Direct miRNA-hybridization assays and their potential in diagnostics

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MicroRNAs (miRNAs) play a significant role in gene regulation and have been shown to be deregulated in various diseases. Specific sets of deregulated miRNAs, termed "miRNA fingerprints", can distinguish diseased from healthy samples. Detecting disease-specific fingerprints could be used in diagnostics and there are significant efforts towards developing miRNA-detection methods for this purpose. These methods would require the ability to detect multiple miRNAs in a direct, quantitative, specific, and rugged manner.

This review discusses potential diagnostic methods for miRNA detection, with the main focus on direct hybridization assays. MiRNA-hybridization assays are categorized into two groups, depending on whether or not they require spatial separation of the labeled probe-miRNA duplex from unbound probe. We find that spatial-separation hybridization assays are able to meet all of our criteria, so they have the most potential for use in diagnostics.

Keywords: Diagnostics; Direct assay; Hybridization assay; MicroRNA (miRNA); MicroRNA detection; miRNA fingerprint; Probe-miRNA duplex; Sequencing-based method; Signature-based method; Spatial separation

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1. Introduction

MicroRNAs (miRNAs) are 18–25-nucleotide non-coding RNA molecules that play an important role in gene regulation. MiRNAs target specific mRNA transcripts by hybridizing to their 3′ untranslated region (UTR). Depending on the level of complementarity between miRNA and UTR, this hybridization results in degradation of the mRNA or inhibition of its translation. MiRNAs have a widespread influence on gene expression, regulating up to one-third of all human genes.

The majority of known miRNAs are involved in biological processes (e.g., cell differentiation, proliferation, and tissue formation) [1–3]. Diseases that affect these processes are often accompanied by a change in cellular miRNA content [4–8]. Up-regulation or down-regulation of specific miRNAs can be characteristic of many human diseases. There are significant efforts directed towards combining sets of such deregulated miRNAs into fingerprints that can be used for disease diagnosis. Fingerprints of fewer than 10 miRNAs allow discrimination of cells with differing phenotypes (e.g., Michael et al. used a fingerprint with as few as two miRNAs to distinguish between cancerous and non-cancerous cells) [9].

For miRNA fingerprints to be used in diagnostics, a suitable detection method is required. Such a method should be able to sense multiple miRNAs with low limits of detection (LODs) and high specificity. In addition, it should be robust, rugged and financially feasible. However, the most important aspect of this method lies in its ability to detect miRNA quantitatively. A detection method with high quantitative accuracy would allow for the analysis of fingerprints that are based on slight deregulations in miRNA levels, rather than the presence or absence of particular miRNA species. Furthermore, because disease fingerprints always involve more than one miRNA species, these detection methods must avoid sequence-related biases in quantitation. Such biases are present in the majority of indirect detection methods, in which pre-amplification or enzymatic/chemical modifications of the miRNA are required [10–12]. These additional steps often result in the loss of miRNA sample and increased assay time. For this reason, the use of direct methods, which do not involve amplification/modification of miRNA, is advantageous.

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Currently, the two main methods for miRNA analysis are microarrays and quantitative reverse transcriptase PCR (qRT-PCR). Microarrays allow simultaneous detection of several hundred miRNAs and qRT-PCR allows detection of low-abundance miRNA species [13,14]. These methods have been essential in identifying candidates of disease-specific miRNA fingerprints; however, due to their indirect nature and the associated lack of robustness in quantitation, their usefulness in the validation of miRNA fingerprints and miRNA-based diagnostics is limited. We must look for other potential methods that best meet our criteria for use in diagnostics.

2. Classification

To ensure specificity, any detection method must be able to sense a target miRNA based on its unique nucleotide sequence. Thus, it is useful to classify miRNA detection methods based on the degree to which their sequence is exploited for their detection. All detection methods can then be classified into three main categories: sequencing, signature and hybridization.

