terms of sensitivity with LoD of about 6 pm [12] and approximately 6 fm [13] respectively.

Boronic acids become attractive ligands for many sensing applications. Separation and self-assembly is possible since they can covalently react with cis-diols to form five- or six-membered cyclic esters. For this view, Liu and co-workers described two electrochemical sensors for miRNA-21 using boronic acid functionalized AuNPs on an electrode surface as transducers of miRNA hybridization.

In their first strategy [14], they proposed a label-free and sensitive method for the detection of miRNAs based on the formation of boronate ester covalent bonds and the dual-amplification of gold nanoparticles (AuNPs). DNA capture probes immobilized on the electrode surface hybridize to target miRNA giving DNA–miRNA heteroduplexes, which can bind to boronic acid functionalized AuNPs. Subsequently, dopamine (also a molecule with vicinal hydroxyls) functionalized AuNPs can be immobilized on the primary AuNPs at the electrode surface again through boronate ester formation. The electrochemically active dopamine then allows generation of a DPV signal proportional to miRNA concentration during the initial hybridization step. As a result, their reported detection limit was 50 fM (Fig. 4.1).

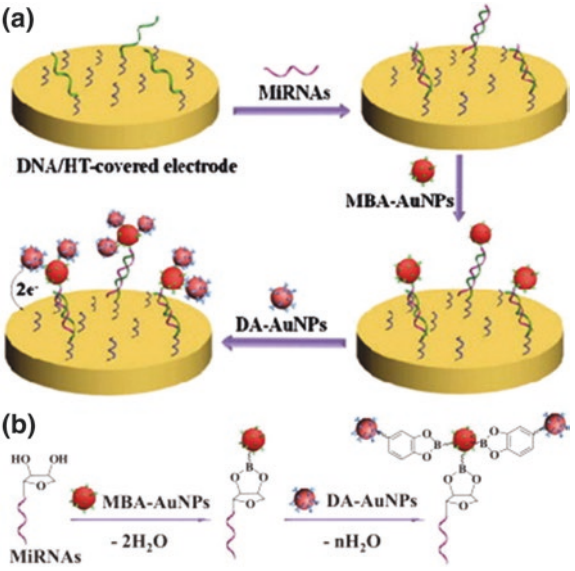

Fig. 4.1 Schematic representation showing the capture of miRNAs by the immobilized DNA probes and the detection of miRNAs by the attachment of MBA-AuNPs and DA-AuNPs (**a**) and the formation of boronate ester bonds between MBA-AuNPs and miRNAs as well as DA-AuNPs (**b**). Reprinted from Ref. [14], Copyright 2013, with permission from Elsevier

In order to improve the detection sensitivity, in their second strategy [15], they replaced the phenyl boronic acid functionalized AuNPs with multifunctional AuNPs carrying biotin as well as phenyl boronic acid moieties on their surface. This replacement allows maintaining the miRNA recognition step (formation of boronate ester bonds) while the biotin tags are used to immobilize streptavidin-conjugated alkaline phosphatase (SA–ALP). After the addition of the 4-aminophenyl phosphate (p-APP) substrate, the enzymatic conversion from p-APP to p-AP occurred in the process. The resulting p-AP could be cycled by a chemical reducing reagent after its electro-oxidization on the electrode (known as p-AP redox cycling) [16], thus enabling an increase in the anodic current of p-AP. The results indicate that the detection limit was 3 fM and the current increased linearly with the miRNA concentration over a range of 10 fM–5 pM. The performance of these systems on samples of biological origin has not been reported.

Wu et al. [17] described a sensor for miRNA-155 employing palladium nanoparticles for immobilization of RNA probes and for amperometric signal enhancement. This assay reached a LoD of approximately 40 amol (~2 pm miRNA in 20 mL sample used for hybridization).

A major limitation of these strategies is the need for tagging miRNA with nanoparticles, which drastically increases sample manipulation before analysis. In order to overcome the difficulty, Zhang and co-workers described an electrochemical strategy with sensitivity (~0.3 zmol) [18]. Their label-free miRNA sensing strategy makes use of oligonucleotide encapsulated AgNCs to convert the hybridization between miRNA and hairpin-structured DNA capture probes into an electric current. No data has been reported about performance of the assay on samples of biological origin.

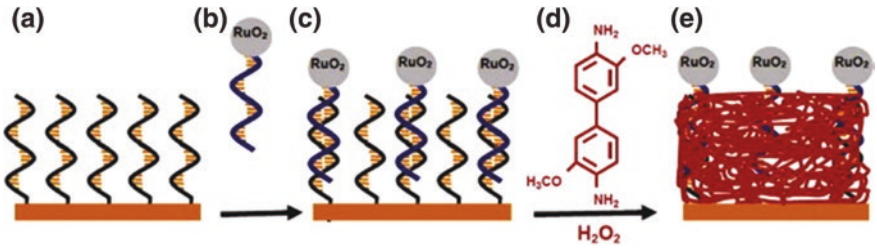

Fig. 4.2 Schematic illustration of the miRNA biosensor based on RuO_2 NP-catalyzed miRNA templated deposition of a thin PBD insulating film. Reprinted with the permission from Ref. [19], Copyright 2011 American Chemical Society

4.1.2 Metal Oxide Nanoparticles

Nucleic acid can guide the deposition of polymer where the phosphate groups serve as templates. Recently, Gao's group developed a simple and sensitive electrical miRNA gene sensor based on the miRNA-guided deposition of insulating poly(3,3′-dimethoxybenzidine) (PDB) polymer film with ruthenium oxide nanoparticles (RuO_2NPs) polymerization method on gold electrodes covered with the mixed monolayers of DNA capture probes (CPs) and 4-mercaptoaniline (MAn) [19]. In the work, RuO_2NPs were coated with 4-(2-aminoethyl) pyridine (AEP), which allows for the label of the oxidized miRNA through the formation of an imine bond between dialdehyde and AEP. In the system, the captured miRNA and the tagged RuO_2NPs act as the templates and catalysts, respectively. Consequently, hybridization with RuO_2-tagged miRNA and incubation in a mixture of 3,3′-dimethoxybenzidine (DB)/H_2O_2 led to the formation of an insulating PDB film and the increase in the electrochemical impedance. The amount and insulating capability of the deposited PDB correlated to the miRNA concentration in the range of 6 fM–2 pM. After incubating the sensing electrode in the mixed DB/H_2O_2 solution for 60 min, a detection limit of 3 fM was obtained by electrochemical impedance spectroscopy (Fig. 4.2).

Ruthenium oxide (RuO_2) nanomaterials have found various applications in nanotechnology. Chemically, RuO_2 is a highly conductive oxide and resistant material toward harsh conditions with strong catalytic activity. These properties make RuO_2 a good candidate for application in electrochemical sensing [20, 21]. As an application in electrochemical detection, Peng et al. [4] reported application of RuO_2NPs in electrochemical detection of target miRNAs. In this study, they conjugated target miRNA molecules with RuO_2 NPs and, after hybridization with a pre-immobilized probe on a gold-film electrode, an H_2O_2/aniline mix solution was added. Finally, formation of polyaniline on miRNA-probe double strands by reaction with RuO_2 NP-assisted H_2O_2 produced a voltammetric current peak. The principle of this method is outlined in Fig. 4.3.

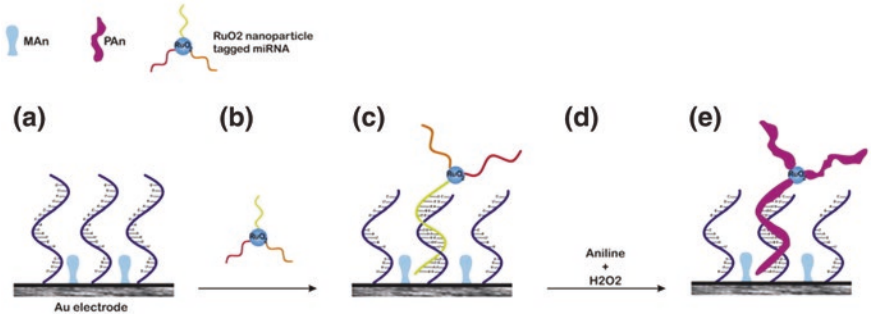

Fig. 4.3 RuO_2 nanoparticle-assisted electrochemical detection of miRNAs Reproduced from Ref. [4] by permission of The Royal Society of Chemistry (RSC) on behalf of the Centre National de la Recherche Scientifique (CNRS) and the RSC

4.1.3 Quantum Dots

Quantum dots (QDs) are defined as one of the most favorable nanomaterials in nucleic-acid and single-molecule detection [22], due to its high quantum yield. Wang et al. [23] demonstrated a hybrid procedure involving a molecular beacon (MB)-based structure tagged with QDs. In this structure, QDs were used as the electroactive labels in miRNA detection. Wang et al. reported that their LOD was about 0.32 aM.

Electrochemical QDs are known to not only have excellent signal amplification feature, but can also display unique voltammetric signals depending on the compositions of QDs. Therefore, electrochemical QDs are good candidates as barcodes for sensitive detection of oligonucleotides [23]. Zhu et al. combined the high base-mismatch selectivity of ligase chain reaction (LCR) and the remarkable voltammetric signature of electrochemical QDs barcodes to report a label-free and PCR-free electrochemical method for multiplexed evaluation of miRNA in a single-tube experiment. They labeled reporting probes of RP1 and RP2 with PbS and CdS QDs to prepare PbS–RP1 and CdS–RP2 conjugates, and two capture probes of CP1 and CP2 were co-immobilized on magnetic beads (MBs) to fabricate MB–CP1CP2 conjugate. The miRNA samples were simply incubated with MB–CP1CP2, PbS–RP1, and CdS–RP2 conjugates and then added with T4 DNA ligase. After release of the disjoined QDs barcodes from the MB conjugates, two target miRNAs of miRNA-155 and miRNA-27b were simultaneously detected by square wave voltammetry with linear ranges of 50 fM–30 pM, and 50 fM–1050 pM and limits of detection (LODs) of 12 fM and 31 fM (S/N = 3), respectively. The method fulfilled the assay in less than 70 min, and showed acceptable testing recoveries for the determination of miRNAs in the biological matrix. Currently, there are rare reports of electrochemical multiplexed quantification of miRNA. The method is likely to provide a new platform for identification of multiple miRNA in a simple manner [24] (Fig. 4.4).

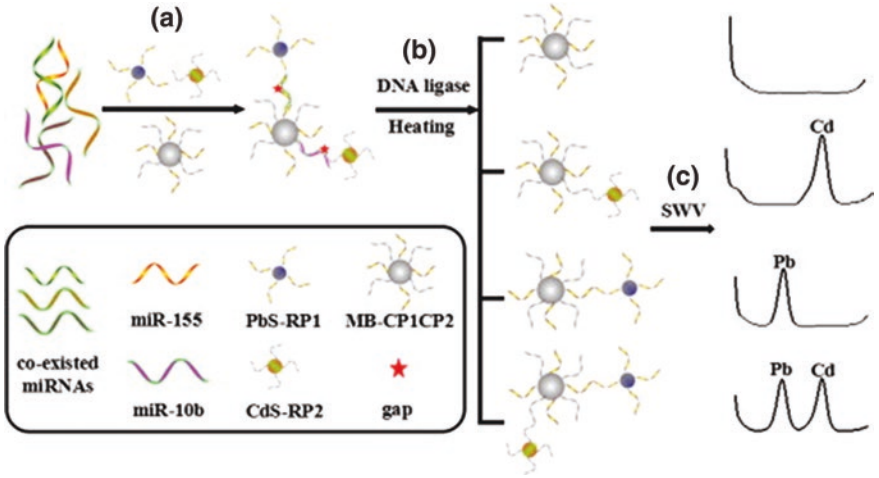

Fig. 4.4 Schematic illustration of the simultaneous electrochemical detection of multiple miRNA targets. Reprinted from Ref. [24], Copyright 2014, with permission from Elsevier

4.1.4 Carbon Nanomaterials

Sensitivity is challenging for the development of high performance electrochemical genosensors, due to the unique characterization of miRNA. In order to enhance sensitivity, carbon nanotubes (CNTs) are frequently reported [25] to increase the electroactive area and decrease the electrical resistance of the working electrodes leading to 3D conductive materials [26–28]. The electrodes are nanosized unique tubular structures with high length/diameter ratio, in addition to attractive electrical, chemical, mechanical, and structural properties. The metallic, semiconducting and superconducting electron-transport abilities of CNTs and their large capacity to be loaded with various biomolecules make them excellent candidates in electrochemical biosensing. In this way, they were used as an interpenetrated network to detect target miRNA through a ‘‘signal-on’’ method [29]. The method takes advantage of embedding the quinine group in the polymeric backbone of poly-[5-hydroxy-1,4-naphthoquinone]-copolymer-[3-(5-hydroxy-1,4-dioxo-1,4-dihydronaphthalen-2(3)-yl) propanoic acid], poly(JUG-coJUGA), in order to achieve higher electroactivity of the conducting polymer followed by deposition of this modified polymer on the multi-walled CNTs (MWCNTs) and subsequent application of this interpenetrated network in the sensing of target miRNA [29].

CNTs have also been widely used in reagentless formats [30–32]. In this paper, they described a label-free and reagentless miRNA sensor based on an interpenetrated network of CNTs and electroactive polymer. The nanostructured polymer film presents well-defined electroactivity in neutral aqueous medium; in the cathodic potential domain from the quinone group embedded in the polymer backbone. Addition of miRNA-141 target (prostate cancer biomarker) gives a “signal-on”

response, i.e., a current increase due to enhancement of the polymer electroactivity. By contrast, noncomplementary miRNA, such as miRNA-103 [33] and miRNA-29b-1 [34], do not lead to any significant current change. A very low detection limit of 8 fM is achieved with this sensor.

They built a sensitive and selective sensor based on a carbon nanotubes field-effect transistor (CNTs-FET); with the surface functionalized with protein p19, which binds with nanomolar affinity to 21–23 base-pair long double-stranded RNAs (dsRNA) through a combination of electrostatic and hydrogen bonding interactions with the sugar–phosphate backbone. In this strategy, the RNA capture probes complementary to miRNA do not need to be immobilized on the electrode surface but can simply be mixed with the sample solution just before analysis, alleviating the stability concern. A record sensitivity corresponding to the detection of 0.01 zmol miRNA (1 am in 10 mL sample solution) was reached using this strategy. No data has been reported about the performance of this sensor on samples of biological origin.

4.1.5 Other Nanomaterials

Polyaniline nanowire (PAn-NWs) have many applications in electrochemical-based sensing. Its main characteristic is that, under certain conditions, they can be grown in the form of an interwoven NW network [35–38]. The electrochemical conductivity of these NWs is due to the protonation of the constructing polymer. However, its conductivity depends on its electrochemical redox state, humidity, pH, the type of anions in the solution, and temperature. The conductivity of PAn is typically in the range 2–10 S/cm [35, 39]. This high conductivity has been exploited in research to detect miRNA targets. Based on this research, a simplified and sensitive electrical biosensor is proposed to directly detect target miRNA in a range from 10 fM to 20 pM [40]. Fan et al. [40] introduced a detection of miRNA using target-guided formation of conducting polymer nanowires in nanogaps. Hybridization and electrical detection of the sensing procedure was depicted in Fig. 4.9. In their study, peptide nucleic acid (PNA) was used as a capture probe while PAn-NWs were used as conducting polymer nanowires. The PNA was immobilized in nanogaps of a pair of interdigitated microelectrodes and hybridization was performed with their complementary target miRNA. Then, deposition of PAn-NWs was carried out by an enzymatically catalyzed method, where the electrostatic interaction between anionic phosphate groups in miRNA and cationic aniline molecules was exploited to guide the formation of the PAn-NWs onto the hybridized target miRNA. The conductance of the deposited PAn-NWs correlates directly to the amount of hybridized miRNA. This approach directly utilized chemical ligation and amplification for signal read-out and thus eliminated the use of labeling probes, which greatly simplifies the detection procedure. A lower detection limit could be achieved with longer target nucleic acids, because the

bridging of nanogaps by the PAn-NWs can be achieved with fewer long nucleic acid molecules. The dynamic range for the biosensor was from 10 fM to 20 pM with a detection limit of 5.0 fM.

Bartosik et al. [41] developed an electrochemical miRNA genosensor relying on magnetic bead-based DNA/miRNA hybridization and labeling of electroactive Os(VI)L. In this work, miRNA was labeled with Os(VI)bipy and hybridized with biotinylated DNA capture probe attached to the streptavidin magnetic beads. The labeled miRNA was then detected at a hanging mercury drop electrode at femtomolar level due to the electrocatalytic nature of the peak from the Os(VI)bipy labeled detection of miRNA (Fig. 4.5).

A concatamer-based hybridization chain reaction method was developed by Hong et al. [42]. In this method, a hairpin capture probe comprising of the sequence complementary to the target miRNA in the loop is immobilized onto the sensor surface. In the absence of target miRNA, the capture probe exists predominantly in hairpin form which prohibits the binding of two auxiliary probes complementary to the end section of the hairpin probe. However, in the presence of target miRNA, the stem loop structure of capture probe is unfolded and the DNA concatamers can hybridize with the terminus of a DNA capture probe. The long DNA structures on the surface can electrostatically adsorb a large amount of positively charged redox indicator $[Ru(NH_3)_6]^{3+}$ added into the solution. Differential pulse voltammetry (DPV) was used to detect the adsorbed redox indicator with the peak current proportional to the miRNA concentration in the sample. The proposed assay can detect as low as 100 aM target miRNA-21. Furthermore, fulfilling this technology allowed absolute quantification of miRNA-21 in just 8 mL human serum without RNA isolation and the results were validated by qRT-PCR [43].

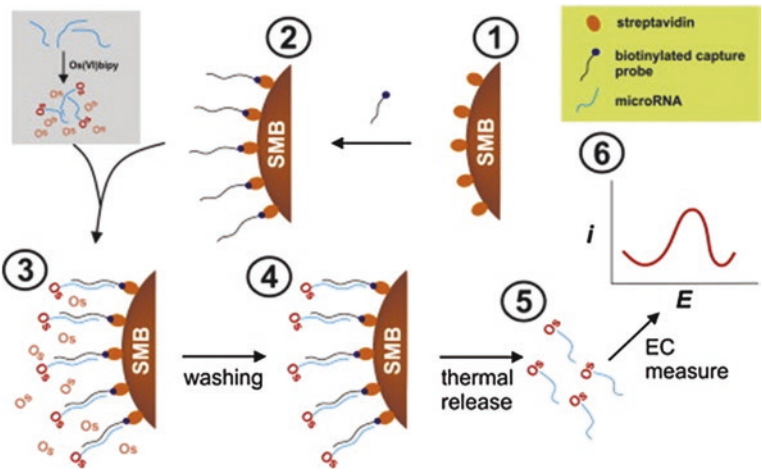

Fig. 4.5 Scheme of detection of miRNA with specific sequence using magnetic beads. Reprinted from Ref. [41], Copyright 2014, with permission from Elsevier

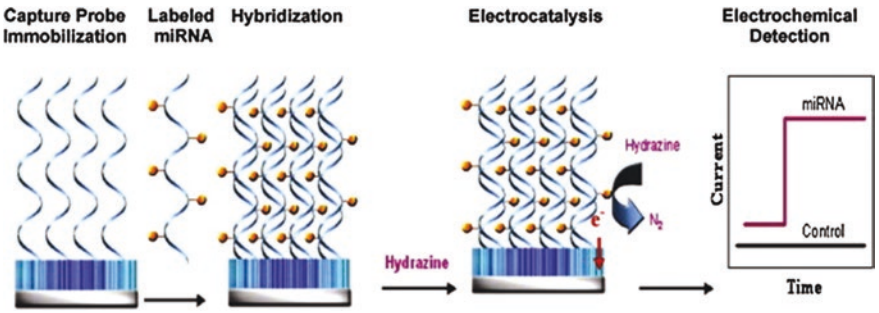

Fig. 4.6 Schematic representation of an miRNA assay using electrocatalytic label. Reprinted from Ref. [45], Copyright 2007, with permission from Elsevier

Zhang et al. [44] reported a label-free direct detection of miRNAs with Silicon nanowire (SiNWs) biosensors. They represented a label-free and direct hybridization assay for ultrasensitive detection of miRNA using a SiNWs device. Peptide nucleic acids (PNAs), which serve as a receptor to recognize miRNA directly without labeling the target miRNA, were immobilized on the surface of the SiNW device. Electrical measurements for the sensing experiments were carried out by detecting the resistance change in SiNWs before and after PNA–miRNA hybridization.

Gao and Yu [45] reported a direct labeling miRNA with an electrocatalytic moiety and its application in ultrasensitive miRNA assays. In this paper, miRNA is directly labeled with $Ru(PD)_2Cl_2$(PD = 1,10-phenanthroline-5,6-dione), through coordinative bonds with purine bases in the miRNA molecule. The electrode was placed in a moisture saturated environmental chamber maintained at 30 °C. A 2.5 μl aliquot of hybridization solution, containing the desired amount of labeled miRNA, was uniformly spread onto the electrode. It was then rinsed thoroughly with a blank hybridization solution at 30 °C after a 60-min hybridization period. The hydrazine electrooxidation current was measured amperometrically at 0.10 V in vigorously stirred PBS containing 5.0 mM hydrazine. At low miRNA concentrations, smoothing was applied after each amperometric measurement to remove random noise and electromagnetic interference. Amplification from the electrocatalytic oxidation of hydrazine greatly enhances the detectability of the approach (Fig. 4.6).

4.2 Enzyme-Assisted Electrochemical Signal Amplification

The introduction of enzymes into a nucleic acid biosensor leads to a 1:1 hybridization event associated with numerous enzymes catalytic response, which sharply amplify the detection signal and improve the sensitivity of the assay. Enzyme is used to develop signal amplification, target amplification, or probe amplification strategies to improve the LOD of the hybridization assays.

