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REVIEW

Advanced methods for microRNA biosensing: a problem-solving perspective

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Abstract

MicroRNAs (miRNAs) present several features that make them more difficult to analyze than DNA and RNA. For this reason, efforts have been made in recent years to develop innovative platforms for the efficient detection of microRNAs. The aim of this review is to provide an overview of the sensing strategies able to deal with drawbacks and pitfalls related to microRNA detection. With a critical perspective of the field, we identify the main challenges to be overcome in microRNA sensing, and describe the areas where several innovative approaches are likely to come for managing those issues that put limits on improvement to the performances of the current methods. Then, in the following sections, we critically discuss the contribution of the most promising approaches based on the peculiar properties of nanomaterials or nanostructures and other hybrid strategies which are envisaged to support the adoption of these new methods useful for the detection of miRNA as biomarkers of practical clinical utility.

Keywords MicroRNA · Biosensing · Surface plasmon resonance · Electrochemistry · Fluorescence · Microfluidics

Introduction

MicroRNAs (miRNAs) are small, noncoding RNAs that have emerged as a novel and rapidly expanding area of interest in clinical diagnostics [1]. MiRNAs post-transcriptionally regulate the expression of thousands of genes and have been found to modulate many cellular processes [2]. They also show promise as biomarkers for critical diseases [3]. It is for those reasons that miRNAs' expression profile is gaining popularity and rapidly increasing its importance in clinical context [1].

MiRNAs present several features that make them more difficult to analyze than DNA and RNA [4]. The specific pathway of miRNA biogenesis is responsible for some of such features (Fig. 1). MiRNAs are initially transcribed in the cell nucleus as long precursors (1–3 kb), called primary miR (pri-miRNA), that are enzymatically processed into approximately 70–100-nt long stem-loop structures called precursor miR (pre-miRNA). Pre-miRNAs are then exported into the cell cytoplasm where an enzymatic process creates 19–23-nt long mature miRNAs. Mature miRNAs can regulate the gene expression by interacting with messenger RNA (mRNA). The translation repression or mRNA target degradation is thus obtained in consequence of an imperfect or perfect complementarity, respectively.

The short sequence of mature miRNAs introduces limitations in the design of probes used by hybridization-based assays and biases in miRNA detection caused by the significant variance of melting temperatures (T_m) due to GC content heterogeneity. The specific biogenetic processes leading to the formation of mature miRNAs produces sequences with a relatively high degree of homology thus introducing additional challenges in the design of selective assays. The features mentioned above are combined with the variable and low abundance of miRNAs in body fluids and tissues (Fig. 2) [5, 6]. The concentration of miRNAs in body fluids ranges from femtomolar to picomolar in normal physiologic conditions and can be significantly decreased or increased under pathological conditions. The concentration of miRNAs also depends on the sample type and the extraction method adopted [4].

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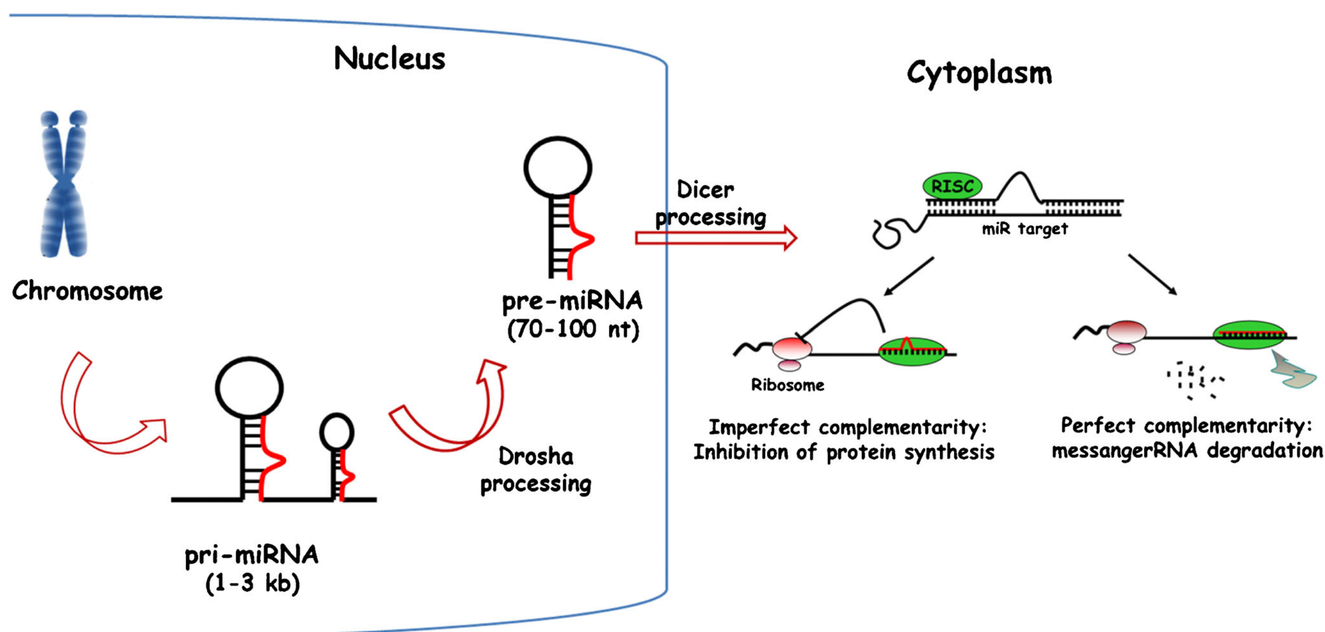

Fig. 1 miRNA pathways: biogenesis and function

Extensive research efforts have been deployed over the last 10 years to develop innovative methods for the sensitive and specific detection of miRNAs. Excellent review papers summarize recent progresses in the field with a specific focus on the developed methods and largely referring to both enzyme-based amplification and recycling strategies as well as nanomaterial-based approaches [7–9]. However, different critical aspects, not explicitly reviewed in already published papers, play a central role in miRNA detection. For this reason, here we will specifically discuss issues related to miRNA biosensing on a problem-solving perspective. The strength and weakness of critically selected strategies for miRNA detection will be discussed with a focus on both challenging problems to be faced in miRNA detection and quantification as well as the potential for clinical application. The latter aspect is highlighted since only a relatively limited number of papers describing innovative methods report on the detection of miRNAs in clinical samples or biological fluids. Conventional methods for the detection of miRNA including northern blotting [10], qRT-PCR [11, 12], and microarrays [13, 14] will not be discussed here, while attention will be devoted to biosensors and biosensor-related platforms.

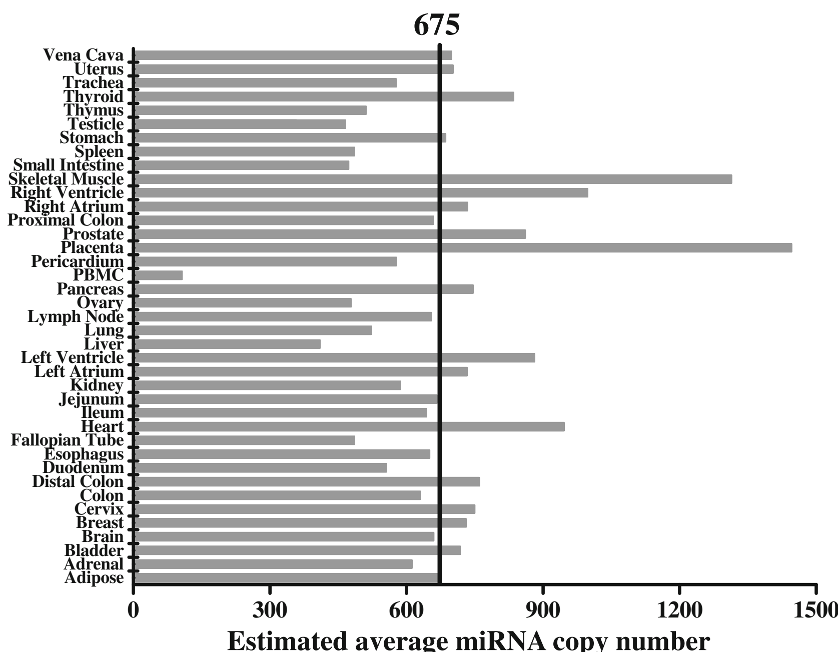

Fig. 2 The abundance of miRNAs in all tissues represented by the estimated copy number per cell (assuming 30 pg of total RNA in each cell). Adapted from data [5]

Limit of detection and dynamic range in miRNA biosensing

MiRNAs represent a small fraction of the total RNA available in biological samples (about 0.01%), and their expression level in cells may vary up to a factor of 10^5 [15]. Such features explain why an effective miRNA detection method should operate with limits of detection and dynamic range that depend on miRNA expression levels in the samples being analyzed. Small changes in the expression of abundant miRNAs will have a significant impact on the level of the target protein. Therefore, a limited dynamic range will be required for similar cases. For example, the detection of a twofold change in miRNA expression level may be adequate to investigate liver-specific miR-122 that accounts for more than half of all miRNAs expressed in hepatocytes [16]. On the other hand, low-abundant miRNAs may change their expression level by order of magnitudes to translate into a biological effect. However, the ability to detect low-abundant miRNAs that may serve as biomarkers of diseases is highly important for both research and clinical diagnostic purposes.