2.1. Sequencing

Sequencing-based methods identify the nucleotide sequence of miRNA base-by-base by using a DNA-sequencing method. The classical Sanger method uses DNA chain-terminating dideoxynucleotide bases that terminate DNA strand elongation. The four dideoxynucleotide bases are each labeled with a different fluorophore allowing for identification of each sequential base (Fig. 1). Next-generation sequencing is a much more high-throughput technique that parallelizes this process, lowering the cost of sequencing. There are several different forms of next-generation sequencing; however, most of them still use the four dye-labeled terminating bases, allowing detection of each individual base in the sequence. Sequencing-based miRNA detection methods increase confidence in target specificity due to the larger set of information provided for each miRNA species. These methods are thus less prone to false-positive results. Current sequencing methods all require some form of modification to miRNA prior to analysis [15–17], so they do not meet our requirement of being direct. Nanopore-based sequencing techniques have the potential to be direct, but this technology is not yet sufficiently developed to be feasible in any practical application [18,19].

2.2. Signature

Signature-based methods detect target miRNA *via* information retrieved by different kinds of spectroscopic techniques. These methods do not specifically identify the nucleotide sequence of miRNA, but rather associate it with a physical characteristic (e.g., mass, charge, or structure of a molecule). For example, Driskell et al. used

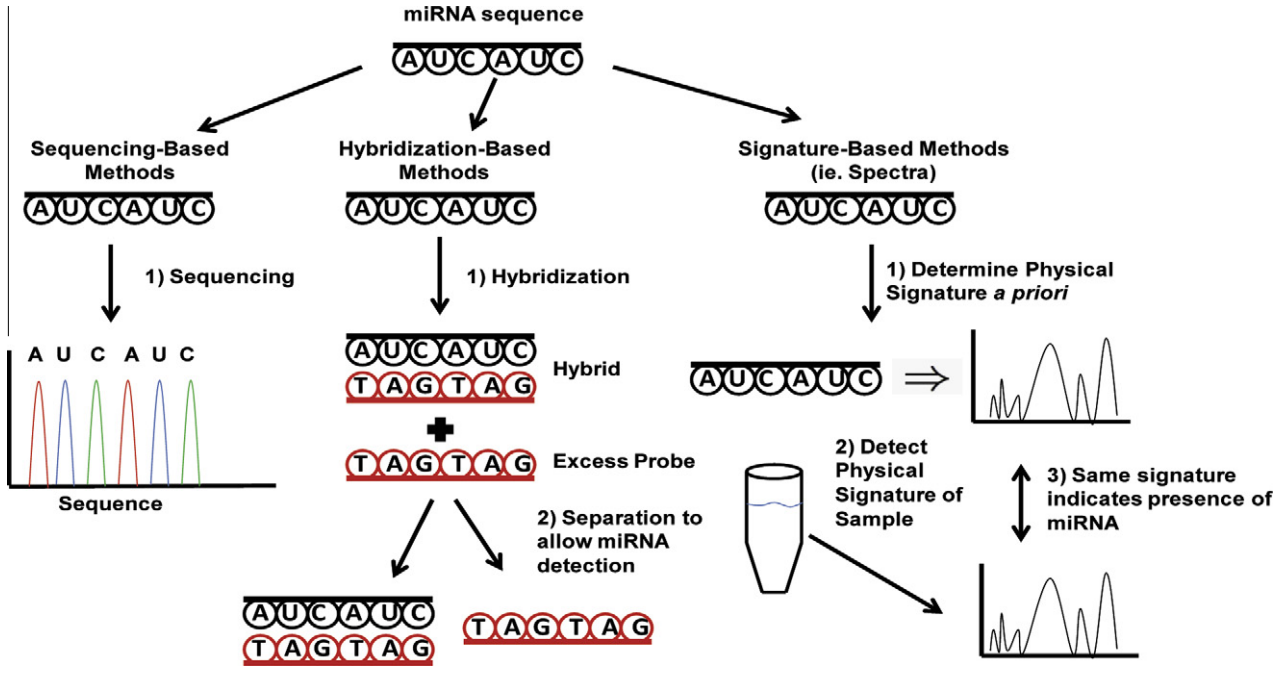

Figure 1. Classification of all miRNA-detection techniques based on how the miRNA sequence is exploited in the analysis.