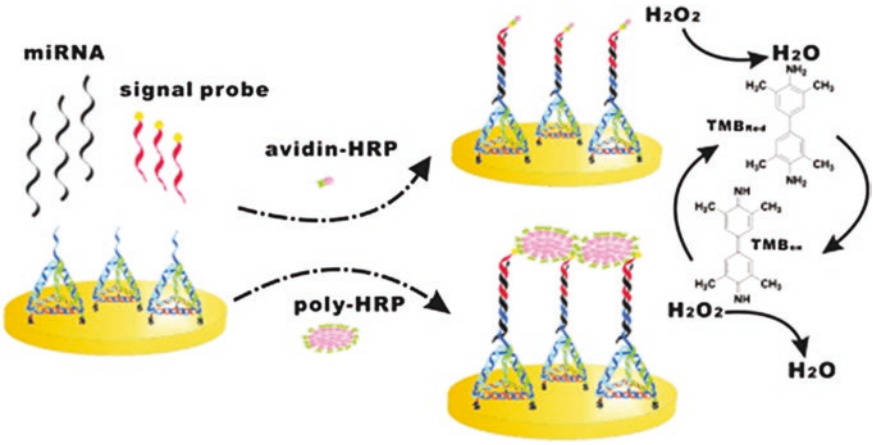

Fig. 4.7 miRNA detection with the tetrahedron-based electrochemical miRNA sensor using enzyme-based signal transduction (either avidin-HRP or high-activity poly-HRP) Reprinted with permission from Ref. [47], Copyright 2012, with permission from Elsevier

4.2.1 Horseradish Peroxidase (HRP)

HRP is the enzyme most used in bioanalysis. Yin et al. developed a highly sensitive miRNA biosensor using a molecular beacon and bio-barcode voltammetric assay with HRP reduction of benzoquinone as the reporter [46]. An LNA integrated molecular beacon probe was modified on graphene and dendritic gold nanostructure modified glassy carbon electrode (GCE). The miRNA binding to the molecular beacon probe releases the end of the beacon to bind a bio-barcode based on HRP reporter. Then, with specific interaction between biotin and streptavidin, the streptavidin–HRP can be immobilized on the electrode surface to catalyze the oxidation reaction of hydroquinone by H_2O_2 to form benzoquinone and enhance the electrochemical reduction signal of benzoquinone for detection [9].

Wen et al. reported an miRNA detection strategy that enables attomolar detection limits for target miRNAs and discriminates single-base mismatched strands by using a tetrahedral DNA nanostructure for optimum spatial control of the DNA probe immobilization and avidin-HRP or poly-HRP catalyzing H_2O_2 in the presence of 3,3′,5,5′-tetramethylbenzidine (TMB) as an electron-shuttle [47] (Fig. 4.7).

4.2.2 Alkaline Phosphatase (ALP)

ALP is the enzyme used in widespread bioanalysis investigations. Wang et al. [48] reported a photoelectrochemical (PEC) biosensor for sensitive and specific detection of mRNA based on Bi_2S_3 nanorods and ALP enzymatic signal amplification.

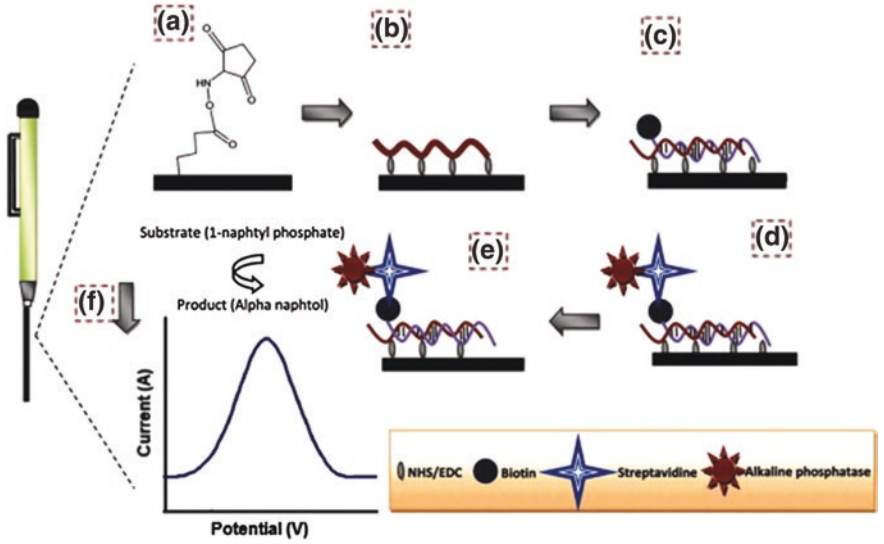

Fig. 4.8 Sketch of steps involved in **a** surface activation of PGE with NHS/EDC, **b** probe immobilization onto the surface of PGE, **c** hybridization of probe with biotinylated target, **d** Ex-Ap–biotin interaction, **e** enzyme substrate interaction and production of a-NAP and **f** voltammetric measurement of oxidation signal of a-NAPL. Reprinted from Ref. [50], Copyright 2012, with permission from Elsevier

Using the catalytic effect of ALP on L-ascorbic acid 2-phosphate trisodium salt (AAP), ascorbic acid (AA) was in situ generated and used as an electron donor. A signal-on protocol was successively achieved for miRNA detection due to the dependence of photocurrent response on the concentration of electron donor of AA. Under amplification of the immunogold-labeled streptavidin (SA-AuNPs), a low detection limit of 1.67 fM was obtained with this setup. Bettazzi et al. [49] reported an electrochemical detection of miRNA-222 by use of a magnetic bead-based bioassay and enzyme amplification. The proposed bioassay immobilized biotinylated DNA capture probes on streptavidin-coated paramagnetic beads. After incubating with biotinylated miRNA, the beads were hybridized with streptavidin–ALP and exposed to the appropriate enzymatic substrate to produce an electrochemical signal for detection. Notably, by using a compact microfluidic device, it enables multiplexed analysis of eight different samples with a detection limit of 7 pM.

Kilic et al. [50] reported an electrochemical-based detection of miRNA-21 in breast cancer cells. In their study, the proposed enzymatic detection method was detailed and compared with the conventional guanine oxidation-based assay in terms of detection limit and specificity; the oxidation signal of enzymatic reaction product (alpha naphthol, a-NAP), which is expected to be produced in the presence of hybrid, was detected by differential pulse voltammetry on a disposable PGE. They claimed that for the first time an enzyme-based biosensor was designed for detection of miRNA from cell lysates without any modification of the sample. As a positive control, total RNA isolated from a breast cancer cell line that

contains unregulated miRNA-21 was used for investigation. The specificity of the assay was proven by noncomplementary studies using miRNA-21-free total RNA samples. The proposed enzyme-based assay seems to provide more reproducible results for detection of miRNA than the conventional guanine oxidation-based method for cell lysates. Additionally, the method can unambiguously distinguish between miRNA-21; including samples and miRNA-21 free samples, while detection is impossible with the guanine-based method due to very low signal (Fig. 4.8).

4.2.3 Other Enzymes or Molecules

Recently, several papers have reported the use of enzymatic reaction for recognition of a hybridization event between probe and miRNA targets. In this way, Pohlmann and Sprinzl [51] proposed an electrochemical detection of miRNAs via gap hybridization assay. They introduced a method for detection of mature miRNAs based on four components DNA/RNA hybridization and electrochemical detection using esterase 2-oligodeoxynucleotide (EST2-ODN) conjugates. Due to complementary binding of miRNA to a gap built of capture and detector oligodeoxynucleotides, the reporter enzyme is brought to the vicinity of the electrode and enzymatically produces an electrochemical signal (Fig. 4.9).

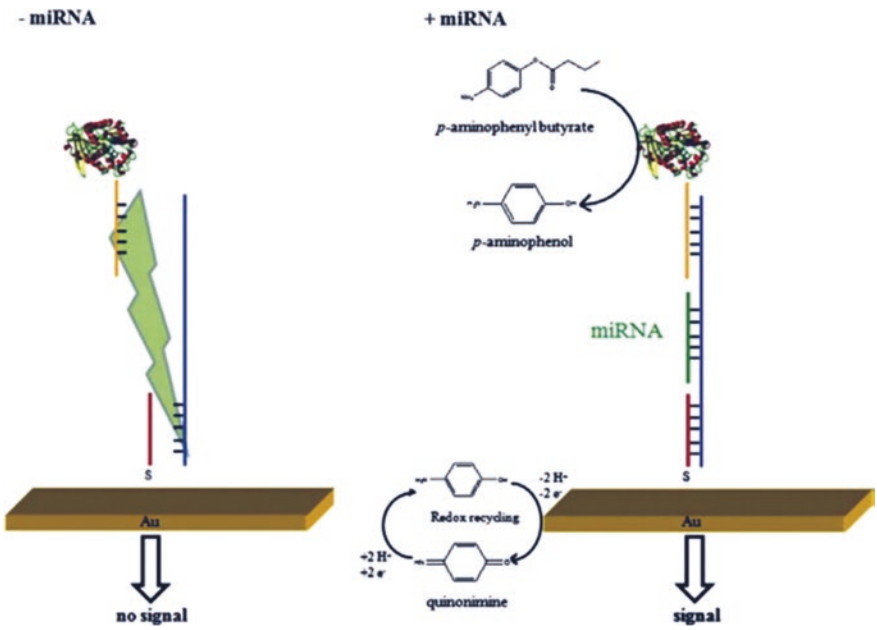

Fig. 4.9 Electrochemical detection of gap hybridization among immobilized capture ODN (*red*), miRNA (*green*), EST2-miR conjugate (*yellow*), and complementary RNA probe (*blue*). Reprinted with permission from Ref. [51], Copyright 2010 American Chemical Society

Zhou et al. proposed a simple, sensitive, and label-free method for miRNAs biosensing based on mimic enzyme catalysis signal amplification. They synthesized carboxylic graphene–hemin hybrid nanosheets and used them to catalyze the oxidation reaction of hydroquinone in the presence of H_2O_2, due to the intrinsic peroxidase-like activity of hemin on the carboxylic graphene surface. The electrochemical reduction current of the oxidative product of benzoquinone was dependent on the hybridization amount of miRNAs and used to monitor the miRNAs hybridization event. Under optimal detection conditions, the current response was proportional to the logarithm concentration of miRNA-159 from 0.5 pM to 1.0 nM with the detection limit of 0.17 pM (S/N = 3). The fabricated biosensor showed high reproducibility and detection selectivity [52].

An innovative concept for miRNA detection is based on the molecular recognition properties of the RNA-binding protein p19, which is from the Carnation Italian ringspot virus [53, 54]. The p19 homodimers bind with nanomolar affinity to 21–23 base-pair long double-stranded RNAs (dsRNA) through a combination of electrostatic and hydrogen bonding interactions with the sugar–phosphate backbone. Accordingly, binding to dsRNA is sequence independent and p19 can be used for recognition of miRNA hybridized to suitable RNA probes. In this study, an electrochemical biosensor for detection of miRNA-21 using the oxidation signal of protein 19 (p19) as a molecular caliper was designed. For the first time based on this property, the proposed method enables detection of miRNA-21 in a direct, rapid, sensitive, inexpensive, and label-free way. The detection of miRNA-21 was achieved at picomolar sensitivity through the changes in intrinsic p19 oxidation signals observed at +0.80 V with differential pulse voltammetry (DPV) and the specificity of the designed sensor was proven by control studies [55].

Berezovski and co-workers proposed a strategy to transduce miRNA hybridization into an electrochemical signal [56]. They immobilized RNA capture probes on AuNP-modified screen-printed electrodes allowing miRNA detection in three different modalities: direct hybridization with target miRNA, p19 binding, and protein displacement. In the direct hybridization modality, target miRNA hybridizes to the immobilized RNA-capture probes causing a modulation of the electrical signal observed by scanning wave voltammetry (SWV). Ultrasensitive detection (LoD = 0.15 zmol or 5 am in 30 mL sample solution) can be reached in the protein-based modality by adding p19 protein to the preformed hybrid. Binding of the bulky p19 largely enhances shielding of the electrode surface compared to simple miRNA hybridization and results in signal amplification. This sensing strategy is appealing because of its high sensitivity, direct miRNA detection on small sample volumes, no need for miRNA labeling or PCR amplification, and low cost of electrodes. However, nonspecific adsorption of serum proteins, yeast tRNA and p19 on the sensors surface was mentioned to likely affect quantification. This limitation should be taken into consideration in the future method development. Finally, in the protein displacement mode, the initial state for the sensor corresponds to full-coverage of p19 protein on the electrode surface. In the presence of target miRNA in solution, addition of the complementary RNA probe produces dsRNA. As a consequence, p19 is released from the electrode surface by competition with the

immobilized dsRNA. In this mode, the LoD reached was, however, much higher (1.5 fmol, 50 pm in 30 mL sample solution). Another possible limitation of quantification strategies employing protein p19 is the reduced stability of RNA probes compared to DNA probes. An alternative approach that tries to minimize this problem was proposed by Mulchandani and co-workers [57].

Labib et al. [58] endeavored to develop a DNA four-way junction-based electrochemical sensor (4J-SENS) for ultrasensitive miRNA analysis. The developed sensor can be operated within the dynamic range from 10 aM to 1 fM and detect as low as 2 aM of miRNA-122 (∼36 molecules per sample), without PCR amplification.

4.3 Nucleic Acid Strand Amplification Electrochemical miRNA Detection

As a typical target amplification technique, PCR is replication of a specific DNA fragment of interest to a high enough concentration to be detected using conventional approaches. However, the expensive thermal cycler limits its application in point-of-care settings. Isothermal target amplification methods including helicase-dependent amplification (HDA) [59, 60], strand displacement amplification (SDA), loop-mediated isothermal amplification (LAMP) [61–64], and recombinase polymerase amplification (RPA) [65] have emerged as alternatives to PCR; attracting intense interest in nucleic acids analysis. Besides the target amplification, probe amplification strategies, such as rolling circle amplification (RCA) [66–69] in which the amount of the target remains the same but the probe sequence is replicated, are also employed to develop nucleic acid biosensors.

Duplex-specific nuclease (DSN) displays a strong preference for cleaving double-stranded DNA or DNA in DNA:RNA heteroduplexes and is practically inactive toward single-stranded DNA, or single-or double-stranded RNA [70]. It shows good capability to discriminate between perfectly and nonperfectly matched (up to one mismatch) short duplexes and is widely applied in the molecular biology field, including full-length cDNA library normalization, subtraction, quantitative telomeric overhang determination, and genomic single-nucleotide polymorphism detection. Based on this property, Gao's group developed a highly sensitive and selective label-free miRNA genosensor, based on hybridized target miRNA strands initiated cleavage of hybridized DNA capture probes (CPs) by a DSN on a gold electrode [54]. Briefly, the hybridized CPs in the miRNA-CP duplexes are simultaneously cleaved by the DSN, releasing the target miRNA strands back to the sample solution. The released target miRNA strands again hybridize with the remaining CPs on the electrode, thus forming an isothermal amplification cycle. The distinct difference in electrochemical impedance between a control and the DSN cleaved genosensor allows label-free detection of miRNA down to femtomolar levels. The mismatch discrimination ability of the DSN permits miRNA expression to be profiled with high selectivity. Moreover, it can be easily applied to all miRNAs, because DSN enzyme has no requirement for specific recognition sequence [71] (Fig. 4.10).

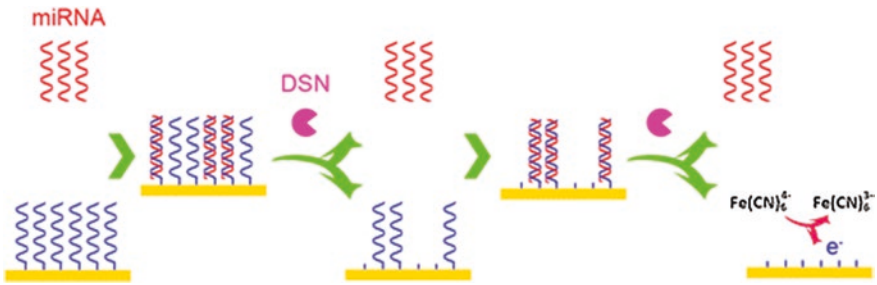

Fig. 4.10 Schematic illustration of working principle of the label-free electrochemical biosensor. Reprinted with permission from Ref. [71], Copyright 2013 American Chemical Society

An ultrasensitive miRNA-16 biosensor was designed by combining rolling circle amplification (RCA), QDs tagging, and anodic stripping voltammetric detection (ASV). The LNA-MB probes immobilized on the Au electrode surface react with the target miRNA to open up MB to release the RCA primer. After the RCA primer binding RCA template, dNTPs and Phi29 DNA polymerase were introduced to initiate the RCA reaction. The obtained RCA product was a long single-strand DNA containing thousands of repeated sequences for linear periodic hybridization with the QDs-modified detection probes. The Cd^{2+} released by dissolving QDs attaching to the resulting electrode was quantified by ASV [72] (Fig. 4.11).

An electrochemical biosensor without the assistance of enzyme for highly sensitive miRNA detection was developed by combining a catalyzed hairpin assembly reaction (CHA) and hybridization chain reaction (HCR). First, the hairpin-shaped capture probe H1 immobilized on the electrode surface was opened by a target. In the presence of another hairpin probe H2, hybridization of H1–H2 resulted in the release of target from the H1-target complex by a strand-displacement reaction. The released target further hybridized with the remaining capture probe H1. After the target recycling process, H1–H2 complex was achieved with an exposed stem of H2. Then, the exposed stem of H2 served as initiator to trigger an HCR event, yielding a long double-strand (dsDNA) molecule. Ultimately, numerous methylene blue acting as redox probes intercalated into the minor groove

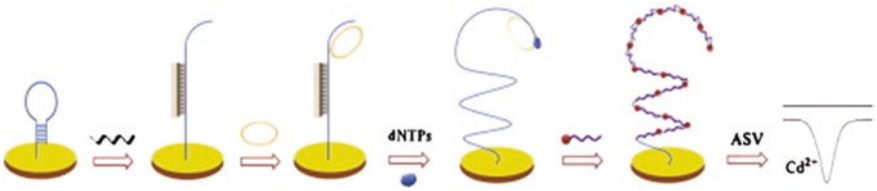

Fig. 4.11 Schematic representation of the designed strategy for miRNA detection. Reprinted from Ref. [72], Copyright 2013, with permission from Elsevier

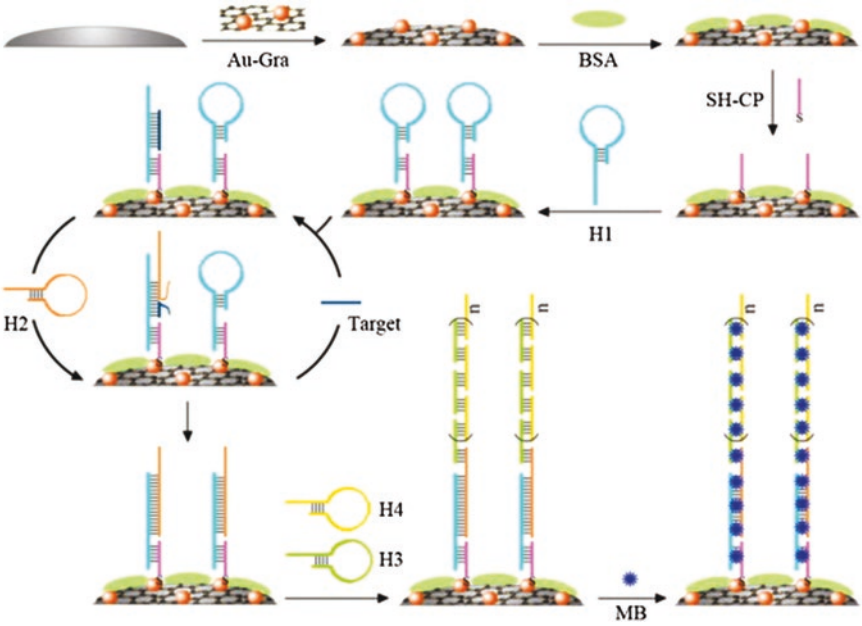

Fig. 4.12 Schematic illustration of electrochemical miRNA biosensor-based on enzyme-free dual signal amplification of CHA and HCR. Reprinted from Ref. [73], Copyright 2014, with permission from Elsevier

of the long dsDNA polymers to achieve an amplified electrochemical signal. The proposed miRNA biosensor achieved a linear range from 10 fM to 1 nM with a wide dynamic range of six orders of magnitude [73] (Fig. 4.12).