Considerable efforts have been made to enhance the sensitivity for detection of miRNAs exploiting the chemical and physical properties of nanostructured materials [17]. Because of their biological compatibility, high surface area, chemical stability, and excellent catalytic activity, the use of nanomaterials has considerably improved performances in terms of intensity of the detected signal and dynamic range. Their use in platforms for optical and electrochemical sensing of miRNA has been gaining merit in the field [18, 19].

Nanomaterial-based miRNA detection strategies can be classified into three different groups: assays using nanoparticles dispersed in solution, approaches using nanostructured materials or surfaces, and strategies based on the combination of nanomaterials and enzymatic reactions.

Gold nanoparticles (AuNPs), silver nanoclusters (AgNCs), quantum dots (QDs), and carbonaceous materials are mostly exploited in optical as well as electrochemical readout [20]. In particular, functionalized AuNPs are largely used to detect nucleic acids with high sensitivity [21–24]. Micrometer-sized gold shells with embossed nanostructures can be used in combination with laser desorption ionization mass spectrometry to achieve a femtomolar detection of miRNAs with no need for enzymatic amplification steps and target labeling (Fig. 3) [25]. The method can detect miR-122 at a concentration ranging from 1 nM to 0.1 fM and can operate with Huh 7 cells crude extract.

Surface plasmon resonance (SPR) sensitivity has been shown to greatly benefit from the use of AuNPs in detecting nucleic acids [26, 27]. MiR-16, miR-23b, and miR-122b can be detected with 10 fM sensitivity using SPR in combination with a sandwich-like assay obtained using locked nucleic acid (LNA) probes, enzymatic polyadenylation of the miRNA targets hybridized to the LNA probe, and poly(T)-functionalized AuNPs [28].

The beneficial role of nanoparticles in enhancing SPR-based miRNA detection can be quantified comparing performances of the nanoparticle-enhanced detection with the direct SPR detection of miRNAs. The direct detection of miR-122 has been performed with a limit of detection of 100 pM using an SPR platform with diffraction grating couplers [29]. The limit of detection has been pushed down to 2 pM by introducing an amplification step in the assay based on the use of an S9.6 mouse monoclonal antibody able to target the formed RNA/DNA duplex.

A sandwich-like assay using a streptavidin–oligonucleotide complex to amplify the SPR signal allows the detection of miR-122 with a limit of detection of 1.7 fM [30]. In this case, the practical applicability of the assay has been evaluated using samples from a breast tumor.

A sequence-independent enhancing strategy has been instead obtained using boronic acid functionalized AuNPs (PBA-AuNPs) [31]. PBA-AuNPs preferentially bind cis-diol moieties of miRNA targets.

Plasmonic nanostructures such as nanoaggregates, nanoassemblies, or magnetic nanomaterials have been employed for surface-enhanced resonance Raman scattering (SERS) detection of nucleic acids [32]. SERS-based detection of miRNAs can be achieved through label-free detection [33], direct hybridization [34], or signal amplification [35] assay format. In the latter case, the remarkably high enhancement factor (up to 10^{11}) of Raman scattering has made SERS a powerful technique for the direct and simultaneous detection of multiplex miRNA, providing narrow fingerprint profiles for individual miRNA molecules and enabling simultaneous detection of multiple analytes [36–38]. Composite nanomaterials, such as bimetallic hybrid nanomaterials, have been also used as magnetic SERS substrates capable to catch target molecules from samples [39]. In a recent report, Pang et al. [40] used DNA conjugated Fe3O4@Ag core–shell magnetic NPs for miRNA capture and duplex-specific nuclease (DSN) signal amplification. Compared to other SERS biosensors [41], higher sensitivity is achieved with LOD 300 aM.

Different nanostructured materials including Au, Pd, Pt, OsO_2, and RuO_2 can be used to enhance the sensitivity and dynamic range of electroanalytical platforms for miRNA detection [19, 42]. Wu and coworkers [43] devised a stepwise fabrication protocol for nafion, thionin (Thi), and Pd nanoparticles used to fabricate a layer onto which target biomolecules are immobilized. The biosensor provided good electrocatalytic activity toward H_2O_2 and enhanced the amperometric response in detecting miR-155 with limit of detection of 1.87 pM and a linear response in the 5.6–5.6 × 10^5 pM range.

Ferrocene (Fc)-capped AuNPs/streptavidin conjugates can be used for the voltammetric detection of the expression levels

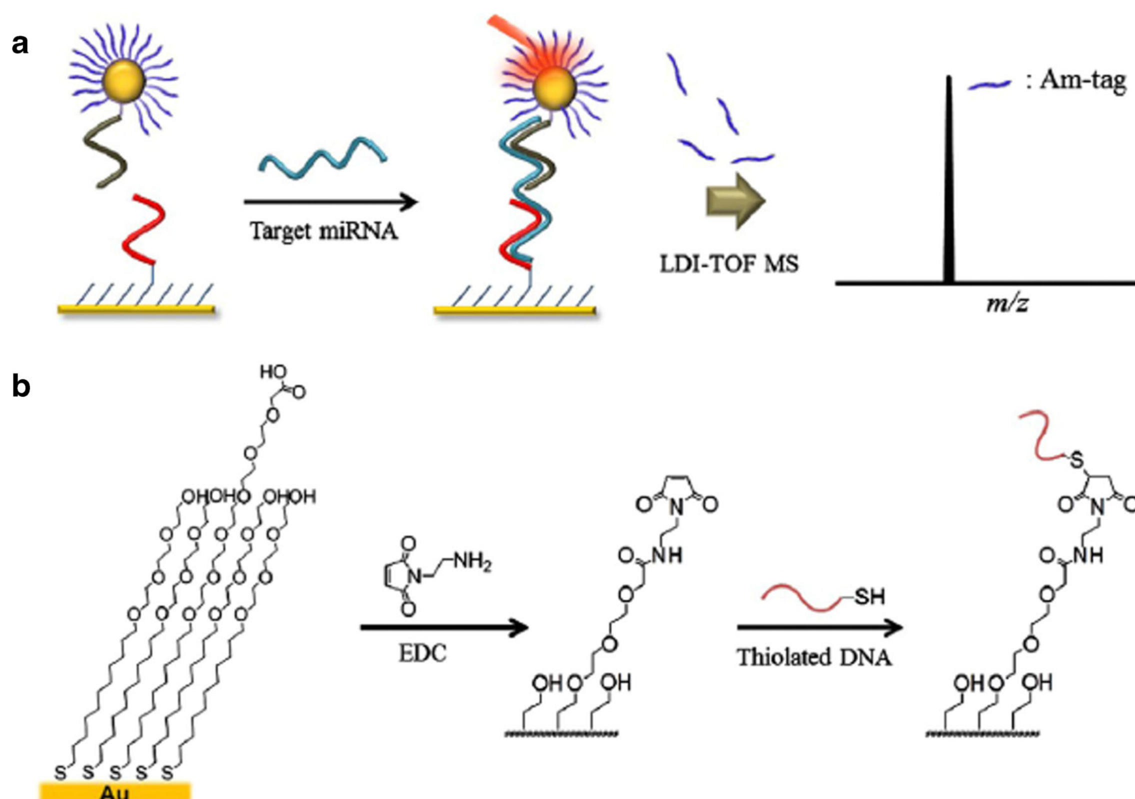

Fig. 3 **a** Signal amplification strategy for ultrasensitive detection of miRNAs using Am-tag and LDI–TOF-MS. For details, see the main text. **b** The structure of self-assembled monolayers on gold and chemical modifications employed to prepare capture DNA-presenting surfaces. The carboxylic acid-presenting monolayer is coupled to *N*-aminoethylmaleimide, followed by thiolated DNA. Reproduced with permission from [25]

of miR-182 in sera extracted from glioma patients [44]. In this case, the competition between a biotinylated short-stranded RNA and the miRNA target for the hybridization with an oligonucleotide probe is exploited to quantify the miRNA target with 10 fM sensitivity in 10 μL solution. The measured anodic peak current is inversely proportional to the concentration of the target with a linear response in the 1-fM to 2-pM range.