surface-enhanced Raman scattering (SERS) to create sequence-specific spectra of miRNA [20]. Even though there are efforts under way to adopt other spectroscopic techniques {e.g., mass spectrometry (MS) [21] and circular dichroism [22]} for miRNA detection, none are yet feasible in diagnostics.

Unfortunately, all these methods have an inherent limitation of being prone to false-positive results, because non-identical miRNAs with some similarities can produce indistinguishable signatures. Furthermore, as various classes of molecules can potentially mimic miRNA signatures, this limitation becomes even more detrimental when working with complex biological samples. In these cases, a significant effort would have to be made to validate the specificity of each individual signature. This currently prevents these methods from being used for practical miRNA analysis.

2.3. Hybridization

Hybridization-based methods employ a complementary probe to detect specific miRNA. Designing hybridization probes requires *a priori* knowledge of the target-miRNA sequence. Also, careful consideration of melting temperatures and chemical composition of the probes is required to prevent hybridization to miRNAs that differ by one or two nucleotides. It is desirable to keep such false positives to a minimum, as there are families of miRNAs that differ by only a single base. It has been shown that the use of locked nucleic-acid (LNA) bases in probes can equalize melting temperatures of multiple miRNAs, allowing increased specificity. Furthermore, current hybridization assays can be direct and highly sensitive. There are techniques available that allow for simultaneous analysis of multiple miRNAs in a robust, efficient manner. As long as certain considerations are taken into account, hybridization assays can meet all of the established criteria:

(1) quantitative;
(2) high sensitivity;
(3) ability to analyze multiple miRNAs simultaneously; and,
(4) robustness, ruggedness, and financial feasibility.

As methods based on sequencing and signature do not meet these chosen criteria, we do not review them further. This review focuses instead on direct hybridization-based assays that are currently feasible for use in diagnostics.

3. Hybridization assays

Every hybridization assay requires the binding of the target miRNA to a complementary probe, composed of DNA, RNA, LNA or PNA. The presence and the quantity of miRNA are inferred from the detection of such duplexes. There are two basic ways of detecting the presence of probe-miRNA hybrids, as duplexes can be distinguished from non-bound probe by detecting hybridization-dependent signal changes or through their spatial separation. This allows us to categorize all hybridization-based assays into two basic groups: detection that requires no spatial separation and spatial separation-based detection (Fig. 2). In the former category, duplexes are detected by measuring changes in hybridization-dependent parameters (e.g., fluorescence or electrical conductivity of the sample). In the latter category, duplexes can be spatially separated using certain techniques (e.g., immobilization or electrophoresis).

We next focus on the advantages and the limitations of each of these categories, highlighting methods with the most potential for diagnostics.

3.1. Non-spatial separation methods

3.1.1. Electrochemical detection. The use of electrochemical detection in hybridization assays is a relatively recent technique that takes advantage of a change in circuit properties upon miRNA hybridization. In all electrochemical techniques, miRNA hybridizes to a complementary probe that is immobilized on an electrode or a nanowire. Depending on which technique is used, miRNA-binding causes the promotion of oxidation on an electrode or a change in the conductance of a nanowire.

Though indirect, the first electrochemical miRNA-detection method was developed in 2006 (Gao et al.), and took advantage of a catalyzed oxidation reaction [23]. In this initial design, a ligation reaction was required between an oxidation reagent and the target miRNA (Fig. 3).