References

1. Hamidi-Asl E et al (2013) A review on the electrochemical biosensors for determination of microRNAs. Talanta 115:74–83
2. Tosar JP, Branas G, Laiz J (2010) Electrochemical DNA hybridization sensors applied to real and complex biological samples. Biosens Bioelectron 26(4):1205–1217
3. Gao ZQ, Yang ZC (2006) Detection of microRNAs using electrocatalytic nanoparticle tags. Anal Chem 78(5):1470–1477
4. Peng Y, Yi G, Gao Z (2010) A highly sensitive microRNA biosensor based on ruthenium oxide nanoparticle-initiated polymerization of aniline. Chem Commun 46(48):9131–9133
5. Tiwari PM, Vig K, Dennis VA, Singh SR (2011) Functionalized gold nanoparticles and their biomedical applications. Nanomaterials 1(1):31–63
6. Yang W-J et al (2008) Quantification of microRNA by gold nanoparticle probes. Anal Biochem 376(2):183–188
7. Barad O et al (2004) MicroRNA expression detected by oligonucleotide microarrays: system establishment and expression profiling in human tissues. Genome Res 14(12):2486–2494

8. Wang J et al (2012) Direct quantification of microRNA at low picomolar level in sera of glioma patients using a competitive hybridization followed by amplified voltammetric detection. Anal Chem 84(15):6400–6406
9. Yin H et al (2012) Electrochemical determination of microRNA-21 based on graphene, LNA integrated molecular beacon, AuNPs and biotin multifunctional bio bar codes and enzymatic assay system. Biosens Bioelectron 33(1):247–253
10. Zhou Y et al (2012) MicroRNA-21 detection based on molecular switching by amperometry. New J Chem 36(10):1985–1991
11. Yin H et al (2012) An electrochemical signal 'off-on' sensing platform for microRNA detection. Analyst 137(6):1389–1395
12. Zhou Y et al (2012) Amplified electrochemical microRNA biosensor using a hemin-G-quadruplex complex as the sensing element. Rsc Advances 2(18):7140–7145
13. Meng X et al (2013) Electrochemical determination of microRNA-21 based on bio bar code and hemin/G-quadruplet DNAenzyme. Analyst 138(12):3409–3415
14. Xia N et al (2013) Label-free and sensitive strategy for microRNAs detection based on the formation of boronate ester bonds and the dual-amplification of gold nanoparticles. Biosens Bioelectron 47:461–466
15. Liu L et al (2014) Highly sensitive and label-free electrochemical detection of microRNAs based on triple signal amplification of multifunctional gold nanoparticles, enzymes and redox-cycling reaction. Biosens Bioelectron 53:399–405
16. Xia N et al (2013) Comparing the performances of electrochemical sensors using p-aminophenol redox cycling by different reductants on gold electrodes modified with self-assembled monolayers. Electrochim Acta 109:348–354
17. Wu X et al (2013) A novel label-free electrochemical microRNA biosensor using Pd nanoparticles as enhancer and linker. Analyst 138(4):1060–1066
18. Dong H et al (2012) Trace and label-free microRNA detection using oligonucleotide encapsulated silver nanoclusters as probes. Anal Chem 84(20):8670–8674
19. Peng Y, Gao Z (2011) Amplified detection of MicroRNA based on ruthenium oxide nanoparticle-initiated deposition of an insulating film. Anal Chem 83(3):820–827
20. Govindaraj M, Muthukumar M, Raju GB (2010) Electrochemical oxidation of tannic acid contaminated wastewater by RuO2/IrO2/TaO2-coated titanium and graphite anodes. Environ Technol 31(14):1613–1622
21. Yang F et al (2011) Determining the behavior of RuOx nanoparticles in mixed-metal oxides: structural and catalytic properties of RuO2/TiO2(110) surfaces. Angewandte Chemie-International Edition 50(43):10198–10202
22. Dolatabadi JEN et al (2011) Optical and electrochemical DNA nanobiosensors. Trac-Trends Analy Chem 30(3):459–472
23. Dong H et al (2012) Highly sensitive multiple microRNA detection based on fluorescence quenching of graphene oxide and isothermal strand-displacement polymerase reaction. Anal Chem 84(10):4587–4593
24. Zhu W et al (2014) A label-free and PCR-free electrochemical assay for multiplexed microRNA profiles by ligase chain reaction coupling with quantum dots barcodes. Biosens Bioelectron 53:414–419
25. Wang J (2005) Carbon-nanotube based electrochemical biosensors: a review. Electroanalysis 17(1):7–14
26. Peigney A et al (2001) Specific surface area of carbon nanotubes and bundles of carbon nanotubes. Carbon 39(4):507–514
27. Kulesza PJ et al (2006) Fabrication of network films of conducting polymer-linked polyoxometallate-stabilized carbon nanostructures. Electrochim Acta 51(11):2373–2379
28. Acevedo DF et al (2008) Fabrication of an interpenetrated network of carbon nanotubes and electroactive polymers to be used in oligonucletide biosensing. Electrochim Acta 53(11):4001–4006

29. Tran HV et al (2013) Label-free and reagentless electrochemical detection of microRNAs using a conducting polymer nanostructured by carbon nanotubes: application to prostate cancer biomarker miR-141. Biosens Bioelectron 49:164–169
30. Xu Y et al (2004) Electrochemical impedance detection of DNA hybridization based on the formation of M-DNA on polypyrrole/carbon nanotube modified electrode. Anal Chim Acta 516(1–2):19–27
31. Xu Y et al (2006) Impedance DNA biosensor using electropolymerized polypyrrole/multiwalled carbon nanotubes modified electrode. Electroanalysis 18(15):1471–1478
32. Cai H et al (2003) Indicator free DNA hybridization detection by impedance measurement based on the DNA-doped conducting polymer film formed on the carbon nanotube modified electrode. Electroanalysis 15(23–24):1864–1870
33. Chen H-Y et al (2012) miR-103/107 promote metastasis of colorectal cancer by targeting the metastasis suppressors DAPK and KLF4. Cancer Res 72(14):3631–3641
34. Fabbri M et al (2007) MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. Proc Natl Acad Sci USA 104(40):15805–15810
35. Song E, Choi J-W (2013) Conducting polyaniline nanowire and its applications in chemiresistive sensing. Nanomaterials 3(3):498–523
36. Jaymand M (2013) Recent progress in chemical modification of polyaniline. Prog Polym Sci 38(9):1287–1306
37. Hatamzadeh M, Mahyar A, Jaymand M (2012) Chemical modification of polyaniline by N-grafting of polystyrenic chains synthesized via nitroxide-mediated polymerization. J Braz Chem Soc 23(6):1008–1017
38. Zhao G-Y, Li H-L (2008) Preparation of polyaniline nanowire arrayed electrodes for electrochemical supercapacitors. Microporous Mesoporous Mater 110(2–3):590–594
39. Zhu NN et al (2006) Electrochemically fabricated polyaniline nanowire-modified electrode for voltammetric detection of DNA hybridization. Electrochim Acta 51(18):3758–3762
40. Fan Y et al (2007) Detection of microRNAs using target-guided formation of conducting polymer nanowires in nanogaps. J Am Chem Soc 129(17):5437–5443
41. Bartosik M et al (2014) Magnetic bead-based hybridization assay for electrochemical detection of microRNA. Anal Chim Acta 813:35–40
42. Hong C-Y et al (2013) Ultrasensitive electrochemical detection of cancer-associated circulating microRNA in serum samples based on DNA concatamers. Biosens Bioelectron 50:132–136
43. Degliangeli F, Pompa PP, Fiammengo R (2014) Nanotechnology-based strategies for the detection and quantification of microRNA. Chemistry 20(31):9476–9492
44. Zhang G-J et al (2009) Label-free direct detection of MiRNAs with silicon nanowire biosensors. Biosens Bioelectron 24(8):2504–2508
45. Gao Z, Yu YH (2007) Direct labeling microRNA with an electrocatalytic moiety and its application in ultrasensitive microRNA assays. Biosens Bioelectron 22(6):933–940
46. Guo L, Chen F (2014) A challenge for miRNA: multiple isomiRs in miRNAomics. Gene 544(1):1–7
47. Wen YL et al (2012) DNA nanostructure-based Interfacial engineering for PCR-free ultrasensitive electrochemical analysis of microRNA. Sci Rep 2:867
48. Wang M et al (2014) Signal-on photoelectrochemical biosensor for microRNA detection based on Bi2S3 nanorods and enzymatic amplification. Biosens Bioelectron 53:232–237
49. Bettazzi F et al (2013) Electrochemical detection of miRNA-222 by use of a magnetic bead-based bioassay. Anal Bioanal Chem 405(2–3):1025–1034
50. Kilic T et al (2012) Electrochemical based detection of microRNA, mir21 in breast cancer cells. Biosens Bioelectron 38(1):195–201
51. Pohlmann C, Sprinzl M (2010) Electrochemical detection of microRNAs via gap hybridization assay. Anal Chem 82(11):4434–4440

52. Zhou Y et al (2014) Investigation of the effect of phytohormone on the expression of microRNA-159a in *Arabidopsis thaliana* seedlings based on mimic enzyme catalysis systematic electrochemical biosensor. Biosens Bioelectron 54:244–250
53. Nasheri N et al (2011) An enzyme-linked assay for the rapid quantification of microRNAs based on the viral suppressor of RNA silencing protein p19. Anal Biochem 412(2):165–172
54. Ren YQ, Deng HM, Shen W, Gao ZQ (2013) A highly sensitive and selective electrochemical biosensor for direct detection of microRNAs in serum. Anal Chem 85(9):4784–4789
55. Kilic T, Topkaya SN, Ozsoz M (2013) A new insight into electrochemical microRNA detection: a molecular caliper, p19 protein. Biosens Bioelectron 48:165–171
56. Labib M et al (2013) Three-mode electrochemical sensing of ultralow microRNA levels. J Am Chem Soc 135(8):3027–3038
57. Ramnani P et al (2013) Electronic detection of microRNA at attomolar level with high specificity. Anal Chem 85(17):8061–8064
58. Labib M et al (2013) Four-way junction formation promoting ultrasensitive electrochemical detection of microRNA. Anal Chem 85(20):9422–9427
59. Andresen D, von Nickisch-Rosenegk M, Bier FF (2009) Helicase-dependent amplification: use in OnChip amplification and potential for point-of-care diagnostics. Expert Rev Mol Diag 9(7):645–650
60. Jeong Y-J, Park K, Kim D-E (2009) Isothermal DNA amplification in vitro: the helicase-dependent amplification system. Cell Mol Life Sci 66(20):3325–3336
61. Han E-T (2013) Loop-mediated isothermal amplification test for the molecular diagnosis of malaria. Expert Rev Mol Diag 13(2):205–218
62. Mori Y, Notomi T (2009) Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. J Infect Chemother 15(2):62–69
63. Parida M et al (2008) Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. Rev Med Virol 18(6):407–421
64. Njiru ZK (2012) Loop-mediated isothermal amplification technology: towards point of care diagnostics. Plos Negl Trop Dis 6(6)
65. Santiago-Felipe S et al (2014) Recombinase polymerase and enzyme-linked immunosorbent assay as a DNA amplification-detection strategy for food analysis. Anal Chim Acta 811:81–87
66. Zhao W et al (2008) Rolling circle amplification: applications in nanotechnology and biodetection with functional nucleic acids. Angewandte Chemie-International Edition 47(34):6330–6337
67. Stougaard M et al (2011) Strategies for highly sensitive biomarker detection by Rolling Circle Amplification of signals from nucleic acid composed sensors. Integrative Biology 3(10):982–992
68. Demidov VV (2002) Rolling-circle amplification in DNA diagnostics: the power of simplicity. Expert Rev Mol Diag 2(6):542–548
69. Kobori T, Takahashi H (2014) Expanding possibilities of rolling circle amplification as a biosensing platform. Anal Sci 30(1):59–64
70. Yin B-C, Liu Y-Q, Ye B-C (2012) One-Step, multiplexed fluorescence detection of micrornas based on duplex-specific nuclease signal amplification. J Am Chem Soc 134(11):5064–5067
71. Dong H et al (2013) MicroRNA: function, detection, and bioanalysis. Chem Rev 113(8):6207–6233
72. Wang D et al (2013) Molecular beacon structure mediated rolling circle amplification for ultrasensitive electrochemical detection of microRNA based on quantum dots tagging. Electrochem Commun 33:80–83
73. Wu X et al (2014) Dual signal amplification strategy for enzyme-free electrochemical detection of microRNAs. Sens Actuators B Chem 203:296–302

Chapter 5
miRNA Optical Detection

Abstract miRNA is a kind of attractive candidates, as biomarkers, for early cancer diagnosis. Therefore, simple and novel strategies for miRNA detection with high sensitivity and selectivity have great significance not only for the studies of its biological functions but also for clinical cancer diagnosis. Various reliable optical miRNA detection methods have become a class of attractive and paramount for miRNA expression analysis, due to its high sensitivity, wide dynamic range, and multiplexing capabilities. The progress of nanotechnology and nanoscience allows nanomaterial-based signal amplification to develop highly sensitive and selective biosensors for in situ or online detection of miRNA. Molecular biology-based strategies for miRNA detection by amplifying the target or probe with high detection sensitivity are applied in identifying and detecting nucleic acids analysis.

Keywords Optical detection · Signal amplification · Noble metal nanoparticles · Transition metal dichalcogenides · Quantum dots · Canbon nanomaterials · Target or probe amplification

5.1 Nanomaterials-Based miRNA Optical Detection

The development of nanotechnology and nanoscience renders nanomaterial-based signal amplification great promise in developing highly sensitive and selective biosensors for in situ or online detection of miRNA. Nanomaterials-based biosensors exhibit the significant advantages with high sensitivity, wide dynamic range, and multiplexing capabilities compared to bulk materials-biosensors. Nanoparticles (NPs)-based biosensors show the significant advantages as follows: (i) The NPs allow design of low cost and minimized equipment in point-of-care diagnostics due to the small sizes breaking through the limitation of structure miniaturization. (ii) The direct contact between NPs and the environment lead to accelerated

Haifeng Dong and Xiangdan Meng contributed together to this chapter.

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signal transduction, enhanced ability of rapid analysis, and lower detection limits. (iii) The utilization of nanomaterials also gives rise to a new research field and concepts including reagent-less biosensing, biomimetics, and in vivo detection with long-term stability and less cytotoxicity. In an optical biosensor, various nanomaterials-based fluorescent reporters and quenchers are explored to construct the miRNA biosensor. For example, the great fluorescent properties of quantum dots (QDs) [1] or AgNCs [2] are used as fluorescent reporters, while fluorescence quenching behaviors of gold nanoparticles [3], carbon materials [4], graphene oxide [5], or analogous nanostructures(e.g., MoS_2, WS_2, etc.) [6] are employed as efficient quenchers to develop a miRNA biosensing platform. In addition, many nanomaterials have been explored to obtain the amplified detection signal and stabilize recognition probes by using NPs as carriers or tracers, catalysts or electronic conductors in the detection of miRNA [7].

5.1.1 Noble Metal Nanoparticles

Gold nanoparticles (AuNPs) have qualified in diverse applications to bioassay detection, due to physical/chemical properties including surface/surface plasmon resonance and excellent fluorescence quenching, as well as easily controllable size distribution, high stability and compatibility with biomaterials, such as nucleic acids and proteins. A colorimetric assay for convenient detection of miRNA has gained increasing attention because it minimizes or eliminates the necessity of using expensive and complicated instruments with a high sensitivity and wide dynamic range, being easily monitored with the naked eye [8]. Among the many colorimetric sensing strategies, AuNPs-based detection is desirable because of the high extinction coefficients and strong distance-dependent optical properties of the nanoparticles [9]. Mirkin and coworkers [10] designed a scanometric miRNA platform for the detection of relatively low-abundance miRNAs with high specificity and reproducibility. The scanometric miRNA system is able to detect miRNA of 1 fM in serum with single-nucleotide mismatch specificity.

Gao and coworkers [11] have demonstrated that AuNP networks in conjunction with the duplex-specific nuclease (DSN) can be utilized to devise a simple and yet ultrasensitive colorimetric assay for label-free detection of miRNAs (Fig. 5.1). Hybridization of a target miRNA introduces the target miRNA strand into the AuNP networks through forming a miRNA-capture probe (CP) duplex, then the DSN cleaves the CP in the miRNA-CP duplex, releasing the miRNA strand together with the AuNP into the sample solution. The released miRNA strand again hybridizes with the AuNP networks, thus forming an isothermal amplification cycle through which one target miRNA strand cleaves millions of AuNPs from the AuNP networks during incubation. A sensitive colorimetric detection of the target miRNA is realized after a sufficient period of incubation in the dynamic range of 0.2 fM to 10 pM, with a detection limit of 0.1 fM.

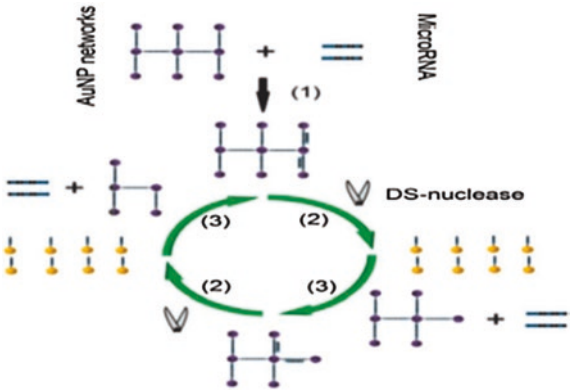

Fig. 5.1 Scheme for a real-time colorimetric assay for label-free detection of miRNA, down to sub-fetomolar levels. Reproduced from Ref. [11] by permission of The Royal Society of Chemistry 2013

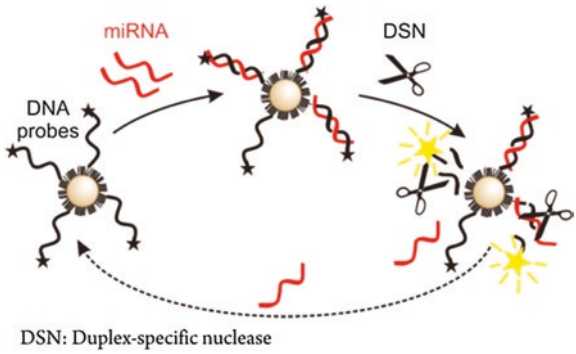

Fig. 5.2 Scheme for absolute and direct miRNA quantification using DNA-AuNPs nanoprobes. Reprinted with permission from Ref. [15]. Copyright 2014 American Chemical Society

Since Mirkin and coworkers reported that DNA-modified AuNPs (DNA-AuNPs) are a novel nanoprobe system for biomolecules detection for the first time in 1996 [12], a variety of high-sensitive biomolecules detection based on a DNA-AuNPs nanoprobe have been developed since that time [13, 14]. Degliangeli et al. [15] have proposed a strategy for miRNA quantification based on DNA-AuNPs probes. As shown in Fig. 5.2, they designed a fluorescently labeled DNA probe immobilized on PEGylated AuNPs, whose fluorescence is quenched. In the presence of target miRNA and the endonuclease DSN, DNA–RNA heteroduplexes are formed and the DNA strands are cleaved via enzymatic hydrolysis resulting in the fluorophores moving away from the AuNPs surface, yielding a fluorescence signal. This strategy allows absolute and direct quantification of as little as 0.2 fmol of miR-203.

Surface-enhanced Raman spectroscopy (SERS) utilizes nanostructured metal surfaces, primarily silver and gold, that generate plasmon resonances in the visible spectral region where Raman scattering is excited to a different state. The phenomenon provides the basis for a powerful analytical technique offering both quantitative and qualitative molecular information about biomolecules.

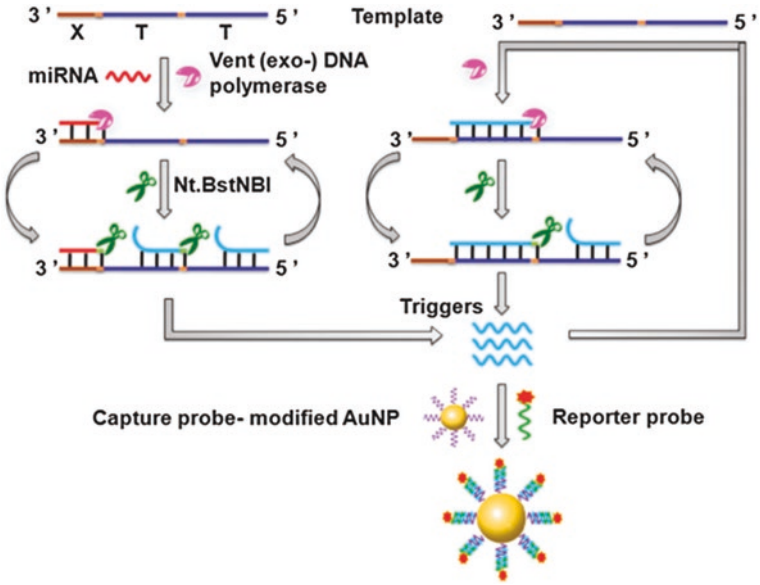

Fig. 5.3 Scheme for surface-enhanced Raman spectroscopy (SERS) for simultaneous sensitive detection of multiple miRNAs in lung cancer cell. Reproduced from Ref. [19] by permission of The Royal Society of Chemistry 2014

Low detection limits, narrow spectral bandwidths, the ability to quench fluorescence, and the capacity to be used with or without optical labels make SERS a good choice for biomolecules [16]. Widespread application of SERS-based sensors, however, has been limited due to an inherent lack of spectral detail, reproducibility [17], or a means of statistical analysis for quantitative analysis [18]. In order to overcome these issues, a series of SERS-based strategies for miRNA detection have been developed. Highly reproducible silver nanorod SERS substrates along with a straight forward least squares (LS) technique have been employed for the quantitative determination of the relative ratios of the four nucleotide components: A, C, G, and T/U before and after hybridization using a clinically relevant miRNA sequence. This study highlights a potentially powerful advancement for miRNA label-free hybridization analysis [18]. Meanwhile, Zhang group [19] have developed circular exponential amplification reaction (EXPAR)-based SERS for simultaneous sensitive detection of multiple miRNAs in nonsmall cell lung cancer cells with a detection limit of as low as 0.5 fM (Fig. 5.3).

5.1.2 Silver Nanoclusters (AgNCs)

The remarkable optical properties of silver nanoclusters (AgNCs) have stimulated extensive interest for their potential use as fluorescent labels for

microscopic imaging and as sensors for the detection of biomolecules [20]. Using the properties of DNA–silver nanoclusters (DNA/AgNC), a DNA/AgNC probe for miRNA detection without pre- or postmodification, addition of extra enhancer molecules or labeling was designed [21]. From this point, DNA/AgNC probes have attracted prominent attention for miRNAs detection as a new, simple, inexpensive, and instant technique. Numerous studies have applied DNA as a scaffold for nanocluster formation, focusing on the photoluminescence aspects of the DNA-templated silver nanoclusters (DNA/AgNCs) for biosensing application [22–24]. Bjerrum and Yang [25] have developed a strategy based on various fluorescent DNA/AgNCs probes for multiplex miRNA detection in solution. For the creation of spectrally different DNA/AgNCs probes, the emitters are encapsulated in nine different DNA-12nt scaffolds, which is necessary, and with the tethered target-sensing DNA sequences (also crucial) to tune the fluorescence across the visible to infrared region (Fig. 5.4). In this study, they obtain three spectrally distinctive emitters of each DNA/AgNCs probe such as green, red, and near-infrared (NIR) fluorescence. This study shows a proof of concept for a rapid, one-step, in-solution multiplex miRNA detection method.