Gao's group [45–47] was the first to develop electrochemical methods for miRNA analysis, using OsO_2 and RuO_2 nanoparticles as electroactive miRNA labels. The electrocatalytic nanoparticle tags (ENT) strategy they developed is based on the amplified chemical ligation utilizing indium tin oxide electrodes and isoniazid-modified OsO_2 nanoparticles (NPs). MiRNA is oxidized to dialdehyde and then hybridized to surface-immobilized oligonucleotide capture probes. A ligation reaction is used to tag miRNAs with the OsO_2 NPs. OsO_2 NPs induce a signal enhancement producing a detection limit of 80 fM for miRNA with a 0.3–200-pM dynamic range. The method provides a sensitive detection of miRNA but suffers from the complexity of the assay and the needs for expensive reagents for signal amplification.

In 2010, Gao et al. [48] described a miRNA biosensor based on RuO_2 NP-guided formation of polyaniline (PAn) nanowires in the presence of H_2O_2. In this work, gold electrodes covered with the mixed monolayers of peptide nucleic acid (PNA) and 4-mercaptoaniline (MAn) were used for the capture of miRNAs. PNA capture probes are widely used in nucleic acid biosensing as a consequence of the higher affinity to complementary sequence they show compared with oligonucleotide probes [49]. The neutral PNA backbone can alleviate the electrostatic absorption of cationic aniline on the sensing surface, thus producing a clean background with high signal/noise ratio; 2.0 fM limit of detection and 5.0 fM to 2.0 pM dynamic range were achieved with square wave voltammetry detection. The same performances in terms of limit of detection and dynamic range are obtained by exploiting the hybridized miRNA-templated deposition of an insulating polymer film [50]. The hybridization of miRNA to neutral morpholino capture probes converts to anionic the surface of the biosensor thus allowing the deposition of an insulating polymer film, poly(3,3′-dimethoxybenzidine) (PDB), obtained by the horseradish peroxidase-catalyzed polymerization of 3,3′-dimethoxybenzidine in the presence of H_2O_2. Other assays providing the electrochemical detection of miRNA with larger dynamic range (1 fM to 100 nM [51], 10 aM to 1 μM [52]) and better limit of detection (0.37 fM [51], 5 aM [52]) have been described, further demonstrating that metallic

nanoparticles can be used to achieve an ultrasensitive electrochemical detection of miRNAs with a wide dynamic range.

Nuclease-assisted amplification has been frequently implemented in miRNA detection methods for its capacity to specifically digest DNA in DNA/RNA duplex while leaving the RNA molecule intact [53–55]. MiRNA optical biosensing greatly benefits from nuclease-assisted amplification methods such as DSN [56]. The combination of DSN-assisted target recycling amplification and pyrene excimer switching has been shown to detect let-7 miRNA family members in cell extracts and blood samples with a detection limit of 0.58 fM and large dynamic range (1 fM to 10 nM) [57].

Signal amplification with nanomaterials is attractive to enhance sensitivity in miRNA detection [20]. Nanomaterial-based sensing strategies for electrochemical [58, 59] or fluorescence [60] detection of miRNA provide among the most performing approaches. Nanomaterials are usually employed as electrode-supporting substrates, vehicles for signal elements, markers or catalysts, or mediators to control the electron transfer process. Sensitive impedimetric detection of miR-222 has been obtained using nanostructured carbon electrodes and enzyme-decorated liposomes [61]. The assay uses DNA capture probes immobilized onto gold-nanostructured carbon surfaces. The miRNA target–probe hybrid is then exposed to enzyme-decorated liposomes that generate the detected impedimetric signal when exposed to alkaline phosphatase. MiR-222 is detected with LOD = 1.7 pM and 1.70–900 pM dynamic range. The direct detection of miR-221 is obtained using biotinylated polythiophene film deposited on gold screen-printed electrodes. A layer-by-layer deposition of streptavidin and biotinylated capture probes provides the detecting surface [62]. Electrochemical impedance spectroscopy has been used to detect miR-221 with LOD = 0.7 pM and 1–100 pM dynamic range. Miao et al. [63] established a label-free platform for miR-155 detection based on the fluorescence quenching of positively charged (+)AuNPs to silver nanoclusters (AgNCs). Taking advantage of the DSN-assisted target amplification and quenching efficiency of (+)AuNPs, 33.4 fM limit of detection and 100 fM to 1.0 pM dynamic range are obtained for miRNA-155 detection with capabilities for the evaluation of the expression levels of miRNA-155 in clinical serum samples from cancer patients.

Besides metallic nanoparticles, considerable attention has been paid to implement other functional nanomaterials, such as QDs and carbon-based nanomaterials, in miRNA detection assays. The exploitation of QD signal for the direct detection of miRNAs can be obtained in different ways, including the quenching of the fluorescence intensity via Förster resonance energy transfer fluorescence triggered by the hybridization of miRNA target to DNA capture probes immobilized on the surface of QDs. The limit of detection of the assay for miR-21 is 10 fM [64]. However, it is 10 times higher in 2% bovine serum.

QDs combined with DSN amplification introduce three orders of magnitude amplification and provide a direct and accurate quantification of miR-21 in extracts from human cancer cell lines [65].

Multiwalled carbon nanotubes (MWCNTs) and reduced graphene oxide (GO) have high affinity for ssDNA and lower affinity for DNA/RNA heteroduplexes. The hybridization of target miRNA to the DNA capture probes immobilized on such carbonaceous material induces a conformational reorganization of the duplexes with a significant increment in measured current [66, 67]. The limit of detection of such assay for miR-29b-1 and miR-141 has been estimated between 5 and 10 fM.

MiRNA assays analogous to those discussed before and exploiting the quenching of labeled PNA probes can be also designed by using carbon nitride nanosheet (CNNS). In this case, the acquisition of the fluorescence radiated by labeled PNA probes adsorbed on CNNS upon hybridization with complementary miRNA target is used to detect miRNA in the complex medium [68].

The combination of different nanomaterials can improve performances of assays and provides new opportunities for the amplification of detected signals. In this case, the coupling of light excitation and electrochemical detection in photoelectrochemistry provides sensitive miRNA biosensing, thanks to the low background current [69]. An example of a similar strategy is represented by the combination of (SWCNTs) and DNA-modified QDs. A photoelectrochemical biosensing platform using such a reactive surface, combined with endonuclease-based amplification, has been reported to detect miR-7f with 34 fM limit of detection and 50 fM to 100 pM linear response range [70].

Selectivity in miRNA biosensing

The selective detection of miRNAs is a challenging task because mature miRNAs are highly heterogeneous both in sequence and length. A close sequence similarity is often found among miRNAs belonging to the same family—sequences deriving from common precursors and showing similar physiological function [71]. It has been suggested that miRNA isoforms (also called isomiRs), due to variations in precursor processing by Drosha and/or Dicer enzymes, might exhibit different biological properties, thus introducing a potential justification for the heterogeneity [72]. In addition, mature miRNAs are found in combination with precursor sequences (pre-miRNAs) which are considered as independent miRNA genes from which mature miRNA sequences are excised. RNAs extracted from biological samples include variable amounts of both pri- and pre-miRNA.

The most comprehensive miRNA database (miRBase. Release 22) contains 38,589 entries representing hairpin

precursor miRNAs, expressing 48,885 mature miRNA products, in 271 species (http://www.mirbase.org/). Some of the miRNA genes are categorized into different miRNA families on the basis of similarities found in their sequences. MiRNAs belonging to the same family are supposed to derive from the identical ancestor in the phylogenetic tree and to execute similar biological functions [73].

In spite of this complex scenario, the detection of multiple miRNAs has been proposed as an effective way to fingerprint cancerous cells [74] and the definition of the expression level of selected miRNAs a promising tool for the prediction and prognosis of important diseases [75].

Methods for miRNA detection should be able to detect mismatches that differentiate among different miRNA family members.

The treatment of biological samples before the analysis aimed at isolating mature miRNA sequences to be detected can significantly affect the successful detection of miRNAs. In this respect, commercial kits are available to efficiently separate mature miRNAs from other RNA species, including pri-miRNA and pre-miRNA [76].