In 2009, Yang et al. designed a direct electrochemical-detection method by constructing microelectrodes decorated with immobilized PNA probes [24]. In PNA, the negative sugar-phosphate backbone is replaced with a neutral peptide backbone. Binding of miRNA to these PNA probes resulted in an accumulation of negative charge on the electrode, which, in turn, attracted Ru^{3+} redox reporter. Electrochemical reduction of Ru^{3+} to Ru^{2+} resulted in a detectable change in electrical current. With the use of a signal-amplification technique, an impressive LOD of 10 aM was achieved. The signal-amplification technique involved the addition of ferricyanide, which oxidizes the Ru^{2+} back to $Ru^{3+}$and allows for a single ruthenium atom to interact with miRNA multiple times. They were able to measure the quantity of mir21 and mir205 from total-RNA extracts of various cell lines. Unfortunately, signal-amplification techniques often improve LOD at the expense of quantitative accuracy, as it is difficult to control the number of amplification events per each signal event precisely. Furthermore, in this particular method, the great sensitivity was also accompanied by a relatively narrow dynamic range of two orders of magnitude. Since miRNA levels can potentially range over four orders of magnitude, this method in its current format is hardly suitable for practical measurement of deregulated miRNA in tissue samples.

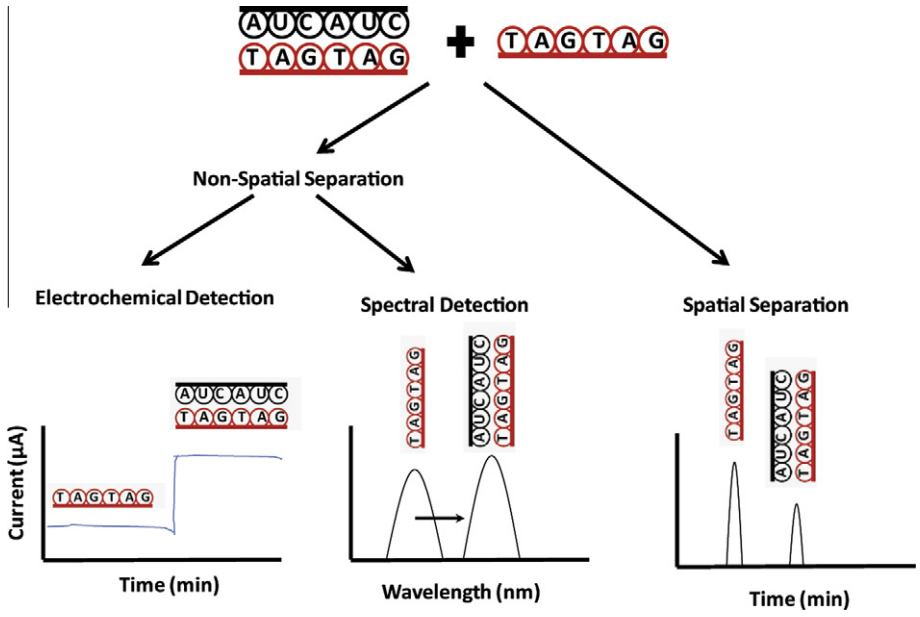

Figure 2. Hybridization assays classified based on how the signals from the hybrid and excess unbound probe are separated.

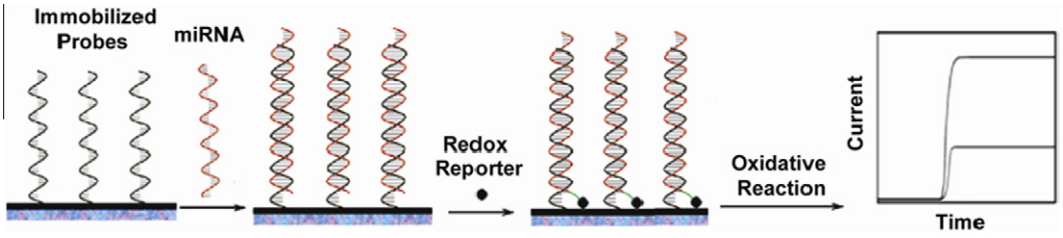

Figure 3. Electrochemical-detection technique. miRNA is hybridized to immobilized probe, allowing for accumulation of redox reporter, causing a detectable change in circuit properties (Adapted with permission from [23]. Copyright [2006] American Chemical Society).