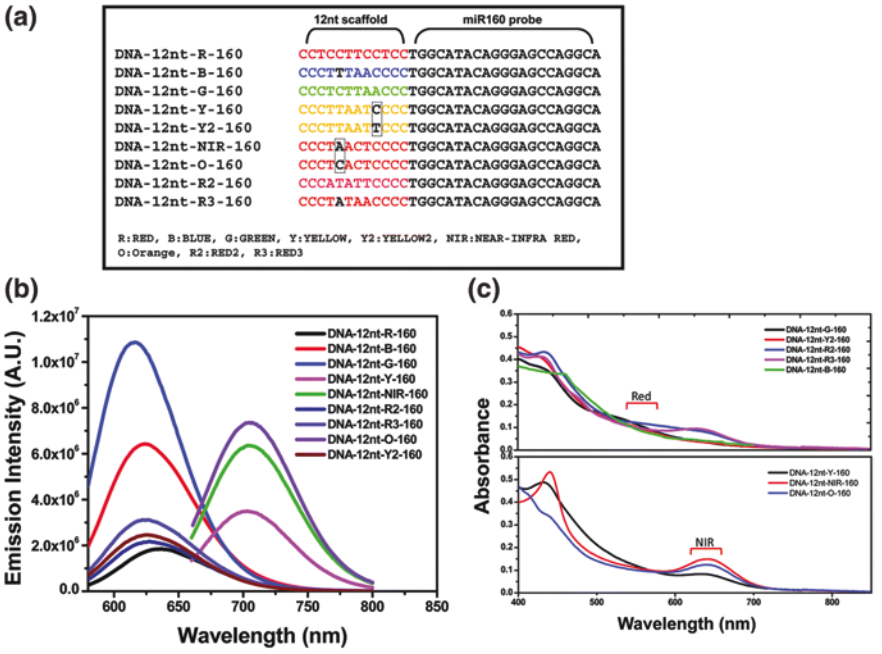

Fig. 5.4 DNA sequences of nine DNA/AgNCs probes with their abbreviations (**a**), the maximum emission spectra of each DNA/AgNC probes (**b**) and absorption spectra of eight DNA/AgNCs probes (**c**). Reproduced from Ref. [25] by permission of The Royal Society of Chemistry 2014

5.1.3 Quantum Dots

QDs, a novel semiconductor nanocrystal, have advantages over traditional fluorophores for biosensing application. Especially for miRNA detection, due to their outstanding optical properties, such as high quantum yields, broad absorption spectra, narrow and symmetric size-tunable emission and strong resistance to photobleaching [26]. The combination of QDs with single-molecule detection (e.g., nucleic acids, proteins, and small molecules) enables the development of QD-based biosensors with distinct advantages including high signal-to-noise ratios, low sample consumption, and high sensitivity [27]. In particular, QDs hold great promise as fluorescence resonance energy transfer (FRET) [28] or electrochemiluminescence resonance energy transfer (ERET) [29] donors in various biosensors for miRNA detection. Zhang and coworkers [28] developed a QD-based miRNA nanosensor for a point mutation assay with primer generation-mediated rolling circle amplification (PG-RCA), and further applied it to analyze the point mutation of mir-196a2 in real samples. As shown in Fig. 5.5, in the presence of mir-196a2T, the mir-196a2T-specific linear padlock probe forming a circular template and mir-196a2T as a primer can initiate the RCA reaction with polymerase, producing a long repeat sequence, and with the nicking enzyme Nb.BsmI, an exponential amplification can be performed. In the presence of Nt.BstNBI, a double-stranded DNA (dsDNA) consisted of biotin/Cy5-labeled capture probes and RCA product with a recognition site of the Nt.BstNBI was cleaved, leading to the separation of Cy5 from biotin and the release of the RCA product, and triggering the

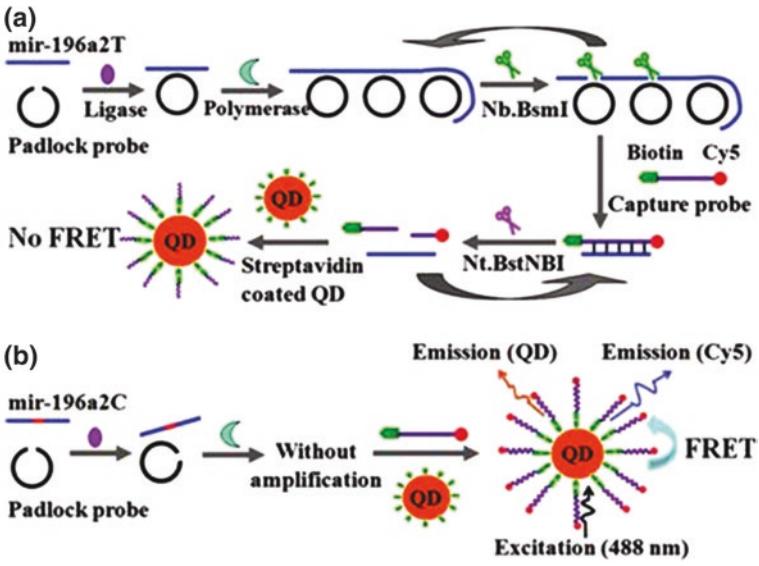

Fig. 5.5 Scheme for a quantum dot-based miRNA nanosensor for point mutation assays. Reproduced from Ref. [28] by permission of The Royal Society of Chemistry 2014

next cleavage of capture probes. The higher the mir-196a2T concentration, the lesser the Cy5 molecules are absorbed onto the surface of the streptavidin-coated QD, resulting in the FRET efficiency between the QD donor and Cy5 acceptors reduced and fewer Cy5 counts being detected in this application. While in the presence of mir-196a2C, amplification reaction and cleavage of capture probes are not triggered. The biotin/Cy5-labeled capture probes will be assembled on the surface of QDs to form the QD–capture probe–Cy5 complex through a specific streptavidin–biotin interaction, resulting in the occurrence of FRET between the QD donor and Cy5 acceptors and, consequently, the detection of Cy5 counts.

Ju et al. [29] designed a distance-dependent ERET system based on CdTe nanocrystals and Au nanoclusters (Au NCs) with the aid of ligase for a highly selective detection of miRNA (Fig. 5.6). Au NCs functionalized hairpin DNA (DNA–AuNCs) composite can be bound to the carboxylated CdTe nanocrystals via amide reaction on a glass carbon electrode. The strong interaction between CdTe nanocrystals and AuNCs led to the electrochemiluminescence (ECL) quenching of CdTe nanocrystals. In the presence of assistant DNA and miRNA, the ligase can selectively ligate both of them on the strand of the hairpin DNA to form long DNA–RNA heteroduplexes. Thus, the ECL signal was recovered due to the blocking of the ERET. As a comparison, when directly opening the hairpin DNA by the target, the ECL emission signal is weak owing to the presence of the ERET effect at the short distance. Based on the distance-dependent ERET, a "signal on" ECL system was utilized for the detection of miRNA in six orders of magnitude linear range with LOD of 21.7 fM and an excellent sequence specificity.

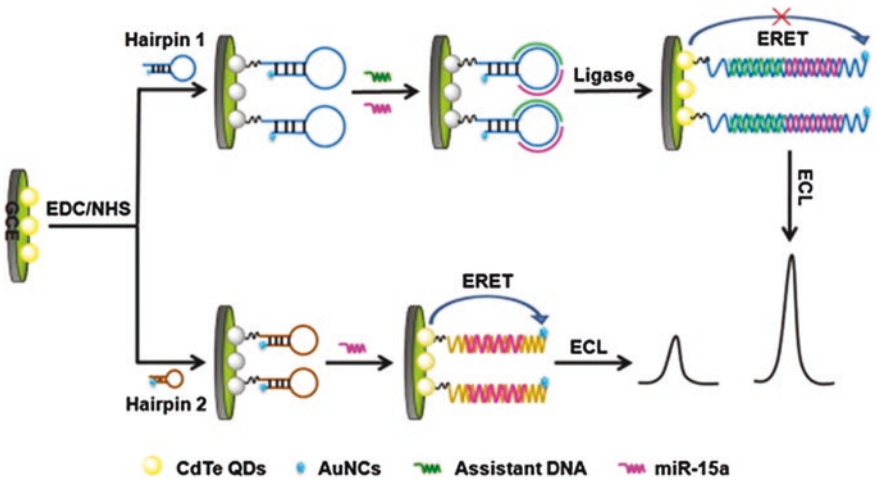

Fig. 5.6 Scheme for highly selective detection strategy of miRNA based on the distance-dependent ERET between CdTe nanocrystals and Au nanoclusters. Reprinted from Ref. [29]. Copyright 2013, with permission from Elsevier

5.1.4 Carbon Nanomaterials

The excellent fluorescence quenching properties of carbon nanomaterials have been widely used in biosensors. Carbon nanotubes (CNTs), as a typical one-dimensional (1D) nanomaterial, have been applied in a huge scientific field due to their unique mechanical, electronic, and physicochemical properties. Furthermore, a variety of works exhibit that CNTs have promising applications for biomolecules detection based on the small size with large surface area, excellent electron transfer ability and ability of biomolecule immobilization [30, 31]. Yang et al. [32] have proposed an effective self-assembled single-wall carbon nanotube (SWNT) complex with a fluorophores-modified DNA probe, based on the excellent fluorescence quenching of SWNT, and exhibiting the feasibility of SWNT in recognizing and detecting specific DNA sequences in a single step (Fig. 5.7).

Graphene oxide (GO), a two-dimensional (2D) nanomaterial, has shown unique and excellent electronic, thermal, and mechanical properties. Graphene is a one-atom thick sheet of sp^2 hybridized carbon network arranged in a perfect honeycomb lattice. GO has been applied particularly for biosensors due to its extraordinary distance-dependent fluorescence quenching property. GO interacts with single-stranded oligonucleotides through π–π stacking interaction with nucleobases and efficiently quenches fluorescence of dyes present nearby. Dong and coworkers [33] have proposed a highly sensitive multiple miRNA detection method based on the GO fluorescence quenching and isothermal strand-displacement polymerase reaction (ISDPR). As shown in Fig. 5.8, in the absence of specific targets, the strong interaction between ssDNA and GO led to the fluorescent ssDNA probe exhibiting minimal background fluorescence. Upon the recognition

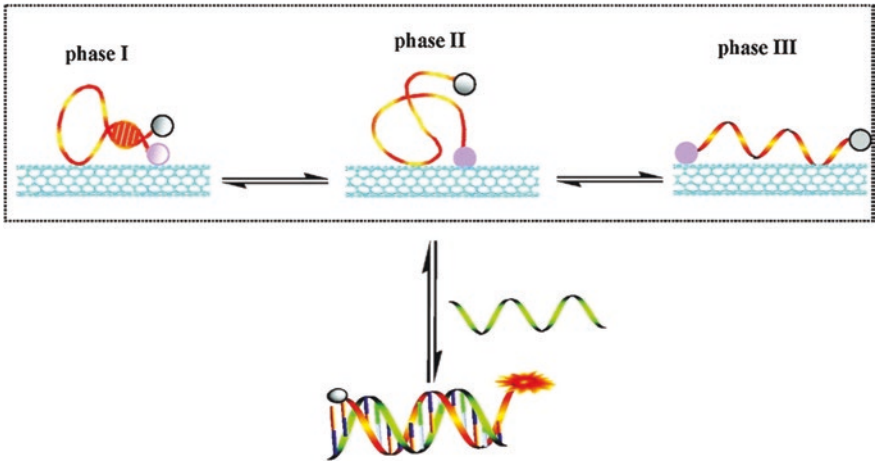

Fig. 5.7 Schematic drawings of target-induced fluorescence change of the ssDNA-FAM-SWCNTs complex from Ref. [32]. Reprinted with permission from Ref. [32]. Copyright 2008 American Chemical Society

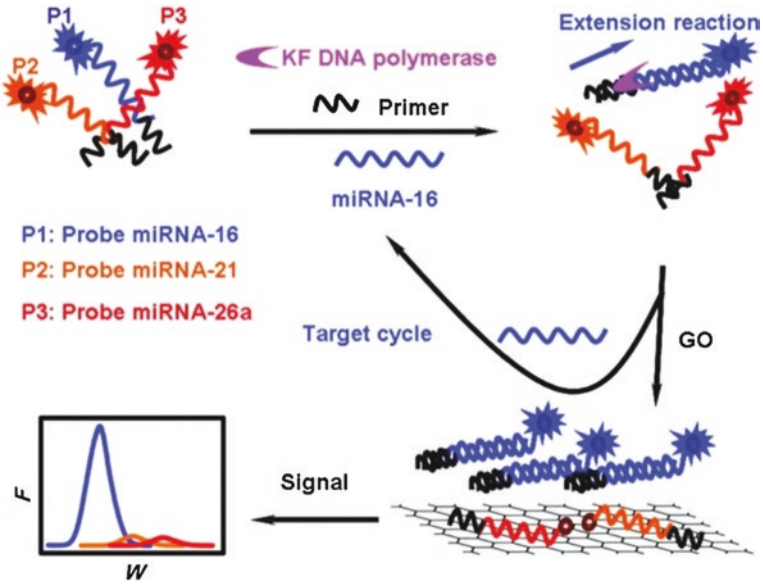

Fig. 5.8 Scheme for highly sensitive multiple miRNA detection method based on the GO fluorescence quenching and strand-displacement polymerase reaction (ISDPR). Reprinted with permission from Ref. [33]. Copyright 2012 American Chemical Society

of specific target miRNA, an ISDPR was triggered to produce numerous massive specific DNA-miRNA duplex helixes, and a strong emission was observed due to the weak interaction between the DNA-miRNA duplex helix and GO. This miRNA biosensor exhibited LOD down to 2.1 fM with a linear range of four orders of magnitude. Furthermore, the large planar surface of GO allows simultaneous quenching of several DNA probes with different dyes and produces a multiple biosensing platform with high sensitivity and selectivity, which has promising application in profiling the pattern of miRNA expression and biomedical research.

5.1.5 Transition Metal Dichalcogenides and Carbon Nitride Nanosheet

Transition metal dichalcogenides (TMD) (e.g., MoS_2 [34], WS_2 [6], etc.) and carbon nitride nanosheet [35] are 2D layered nanomaterials analogous to graphene and have attracted great attention due to their carrier mobility, optoelectronics, and catalytic properties. These properties have led to the idea that TMD nanosheets can hold great potential in biomedical applications because surfactants may cause denaturation of proteins and oxidation can alter the semiconductive property of the nanostructures. Jiang Jianhui and his group [6] developed a new strategy for miRNA detection by combining the super fluorescence quenching ability of the

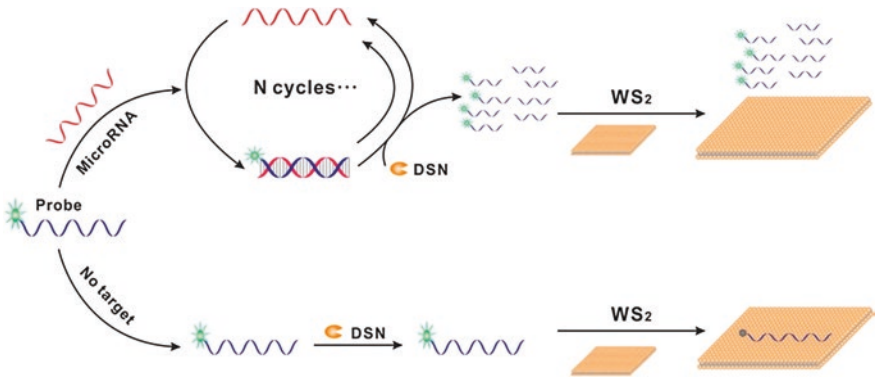

Fig. 5.9 Scheme for highly sensitive and selective strategy for miRNA detection based on WS_2 nanosheet-mediated fluorescence quenching and DSNSA. Reprinted with permission from Ref. [6]. Copyright 2014 American Chemical Society

WS_2 nanosheet and duplex-specific nuclease signal amplification (DSNSA), with a detection limit of 300 fM. As shown in Fig. 5.9, upon the addition of target miRNA, the ssDNA probe hybridizes to a target miRNA to form a heteroduplex as the substrate for triggering the DSNSA. After the product from the DSNSA reaction is incubated with WS_2 nanosheets, the cleaved short FAM-linked oligonucleotide fragments will not be adsorbed on the nanosheets due to the weak affinity and retain a strong fluorescence signal. In contrast, in the absence of the DSNSA reaction, the ssDNA probes remain intact and their fluorescence is almost entirely quenched due to their strong affinity to WS_2 nanosheets.

5.1.6 Other Nanomaterials

Recently, a variety of novel fluorescent probes have been employed for miRNA detection. Owing to a superior electronic property, light-harvest nature, and effective optical signal amplification, conjugated polymers (CPs) have received worldwide attention as biological sensors that either have a change in fluorescence or a change in color when the molecules of interest are present [36]. Li and coworkers [37] have established a 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 for miRNA detection. Upon addition of ssDNA, the DNA/PMNT solution color accordingly becomes red. When miRNA is hybridized with ssDNA and then mixed with the PMNT, due to the triplex structure of DNA/miRNA/PMNT, the color of the triplex solution is orange. As a result of the chemoselectivity, tunable kinetics, and fluorogenic nature, bioorthogonal tetrazine is well known for being used as a fluorophore for molecular tagging or labeling in many applications. Devaraj's group [38] developed a transfer reaction between 7-azabenzonorbornadiene derivatives and fluorogenic tetrazines facilitating turnover

amplification of the fluorogenic response in nucleic acid-templated reactions with LOD for DNA and miRNA as low as 0.5 and 5 pM, respectively.

5.2 Target or Probe Amplification-Based miRNA Optical Detection

In order to improve the detection sensitivity, molecular biological techniques amplifying the target or probe are applied in identifying and detecting nucleic acids analysis. Molecular biology-based strategies for miRNA detection are always accompanied by enzyme signal amplification, e.g., endonuclease, polymerase, and exonuclease, which have attracted considerable attention for producing a strong detectable signal in the analysis of trace target miRNA. For the target amplification, reverse transcription polymerase chain reaction (RT-PCR) [39] can render a 10^8–10^9-fold amplification of a specific target to high concentration detected by conventional methods. However, the expensive thermal cycle instrument limits its expansion application in point-of-care diagnostics. Isothermal amplification techniques free of thermal cycle instrument: including ligase chain reaction (LCR) [40], rolling circle amplification (RCA) isothermal reaction [41], loop-mediated isothermal amplification (LAMP) [42], and EXPAR [43] have emerged as alternative nucleic acids strand amplification techniques in bioanalysis.

5.2.1 LCR

LCR is employed for two pairs of oligonucleotide probes which are adjacent and perfectly complementary to target miRNA or the ligated DNA strand for performing ligation-based exponential amplification [44]. Upon hybridization to a target miRNA strand, the adjacent probes are ligated by a specific ligase [45] (e.g., T4 RNA ligase 2 [46]) to form a long DNA strand. Afterwards, the ligation products can serve as templates for the subsequent thermal cycles, leading to an exponential amplification process and achieving high specificity to discriminate one-base mutation in targets and with high sensitivity [47]. With a LCR utilized for exponential amplification of miRNA and lambda exonuclease-assisted cationic conjugated polymer (CCP) for fluorescent signal detection, a homogeneous and sensitive detection of miRNA has been proposed (Fig. 5.10) [44]. The method is sensitive enough to detect 0.1 fM target miRNA and specific to discriminate a one-base difference of miRNAs. On the basis of enzymatic ligation of DNA stem-loop probes, a variety of PCR miRNA detection methods [40, 48] are presented to reduce nonspecific ligation at least 100-fold with a wide dynamic range and single-base mismatch discrimination among miRNA sequences with a low limit of detection. These strategies will be detailed in the following RT-PCR for miRNA detection.

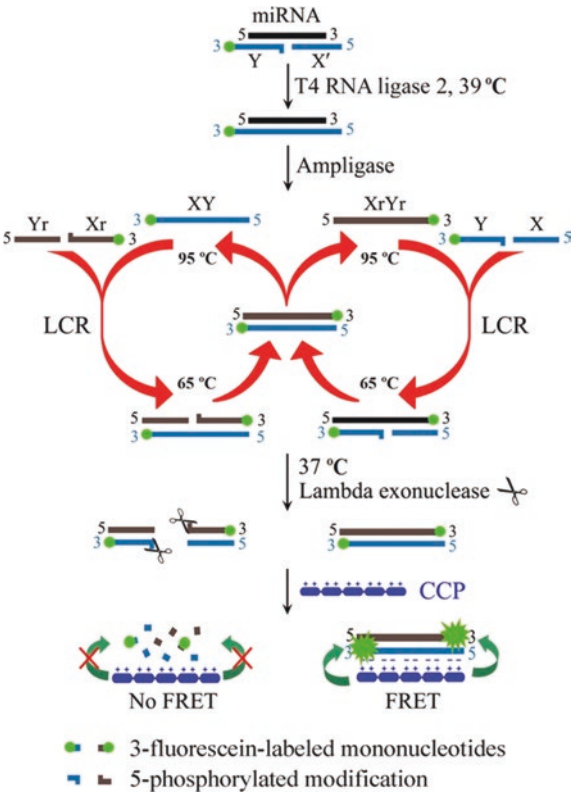

Fig. 5.10 Scheme for sensitive miRNA detection with LCR and lambda exonuclease-assisted cationic conjugated polymer biosensing. Reprinted with permission from Ref. [44]. Copyright 2014 American Chemical Society

5.2.2 RT-PCR

An RT-PCR miRNA detection method can overcome poor sensitivity and low throughput of conventional technologies. Furthermore, a series of sensitive and specific quantitative RT-PCR (qRT-PCR) techniques have been developed and optimized for miRNA detection. For example, a real-time method based upon a reverse transcription (RT) reaction with a stem-loop primer followed by a TaqMan PCR analysis has been designed [49] for total RNA from cells. This study has indicated that stem–loop RT–PCR can be used for the quantification of other small RNA molecules with better specificity and efficiency. Li and coworkers [48] have proposed an ultrasensitive quantification of mature miRNAs by real-time PCR based on ligation of a ribonucleotide-modified DNA probe. As shown in Fig. 5.11, in the presence of target miRNA (let-7a), probe A-M and probe B (which contains a universal sequence used for PCR amplification (green), a target-specific sequence (blue), and a stuffer sequence in between (red), respectively), hybridize with the half sequence of the target miRNA and are immediately adjacent to each other. Therefore, the probe can be ligated by the catalysis of T4 RNA ligase 2. The ligation product is then amplified by PCR with the universal forward primer and

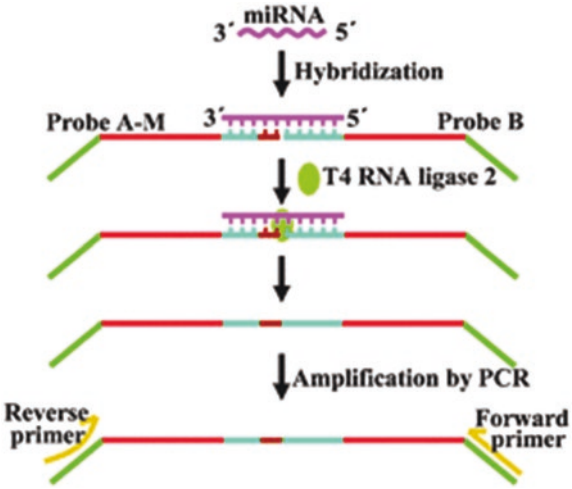

Fig. 5.11 Scheme for ultrasensitive quantification of mature miRNAs by real-time PCR based on ligation of a ribonucleotide-modified DNA probe. Reproduced from Ref. [48] by permission of The Royal Society of Chemistry 2011

reverse primer. SYBR Green I is utilized as the fluorescent dye for real-time detection of PCR products. With PCR amplification of the ligated DNA probe, as low as 0.2 fM target miRNAs can be detected with high specificity.