Different strategies have been investigated with the specific aim to improve the selective detection of miRNAs. They exploit molecular biology techniques (e.g., in situ hybridization—ISH [77] or enzymatic amplification [78–81]) or nanotechnology-based approaches [18]. Such strategies are applied to extend the length of the capture probe thus increasing the melting temperature (T_m) of the probe–miRNA duplex thus optimizing miRNA hybridization conditions [82]. Alternative strategies aim at extending the length of the duplex with additional base pairs [83]. Molecular beacons (MB) are sometimes used to distinguish between pre-miRNAs and mature miRNAs [84, 85].

The identification of efficient probes is essential in achieving selective discrimination of single-base mismatched miRNAs. In this context, the use of synthetic DNA analogues such as PNAs [86–88], LNAs [83, 89], or phosphorodiamidate morpholino oligos (PMO) [90] has been shown to improve selectivity in miRNA detection [91, 92].

ISH is widely used for the detection of miRNA expression profiles [93, 94]. The introduction of LNA probes [95] improves ISH performance as a consequence of the higher affinity of LNAs for the complementary RNA sequences compared to conventional RNA or DNA [84].

MiRNA detection may also benefit from the implementation of new signaling probes such as those obtained using the thermostable enzyme esterase 2 (EST2) [96]. EST2 offers the possibility to perform site-specific modification of probes and provides superior performances to common enzyme labels such as alkaline phosphatase and horseradish peroxidase. EST2–oligodeoxynucleotide conjugates provide enzyme-mediated electrochemical responses to obtain a selective detection of miRNA-16 within a mixture of other miRNAs [97].

The isothermal enzymatic amplification introduces some advantages in the selective detection of nucleic acid target sequences [98, 99]. In particular, isothermal amplification combined with the use of GO has been shown to significantly improve performances in miRNA detection allowing the simultaneous detection and discrimination of highly similar let-7 miRNA sequences. In this case, advantages come from both the quenching action of GO on dye-labeled ssDNA probes as well as the reduced affinity of GO surfaces toward dsDNA compared to ssDNA [100, 101]. GO surface inhibits the nuclease degradation of adsorbed ssDNAs [102]. The combination of such effects with the cyclic enzymatic amplification method (CEAM) has been investigated to develop a new miRNA detection assay in complex biological samples using *DNase I* endonuclease and labeled ssDNA fluorescent probes. ssDNA fluorescent probe spontaneously adsorbs on the GO surface causing the quenching of the fluorescence and preventing the probe degradation from DNase I. The duplex formed after the hybridization between target miRNA and ssDNA probe weakly binds to GO and is released in solution where DNase I degrades the ssDNA while leaving unaltered the miRNA sequence (Fig. 4) [103]. A similar approach has been applied using *RsaI* endonuclease. In this case, the selectivity of the assay has been evaluated by detecting cDNAs from miR-141, miR-21, let-7d, and miR-122, respectively [104].

Multiple approaches based on the use of GO nanoassemblies have been investigated with the aim to address both the selectivity and sensitivity challenges in miRNA detection. Some of them exploit hybridization chain reactions [105] and enzyme or enzyme-free amplification methods [106, 107]. The insertion of multiple mismatches at specific positions of the probe strands immobilized on GO surface increases the selectivity in miR-10a and miR-10b detection [108]. The enhanced selectivity is counterbalanced by a loss of signal intensity that may be compensated by the use of an endonuclease amplification step.

Nanopore-based electronic detection offers new possibilities for the selective detection of miRNAs, thanks to its inherent size selectivity. Molecules with different sizes give rise to detectable different ionic current signatures when passing through a single nanopore [109, 110].

In particular, it has been demonstrated that nanopore-based approaches can be exploited for the detection of liver-specific miR-122a sequences isolated from tissues thus exceeding the selectivity of conventional methods [111]. The selective detection is obtained in this case by first allowing the target miRNA to hybridize the probe sequence. The formed duplex is then bound to protein p19-functionalized magnetic beads. p19 specifically binds to dsRNA in a size-specific manner [112]. The duplex is then electronically detected using the nanopore technology. Separation methods of single-base mismatch in miRNAs based on the DNA probe is not practical in

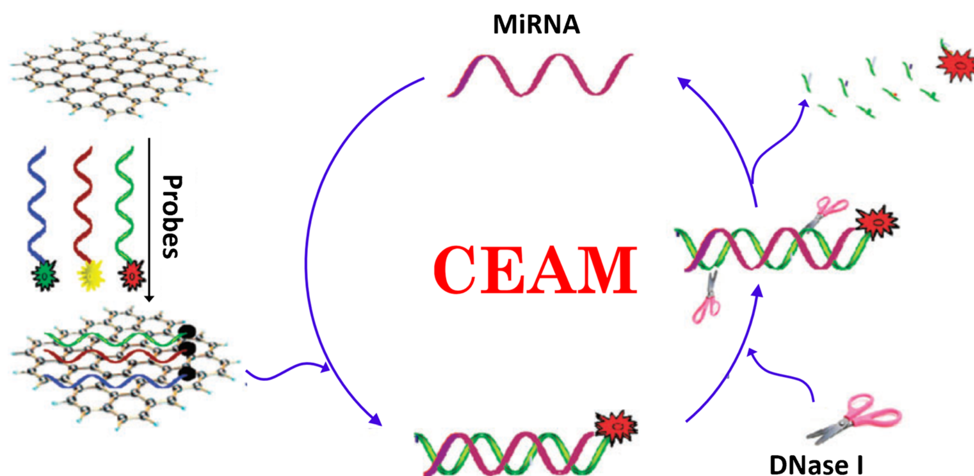

Fig. 4 Working principle of cyclic enzymatic amplification method for multiplex miRNA analysis based on graphene oxide–protected and quenched ssDNA probes. Reproduced with permission from [103]

real-time detection to achieve the needed discrimination accuracy for real samples which include various RNA species such as miRNAs, mRNAs, and tRNA. To overcome such limitation, probes different than DNA, such as polycationic peptide–PNA probes, have been used in combination with nanopore-based detection [113]. Cationic probes, when hybridized with miRNA, form a dipole complex which can be captured by nanopores using a voltage polarity that is opposite to the polarity used to capture negatively charged nucleic acids. As a result, nontarget species are driven away from the nanopore thus allowing miRNA detection without interference. Nanopore technology has benefited also from PNA-enhanced capability to selectively detect point mutations by targeting let-7b and let-7c [114]. Nanopore-based sensing technology has been also used to detect circulating miR-155 in lung cancer patients differentiating between members of the same miRNA family. In this case, a modification of the nanopore-based approach has been introduced using the alpha-haemolysin protein as a 2-nm-wide nanopore for the direct detection of miRNA from tissue [115]. A 3′ and 5′ poly$(dC)_{30}$-labeled capture probe was used to target miR-155 and the characteristic change in the nanopore conductance measured. The method can distinguish the relative levels of miR-155 of healthy and lung cancer patients in close agreement with RT-PCR-based measurements.

Multiplexing capability of miRNA sensing platforms

MiRNA expression profile is depending on physiological conditions and changes over time. One single miRNA can control numerous functions and can be relevant for different diseases, while one selected disease is frequently correlated to the expression of multiple miRNAs.

Several pieces of evidence have proved that an aberrant miRNA expression establishes a singular, exclusive, and specific pattern for each type of cancer, reflecting the onset and the progression of the tumor disease [116].

The abovementioned considerations justify how important is the high-throughput capability of miRNA detection platforms. High-throughput detection also helps in reducing the cost and complexity of the assay, being significant drivers for clinical diagnostic applications.

The multiplex detection can be obtained through the spatially resolved patterning of different miRNA capture probes, the sequential separation of miRNAs before detection, or through the use of distinguishable tags.

Among the different methods under investigation for miRNA detection, some of the most promising in terms of high-throughput capability involve the optical or electronic signal transduction [15].

GO fluorescence quenching property has been applied to design a detection system which exploits dye-labeled PNA probes and nanosized GO (NGO) for the real-time monitoring of multiple miRNAs in living cells [101, 117]. The miRNA target hybridization to dye-labeled PNA causes the probe detachment from the NGO surface and quenched fluorescence recovery. PNA probes are used in this case due to the lower fluorescence background and the more stable binding with NGO compared to DNA probes. This so-called PANGO detection system allows high-throughput monitoring of samples containing multiple miRNAs with low background and almost no cross-reactivity [118]. A similar detection system is obtained using nanoporous metal–organic frameworks (MOFs) and labeled PNA probes [119]. Quenched dye-labeled PNA probes are released from nano-MOF after the miRNA target hybridization. The multiplexing capability of the assay has been tested against miRNAs expressed in cancer cell lines (miR-21, miR-96, and miR-125b) [120]. Enzymatic amplification methods may be combined to enhance the sensitivity of assays [103, 121], while large GO surfaces make it possible to quench several probes tagged with different dyes thus increasing the multiplying capacity of the platform.