Other forms of electrochemical detection [25,26] involve the use of nanowires to sense target miRNA. Fan et al. designed an electronic circuit where a conducting polyaniline nanowire was interrupted by nm-sized gaps [25], which were decorated with neutral PNA-hybridization probes. The negatively-charged target miRNA, upon hybridization with the probes, interacted with cationic anilines and increased the conductance of the electronic circuit. Electrical conductance of the nanowire directly correlated with the amount of hybridized miRNA. This allowed Fan et al. to detect miRNAs at concentrations as low as 5 fM. Zhang et al. (2009) developed a similar method, where a PNA-decorated silicon nanowire was used [26]. In this technique, hybridization of negatively-charged miRNA affected the semi-conductor properties of the silicon nanowire, resulting in an increased resistance of the circuit in a miRNA-concentration-dependent manner. This method also showed great sensitivity, with an LOD of 1 fM. Unfortunately, the complicated manufacturing process of these nanocircuits has, so far, prevented them from being used for simultaneous detection of multiple miRNAs. However, Zhang et al. did express an interest in developing nanocircuit-based miRNA arrays and reports on their progress are anticipated with interest.

The main advantage of electrochemical methods lies in their impressive LODs, which can be as low as 10 aM of miRNA (in as little as 10 ng of sample). In general, LODs of these methods are better than the limits of all other types of miRNA-hybridization assay. This allows for electrochemical methods to avoid the use of miRNA amplification by PCR. The electrochemical methods also possess high specificity, as they are able to distinguish miRNAs with 1-nucleotide accuracy.

Unfortunately, the use of electrochemical methods in diagnostics will require researchers to overcome some major limitations. In most of the examples described, only a single miRNA was detected. Currently, efforts in multiplexing miRNA targets are hindered by the narrow dynamic range of detection or the complicated manufacturing process of the chips. Also, all of the electrochemical methods are currently incompatible with crude biological samples. Various components of cell lysate may interact with the nanostructures in unforeseen ways and cause false-positive or false-negative readings.

Thus, the use of miRNA extraction kits is required, which can introduce different quantitation biases and increase overall assay time. This makes electrochemical-detection methods less rugged, as strict control over clinical standards has to be implemented. Lastly, as the chips have a limited lifespan, their complex manufacturing process is a major limitation in terms of cost associated with analysis.

3.1.2. Spectral detection. Spectral detection methods require a change in absorbance, fluorescence, refractive index or reflectivity of the sample to occur upon binding of target miRNA to its complementary probe. The most common spectral detection technique involves the use of molecular beacons (MBs). MBs are constructs that take advantage of phenomena [e.g., quenching or fluorescence resonance energy transfer (FRET)]. MBs consist of four functional parts:

(1) target miRNA-hybridization sequence (similar to a hybridization probe);
(2) complementary sequences at the 5′ and 3′ ends;
(3) a terminal fluorophore; and,
(4) a terminal quencher/acceptor (Fig. 4).

When the target miRNA is absent, a stem loop is formed through hybridization of the 5–6 nucleotide-long complementary components at the ends of the construct. In this conformation, the fluorophore and the quencher are brought into close proximity, so the absence of target miRNA results in the absence of fluorescence signal. However, when a target miRNA hybridizes to the MB, it interferes with the stem-loop structure and results in a spatial separation of the fluorophore and the quencher, so the presence of target miRNA results in an increase in fluorescence signal (Fig. 4).