5.2.3 RCA

As a result of simplicity, robustness, specificity, and high sensitivity, RCA has become increasingly popular in the detection of DNA [50], RNA [51], and proteins [52]. 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 RCA reaction. This reaction is an isothermal enzymatic replication process that uses certain DNA or RNA polymerases to generate long single-stranded DNA or RNA with tandem repeats of the complementary sequence of a single-stranded circular DNA template [53]. Four components are essential for a typical RCA reaction: (1) DNA polymerase (e.g., Phi29 DNA polymerase) including a suitable buffer (usually provided by manufacturer along with the polymerase); (2) a short DNA or RNA primer; (3) a circular DNA template; and (4) deoxynucleotide triphosphates (dNTPs) [54]. 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 [51]. On the basis of RCA and a T7 exonuclease-assisted cyclic enzymatic amplification, a dual amplification strategy for miRNA detection has been proposed (Fig. 5.12) [55]. In the presence of target miRNA, the circular template permits replication assisted to phi29 DNA polymerase forming a long ssDNA with numerous copies of the complementary sequence of the

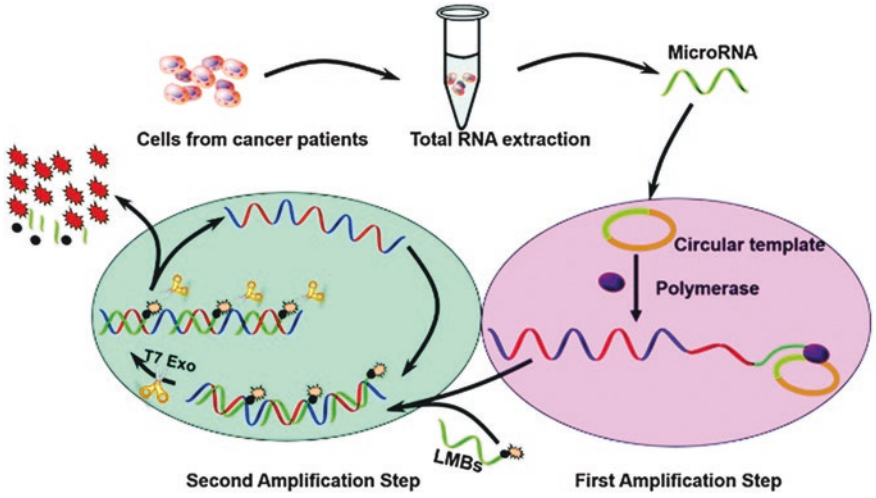

Fig. 5.12 Scheme for a dual amplification strategy for sensitive and selective miRNA detection using a T7 exonuclease-assisted cyclic enzymatic amplification method coupled with RCA. Reproduced from Ref. [55] by permission of The Royal Society of Chemistry 2014

circular template, which can hybridize with thousands of linear molecular beacons (LMB). The LMB from the duplex is digested by T7 exonuclease (T7 Exo) and the fluorophore is separated from the quencher to emit fluorescence when the RCA product is intact and initiates a new cleavage process achieving cyclic enzymatic amplification.

5.2.4 LAMP

LAMP, a novel nucleic acid amplification method, is one of the most potential candidates for simple and rapid biomolecular detection [56], which relies on auto cycling strand displacement DNA synthesis performed by the enzyme *Bst* polymerase [57]. LAMP is a simple, rapid, and cost-effective nucleic acid method, and the resulting amplicons can be detected by metal indicator [58] or SYBR Green I [59] after reaction. LAMP can amplify a few copies of the target to 10^9-fold in short time, even when large amounts of nontarget DNA are present, and has been applied to detect a variety of viral pathogens [60]. Recently, Fang et al. [61] have designed a LAMP-based detection system into one microfluidic chip with a detection limit of 10 fg of DNA sample within 1 h using only the naked eye as a detector. The detailed mechanism of LAMP for miRNA detection is illustrated in Fig. 5.13. Four probes are employed in the LAMP reaction: a template DNA (containing the sequences of B3, B2, B1, F1c, F2c and complementary sequence of target miRNA), forward inner primer (FIP, F1c, a TTTT spacer and the sequence F2 complementary to F2c), backward inner primer (BIP), and outer

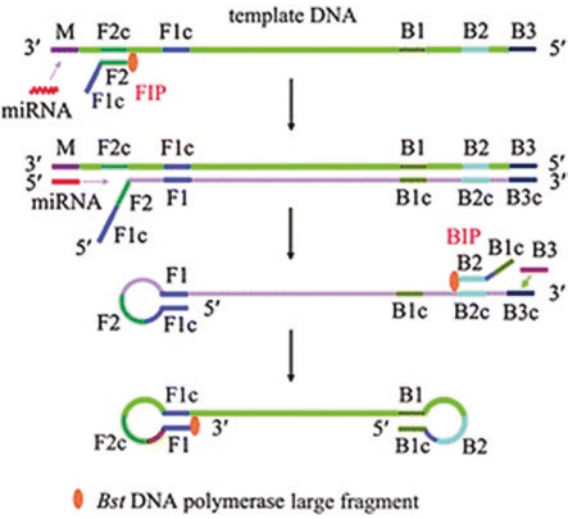

Fig. 5.13 Scheme for one-step ultrasensitive detection of miRNAs with LAMP. Reproduced from Ref. [59] by permission of The Royal Society of Chemistry 2011

primer B3 with modifications. In the presence of *Bst* polymerase, FIP and miRNA hybridize the template DNA and extend, generating a FIP-linked single-strand DNA (ssDNA), which can form a stem-loop structure. Then, BIP and primer B3 hybridize to the ssDNA performing the primer extension and releasing a BIP-linked ssDNA; which can form double stem-loop structures. Based on the strand extension and strand displacement, several cycles of strand extension can proceed, achieving cyclic signal amplification. Finally, the LAMP products can then be detected using SYBR Green I.

5.2.5 EXPAR

As an alternative amplification technique, the EXPAR has obtained great attention for low-abundance miRNA detection due to its high sensitivity, low cost, and good tolerance to the inhibitory components in the clinical samples. By the combination of polymerase strand extension and single-strand nicking, EXPAR has the intrinsic merits of high amplification efficiency (its isothermal nature and rapid amplification kinetics), which can provide 10^6–10^9-fold amplification within minutes [62]. EXPAR was first proposed by Galas and coworkers for the rapid and efficient amplification of short oligonucleotides sequence by a combination of polymerase strand extension and single-strand nicking [63]. Some improved approaches combining MB [64], silver nanocluster[22] or SYBR Green I [65] with EXPAR have been successively developed by different research groups for miRNA detection. Duan et al. [66] have developed an ultrasensitive detection of miRNA at the single-cell level based on hairpin-mediated quadratic enzymatic amplification (HQEA). As shown in Fig. 5.14, in the presence of the target miRNA, the loop region of MB probe is opened and hybridized, resulting in recovery of the

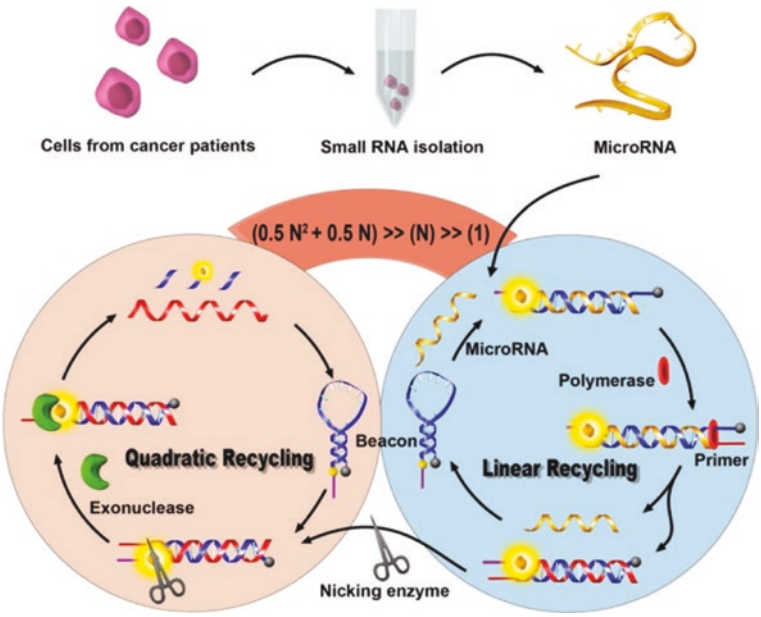

Fig. 5.14 Scheme for ultrasensitive detection of miRNAs at the single-cell level and in breast cancer patients using quadratic isothermal amplification. Reprinted with permission from Ref. [66]. Copyright 2013 American Chemical Society

fluorescent signal. Next, a primer anneals with the opened MB and allows polymerization with Bst polymerase, which displaces the target miRNA and forms a DNA duplex according to the MB probe. The displaced miRNA is free to bind to another beacon, triggering the next cycle and achieving the fluorescent signal amplification. By introducing the nicking enzyme and exonuclease, the beacon is cleaved and dissociated, achieving a quadratic signal amplification. Thus, once initiated, many copies of miRNA are generated in the first amplification cycle, and the nicking enzyme and exonuclease produce multiple copies of the newly recycled target DNA in the second cycle, enhancing the signal. This method exhibits ultrasensitivity with LOD of 10 fM at 37 °C and 1 aM at 4 °C (reaction temperature) for miRNA-21.

5.3 Probe Design Strategy Techniques in miRNA Optical Detection

Due to the short size of miRNAs, the traditional DNA oligonucleotide probe shows inefficiency in miRNAs detection. Probe design strategies have also been significantly improved in recent years; peptide nucleic acids (PNAs) and locked nucleic acids (LNA) oligonucleotide probes with considerable enhanced

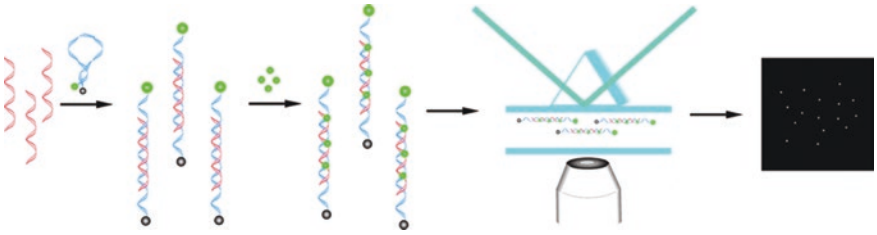

Fig. 5.15 Schematic illustration of the solution-based hybridization assay for the direct quantification of miRNAs with total internal reflection fluorescence microscopy (TIRFM). Reprinted with permission from Ref. [71]. Copyright 2014 American Chemical Society

sensitivity in detecting small RNA have been increasingly used in miRNA detection. PNAs are DNA analogues in which the anionic phosphate backbone is replaced by a neutral charge backbone, so that the less repulsion between PNA and RNA results in increased melting temperature and subsequently enhanced hybridization efficiency [67]. For miRNA detection, LNA (bicyclic RNA analogues)-based probes have shown great advantages to enable specific identification of highly similar sequences, such as miRNA family members and single mutations due to the remarkable affinity and specificity of LNA to miRNA [68, 69]. Várallyay et al. [70] describe an improved protocol for miRNA northern blot analysis using an LNA-modified oligonucleotide probe with a short detection time. As shown in Fig. 5.15, Ho et al. [71] have designed a strategy to differentiate the expression of a nasopharyngeal carcinoma (NPC) upregulator hsa-mir-205 (mir-205) in serum using an LNA -modified MB as the CP for overcoming the background matrix interference in serum and enhancing sensitivity and specificity. The LOD of this method is as low as 500 fM, and exhibits the capability of differentiating NPC stages by the level of mir-205 detection in serum with only 10 μL of serum in a short time.

References

1. Cao H, Liu S, Tu W, Bao J, Dai Z (2014) Chem Commun 50:13315–13318. doi:10.1039/c4cc06214k
2. Dong H, Jin S, Ju H, Hao K, Xu LP, Lu H, Zhang X (2012) Anal Chem 84:8670–8674. doi:10.1021/ac301860v
3. Tu Y, Wu P, Zhang H, Cai C (2012) Chem Commun 48:10718–10720. doi:10.1039/c2cc35564g
4. Wang L, Cheng Y, Wang H, Li Z (2012) Analyst 137:3667–3672. doi:10.1039/c2an35396b
5. Ryoo S-R, Lee J, Yeo J, Na H-K, Kim Y-K, Jang H, Lee JH, Han SW, Lee Y, Kim VN, Min D-H (2013) ACS Nano 7:5882–5891. doi:10.1021/nn401183s
6. Xi Q, Zhou DM, Kan YY, Ge J, Wu ZK, Yu RQ, Jiang JH (2014) Anal Chem 86:1361–1365. doi:10.1021/ac403944c
7. Dong H, Lei J, Ding L, Wen Y, Ju H, Zhang X (2013) Chem Rev 113:6207–6233. doi:10.1021/cr300362f
8. Zhang Y, Hu J, Zhang CY (2012) Anal Chem 84:9544–9549. doi:10.1021/ac3024087

9. Liu J, Lu Y (2006) Nat Protoc 1:246–252. doi:10.1038/nprot.2006.38
10. Alhasan AH, Kim DY, Daniel WL, Watson E, Meeks JJ, Thaxton CS, Mirkin CA (2012) Anal Chem 84:4153–4160. doi:10.1021/ac3004055
11. Shen W, Deng H, Ren Y, Gao Z (2013) Chem Commun 49:4959–4961. doi:10.1039/c3cc41565a
12. Mirkin CA, Letsinger RL, Mucic RC, Storhoff JJ (1996) Nature 382:607–609
13. Nam J-M, Stoeva SI, Mirkin CA (2004) J Am Chem Soc 126:5932–5933
14. Hill HD, Mirkin CA (2006) Nat Protoc-Electron Ed- 1:324
15. Degliangeli F, Kshirsagar P, Brunetti V, Pompa PP, Fiammengo R (2014) J Am Chem Soc 136:2264–2267. doi:10.1021/ja412152x
16. Hudson SD, Chumanov G (2009) Anal Bioanal Chem 394:679–686. doi:10.1007/s00216-009-2756-2
17. Driskell JD, Seto AG, Jones LP, Jokela S, Dluhy RA, Zhao YP, Tripp RA (2008) Biosens Bioelectron 24:923–928. doi:10.1016/j.bios.2008.07.060
18. Abell JL, Garren JM, Driskell JD, Tripp RA, Zhao Y (2012) J Am Chem Soc 134:12889–12892. doi:10.1021/ja3043432
19. Ye LP, Hu J, Liang L, Zhang CY (2014) Chem Commun 50:11883–11886. doi:10.1039/c4cc05598e
20. Patel AS, Mohanty T (2013) J Mater Sci 49:2136–2143. doi:10.1007/s10853-013-7906-4
21. Yang SW, Vosch T (2011) Anal Chem 83:6935–6939. doi:10.1021/ac201903n
22. Liu YQ, Zhang M, Yin BC, Ye BC (2012) Anal Chem 84:5165–5169. doi:10.1021/ac300483f
23. Zhang M, Liu YQ, Yu CY, Yin BC, Ye BC (2013) Analyst 138:4812–4817. doi:10.1039/c3an00666b
24. Xia X, Hao Y, Hu S, Wang J (2014) Biosens Bioelectron 51:36–39. doi:10.1016/j.bios.2013.07.036
25. Shah P, Thulstrup PW, Cho SK, Bhang YJ, Ahn JC, Choi SW, Bjerrum MJ, Yang SW (2014) Analyst 139:2158–2166. doi:10.1039/c3an02150e
26. Li J, Zhu J-J (2013) The Analyst 138:2506. doi:10.1039/c3an36705c
27. Zhang C-Y, Yeh H-C, Kuroki MT, Wang T-H (2005) Nat Mater 4:826–831. doi:10.1038/nmat1508
28. Zeng YP, Zhu G, Yang XY, Cao J, Jing ZL, Zhang CY (2014) Chem Commun 50:7160–7162. doi:10.1039/c4cc02034k
29. Cheng Y, Lei J, Chen Y, Ju H (2014) Biosens Bioelectron 51:431–436. doi:10.1016/j.bios.2013.08.014
30. Li F, Peng J, Wang J, Tang H, Tan L, Xie Q, Yao S (2014) Biosens Bioelectron 54:158–164. doi:10.1016/j.bios.2013.10.061
31. Tian Q, Wang Y, Deng R, Lin L, Liu Y, Li J (2014) Nanoscale 7:987–993. doi:10.1039/c4nr05243a
32. Yang R, Jin J, Chen Y, Shao N, Kang H, Xiao Z, Tang Z, Wu Y, Zhu Z, Tan W (2008) J Am Chem Soc 130:8351–8358
33. Dong H, Zhang J, Ju H, Lu H, Wang S, Jin S, Hao K, Du H, Zhang X (2012) Anal Chem 84:4587–4593. doi:10.1021/ac300721u
34. Zhu C, Zeng Z, Li H, Li F, Fan C, Zhang H (2013) J Am Chem Soc 135:5998–6001. doi:10.1021/ja4019572
35. Wang Q, Wang W, Lei J, Xu N, Gao F, Ju H (2013) Anal Chem 85:12182–12188. doi:10.1021/ac403646n
36. Lee K, Povlich LK, Kim J (2010) Analyst 135:2179–2189. doi:10.1039/c0an00239a
37. Zhang Y, Li Z, Cheng Y, Lv X (2009) Chem Commun 22:3172–3174. doi: 10.1039/b904579a
38. Wu H, Cisneros BT, Cole CM, Devaraj NK (2014) J Am Chem Soc 136:17942–17945. doi:10.1021/ja510839r
39. Varkonyi-Gasic E, Wu R, Wood M, Walton EF, Hellens RP (2007) Plant methods 3:12. doi:10.1186/1746-4811-3-12
40. Li J, Yao B, Huang H, Wang Z, Sun C, Fan Y, Chang Q, Li S, Wang X, Xi J (2009) Anal Chem 81:5446–5451. doi:10.1021/ac900598d

41. Jonstrup SP, Koch J, Kjems J (2006) RNA 12:1747–1752. doi:10.1261/rna.110706
42. Lenarcic R, Morisset D, Pirc M, Llop P, Ravnikar M, Dreo T (2014) PLoS ONE 9:e96027. doi:10.1371/journal.pone.0096027
43. Bi S, Zhang J, Hao S, Ding C, Zhang S (2011) Anal Chem 83:3696–3702. doi:10.1021/ac200096b
44. Yuan Z, Zhou Y, Gao S, Cheng Y, Li Z (2014) ACS Appl Mater Interfaces 6:6181–6185. doi:10.1021/am500883q
45. Luo J, Bergstrom DE, Barany F (1996) Nucleic Acids Res 24:3071–3078. doi:10.1093/nar/24.15.3071
46. Yan J, Li Z, Liu C, Cheng Y (2010) Chem Commun 46:2432–2434. doi:10.1039/b923521c
47. Zhang P, Zhang J, Wang C, Liu C, Wang H, Li Z (2014) Anal Chem 86:1076–1082. doi:10.1021/ac4026384
48. Zhang J, Li Z, Wang H, Wang Y, Jia H, Yan J (2011) Chem Commun 47:9465–9467. doi:10.1039/c1cc13466c
49. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, Lao KQ, Livak KJ, Guegler KJ (2005) Nucleic Acids Res 33:e179. doi:10.1093/nar/gni178
50. Dong H, Wang C, Xiong Y, Lu H, Ju H, Zhang X (2013) Biosens Bioelectron 41:348–353. doi:10.1016/j.bios.2012.08.050
51. Cheng Y, Zhang X, Li Z, Jiao X, Wang Y, Zhang Y (2009) Angew Chem 48:3268–3272. doi:10.1002/anie.200805665
52. Jin G, Wang C, Yang L, Li X, Guo L, Qiu B, Lin Z, Chen G (2015) Biosens Bioelectron 63:166–171. doi:10.1016/j.bios.2014.07.033
53. Lin C, Xie M, Chen JJ, Liu Y, Yan H (2006) Angew Chem 45:7537–7539. doi:10.1002/anie.200602113
54. Ali MM, Li F, Zhang Z, Zhang K, Kang DK, Ankrum JA, Le XC, Zhao W (2014) Chem Soc Rev 43:3324–3341. doi:10.1039/c3cs60439j
55. Cui L, Zhu Z, Lin N, Zhang H, Guan Z, Yang CJ (2014) Chem Commun 50:1576–1578. doi:10.1039/c3cc48707e
56. Kim J, Easley CJ (2011) Bioanalysis 3:227–239. doi:10.4155/bio.10.172
57. Zhu RY, Zhang KX, Zhao MQ, Liu YH, Xu YY, Ju CM, Li B, Chen JD (2009) J Microbiol Methods 78:339–343. doi:10.1016/j.mimet.2009.07.006
58. Tomita N, Mori Y, Kanda H, Notomi T (2008) Nat Protoc 3:877–882. doi:10.1038/nprot.2008.57
59. Li C, Li Z, Jia H, Yan J (2011) Chem Commun 47:2595–2597. doi:10.1039/c0cc03957h
60. Yan L, Zhou J, Zheng Y, Gamson AS, Roembke BT, Nakayama S, Sintim HO (2014) Mol BioSyst 10:970–1003. doi:10.1039/c3mb70304e
61. Fang X, Liu Y, Kong J, Jiang X (2010) Anal Chem 82:3002–3006. doi:10.1021/ac1000652
62. Yu Y, Chen Z, Shi L, Yang F, Pan J, Zhang B, Sun D (2014) Anal Chem 86:8200–8205. doi:10.1021/ac501505a
63. Van Ness J, Van Ness LK, Galas DJ (2003) Proc Natl Acad Sci USA 100:4504–4509. doi:10.1073/pnas.0730811100
64. Wang GL, Zhang CY (2012) Anal Chem 84:7037–7042. doi:10.1021/ac3012544
65. Jia H, Li Z, Liu C, Cheng Y (2010) Angew Chem Int Ed 49:5498–5501. doi:10.1002/anie.201001375
66. Duan R, Zuo X, Wang S, Quan X, Chen D, Chen Z, Jiang L, Fan C, Xia F (2013) J Am Chem Soc 135:4604–4607. doi:10.1021/ja311313b
67. Zhang GJ, Chua JH, Chee RE, Agarwal A, Wong SM (2009) Biosens Bioelectron 24:2504–2508. doi:10.1016/j.bios.2008.12.035
68. Jorgensen S, Baker A, Moller S, Nielsen BS (2010) Methods 52:375–381. doi:10.1016/j.ymeth.2010.07.002
69. Østergaard ME, Hrdlicka PJ (2011) Chem Soc Rev 40:5771–5788
70. Várallyay É, Burgyán J, Havelda Z (2008) Nat Protoc 3:190–196
71. Ho SL, Chan HM, Ha AW, Wong RN, Li HW (2014) Anal Chem 86:9880–9886. doi:10.1021/ac5025182

Chapter 6
Other Emerging miRNA Detection Strategies

Abstract A number of specialized miRNA detection strategies have been recently reported, including surface plasmon resonance (SPR), microfluidic technique, next-generation sequencing, laser ablation (LA) inductively coupled plasma mass spectrometry (ICPMS), and so on. With a sensitive, rapid, and on-site analysis, SPR is an attractive alternative to conventional techniques in miRNA detection. Referring to small sample volume and rapid detection for POC diagnosis, microfluidic technique is a commendable choice. The possibility of new miRNA discovery is the major advantage of next-generation sequencing. And using LA-ICPMS for miRNA detection makes multiplexed, sensitive, and quantitative analysis possible with the sensitivity comparable to radioactive detection. For all these advantages, it is believed that in the future, these new technologies will emerge as an efficient and alternative tool for daily miRNA analysis.