Zhu et al. [122] described a PCR-free electrochemical assay for the multiplexed detection of miRNAs relying on the selectivity of ligase chain reaction and the voltammetric signature of electrochemical QD barcodes. The assay uses two reporting probes tagged to PbS and CdS QDs, respectively, and two capture probes co-immobilized on magnetic beads. After the incubation of miRNAs and the addition of T4 DNA ligase, the covalent bond of only perfectly complementary miRNA targets is obtained. The simultaneous square wave voltammetry detection of miR-155 and miR-10b is thus performed (Fig. 5).

QDs are also used in FRET-based biosensing of multiple miRNA detection. The multiplexed miRNA assay is based on the use of different QDs conjugated with short oligonucleotides complementary to luminescent Tb-conjugated oligonucleotide strands. Sequences are designed to obtain a stable interaction between the Tb-conjugated and QD-immobilized oligonucleotides only in the presence of miRNA. MiRNA triggers the formation of a stable miRNA-Tb-reporter double-stranded RNA/DNAs which can then hybridize to the QD-immobilized oligonucleotide. The presence of the selected miRNA brings Tb and the different QDs close enough to enable the different FRET processes [123].

Multiplexed fluorescence detection of miRNA [124] has been largely applied, even if the spectral overlap limits the implementation of this method to high-level multiplex quantification [125]. The multianalyte capability of inductively coupled plasma-mass spectrometry (ICP-MS) [126] has been applied to develop a version of DSN-mediated target recycling in which lanthanide-tagged probes are immobilized on streptavidin-coated magnetic particles. In the presence of the miRNA target, a DNA/miRNA heteroduplex is formed, and as a consequence of DSN action [127], the lanthanide-tagged DNA probe released in solution and quantified by ICP-MS [128].

Silicon photonic microring resonators offer another platform for the multiplexed label-free detection of miRNAs. The multiplexing capability arises from differentially functionalized microrings available on the silicon chip surface. Sequence-specific responses are detected at appropriate microrings only when the complementary miRNA interacts with the functionalized sensor array. Microring arrays can be used to quantify the expression level of multiple miRNAs from clinically relevant samples within a data acquisition time of 10 min [129]. Anti-DNA/RNA antibody has been combined with the intrinsic sensitivity of silicon photonic microring resonators to improve LOD. The sensor chip surface modified to expose multiple, sequence-specific capture probes has been treated with antibody for DNA/RNA duplex formed from standard solutions as well as from the total RNA extract from mouse brain tissue without the qRT-PCR amplification procedure. The assay can detect miRNAs at concentration as low as 10 pM (350 amol) [130].

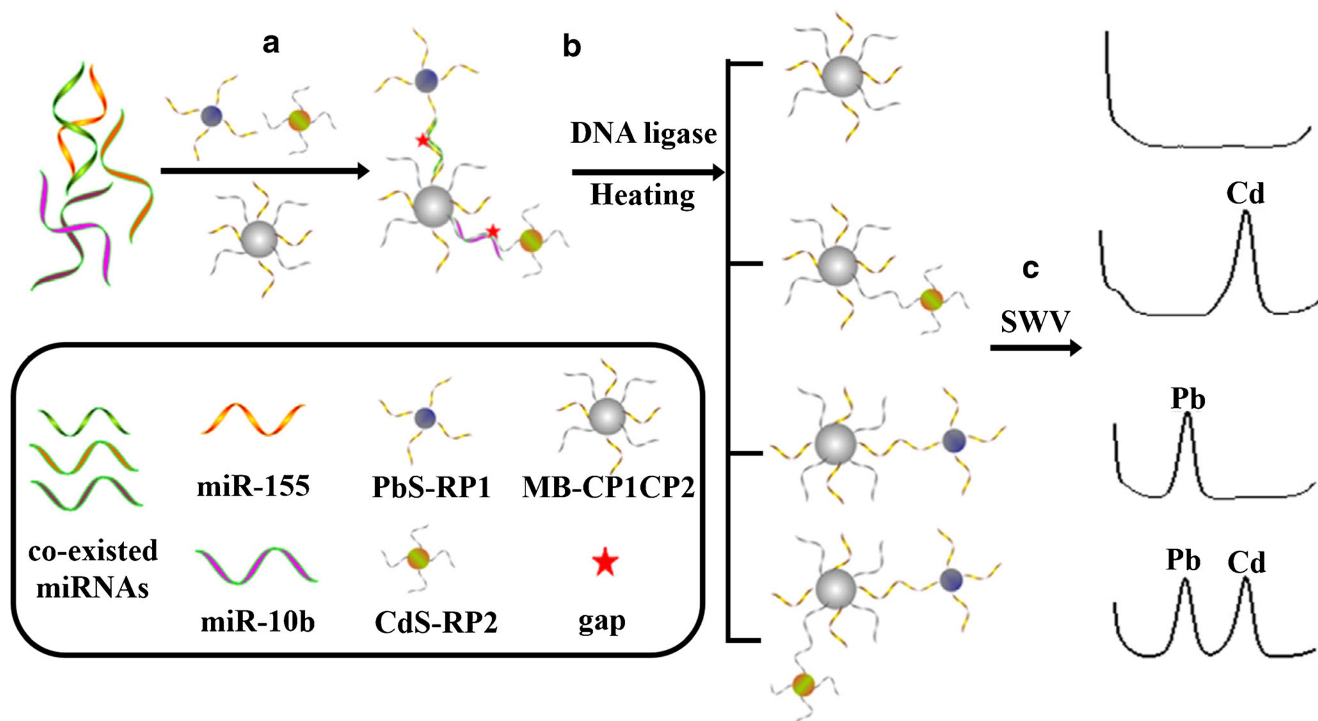

Fig. 5 Schematic illustration of the simultaneous electrochemical detection of multiple miRNA targets: **a** incubation of the testing samples with MB–CP1CP2, PbS–RP1, and CdS–RP2 conjugates; **b** addition of the T4DNA ligase to the resultant MBs and heating at 90 °C for 30 min; and **c** stripping analysis of the joined QD barcodes and the voltammetric signal, indicating the levels and pattern of the target miRNAs. Reproduced with permission from [122]

SPR imaging (SPRI) is a powerful biosensing method to detect miRNA with ultrasensitivity and multiplexing capability. SPRI detection can be implemented with different array configurations [131] and can provide highly sensitive detection when combined with nanostructured enhancers. Recently, a universal nanostructured enhancer has been used to detect and quantify with SPRI miR-422, miR-223, miR-126, and miR-23a in human serum samples [132].

Capillary electrophoresis (CE) also holds the potential for direct, sensitive, and high-throughput detection of miRNAs [133, 134]. High-throughput is in particular linked to CE laser-induced fluorescence (LIF) method capability to detect differently labeled ssDNA probes [135]. Different CE-based strategies for miRNA detection have been established such as protein-facilitated affinity CE (ProFACE) [136], combination of isothermal exponential amplification reaction (EXPAR) with CE-based single-strand conformation polymorphism (CE-SSCP) [137], and capillary electrophoresis–electrospray ionization–mass spectrometry (CE-ESI-MS) [138]. Among these, a CE-based assay called direct quantitative analysis of multiple miRNAs (DQAMmiR) allows the simultaneous analysis of up to 25 different miRNAs, with the possibility to adapt the method for clinical applications with an overall assay time of about 20 min [139–141].

Detection of miRNAs from different biological samples

The expression profile of miRNAs is tissue- and organ-specific [142]. An important aspect concerning the use of miRNAs as clinical biomarkers is related to the identification and validation of the best biological sample source to be used for the analysis. Biological samples include tissue, blood, plasma or serum, and other fluids such as saliva, urine, seminal fluid, cerebrospinal fluid, and tears [6]. After the discovery of circulating miRNAs [143], efforts have been paid in validating panels of miRNAs associated with distinct physiological and pathological processes. The direct analysis of patient samples could mitigate sample loss and provide rapid and flexible assays. The direct analysis of biological fluids helps also in eliminating bias introduced by procedures adopted to extract miRNAs from biological samples [144].