MBs have been used extensively with miRNA in recent years [27–30]. Unfortunately, all MB methods suffer from an inherent lack of sensitivity and poor LOD due to incomplete quenching in the absence of miRNA. Several protocols were developed to improve the sensitivity of MBs. Hartig, in 2004, used a signal-amplifying ribozyme instead of the typical MB [31]. The target miRNA binds to the ribozyme and causes its structural change, activating the cleavage of a fluorophore/quencher-labeled substrate. This cleavage releases the fluorophore, which, in turn, produces a detectable signal. Signal amplification was achieved by multiple substrates being cleaved by a single activated ribozyme. They were able to achieve an LOD of 5 nM.

Kang et al. showed the versatility of MBs by detecting two miRNA species within single live cells [28]. Mir26a and mir206 were detected from individual mouse myoblast cells simultaneously using two differently labeled fluorophores. Confocal microscopy was used to monitor the two miRNA species through myogenesis. Thus, MBs can be used *in vivo* to detect miRNA, which, in turn, can help understand the role of miRNA in cellular processes.

There are also examples of low-LOD spectral techniques that do not require the use of MBs. Neely et al. used a single-molecule detector, LNA-DNA probes, and dual fluorophores to achieve an LOD of 500 fM [32]. Locked nucleic acids (LNAs) are RNA analogs that have decreased flexibility of backbone, and, as a result, increased binding strength between the probe and the miRNA. This allowed Neely et al. to fit two short hybridization probes onto a single target. Each of the two probes was decorated with a different fluorophore (Fig. 5). After hybridization, the fluorescence of excess non-bound probes was deactivated by a quencher-labeled complement. The sample was then put through a capillary and analyzed in flow. Two detectors were placed along the length of the capillary, one for each fluorophore. To decrease background fluorescence in miRNA detection, only coincident signals from both detectors were recorded. A significant improvement in LOD was achieved (500 fM). Unfortunately, this elaborate instrument design, requiring multiple lasers and complex detector synchronization, poses a large limitation on the ruggedness of the method.

Yin et al. developed a signal-amplification technique with the use of a Taqman probe and a duplex-specific nuclease (DSN) [33]. A Taqman probe is a short DNA oligo with a fluorophore and a quencher at either end, and it requires cleavage to fluoresce. Upon hybridization with target miRNA, the DSN cleaves the Taqman probe, thus breaking the energy-transfer connection between fluorophore and quencher, so the presence of Taqman-miRNA duplex results in an increased fluorescence signal.

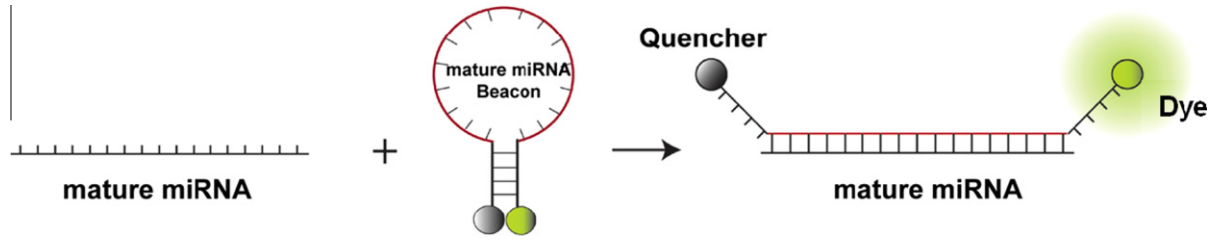

Figure 4. A molecular beacon (MB), which comprises four parts – target miRNA hybridization sequence, complementary 5-6 DNA bases at 5′ and 3′ ends, a fluorophore and a quencher. Upon hybridization, the MB structure opens, allowing fluorescence to occur (Adapted from [27] by permission of Oxford University Press).