Keywords miRNA detection strategies · Surface plasmon resonance · Microfluidic technique · Next-generation sequencing · ICP-MS

6.1 Surface Plasmon Resonance (SPR)

Surface plasmon resonance (SPR), as a charge-density oscillation at the interface between two media, can generate a surface plasmon wave (SPW) with a propagation constant β and with dielectric constants of opposite signs [1]. Since the propagation of the SPW is sensitive to the changes in the refractive index of the dielectric, an SPR-based biosensor is considered as a refractometric device. The binding between analytes and bioreceptors immobilized on the sensing surface causes a local change in refractive index and the propagation constant β and generates a real-time signal in a label-free way (Fig. 6.1).

SPR is an attractive alternative to conventional techniques in miRNA detection[1] with a sensitive, rapid, and on-site analysis [2]. Many developments for the

[1]Haifeng Dong and Conghui Liu contributed together to this chapter.

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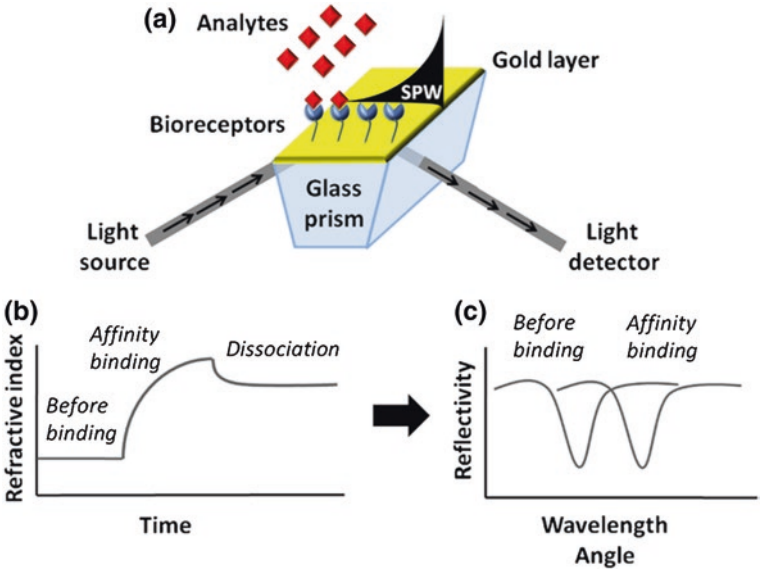

Fig. 6.1 **a** In an SPR biosensor based on a prism coupler, light is totally reflected by a thin gold layer (50 nm) exciting the surface plasmons (SPs) and generates an SPW. **b** The affinity binding between bioreceptors and analytes causes a change in refractive index versus time. **c** Entailing a shift of the reflectivity curve versus wavelength of source light or angle of reflected light. Reprinted from Ref. [1], with kind permission from Springer Science+Business Media

improvement of SPR technology were achieved in terms of sensitivity and specificity [3]. Zhang et al. described a fast and simple method based on a hybrid sandwich-like assay for detection of miR-122 extracted from human breast tumor cells (Fig. 6.2) [4]. MiR-122 was first hybridized from a fully complementary thiolated DNA probe, which was immobilized on the sensor surface using a Biacore X system. After that, a secondary biotinylated DNA probe [linked to streptavidin-oligonucleotide (SON) complex] was hybridized to the target. This method enhances sensitivity, lowers the detection limit down to 17 pM, and shows good reproducibility (CV% = 5.7 %) with PCR-free compounds.

By using SPR technology and a DNA*RNA antibody-based assay, an approach detecting miRNA in less than 30 min at a concentration down to 2 pM has been developed [5]. In addition, the SPR imaging has also been designed for LNA microarrays that are able to detect miRNAs down to 10 fM. There are three steps in the detection and identification of miRNAs (Fig. 6.3): [6]. First, target miRNAs are adsorbed from solution onto a single-stranded LNA microarray (step i). Then, by poly(A) polymerase surface reaction, poly (A) tails are added to the surface bound miRNAs. Finally, the poly (A) tails are hybridized with T30 DNA coated gold nanoparticles (AuNPs) for signal amplification, followed by SPR imaging detection. This ultrasensitive nanoparticle-amplified SPR imaging method can be used for miRNA concentrations determination in a total RNA sample from mouse liver tissue. Using enzymatic silica nanoparticles combined with an SPR imaging

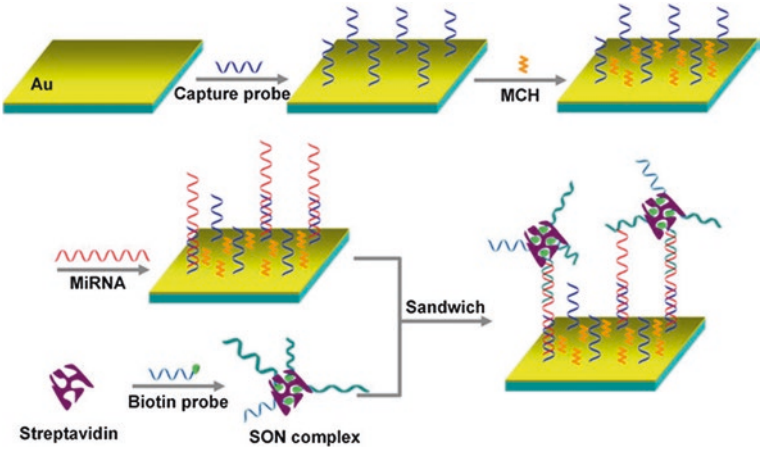

Fig. 6.2 Schematic of the miRNA assay using an SPR biosensor and SON complex for signal amplification. Reprinted from Ref. [4], with kind permission from Springer Science+Business Media

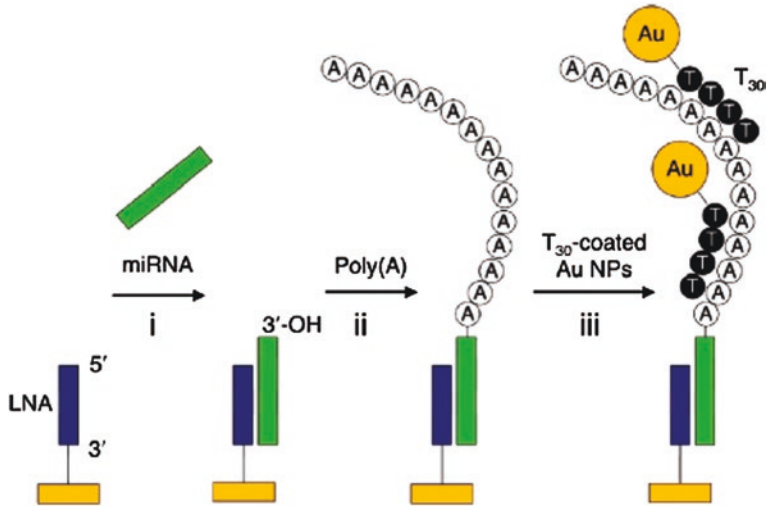

Fig. 6.3 Schematic showing the detection of miRNAs using a combination of surface polyadenylation chemistry and nanoparticle amplified SPRI detection: (i) hybridization adsorption of miRNA onto a complementary LNA array element; (ii) addition of poly(A) tails to the surface bound miRNAs using poly(A) polymerase; and (iii) hybridization adsorption of T30-coated Au nanoparticles to poly(A) tails detected by SPRI measurements. Reprinted with permission from Ref. [6]. Copyright 2006 American Chemical Society

measurement, subpicomolar concentrations of multiple short ssRNA can also be detected for this process [7]. Although the above two assays were used for detection of miRNA from mouse liver, they represented the improvement of analytical performance; which is suitable for future applications to human miRNA detection.

6.2 Microfluidic Technique

A method using a microfluidic device is an attractive choice to meet the requirements of point-of-care (POC) miRNA detection. Using this device can thoroughly shorten the incubation time, due to the straight conveyance of sample molecules to the surface-immobilized probe DNAs by hydrodynamic force [8]. Moreover, compared to microarrays and PCR tubes, smaller sample volumes are required for microchannels. Owing to these properties, microfluidic devices have the possibility to solve problems referring to small sample volume and rapid detection for POC diagnosis, which are difficult with existing technologies.

The power-free microfluidic device allowing self-pumping by poly-dimethylsiloxane (PDMS) air absorption need not connect to an external power supply. Considering the potential for POC applications in the future, it is a significant advantage. The energy stored in degassed PDMS prior to use is the driving force of the power-free microfluidic device [9]. The large amount of air contained by PDMS at atmospheric pressure can be evacuated in a vacuum chamber, and when it is brought back under atmospheric pressure, the air dissolves into the PDMS again. By this air redissolution process, the reduced pressure forces the solutions to be pulled in the outlet reservoir. Furthermore, since in a gas-tight condition the degassed device can be preserved, the device degassing does not need to be performed at the time of the operation [10]. The operation of a power-free microfluidic chip has the properties of compactness, portability, and simplicity, which may overcome the problems of need for an external power source and skilled personnel.

The highly sensitive detection of small and non-labeled miRNAs by surface sandwich hybridization is one of the major challenges existing in the microfluidic approach. In order to solve the issues, two key strategies have been presented to address this. One of them is combination of a laminar flow-assisted dendritic amplification (LFDA) and coaxial stacking-aided sandwich hybridization, and the other depends on the coaxial stacking effect of small DNA and miRNA during sandwich hybridization. Using the power-free microfluidic chip, detection of subattomole miRNAs can be realized in 20 min by both strategies.

LFDA is a signal amplification method for microfluidic analysis of surface-bound molecules [11], which was performed by a device with a pair of microchannels arranged in a Y configuration (Fig. 6.4a). Two amplification reagents, including one fluorescent reagent, were supplied from laminar streams to the surface-bound molecules in LFDA. Both reagents constructed dendritic structures growing over time and amplified fluorescent signals.

LFDA is based on the continuous three-phase reaction at the interaction of two laminar streams and a solid surface. Laminar flow, as a common phenomenon in microchannels, is characterized by low Reynolds numbers [12]. Two different solutions are mixed when they are pumped into a microchannel by the interface diffusion between the two streams (Fig. 6.4b). The components of the two streams are simultaneously and continuously supplied onto the solid surface at the contact line between the two streams and the channel ceiling or floor (Fig. 6.4c) [11].

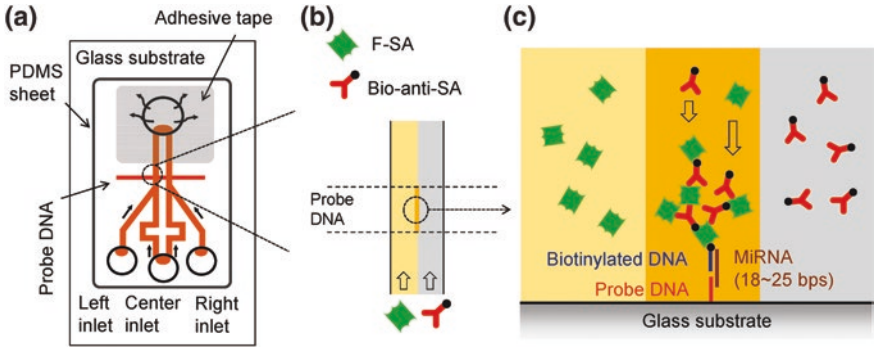

Fig. 6.4 **a** Schematics of the power-free microfluidic device. The width of the DNA probe pattern was 100 μm, and it was located 500 μm downstream of the Y-shaped channel confluent point. **b** Enlarged view of a laminar flow in the microchannel. F-SA and Bio-anti-SA are conveyed by the laminar flow. **c** Enlarged cross-sectional view of the sandwich hybridization and LFDA. F-SA, FITC-labeled streptavidin; Bio-anti-SA, biotinylated antistreptavidin. Reprinted with permission from Ref. [11]. Copyright 2007 American Chemical Society

Coaxial stacking-aided hybridization promotes the affinity and stability of oligonucleotide hybridization [13, 14]. The dipole-induced dipole–dipole interaction between the planar aromatic bases in two contiguous nucleotides is the base stacking. Coaxial stacking at the nick site stabilizes hybridization when two neighboring sequences are annealed on a longer strand. Maeda's group presented a method that targets miRNA detection using sandwich-hybridization, taking advantage of the coaxial stacking effect. In the experiment, a PDMS microfluidic device used with multiple microchannels 100 μm wide and 25 μm high. Amino-labeled probe DNAs were selectively immobilized on an aminated glass surface by glutaraldehyde (Fig. 6.5). This method was allowed to detect miR-21 in 20 min with a 0.5 mL sample volume at a limit of detection (LOD) of 0.62 nM [15], which was far above the criteria for practical application. Therefore, further improvement of the LOD by three orders of magnitude to 0.5 pM was made by adopting LFDA [11], which is a signal amplification method for microfluidic analysis of surface-bound molecules [16].

Generally speaking, the coaxial stacking effect has been proven to be helpful in DNA–RNA sandwich hybridization and has increased detection sensitivity by one order of magnitude. LFDA increased the fluorescent signal by three orders of magnitude. In addition to these strategies, various microfluidic-based strategies have been explored for miRNA detection. Beier's group introduced a microfluidic primer extension assay (MPEA) for miRNA detection on highly flexible microfluidic microarrays, which can analyze any type of lncRNA with the two-tier specificity of the conventional RAKE assay, reduce amounts of input RNA, and increase flexibility [17]. The qPCR-array based microfluidic dynamic array platform developed by Jen et al. can be used in conjunction with multiplexed RT reactions for miRNA gene expression profiling. This approach is highly reproducible and the results correlate closely with the existing singleplex qPCR platform

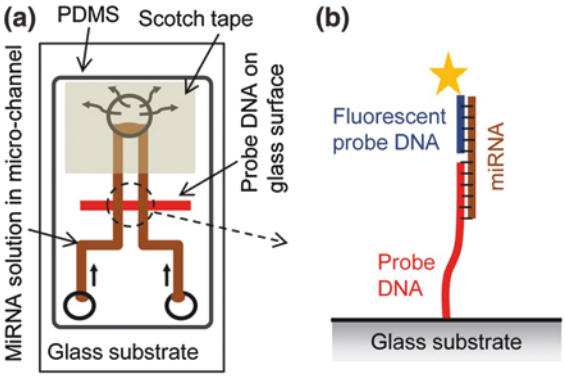

Fig. 6.5 **a** Schematics of the power-free microfluidic device. PDMS absorbs air in the outlet chamber, being a self-standing pumping device. Probe DNA is immobilized onto the glass surface and microchannels convey the sample to the probe. **b** Schematics of sandwich hybridization. Half of the miRNA sequence is complementary to the fluorescent labeled probe DNA and the other half is complementary to the probe DNA immobilized on the glass surface. Reproduced from Ref. [15] by permission of The Royal Society of Chemistry

at a throughput that is 5–20 times higher and a sample and reagent usage that was about 50–100 times lower than conventional assays. They established optimal conditions for using the Fluidigm microfluidic technology for rapid, cost-effective, and customizable arrays for miRNA expression profiling and validation [18]. In 2011, a new approach for highly sensitive detection of miRNA on a microfluidic-assisted microarray was demonstrated. Due to its unmatched resolution, the technique could detect as low as 300 copies of target miRNAs in a sample volume of 1.0 ml. With the greatly improved sensitivity, the amount of total RNA needed in the assay is reduced to only a few nanograms, providing an excellent opportunity for fast and direct miRNA profiling without any labeling and amplification procedure [19].

For further practical applications, optical or electrical devices for signal readouts should be miniaturized and integrated on the same chip. It should also be possible that this technology is combined with existing electronic devices, such as cell phones [20]. The ideal properties for POC diagnosis include rapidity, small sample volume, simple operation, and portability of the device. Hence, further study in commercialization might be helpful to improve global health and healthcare environments even in regions that lack resources.

6.3 Next-Generation Sequencing

Next-generation sequencing, allowing the sequencing of small RNA molecules and the calculation of their expression levels, involves enzymatic generation of a cDNA library, which was followed by extensive parallel sequencing of individual

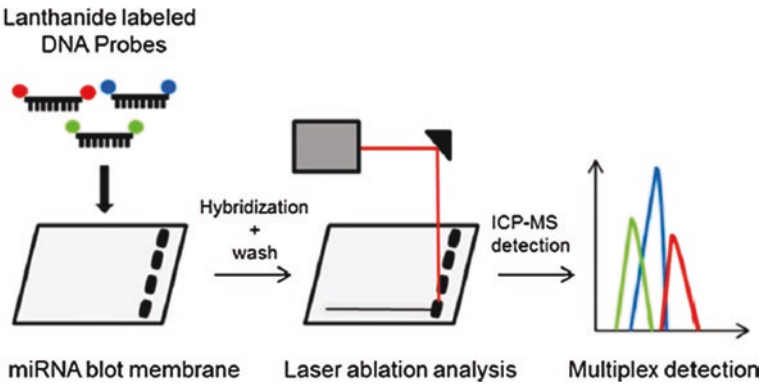

Fig. 6.6 Scheme of miRNAs that were northern blotted and incubated with lanthanide-labeled DNA probes. Reprinted with permission from Ref. [25]. Copyright 2014 American Chemical Society

cDNA molecules. The possibility of new miRNA discovery is the major advantage of next-generation sequencing. Based on this technology, researchers have witnessed a large increase of newly discovered miRNA sequences in the past few years. However, miRBase has introduced a high confidence subset of miRNA entries, since loci annotated as new miRNAs have been identified under inhomogeneous stringency criteria [21]. By comparison of numerous sequencing data sets available in the miRBase library, this entirety is obtained under the constraint of specified criteria for the identification of miRNA.

Bioinformatics analysis of the sequence reads may be used to quantify relative miRNA by a digital approach, which is estimation of the relative abundance of a specific miRNA from multiple reads for its sequence normalized to the total reads in the sample [22]. However, exorbitant price and requirements of highly specialized computational infrastructures and expertise still limit the large-scale application of this promising technology [23, 24].

6.4 ICP-MS

Multiplexed analysis, the detection of several miRNAs in the same sample at a given run, is desirable because it enables the direct comparison of several miRNAs in one analysis, while simultaneously reducing the associated labor and costs. However, it still remains a challenge due to the chemical properties of the reporter tags combined with the narrow size range of miRNAs [25].

Recently, analytical approaches for multiplexed protein analysis based on lanthanide-labeled antibodies including laser ablation (LA) inductively coupled plasma mass spectrometry (ICPMS) of Western blot membranes have been developed, which were combined with ICPMS detection [26]. Bang and co-workers

combined the lanthanide labeled DNA probes with LA-ICPMS to detect miRNAs on Northern blot membranes (Fig. 6.6). The DNA probes used for hybridization had full sequence complementarity to the miRNA targets, ensuring high specificity of the assay. Using LA-ICPMS for miRNA detection makes multiplexed, sensitive, and quantitative analysis possible with sensitivity comparable to radioactive detection (low femtomolar range) [25].