Electrochemical detection of miR-21 has been demonstrated by using esophageal squamous cell carcinoma tumor tissue [145]. The designed detection protocol allows the miR-21 expression level to be defined with pre-miRNAs differentiated from mature miR-21. Other electrochemical platforms with the capacity to operate with saliva and blood samples have been also described [146, 147]. Among them, recently conductive gold-coated magnetic nanoparticles has been used as dispersible electrodes [148] that allow measuring miRNA concentrations from 10 aM to 1 nM from unprocessed blood samples [149]. First, the sensor was challenged by analyzing miR-21 levels in a pool of other RNA sequences present in the total RNA extracted from human lung cancer cells and vesicles and by spiking different amounts of the extracted total RNA into either PBS or 50% whole human blood. Then, the clinical relevance of the method has been demonstrated by measuring and distinguishing small variations in miR-21 expression level in whole unprocessed blood samples of mice with growing tumors (Fig. 6).

SPR biosensors have been also developed for the detection of miRNA in biological samples [29, 150–153]. Recently, an ultralow fouling SPRI system has been reported for the detection of multiple miRNAs in erythrocyte lysate with no need for miRNA extraction and preamplification [154]. The streptavidin SPR signal enhancement has been used in combination with DNA super-sandwich assemblies for the development of a biosensor able to detect miR-21 in total RNA samples from human breast adenocarcinoma MCF-7 cells [155].

The detection of miRNAs from urine samples in establishing bladder cancer diagnosis is obtained using a dual-isothermal cascade-assisted lateral flow assay [156]. The assay integrates base stacking hybridization (BSH) with an exponential isothermal amplification (EXPAR) [157] and a lateral flow PNA-based biosensor. Following the BSH process triggered by the presence of the miRNA target, EXPAR may occur, and amplified DNA is adsorbed on the sample pad of a lateral flow strip. Then, miRNA-specific DNA-functionalized AuNPs are eluted on the conjugate pad of the lateral flow strip where two PNA probes (test and control) were prior immobilized. A visible red line results from the accumulation of AuNPs due to the capture by the immobilized complementary PNA and the miRNA-specific AuNP conjugates on the PNA probe test line. The assay reveals miR-126, miR-182, and miR-152 from urine samples of bladder cancer patients and healthy donors with LOD 0.6 fM.

Detection of miRNAs in microfluidic and point-of-care devices

The integration of nucleic acid detection assays in microfluidic devices represents a highly promising approach for the development of cheap and convenient tools for clinical diagnostic applications [158]. Such tools are expected to operate at the point-of-care and in resource-limited settings.

Droplet microfluidics offers remarkable prospects for the development of chip-based diagnostics [159]. As far as miRNA detection is concerned, the combined use of droplet microfluidics and molecular beacon–assisted isothermal circular-strand-displacement polymerization allows 20 nL droplets of miR-210 solutions 330 pM in concentration to be detected using a total sample volume as low as 200 nL [99]. Droplet microfluidics is inherently compatible with digital bioassays [160], and digital droplet PCR is widely used today to quantify miRNAs expressed in different biological sources [161–164].

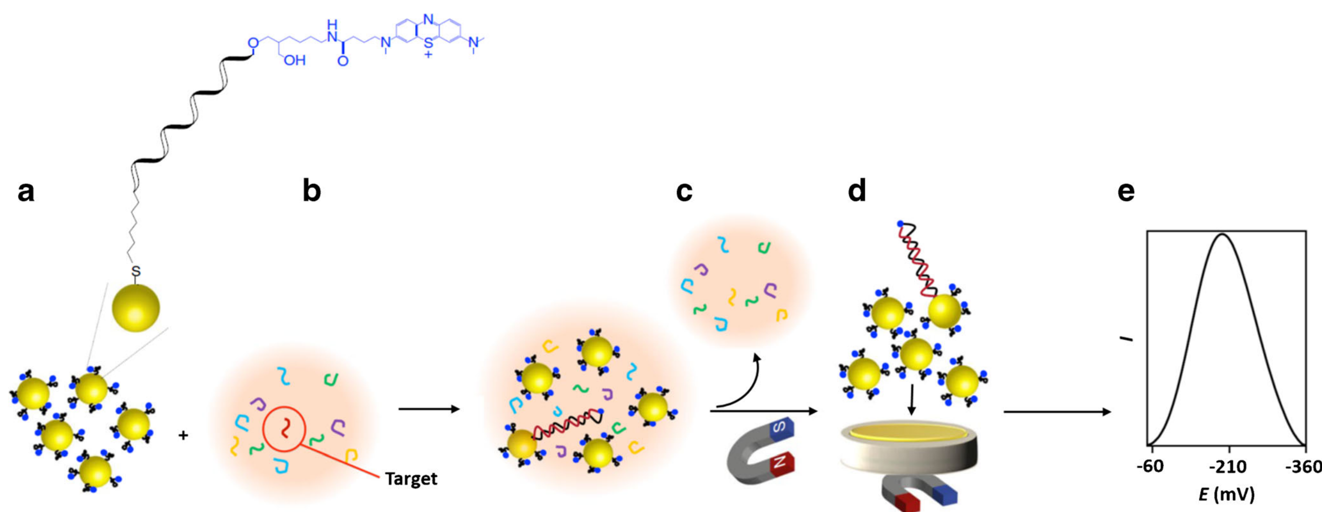

Fig. 6 Schematic representation of the steps involved in the proposed sensing strategy for detecting miRNA. **a**, **b** Excess Au@MNPs, modified with methylene-blue–labeled probe DNA complementary to target miRNA (**a**), are mixed with the sample (**b**). **c** After 30 min, Au@MNPs are separated from the solution magnetically and unhybridized sequences are washed away. **d** Au@MNPs (hybridized and unhybridized) are magnetically collected on the surface of a gold microelectrode. **e** Electrical reconfiguration of Au@MNPs in their heterogeneous network (hybridized and unhybridized) and stable peak currents obtained before and after hybridization is used to measure the amount of target analyte. Reproduced with permission from [149]

Microfluidics-based devices can help in enabling diagnostic testing of patients without the demand of sophisticated facilities [165]. In this regard, PDMS has been widely used to fabricate microfluidic devices for miRNA detection. The combination of such devices with laminar flow-assisted dendritic amplification has been used to develop a power-free device for the easy-to-use and fast detection of miR-16, mir-21, and miR-500 [166]. The implementation of plasmonic sensing in microfluidic devices based on the use of metallic nanoparticles introduces further improvements in miRNA detection [167]. The coupling of surface plasmon resonance biosensor with a microfluidic device provides an efficient platform for the detection of miR-16-5p in urine samples of patients with acute kidney injury, without amplification and labeling of the target molecules [168].

A rapid and accurate quantification of circulating miRNA at ultralow concentration ranging from 10 to 10,000 copies mL^{-1} in ≤ 3 h and with no need for sample pretreatment has been obtained using an Integrated Comprehensive Droplet Digital Detection (IC 3D) approach. IC 3D combines the encapsulation of miRNA target in droplets and their exponential amplification, then microparticles are counted for digital quantification with a 3D counter (Fig. 7) [169]. With this method, let-7a target is absolutely quantified with LOD of 50 copies per mL, directly from the blood of colorectal cancer patients.

Most of the microfluidic-based assays exploit the miRNA capture by probes and operate with crude biological samples, including cell lysates [170], human plasma/serum [171], and human cerebrospinal fluid [172]. The so-called Theranostic one-step RNA detector ("TORNADO") uses Pt nanoparticles functionalized with a thiolated probe complementary to a portion of the miRNA target sequence along with a capture probe immobilized on gold electrodes [172]. The detection system is implemented in a centrifugal microfluidic device that is spun to mix reagents and to form a sandwich-type complex. Pt nanoparticles catalyze the reduction of H_2O_2 to allow the amperometric detection of miR-134 in unprocessed human plasma and cerebrospinal fluid samples.

The use of low-cost and flexible materials is highly desirable for the fabrication of point-of-care devices for the detection of miRNAs in resource-limited settings [173, 174]. The naked eye detection of miR-21 associated with lung cancer is achieved using a polyvinylidene fluoride porous membrane modified with a luminescent reporter (poly(3-alkoxy-4-methylthiophene), PT) and PNA probes [175]. In this case, the orange fluorescence signal, generated when the triplex PT–PNA–miR-21 is formed, allows the implementation of a very simple platform for miRNA detection, which does not require the use of sophisticated equipments and operates with a wide dynamic range (10 nM to 10 mM).