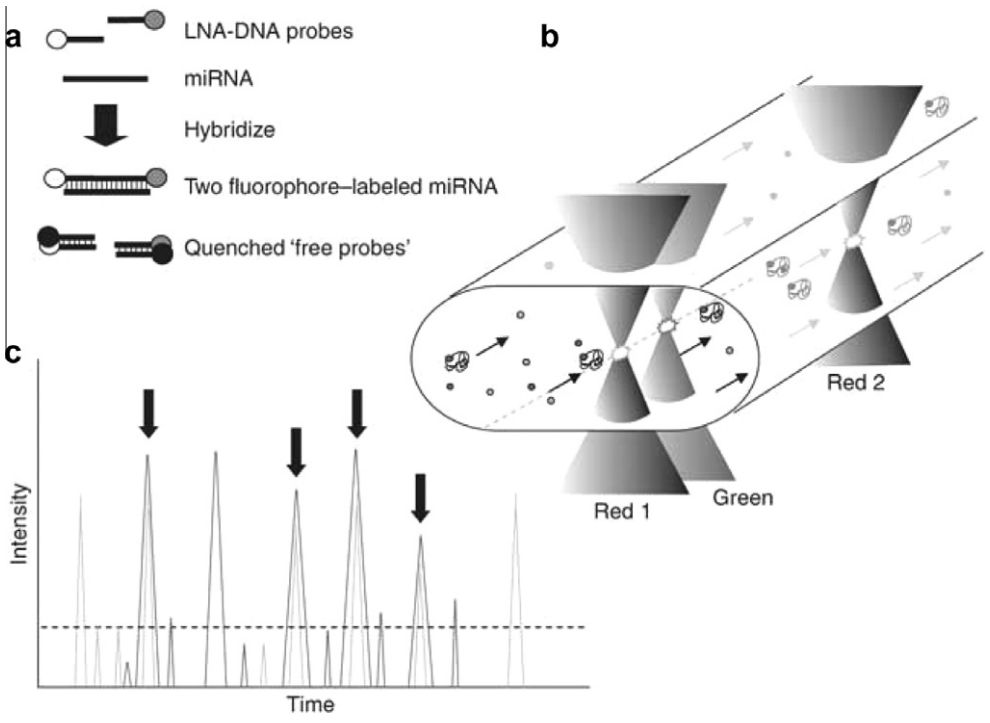

Figure 5. Single-molecule quantitation of miRNA using dual fluorophores. Two labeled probes, each with a different fluorophore, hybridize to miRNA. Sample is run through the capillary and synchronized detection of both fluorophores indicates the presence of miRNA (Reprinted from [32] by permission from Macmillan Publishers Ltd. Copyright [2011]).

Furthermore, cleavage of Taqman releases the target miRNA undamaged, allowing for hybridization to a new Taqman probe to occur. This signal-amplification technique yielded an LOD as low as 100 fM.

In general, spectral techniques have a dynamic range of up to four orders of magnitude, which makes them suitable for detecting multiple miRNAs at different expression levels in practical assays.

Furthermore, the use of multiple fluorophores or microarray format allows simultaneous analysis of multiple miRNA targets. Also, some spectral methods are less prone to non-specific effects of crude biological samples and, as a result, require fewer preparative steps (e.g., RNA extraction). The fact that spectral methods can be applied directly to cell lysates, and even used directly in living cells, increases their versatility. Spectral methods can usually be performed with relatively inexpensive commercial equipment, making them especially attractive for clinical use.

Spectral methods do not have many limitations, but, unfortunately, these limitations significantly affect their potential for use in diagnostics. Due to the high background signal that accompanies fluorophore-quencher systems, the current LODs of classical spectral methods make them unsuitable for analysis of low-abundance miRNA samples (e.g., from fine-needle biopsies or blood). While there are efforts to improve these LODs, they typically result in decreased quantitative accuracy or significantly more complex and cost-inefficient instrumentation.