References

1. Mariani S, Minunni M (2014) Surface plasmon resonance applications in clinical analysis. Anal Bioanal Chem 406:2303–2323
2. Nasheri N, Cheng J, Singaravelu R, Wu P, McDermott MT, Pezacki JP (2011) An enzyme-linked assay for the rapid quantification of microRNAs based on the viral suppressor of RNA silencing protein p19. Anal Biochem 412:165–172
3. Spoto G, Minunni M (2012) Surface plasmon resonance imaging: what next? J Phys Chem Lett 3:2682–2691
4. Zhang D, Yan Y, Cheng W, Zhang W, Li Y, Ju H, Ding S (2013) Streptavidin-enhanced surface plasmon resonance biosensor for highly sensitive and specific detection of microRNA. Microchim Acta 180:397–403
5. Sipova H, Zhang S, Dudley AM, Galas D, Wang K, Homola J (2010) Surface plasmon resonance biosensor for rapid label-free detection of microribonucleic acid at subfemtomole level. Anal Chem 82:10110–10115
6. Fang S, Lee HJ, Wark AW, Corn RM (2006) Attomole microarray detection of microRNAs by nanoparticle-amplified SPR imaging measurements of surface polyadenylation reactions. J Am Chem Soc 128:14044–14046
7. Zhou W-J, Chen Y, Corn RM (2011) Ultrasensitive microarray detection of short RNA sequences with enzymatically modified nanoparticles and surface plasmon resonance imaging measurements. Anal Chem 83:3897–3902
8. Arata H, Hosokawa K, Maeda M (2014) Rapid sub-attomole microRNA detection on a portable microfluidic chip. Anal Sci 30:129–135
9. Hosokawa K, Sato K, Ichikawa N, Maeda M (2004) Power-free poly(dimethylsiloxane) microfluidic devices for gold nanoparticle-based DNA analysis. Lab Chip 4:181–185
10. Hosokawa K, Omata M, Sato K, Maeda M (2006) Power-free sequential injection for microchip immunoassay toward point-of-care testing. Lab Chip 6:236–241
11. Hosokawa K, Omata M, Maeda M (2007) Immunoassay on a power-free microchip with laminar flow-assisted dendritic amplification. Anal Chem 79:6000–6004
12. Atencia J, Beebe DJ (2005) Controlled microfluidic interfaces. Nature 437:648–655
13. Yuan B-F, Zhuang X-Y, Hao, Y-H, Tan Z (2008) Kinetics of base stacking-aided DNA hybridization. Chem Commun 6600–6602
14. Cai S, Lau C, Lu J (2010) Sequence-specific detection of short-length DNA via template-dependent surface-hybridization events. Anal Chem 82:7178–7184
15. Arata H, Komatsu H, Han A, Hosokawa K, Maeda M (2012) Rapid microRNA detection using power-free microfluidic chip: coaxial stacking effect enhances the sandwich hybridization. Analyst 137:3234–3237
16. Arata H, Komatsu H, Han AS, Hosokawa K, Maeda M (2012) Rapid microRNA detection using power-free microfluidic chip: coaxial stacking effect enhances the sandwich hybridization. Analyst 137:3234–3237
17. Vorwerk S, Ganter K, Cheng Y, Hoheisel J, Stahler PF, Beier M (2008) Microfluidic-based enzymatic on-chip labeling of miRNAs. New Biotechnol 25:142–149

18. Jang JS, Simon VA, Feddersen RM, Rakhshan F, Schultz DA, Zschunke MA, Lingle WL, Kolbert CP, Jen J (2011) Quantitative miRNA expression analysis using fluidigm microfluidics dynamic arrays. BMC Genom 12:144
19. Roy S, Soh JH, Gao Z (2011) A microfluidic-assisted microarray for ultrasensitive detection of miRNA under an optical microscope. Lab Chip 11:1886–1894
20. Zhu H, Mavandadi S, Coskun AF, Yaglidere O, Ozcan A (2011) Optofluidic fluorescent imaging cytometry on a cell phone. Anal Chem 83:6641–6647
21. Kozomara A, Griffiths-Jones S (2014) miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res 42:D68–D73
22. Pritchard CC, Cheng HH, Tewari M (2012) MicroRNA profiling: approaches and considerations. Nat Rev Genet 13:358–369
23. Gomes CPC, Cho J-H, Hood L, Franco OL, Pereira RW, Wang K (2013) A review of computational tools in microRNA discovery. Front Genetics 4:81
24. Krzyzanowski PM, Muro EM, Andrade-Navarro MA (2012) Computational approaches to discovering noncoding RNA. Wiley Interdiscip Rev RNA 3:567–579
25. de Bang TC, Shah P, Cho SK, Yang SW, Husted S (2014) Multiplexed microRNA detection using lanthanide-labeled DNA probes and laser ablation inductively coupled plasma mass spectrometry. Anal Chem 86:6823–6826
26. Waentig L, Jakubowski N, Roos PH (2011) Multi-parametric analysis of cytochrome P450 expression in rat liver microsomes by LA-ICP-MS. J Anal At Spectrom 26:310–319

Chapter 7
Intracellular and Organic miRNA In Situ Detection

Abstract The detection methods of miRNA in intracellular or organisms fall into two broad categories: indirect detection and direct analysis. The indirect measurement of the expression levels of miRNAs in cells and tissues involves cells lysis and detection by qRT-PCR, northern blotting, or microarray hybridization. The direct analysis methods are a noninvasive manner for repetitively monitoring and obtaining real-time imaging of the intracellular miRNA by using imaging analysis or in situ hybridization (ISH). Technologies for direct detection of the temporal and spatial expression sequence of specific miRNA in cells or tissues are extremely important for elucidating miRNA biology. The progress of optical imaging techniques with multimodal reporter systems holds great promise for noninvasive and real-time imaging of molecular agent expression in living cell. Recent progress in nanotechnology and imaging detection techniques leads to multifunctional nanoprobe with specific-transfection, tracing, and regulation function in intracellular miRNA detection. ISH holds great promise for visualization of the spatial localization of RNA at the tissue, cellular, and even subcellular level.

Keywords Intracellular miRNA · Organic miRNA · In situ detection · Cell imaging analysis · Functional nanoprobe

The detection of intracellular miRNA is significant in the development of gene therapy and gene medicines. The deficiency of small size and degradation of the mature miRNAs make it difficult to directly transfer the specific miRNA into the cell. A noninvasive manner for repetitively monitoring and obtaining real-time imaging of the intracellular miRNA is required for the analysis of miRNAs in practical clinic application. Efficient gene vectors, including viral [1] and nonviral categories, [2] were usually required to translocate miRNA probe through membrane barrier into the cell. Retrovirus and adenovirus vectors can be effectively transferred via the gene probes (DNA, RNA) into most cell lines. However, the

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high cell toxicity of these vectors easily induces cell-mediated immunity response and limits the broad application [3]. The nonviral vectors show less efficiency compared to viral vector in delivering the gene probe and preventing it from degradation of the cell autoimmunity, but good biocompatibility and large-scale production of nonviral vectors make it promising in gene therapy research [4]. Various nonviral gene vectors such as liposomes, [5] cationic polymers, [6] dendritic polymers, [7] polymeric peptides [8], and nanoparticles [9–11] have been continuously explored for gene therapy.

Recent significant progress in optical imaging techniques with multimodal reporter systems holds great promise for noninvasive and real-time imaging of molecular agent expression in living cell. Various reporter-based optical imaging systems such as luciferase or fluorescent probes, and radionuclide imaging with sodium iodide symporter and herpes simplex virus 1-thymidine kinase (as report) have been developed for endogenous gene expression [12–15]. These emerging miRNA detection strategies provide a better understanding of miRNAs expression profile in human diseases and the function in biological process. Notably, the progress of nanotechnology combining with novel molecular modification and imaging detection technique allows development of multifunctional nanoprobe with specific-transfection, tracing, and regulation function in intracellular miRNA detection.

7.1 Functional Nanoprobe-Based Intracellular miRNA Detection

Generally, an efficient nanovector consists of three functional moieties including specific target cell moieties, tracer, and gene probe. Due to good biocompatibility and larger surface, carbonaceous nanomaterials are always used to develop gene vector. The application of nanosized GO (NGO) in detection of multiple miRNA targets inside the living cells has been reported. Dal-Hee Min and co-workers [16] develop a biosensor for quantitative monitoring of target miRNA expression levels in living cells. This strategy is based on tight binding of NGO with peptide nucleic acid (PNA) probes, resulting in fluorescence quenching of the dye that is conjugated to the PNA, and subsequent recovery of the fluorescence upon addition of target miRNA by forming a DNA/miRNA heteroduplex (Fig. 7.1). The present miRNA sensor allowed the detection of specific target miRNAs, with the detection limit as low as 1 pM and the simultaneous monitoring of three different miRNAs in a living cell. A simple nanocarrier of polyethylenimine-grafted graphene nanoribbon (PEI-g-GNR) was designed by Ju and co-workers as effective gene vector for cellular delivery of a locked nucleic acid-modified molecular beacon for recognition of miRNA [7]. The obtained PEI-g-GNR exhibited good protective properties, efficient transfection efficiency, and negligible cytotoxicity, which can effectively deliver detection of LNA-m-MB into the cells to recognize the target miRNA-21 in HeLa cells. Furthermore, the same group developed a functionalized

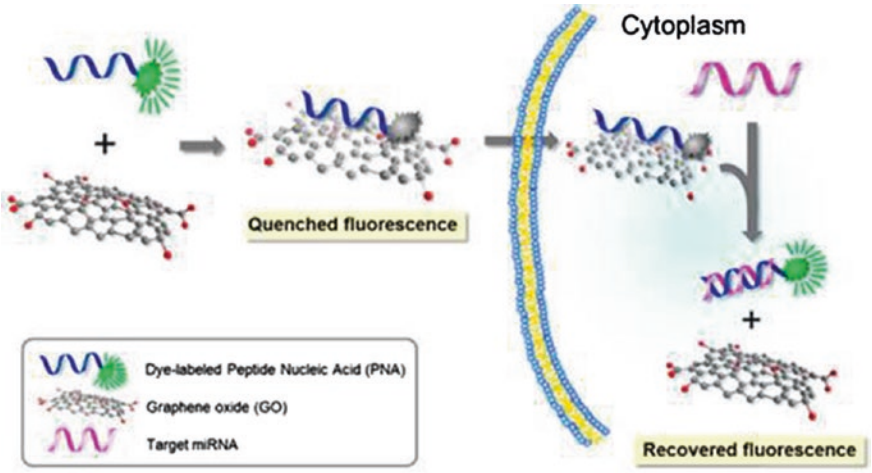

Fig. 7.1 Scheme for quantitative and multiplexed miRNA sensing in living cells based on peptide nucleic acid and nano graphene oxide (PANGO). Reprinted with the permission from Ref. [16]. Copyright 2011 American Chemical Society

graphene oxide probe to mediated adriamycin delivery and miRNA-21 gene silencing to overcome tumor multidrug resistance in vitro [17].

Silicon nanomaterials are another class of widely used nanodelivery compounds. In order to tackle the low-abundant miRNAs in a single cell, Zhu et al. [18] reported a multifunctional mesoporous silicon probe-based programmable nanodevices using DNAzymes to realize stringent miRNAs multiplexed detection and controlled drug release (Fig. 7.2). In this system, the MNAzyme motifs act as three-dimensional gatekeepers to lock the doxorubicin inside the nanocarriers. When nanocarriers were exclusively transfected into the target tumor cells via aptamer-guided recognition, the endogenous miRNAs reopened the gate to release the drug. Notably, the release rates could be spatial–temporally controlled by the modulation of miRNA expression, which provide a general strategy combining chemotherapy and gene therapy for disease diagnosis and prognosis. Zhang et al. [19] have employed a fluorescent silica/metal core–shells nanocomposite as a molecular imaging reporter to detect single miRNA molecules in the lung cancer cells. These metal nanoshells composed of silica spheres with encapsulated $Ru(bpy)_3^{2+}$ complexes as cores and thin silver layers as shells, which display an enhanced emission intensity, shortened lifetime and extended photostability compared to silica spheres alone. Liu et al. [20] designed a multifunctional fluorescent nanoprobe with the AS1411 aptamer as target-cell-specific moieties and a molecular beacon as the detection probe on the surface of the fluorescent dye-doped silica nanoparticles (FSiNPs) for delivery and imaging intracellular miRNA.

Magnetic nanomaterials-based magnetic resonance imaging (MRI) offers great spatial resolution for viewing cells and deep tissues. Yang et al. [21] have synthesized Fe_3O_4@polydopamine coreshell nanocomposites (Fe_3O_4@PDA NCs)

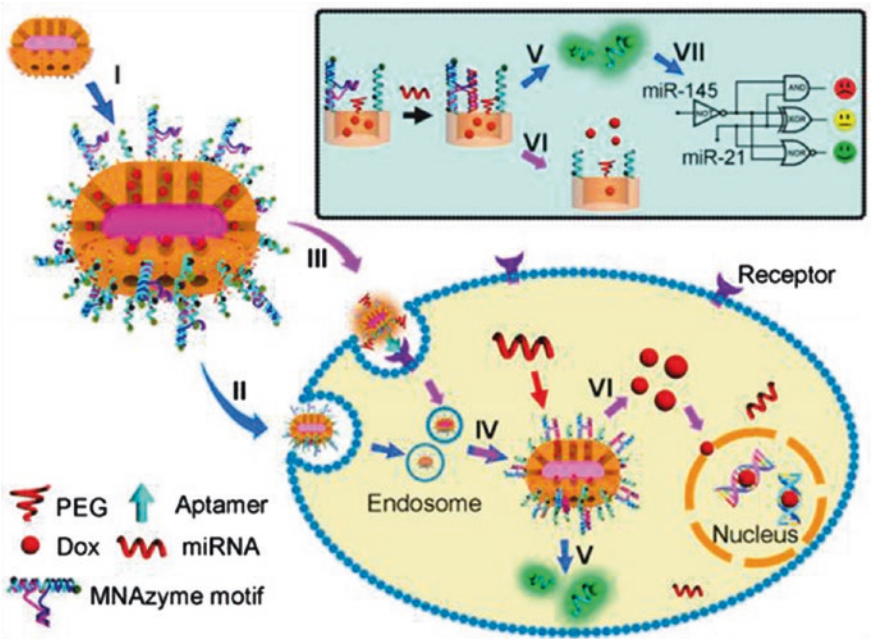

Fig. 7.2 Schematic illustration of in situ amplification of intracellular miRNA with MNAzyme nanodevices for multiplexed imaging, logic operation, and controlled drug release. Reprinted with the permission from Ref. [18]. Copyright 2011 American Chemical Society

using an in situ self-polymerization method. The PDA shell layer delivers the nanocomposites near-infrared absorption, high fluorescence quenching efficiency, and facilitated functional surface. The obtained Fe_3O_4@PDA NCs could acts as theranostic agents for both intracellular mRNA detection and multimodal imaging-guided photothermal therapy. A self-assembling signal on a magnetic fluorescence nanoparticle was self-assembled to a signal on multifunctional system to visualize intracellular miRNAs during neurogenesis using MRI (Fig. 7.3).

In addition, noble metal such as gold nanoparticles have attracted interests in gene vector, and various novel nanomaterials with unique optical or magnetic properties are continuously explored. Some graphene-like fluorescence quenching analogs were reported for bioanalysis. Wu et al. [22] presented a novel strategy by using PNA probes labeled with fluorophores; they were conjugated with nanometal–organic framework (NMOF) as a fluorescence quencher of the labeled PNA and developed for multiplexed miRNAs detection in living cancer cells. A multifunctional SnO_2 nanoprobe for target-cell-specific delivery and imaging with detection of intracellular miRNA was reported by Ju et al. [23] (Fig. 7.4). The multifunctional SnO_2 nanoprobe contains a cell-targeting moiety and a conjugated gene probe to specifically recognize or regulate a target sequence, providing a detection strategy or inhibitor. Using miRNA-21 in HeLa cells as a model, a method for in situ specific detection and regulation of intracellular miRNA is implemented.

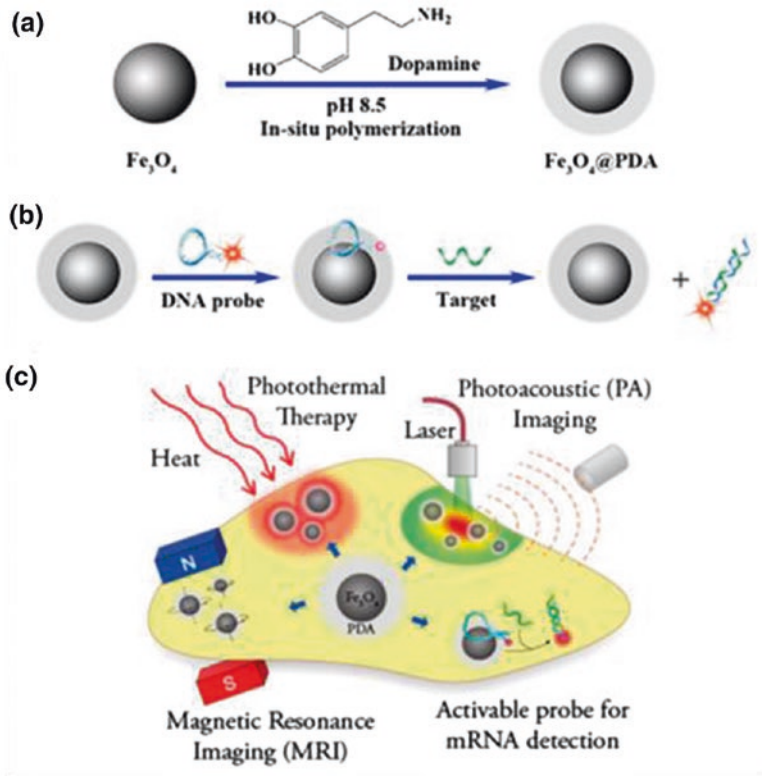

Fig. 7.3 Strategy for multifunctional Fe_3O_4@Polydopamine core/shell nanocomposites for intracellular mRNA detection and imaging-guided photothermal therapy. Reprinted with the permission from Ref. [21]. Copyright 2011 American Chemical Society

7.2 miRNA Imaging Analysis

Bioluminescence imaging has been widely employed to monitor endogenous gene expression and molecular distribution in living animals by detection of light emitted from enzymatic reaction. Luciferase (Fluc), Renilla luciferase (Rluc), and Gaussia luciferase (Gluc) are the three typical luciferase optical reporter proteins. The maximum emission of Fluc loaded at about 562 nm is produced by oxidizing its substrate beetle D-luciferin to oxyluciferin (benzothiazole) [24, 25]. Rluc and Gluc emit bioluminescence light at about 480 nm by catalyzing the oxidation of coelenterazine [26]. Compared to native Rluc or Fluc, Gluc shows a 1000-fold more intensity, and it is the smallest stable luciferase at elevated temperatures, which facilitating it to construct clones and cell transfection [27, 28]. Fluc, meanwhile, possesses better tissue penetration of photons for in vivo imaging in tissue than Rluc and Gluc duo to longer wavelengths emission. Lee et al.

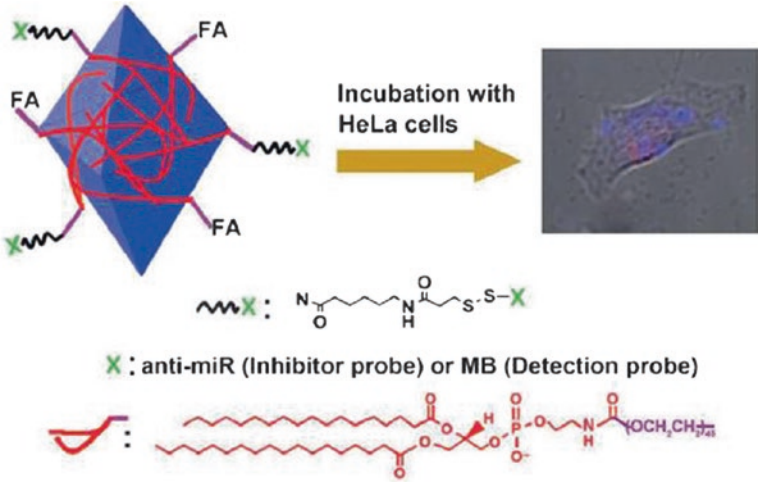

Fig. 7.4 Schematic representation of multifunctional-SnO_2 nanoprobe for target-specific cell imaging and intracellular detection of miRNA. *FA* folic acid, *MB* molecular beacon. Reproduced from Ref. [23] by permission of John Wiley & Sons Ltd

[29] constructed a miRNA-23a specific reporter vector by fusing the 5′-upstream promoter region of primal miRNA-23a to the cassette of pGL3 containing a promoterless Fluc gene to obtain the images of endogenous expression of primal miRNA-23a in HeLa and P19 cells. The highly expressed pattern of a brain-specific primal miRNA-9 during neurogenesis was also observed by the same miRNA-specific reporter vector [30]. By fusing sense and antisense oligonucleotides of the primal miRNA-23a into a cytomegalovirus promoter containing the start codon of the Gluc gene, an optical reporter gene vector can be obtained to view the image of the cleavage of pre-miRNA by the Dicer enzyme is acquired [31]. Under the control of the cytomegalovirus promoter, an imaging system using luciferase reporter has been designed by fusing complementary sequence of mature miRNAs to the downstream of the reporter system to investigate the expression of mature miRNA [32].

Fluorescent proteins with various emission wavelengths, including the green fluorescent protein (GFP) and red-emitting fluorescent proteins (RFP), have been used for in vivo imaging [33]. The high flexibility GFP provides a permanent and heritable label in living cells. By encoding GFP with multitargeting sites of miRNA, lentiviral, or retroviral vectors, in vivo imaging was constructed for viewing [34, 35]. Several target sequences for miRNA-142-3p were fused into the 3′-UTR of a GFP expression cassette driven by the ubiquitously expressed phosphoglycerate kinase promoter to develop a lentiviral vector, providing evidence of miRNA regulation in vivo imaging. A multiple colors system holds a promising platform for monitoring dynamic multiple miRNA functions. By fusing target site for miRNA-133 in the 3′-UTR of a GFP with RFP for normalization, a two-color retroviral vector system for monitoring miRNA-133 in living cells has been reported [36].

The sensitivity of both luciferase-based and fluorescent-protein-based miRNA reporter systems are related to the reporter gene expression level and the pharmacokinetics of probe reporter, which inherently suffer from low sensitivity and the interpretation of cell death. Several alternative signal-on systems were explored to overcome these limitations for in vivo miRNA. MB is a hairpin-shaped single-stranded oligonucleotide hybridization probe containing a loop for complementing a target sequence and stem forming by the self-complementary 5′ and 3′ ends [37]. The unique thermodynamic and relatively low background of MB attract intensive interest in molecular detection of intracellular targets. Several MB-based signal-on imaging strategies have been constructed to detect endogenous miRNAs [7, 23, 38, 39]. In order to improve the sensitivity, molecular biological amplification technique was used to intracellular miRNA detection. Chu et al. [40] have developed a target-primed RCA (TPRCA) strategy for highly sensitive and selective in situ imaging monitor of miRNA expression patterns at the single-cell level. Using two-color fluorescent probes, the obtained TPRCA-based strategy is successfully realized for two miRNAs simultaneous detection differentially expressed in the two cell lines. Li et al. [41] have designed a RCA strategy using toehold-initiated strand-displacement process for in situ amplification of the intracellular target miRNA.