A summary of different aspects of miRNA detection here described is provided in Table 1**.**

Normalization and validation in miRNA detection

The reliable comparison of miRNA expression levels across different samples is a challenge that requires robust

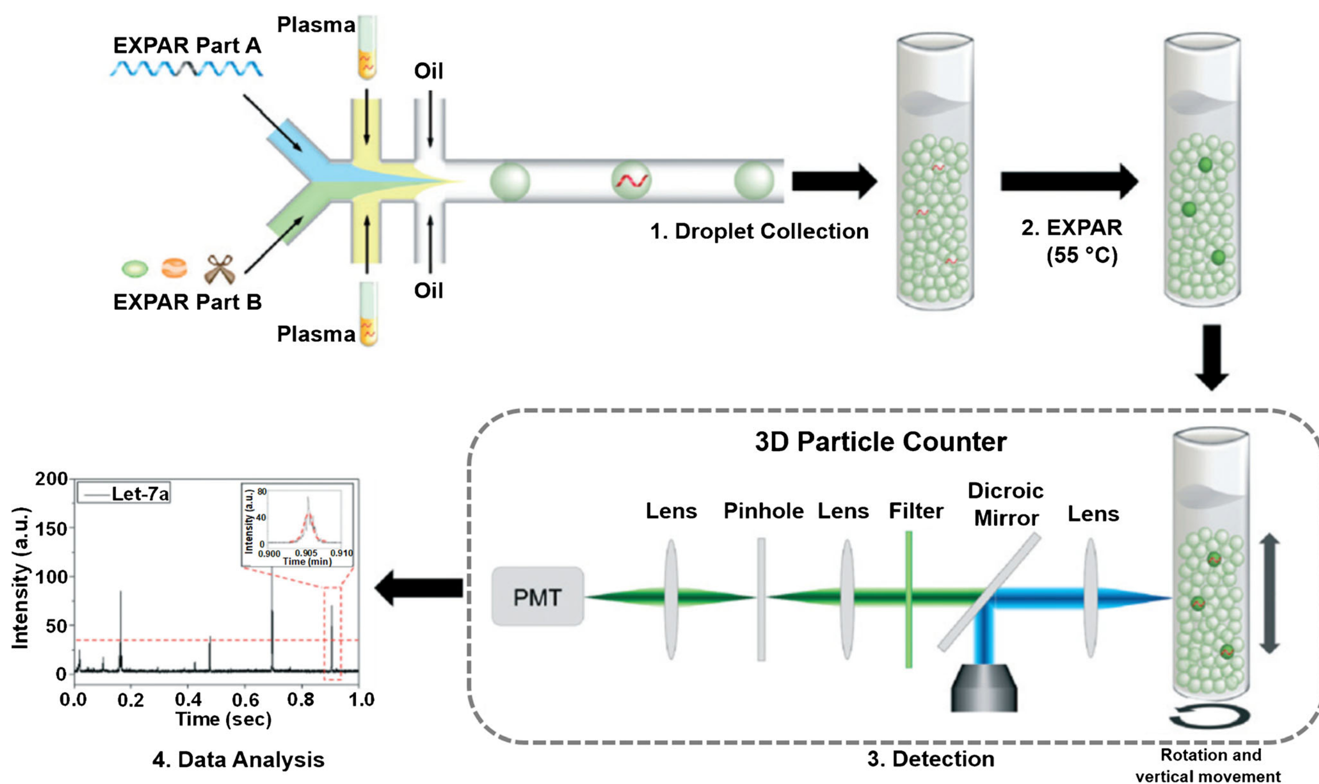

Fig. 7 Schematic diagram of IC 3D technology for miRNA detection. Plasma samples and sensing elements (EXPAR part A and EXPAR part B) are mixed and encapsulated in hundreds of millions of picoliter-sized droplets using a flow-focusing microfluidic device (step 1). Droplets are then collected in a tube and incubated to conduct isothermal exponential amplification (step 2). After incubation, droplets are detected (step 3) and analyzed (step 4) using a high-throughput 3D particle counter that can robustly and accurately detect single fluorescent droplets from milliliter (mL) volumes of sample within several minutes. Reproduced with permission from [169]

procedures for data normalization to be identified. The nature of free circulating miRNAs results in several problems in experimental research which are not encountered when examining miRNA from other sources such as cells and tissues. For this reason, specific considerations must be taken into account for normalization procedures.

Each conventional or innovative technology has advantages and drawbacks to be considered in order to design miRNA experiments. Data can vary across platforms thus requiring careful and critical interpretation of findings [176].

A good normalizer must have (i) invariant expression across all samples, (ii) expression in the sample of interest, (iii) good stability, and (iv) similar extraction efficiency than the target of interest [16, 177].

Small, noncoding RNAs are typically used for data normalization in miRNA profiling experiments, while housekeeping genes are used for normalization of mRNA expression data [178]. Different factors have to be assessed to normalize miRNA expression level data. Some miRNAs show stable expression under different conditions. Such miRNAs represent good alternatives to small RNAs such as U6 in normalization [179, 180].

Additional aspects to be considered in normalization come from variations introduced by pipetting errors and changes in signal background [181]. Unfortunately, universal reference miRNAs are not available yet, even though efforts are paid in identifying them [182, 183].

Specific mention is to be given to normalization of data from free circulating miRNAs. Up to this date, there are no housekeeping miRNAs available for normalization of data obtained from circulating miRNAs. Such samples cannot be normalized against U6 due to its low concentrations in serum and plasma [184]. However, normalization procedures for serum or plasma samples based on sample spiking with mature miRNA from *Caenorhabditis elegans* or plants have been proposed as a way to overcome such problems [185].

To date, there is no gold standard for assessing the validation issue. Standard approaches, such as qRT-PCR, have been successful to quantify miRNAs. However, they can be problematic in some circumstances [186]. They cannot be used for the simultaneous detection of different miRNAs. In addition, there are no standard guidelines for driving and reporting validation procedures. Standardized guidelines should be defined to interpret miRNA data with transparency in reporting.

Table 1 Performances of miRNA detection platforms and assays

Detection platforms and assays		LOD	Dynamic range	Selectivity	Multiplexing capability	Biological specimen	Ref.
Surface plasmon resonance (SPR)	NESPRi LNA probes AuNPs@polyT	10 fM	20 fM to 2 pM	miR-122b miR-16 miR-23b	√		[28]
	Diffraction grating couplers	2 pM	0.1 to 100 nM	miR-122 miR-192	√	Mouse liver tissue	[29]
	Streptavidin–oligonucleotide complex	1.7 fM	0.1 to 100 nM	miR-122 miR-29	√	Breast tumors	[30]
	AuNPs@ DNA/RNA antibody	0.55 pM 0.88 pM 1.19 pM 1.79 pM	0.1 to 100 pM	miR-422 miR-223 miR-126 miR-23a	√	Human serum samples	[132]
	Polymer brushes	0.35 pM 0.39 pM 0.50 pM 0.95 pM	0.1 to 500 pM	miR-16 miR-181 miR-34a miR-125b	√	Erythrocyte lysate samples	[154]
	DNA super-sandwich assemblies and biotin–streptavidin amplification	9 pM	10 pM to 1 µM	miR-21, 1- and 2-base mismatch, noncomplementary sequences and miR-222	√	Human breast adenocarcinoma cell	[155]
Surface-enhanced Raman scattering (SERS)	Duplex-specific nuclease (DSN) amplification, Fe3O4@Ag core–shell magnetic NPs	0.3 fM	1 pM to 10 nM	miR let-7b and let-7 family members	√	Human breast cancer cell lines, cervical cancer cell lines, and human lung adenocarcinoma cell lines	[40]
Electrochemistry	Cyclic voltammetry nafion, thionin (Thi), and Pd nanoparticles	1.87 pM	5.6 to 5.6×10^5 pM	miR-155 and noncomplementary sequences		Human blood serum	[43]
	Amplified voltammetry, ferrocene (Fc)-capped AuNPs/streptavidin conjugates	10 fM	1 fM to 2 pM	miR-182, 1-base mismatch and noncomplementary sequences	√	Serum samples from multiple healthy donors and glioma patients	[44]
	Amperometry, electrocatalytic nanoparticle tags (ENT)	80 fM	0.3 to 200 pM	miR-106 let-7b let-7c		Total RNA extracted from HeLa cells	[45]
	Square wave voltammetry (SWV), PNA probes polyaniline (PAn) nanowires	2 fM	5 fM to 2 pM	miR-720 miR-1248 let-7c let-7a let7-b	√	Total RNAs extracted from HeLa cells and lung cancer cells	[48]
	Electrochemical impedance spectroscopy (EIS), insulating polymer film	2.0 fM	5.0 fM to 2.0 pM	let-7 family let-7c let-7b let-7e.	√	Circulating miRNAs in the blood and miRNAs in total RNA extract from HeLa and lung cancer cells	[50]
	EIS	0.37 fM	1 fM to 100 nM	miR-145 1 and 2 mismatches, and noncomplementary sequences			[51]
	SWV EIS	5 aM	10 aM to 1 µM	miR-21 miR-32 miR-122	√	Human serum	[52]
	EIS, gold-nanostructured disposable carbon electrodes and enzyme-decorated liposomes	0.400 pM	1.70 to 900 pM	miR-222 and noncomplementary miR-16		Spiked human serum samples	[61]
	SQW	8 fM		miR-29b-1			[67]