As shown in Fig. 7.5, binding the target to the toehold domain of the seal probe switches the seal probe to the "activated" circular form via a strand-displacement process. Then, the RCA was triggered and the product hybridizes with 6-carboxyfluorescein (FAM)-labeled probes, leading to a bright spot for detection of each target miRNA. It showed high sequence discriminated capability; no amplification was observed when there is a sequence mismatch between the miRNA and the seal probe, achieving specific miRNA detection. A probe-modified technique

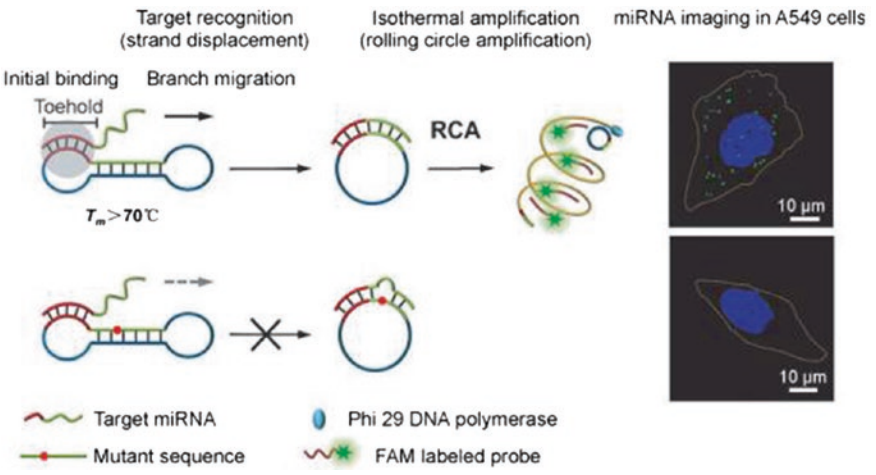

Fig. 7.5 Scheme for toehold-initiated rolling circle amplification for visualizing individual miRNAs in situ in single cells. Reproduced from Ref. [41] by permission of John Wiley & Sons Ltd

can improve the hybridization efficiency and detection sensitivity. Kin Wong et al. [42] have reported an intracellular miRNAs detection strategy using a double-stranded locked nucleic acid (dsLNA) probe, which showed high performance in detecting the dynamic regulation and dose-dependent modulation of miRNAs.

Meanwhile, the significant progress in radionuclide imaging modalities, including the sodium iodide symporter and the MRI reporter gene, gives hope to promising tool for clinical diagnosis and biomedicine [43, 44]. Special attention is paid to develop good MRI label to increase the spatial resolution in radionuclide imaging. A new field termed radiogenomics is associated with gene expression profiles with MRI phenotypes have emerged [45]. By combing quantitative MRI volumetrics with large-scale miRNA expression profiling, a comprehensive radiogenomic analysis is conducted in glioblastoma multiforme [46]. The high periostin and low miRNA-219 expression are demonstrated to significantly relate to the mesenchymal glioblastoma multiforme subtype.

7.3 In Situ Hybridization (ISH)

The study of the physiologic function of miRNA needs to profile miRNA expression at the single-cell level. As a promising alternative option with complete spatial profile of gene expression in single cell, ISH is a powerful technique for visualization of the spatial localization of RNA at the tissue, cellular, and even subcellular level [47–50]. A typical practice for ISH is to modify probes with a reporter, including fluorophores maintaining spatial resolution [51] enzymes catalyzing fluorogenic reactions [52] or radioactively labeled probes detected by autoradiography [53]. By coupling LNA-based ISH system with high-resolution imaging microscopy, Pederson et al. [50] monitored intracellular miRNA-206 localization during differentiation in a single rat myogenic cell. It is well demonstrated that the probe modification, target copy number, and hybridization conditions of stringency could influence the signal of ISH [54]. In contrast to attention paid on the probe modification, by focusing on high-stringency wash conditions based on tetramethylammonium chloride (TMAC) and RNase A, Turner et al. [47] developed an ISH system for miRNA detection. The remarkable affinity and specificity of the probes against RNA targets allow the LNA-ISH system to qualitatively assess miRNA localization patterns and tissue distribution. However, quantitative detection of low abundance miRNAs is less amenable by LNA-ISH system. Various probe modification or label techniques are introduced into an LNA-ISH system to improve the sensitivity [55, 56]. For example, a single-molecule dual-color coincident detection strategy by using two spectrally distinguishable fluorescent LNA–DNA oligonucleotide probes to recognize the target miRNA was developed. The tagged molecules are directly counted for single-molecule detection in a microfluidic, multicolor confocal laser system, achieving sensitivity down to femtomolar concentration of miRNA (500 fM) [57]. Using a fluorescence recovery strategy, the MB probe with LNA–DNA backbones can readily

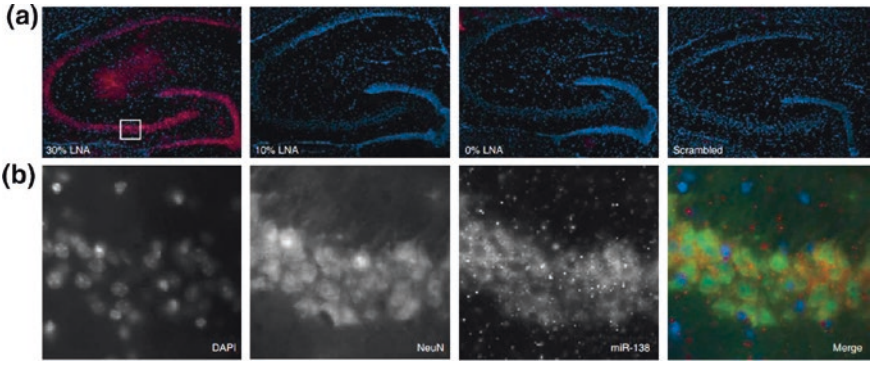

Fig. 7.6 miRNA-138 in situ staining of mouse hippocampus with miR-138 LNA probes containing 30, 10, and 0 % LNA (*red*) as well a scrambled miR-138 30 % LNA probe. Reprinted by permission from Macmillan Publishers Ltd: Ref. [59]. Copyright 1993

distinguish mature- and pre-miRNAs, and reliably quantify miRNA expression with LOD as low as 1 nM [58]. Obernosterer et al. [59] have designed a method for sensitive and specific histological detection of miRNAs using an LNA-ISH strategy under optimized conditions, which could monitor spatial and temporal expression of miRNA-138 miRNAs in mouse hippocampus with high sensitivity and resolution (Fig. 7.6).

References

1. Sinn P, Sauter S, McCray P (2005) Gene therapy progress and prospects: development of improved lentiviral and retroviral vectors–design, biosafety, and production. Gene Ther 12:1089–1098
2. Niidome T, Huang L (2002) Gene therapy progress and prospects: nonviral vectors. Gene Ther 9:1647–1652
3. McNamara JO, Andrechek ER, Wang Y, Viles KD, Rempel RE, Gilboa E, Sullenger BA, Giangrande PH (2006) Cell type–specific delivery of siRNAs with aptamer-siRNA chimeras. Nat Biotechnol 24:1005–1015
4. Hong R, Bai W, Zhai J, Liu W, Li X, Zhang J, Cui X, Zhao X, Ye X, Deng Q (2013) Novel recombinant hepatitis B virus vectors efficiently deliver protein and RNA encoding genes into primary hepatocytes. J Virol 87:6615–6624
5. Karlsen TA, Brinchmann JE (2013) Liposome delivery of microRNA-145 to mesenchymal stem cells leads to immunological off-target effects mediated by RIG-I. Mol Ther 21:1169–1181
6. Wang K, Zhang X, Liu Y, Liu C, Jiang B, Jiang Y (2014) Tumor penetrability and anti-angiogenesis using iRGD-mediated delivery of doxorubicin-polymer conjugates. Biomaterials 35:8735–8747
7. Dong H, Ding L, Yan F, Ji H, Ju H (2011) The use of polyethylenimine-grafted graphene nanoribbon for cellular delivery of locked nucleic acid modified molecular beacon for recognition of microRNA. Biomaterials 32:3875–3882
8. Park K, Lee S, Kang E, Kim K, Choi K, Kwon IC (2009) New generation of multifunctional nanoparticles for cancer imaging and therapy. Adv Funct Mater 19:1553–1566

9. Bonanni A, Chua CK, Zhao G, Sofer ZK, Pumera M (2012) Inherently electroactive graphene oxide nanoplatelets as labels for single nucleotide polymorphism detection. ACS nano 6:8546–8551
10. Neely LA, Patel S, Garver J, Gallo M, Hackett M, McLaughlin S, Nadel M, Harris J, Gullans S, Rooke J (2006) A single-molecule method for the quantitation of microRNA gene expression. Nat Methods 3:41–46
11. Erickson K, Erni R, Lee Z, Alem N, Gannett W, Zettl A (2010) Determination of the local chemical structure of graphene oxide and reduced graphene oxide. Adv Mater 22:4467–4472
12. Gambhir SS, Barrio JR, Herschman HR, Phelps ME (1999) Assays for noninvasive imaging of reporter gene expression. Nucl Med Biol 26:481–490
13. Blasberg RG (2003) In vivo molecular-genetic imaging: multi-modality nuclear and optical combinations. Nucl Med Biol 30:879–888
14. Wang XL, Rosol M, Ge SD, Peterson D, McNamara G, Pollack H, Kohn DB, Nelson MD, Crooks GM (2003) Dynamic tracking of human hematopoietic stem cell engraftment using in vivo bioluminescence imaging. Blood 102:3478–3482
15. Kim KI, Chung J-K, Kang JH, Lee YJ, Shin JH, Oh HJ, Jeong JM, Lee DS, Lee MC (2005) Visualization of endogenous p53-mediated transcription in vivo using sodium iodide symporter. Clin Cancer Res 11:123–128
16. Ryoo S-R, Lee J, Yeo J, Na H-K, Kim Y-K, Jang H, Lee JH, Han SW, Lee Y, Kim VN, Min D-H (2013) Quantitative and multiplexed microRNA sensing in living cells based on peptide nucleic acid and nano graphene oxide (PANGO). ACS Nano 7:5882–5891
17. Zhi F, Dong H, Jia X, Guo W, Lu H, Yang Y, Ju H, Zhang X, Hu Y (2013) Functionalized graphene oxide mediated adriamycin delivery and miR-21 gene silencing to overcome tumor multidrug resistance in vitro. PLoS one 8:e60034
18. Zhang P, He Z, Wang C, Chen J, Zhao J, Zhu X, Li C-Z, Min Q, Zhu JJ (2015) In situ amplification of intracellular microRNA with MNAzyme nanodevices for multiplexed imaging, logic operation, and controlled drug release. ACS Nano 9:789–798
19. Zhang J, Fu Y, Mei Y, Jiang F, Lakowicz JR (2010) Fluorescent metal nanoshell probe to detect single miRNA in lung cancer cell. Anal Chem 82:4464–4471
20. Li H, Mu Y, Qian S, Lu J, Wan Y, Fu G, Liu S (2015) Synthesis of fluorescent dye-doped silica nanoparticles for target-cell-specific delivery and intracellular microRNA imaging. Analyst 140:567–573
21. Lin L-S, Cong Z-X, Cao J-B, Ke K-M, Peng Q-L, Gao J, Yang H-H, Liu G, Chen X (2014) Multifunctional Fe3O4@polydopamine core–shell nanocomposites for intracellular mRNA detection and imaging-guided photothermal therapy. ACS Nano 8:3876–3883
22. Wu Y, Han J, Xue P, Xu R, Kang Y (2014) Nano metal-organic framework (NMOF)-based strategies for multiplexed microRNA detection in solution and living cancer cells. Nanoscale 7:1753–1759
23. Dong H, Lei J, Ju H, Zhi F, Wang H, Guo W, Zhu Z, Yan F (2012) Target-cell-specific delivery, imaging, and detection of intracellular MicroRNA with a multifunctional SnO_2 nanoprobe. Angew Chem Int Ed 51:4607–4612
24. Brasier A, Tate J, Habener J (1988) Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. Biotechniques 7:1116–1122
25. Gould SJ, Subramani S (1988) Firefly luciferase as a tool in molecular and cell biology. Anal Biochem 175:5–13
26. Tannous BA, Kim D-E, Fernandez JL, Weissleder R, Breakefield XO (2005) Codon-optimized Gaussia luciferase cDNA for mammalian gene expression in culture and in vivo. Mol Ther 11:435–443
27. Ottobrini L, Ciana P, Biserni A, Lucignani G, Maggi A (2006) Molecular imaging: a new way to study molecular processes in vivo. Mol Cell Endocrinol 246:69–75
28. Doubrovin M, Serganova I, Mayer-Kuckuk P, Ponomarev V, Blasberg RG (2004) Multimodality in vivo molecular-genetic imaging. Bioconjugate Chem 15:1376–1388
29. Lee JY, Kim S, Jeong JM, Chung J-K, Lee MC, Lee DS (2008) Development of a dual-luciferase reporter system for in vivo visualization of MicroRNA biogenesis and posttranscriptional regulation. J Nucl Med 49:285–294

30. Ko HY, Lee DS, Kim S (2009) Noninvasive imaging of microRNA124a-mediated repression of the chromosome 14 ORF 24 gene during neurogenesis. FEBS J 276:4854–4865
31. Davies BP, Arenz C (2008) A fluorescence probe for assaying micro RNA maturation. Bioorg Med Chem 16:49–55
32. Kim HJ, Kim YH, Lee DS, Chung J-K, Kim S (2008) In vivo imaging of functional targeting of miR-221 in papillary thyroid carcinoma. J Nucl Med 49:1686–1693
33. Wessels JT, Yamauchi K, Hoffman RM, Wouters FS (2010) Advances in cellular, subcellular, and nanoscale imaging in vitro and in vivo. Cytometry Part A 77A:667–676
34. Kato Y, Miyaki S, Yokoyama S, Omori S, Inoue A, Horiuchi M, Asahara H (2009) Real-time functional imaging for monitoring miR-133 during myogenic differentiation. Int J Biochem Cell Biol 41:2225–2231
35. Kato Y, Sawata SY, Inoue A (2010) A lentiviral vector encoding two fluorescent proteins enables imaging of adenoviral infection via adenovirus-encoded miRNAs in single living cells. J Biochem 147:63–71
36. Brown BD, Venneri MA, Zingale A, Sergi LS, Naldini L (2006) Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer. Nat Med 12:585–591
37. Tyagi S, Kramer FR (1996) Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol, 303–8
38. Kang WJ, Cho YL, Chae JR, Lee JD, Choi K-J, Kim S (2011) Molecular beacon-based bioimaging of multiple microRNAs during myogenesis. Biomaterials 32:1915–1922
39. Hwang DW, Song IC, Lee DS, Kim S (2010) Smart magnetic fluorescent nanoparticle imaging probes to monitor microRNAs. Small 6:81–88
40. Ge J, Zhang LL, Liu SJ, Yu RQ, Chu X (2014) A highly sensitive target-primed rolling circle amplification (TPRCA) method for fluorescent in situ hybridization detection of microRNA in tumor cells. Anal Chem 86:1808–1815
41. Deng R, Tang L, Tian Q, Wang Y, Lin L, Li J (2014) Toehold-initiated rolling circle amplification for visualizing individual microRNAs in situ in single cells. Angew Chem Int Ed 53:2389–2393
42. Dean ZS, Riahi R, Wong PK (2015) Spatiotemporal dynamics of microRNA during epithelial collective cell migration. Biomaterials 37:156–163
43. Fox MD, Raichle ME (2007) Spontaneous fluctuations in brain activity observed with functional magnetic resonance imaging. Nat Rev Neurosci 8:700–711
44. Kang JH, Chung J-K (2008) Molecular-genetic imaging based on reporter gene expression. J Nucl Med 49:164S–179S
45. Rutman AM, Kuo MD (2009) Radiogenomics: creating a link between molecular diagnostics and diagnostic imaging. Eur J Radiol 70:232–241
46. Zinn PO, Majadan B, Sathyan P, Singh SK, Majumder S, Jolesz FA, Colen RR (2011) Radiogenomic mapping of edema/cellular invasion MRI-phenotypes in glioblastoma multiforme. PLoS one 6:e25451
47. Thompson RC, Deo M, Turner DL (2007) Analysis of microRNA expression by in situ hybridization with RNA oligonucleotide probes. Methods 43:153–161
48. Levsky JM, Shenoy SM, Pezo RC, Singer RH (2002) Single-cell gene expression profiling. Science 297:836–840
49. Raj A, van den Bogaard P, Rifkin SA, van Oudenaarden A, Tyagi S (2008) Imaging individual mRNA molecules using multiple singly labeled probes. Nat Methods 5:877–879
50. Politz JCR, Zhang F, Pederson T (2006) MicroRNA-206 colocalizes with ribosome-rich regions in both the nucleolus and cytoplasm of rat myogenic cells. Proc Natl Acad Sci 103:18957–18962
51. Schneider M, Andersen DC, Silahtaroglu A, Lyngbæk S, Kauppinen S, Hansen JL, Sheikh SP (2011) Cell-specific detection of microRNA expression during cardiomyogenesis by combined in situ hybridization and immunohistochemistry. J Mol Histol 42:289–299
52. Raap A, Van de Corput M, Vervenne R, Van Gijlswijk R, Tanke H, Wiegant J (1995) Ultrasensitive FISH using peroxidase-mediated deposition of biotin-or fluorochrome tyramides. Hum Mol Genet 4:529–534

53. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Rådmark O, Kim S (2003) The nuclear RNase III Drosha initiates microRNA processing. Nature 425:415–419
54. Nuovo G (1995) In situ PCR: protocols and applications. Genome Res 4:S151–S167
55. Langer PR, Waldrop AA, Ward DC (1981) Enzymatic synthesis of biotin-labeled polynucleotides: novel nucleic acid affinity probes. Proc Natl Acad Sci 78:6633–6637
56. Femino AM, Fay FS, Fogarty K, Singer RH (1998) Visualization of single RNA transcripts in situ. Science 280:585–590
57. Neely LA, Patel S, Garver J, Gallo M, Hackett M, McLaughlin S, Nadel M, Harris J, Gullans S, Rooke J (2005) A single-molecule method for the quantitation of microRNA gene expression. Nat Methods 3:41–46
58. Baker MB, Bao G, Searles CD (2012) In vitro quantification of specific microRNA using molecular beacons. Nucleic Acids Res 40:e13
59. Obernosterer G, Martinez J, Alenius M (2007) Locked nucleic acid-based in situ detection of microRNAs in mouse tissue sections. Nat Protoc 2:1508–1514

Chapter 8
Summary and Prospects

Abstract miRNAs hold great promise in clinical application acting as biomarkers for disease diagnosis and prognosis due to the significant regulating roles in various physiological and pathological processes. The unique characteristics such as small size, highly similar sequences among family members/isoforms, and the low expression profile require efficient tools for rapid, specific, and sensitive detection of miRNAs. Conventional miRNA detection techniques such as qRT-PCR, Northern blotting, and microarrays could meet the detection requirement to some degree. Various emerging reliable technologies in miRNA detection are continuous by incorporating novel materials and techniques owing to the significant progresses in physics, engineering, and biology. Evaluation of the detection methods with high specificity for point-of-care analysis of miRNAs are still in great demand and special attention should be paid to the miniaturization, efficient probe modification techniques, novel physical label-free miRNA biosensors, and the stability and biocompatibility involving the immobilization of the signal probes in miRNA assay.

Keywords Miniaturization device · Probe modification techniques · Label-free biosensor · Stability and biocompatibility

miRNAs play a significant regulating role in various physiological and pathological processes. They can hold great promise in clinical application acting as biomarkers for disease diagnosis and prognosis. The small size of mature miRNAs, highly similar sequences among family members/isoforms, and low expression profile require the creation of efficient tools for rapid, specific, and sensitive detection of miRNAs. Conventional miRNA detection techniques, including qRT-PCR, Northern blotting, and microarrays could meet the detection requirement to some degree. However, miRNA analysis is still challenging due to the unique characteristics of miRNA. Northern blotting can identify specific RNA sequences in a

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solution of mixed RNAs and provide information about the size and expression of predicted miRNAs and precursor miRNAs. qRT-PCR provides an absolute miRNA quantification method with wide dynamic range and high accuracy. miRNA microarrays show advantage of high-throughput-screening capability alongside a low sensitivity and dynamic range. These traits are less expensive as discovery tools. However, they suffer from some deficiency such as being laborious, semi-quantitative, and requiring complicated and labor-intensive sample preparation and expensive equipment or radioactive/toxic labels. Therefore, a more reliable method with high sensitivity and selectivity are in urgent need for detection of miRNA without the time-consuming amplification process and purification step.

Significant progresses in physics, engineering, and biology have introduced various emerging technologies in miRNA detection by incorporating novel materials and techniques such as metallic nanoparticles, semiconductor quantum dots, bioluminescent proteins, novel surface modification, molecular biological technique, etc. The nanoscale sizes of nanoparticles break through the limitation of structure miniaturization, which has high surface areas for improved mass transport, and high loading of receptor molecules for synergistic amplification of the target response. The unique optical, electronic, and catalytic properties of nanomaterials can translate the biorecognition events into an electrochemical or spectroscopic response, resulting in a series of emerging approaches: such as electrochemical assay, SPR, SERS, SiNWs, and nanomachine devices for miRNA detection with high sensitivity, good specificity, multiplexing capability, and easy operation. Furthermore, the molecular biological techniques, such as enzymatic reaction, LCR, RCA, and EXPAR can effectively amplify the target, probe, or hybridization signal for sensitive detection. These techniques exhibit great promise in the detection of miRNAs in cells, circular blood, and tissue samples.

However, there are still great demands for evolving the detection methods for point-of-care analysis of miRNAs. Finding a reliable miRNA profiling technique with high specificity against other family RNAs, minimum sample manipulation, and a wide dynamic range from attomolar to nanomolar concentrations are still a critical requirement. Special attention should be paid to the following:

(i) The trend toward miniaturized devices and sensitive detection make nanomaterials become significant in practice. Nanomaterials can provide a synergic effect among catalytic activity, conductivity, and biocompatibility to accelerate the signal transduction, producing ultrasensitive detection for low-abundance miRNA.
(ii) Exploration of efficient probe modification techniques and molecular biological techniques, such as target cycling, LAN recognition, ISH, and enzyme catalysis is significant to improve the specificity of the assay. Higher specificity is needed for miRNA detection in a complicated sample.
(iii) Novel physical label-free miRNA biosensors based on the unique optical, electronic, and magnetic properties of nanomaterials with total internal reflection fluorescence microscopy. SPR and SERS are supposed to be alternative techniques for miRNA detection, even permitting them to detect miRNA at

the single-molecular level. Microfluidic chips hold great promise for high-throughput analysis of miRNA with enhanced performance, reduced cost, and high sensitivity in single-cell gene expression measurements.

(iv) The study of the physiologic function of miRNA needs to be able to profile miRNA expression at the single-cell level. Powerful techniques, including imaging technology, ISH for visualization of spatial localization of RNA at the tissue, cellular, and even subcellular level are continuously needed in future applications.

(v) In the design of next-generation biosensing methods, stability and biocompatibility involving the immobilization of signal probes should be considered in the future. The novel strategies will provide a powerful tool to not only profile the expression of miRNA, but also act as disease diagnostics and used as a therapy tool to improve human health.