Table 1 (continued)

Detection platforms and assays		LOD	Dynamic range	Selectivity	Multiplexing capability	Biological specimen	Ref.
			1 fM to 1 nM	miR-141			
	EST2–ODN conjugates	2 pM	0.002 to 200 nM	miR-16 miR-21	√	Human breast adenocarcinoma cells	[97]
	SQW Ligase chain reaction (LCR) QDs	12 fM 31 fM	50 fM to 30 pM 50 fM to 1050 pM	miR-155 miR-10b miR-10a miR-27b	√	Spiked human serum samples	[122]
	SQW DNA–Au@MNPs dispersible electrodes	10 aM	10 aM to 1 nM	miR-21 miR-39	√	Unprocessed blood in a xenograft mouse model of human lung cancer	[149]
Fluorescence	DSN amplification, pyrene excimer switching	0.58 fM	1 fM to 10 nM	let-7a let-7b let-7c		Human HeLa cells and blood samples	[57]
	DSN amplification, quenching of (+)AuNPs to silver nanoclusters (AgNCs)	33.4 fM	100 fM to 1.0 pM	miR-155 miR-21 miR-141 let-7° miR-182		Serum samples from healthy donors, lung cancer patients, and breast cancer patients	[63]
	Quantum dots (QDs)	10 fM	1 fM to 10 nM	1-base-mismatched and random sequences, pre-miRNA mature miRNA		Bovine serum samples	[64]
	QDs DSN amplification	42 fM	1 fM to 10 nM	miR-21 miR-148 miR-133a miR-378 miR-423		Human breast cancer cells and noncancerous human embryonic kidney	[65]
	Graphene oxide-protected DNA, cyclic enzymatic amplification method (CEAM)	9 pM	20 pM to 1 nM	let-7 and miR family members	√	Lysate from lung carcinoma cell line and mammary epithelial cells	[103]
	Graphene oxide and DNA nanoassemblies.	225 pM		miR-10b miR-10a		Liquid biopsy mimics and cell extracts	[108]
	PNA Nanosized GO (NGO)	1 pM	0 pM to 1000 nM	miR-21 miR-125b miR-96	√	Early stage breast cancer cell, invasive human breast cancer cell line, late-stage cancer model, and melanoma cell line	[118]
	PNA nanoporous metal–organic frameworks (MOFs)	10 pM	0 to1000 nM	miR-21 miR-96 miR-125b	√	Human breast cancer cell lines	[120]
Photo-electrochemistry	DNA-CdS QDs Single-walled carbon nanotubes (SWCNTs) Cyclic enzymatic amplification	34 fM	50 fM to 100 pM	miR-7f Members of the let-7 family			[70]
Electronical detection	Nanopore sensor protein p19-@ magnetic beads	< 50 pM	50 pM to 5 nM	miR-122a miR-153			[111]
		< 50 pM	50 pM to 5 nM	let-7b let-7c			[115]

Table 1 (continued)

Detection platforms and assays		LOD	Dynamic range	Selectivity	Multiplexing capability	Biological specimen	Ref.
	Nanopore sensor a polycationic peptide-PNA probe			miR-155 miR-21			
	Nanopore sensor based on the α-haemolysin protein		10 nM to 100 nM	miR-155 let-7a let-7b let-7c		Peripheral blood samples from lung cancer patients	[115]
Silicon photonic microring resonator	Anti-DNA:RNA antibodies	10 pM	10 pM to 40 nM	miR-16 miR-21 miR-24-1 miR-26a	√	Mouse brain total RNA	[130]
Capillary electrophoresis (CE)	Confocal time-resolved fluorescence embedded capillary interface CE-CTFR-ECI	12,000 copies per cells		miR-21 miR-125b miR-145	√	Human breast cancer cell line	[141]
Base stacking hybridization (BSH)	Exponential isothermal amplification (EXPAR) and lateral flow PNA biosensor	0.6 fM	1 fM to 100 pM	miR-126 miR-182 miR-152 1-, 1-, and 3-base mismatch	√	Urine samples of bladder cancer patients and healthy donors	[156]
Microfluidics	Digital microfluidics, molecular beacon (MB)–assisted isothermal circular-strand--displacement polymerization (ICSDP)	3.3 amol in 20 nL	165 pM to 4 nM	miR-210 and noncomplementary sequence		Transfected leukemic cell line	[99]
	Microfluidic chip laminar flow-assisted dendritic amplification (LFDA)	48 fM 1.4 pM 140 fM	0.001 to 1000 pM	miR-16 mir-21 miR-500	√		[166]
	Magnetic nanoparticles (MNPs) SPR on capped gold nanoslits	17 fM	1 to 1000 pM	miR-16-5p and 1 mismatch sequence		Urine samples from patients with acute kidney injury	[170]
	Fluorescence Microdroplets high-throughput 3D particle counter	50 copies per mL	1 fM to 1 nM	let-7a let-7b let-7c		Plasma samples from colon cancer patient and healthy donor	[169]
	Electrocatalytic platinum nanoparticles	1 pM	1 pM to 1 μM	miR-134 1-base mismatch and noncomplementary sequences		Unprocessed human plasma and cerebrospinal fluid samples	[172]
Mass spectrometry (MS)	LDI–TOF-MS and nanoengineered microgold shell (lAuS)	0.1 fM	0.1 fM to 1 nM	miR-122	√	Hepatocarcinoma cell line	[25]
	ICP-MS, lanthanide-tagged oligonucleotide and DSN amplification	47.34 fmol 58.69 fmol 0.84 pmol		let-7d miR-21 miR-141	√	Cervical cancer cell line	[128]

Conclusions and perspectives

In consideration of the peculiar features of miRNAs that involve small size, low abundance, and high sequence homology and that put constraints to any detection strategy, numerous efficient and sensitive strategies have been reported.

The gold standard quantitative PCR (RT-qPCR) converts the small miRNA sequences into longer sequences either by adding poly(A) tails [187] or using a stem-loop probe [188]. Compared to PCR, isothermal amplification methods [189, 190] require no thermal cycling and can be conveniently integrated with droplet microfluidics to build platforms for high-throughput miRNA detection [159]. The microfluidic environment reduces sample volume requirements thus minimizing the need to dilute the original sample to comply with the detection system specifications. Sample dilution may preclude the detection of low concentrated miRNAs thus affecting the ability to detect miRNAs freely in the blood [191].

Besides miRNAs, other systems are freely circulating in human blood such as circulating tumor cells [192], vesicles and exosomes [193], circulating tumor DNAs [194], and proteins [195] that may be used to fingerprint tumor characteristics. The translation of miRNA detection into clinical practice will be facilitated with the capacity to monitor concentration changes of different biomarkers.

Nanotechnology-based assays provide impressive sensitivity levels that help in simplifying detection protocols. Some isothermal amplification enzymatic strategies combined with microfluidic-based platforms meet the required sensitivity specification for miRNA analysis even in complex biological matrices and using low miRNA amount. They are also applicable for multiplex analysis.

Unfortunately, most of the described contributions are still at the proof-of-principle level. They have been validated against synthetic or spiked-in samples, do not employ stringent controls in assessing the technology performance, do not adequately evaluate sample purity, and determine LOD taking into account internationally established guidelines. For those reasons, it is not easy to anticipate the extent to which the numerous emerging approaches in miRNA detection will be able to provide a decisive contribution to overcoming issues related to miRNA detection.

Methods with simplified detection schemes to address the sensitivity and selectivity issues in miRNA detection have been developed by exploiting hybrid strategies to improve multiplexing capability. The development of integrated devices for the processing of clinical samples and miRNA detection will be critical to foster further advancement in the field.

The poor standardization of methods used to detect miRNAs in clinical samples remains the most significant challenge. Preanalytical variables such as sampling, storage, and miRNA specimen processing together with analytical procedures such as standardization and data normalization in the absence of validated normalizers introduce discrepancies in miRNA levels detected using different platforms. Such inconsistencies significantly contribute to slowing down the advance of the identification and validation of panels of circulating miRNAs related to the onset and progression of important diseases.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

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