3.2. Spatial separation

There are two basic ways to separate the miRNA-probe duplex physically from the excess probe:

(1) miRNAs are immobilized on a surface through various techniques and are then hybridized with labeled probes; or,
(2) the hybrids are separated from the excess probe based on their inherent physical properties (e.g., mass or charge).

For nucleic acids, one of the most efficient ways to achieve this separation is through use of electrophoretic techniques.

Northern blotting is a gold-standard immobilization technique in miRNA detection. In this technique, the components of total-RNA extracts from biological samples are separated based on size using gel electrophoresis. Afterwards, bands of separated RNA are blotted onto a nitrocellulose membrane. Labeled target-specific DNA probes are then washed over the membrane, allowing them to become immobilized through hybridization. While very popular among researchers, Northern blotting has several limitations when it comes to clinical applications, including long assay time (~24 h),

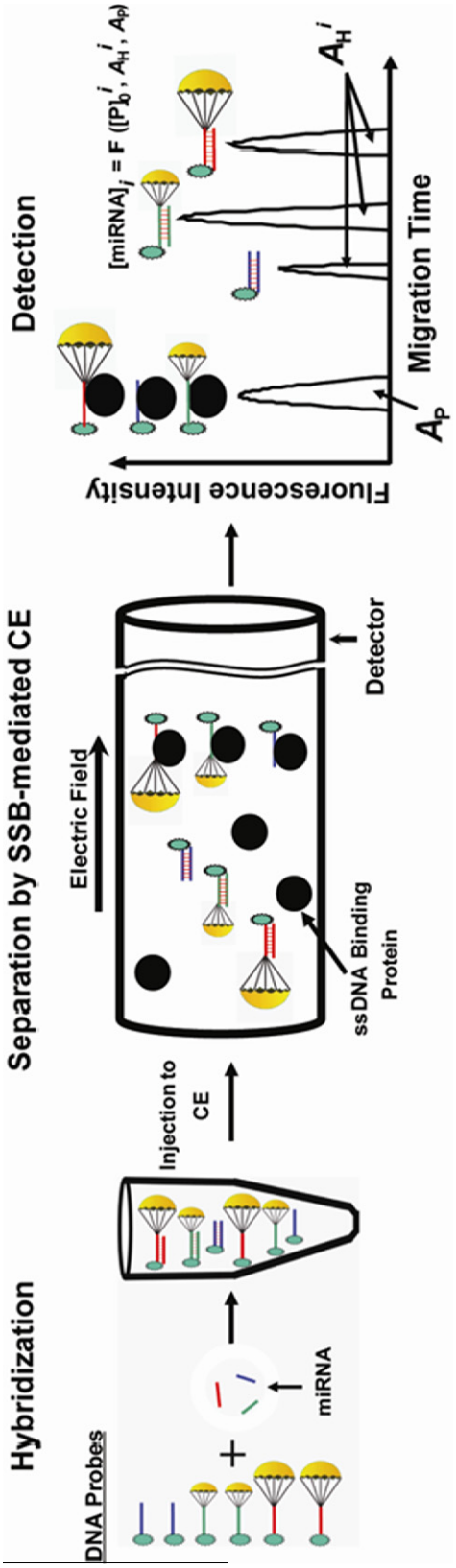

Figure 6. Direct quantitative analysis of multiple miRNAs (DQAMmiR) (Adapted from [40]).

requirement for large samples, limited sensitivity and dependence on radioactive probes.

Variations of Northern blotting were developed to address some of these issues. For example, Varallyay was able to reduce the overall assay time down to 4 h through various improvements, including the use of LNA-hybridization probes [34]. Kim et al. developed a non-radioactive technique through use of LNA probes, a digoxigenin (DIG) label and an improved cross-linking reagent to achieve an LOD of 50 amol [35]. DIG labeling allowed for non-radioactive detection of miRNA by using alkaline-phosphatase (AP)-labeled anti-DIG antibodies. The activity of the enzyme, AP, could then be measured to detect the presence of target miRNA. Though LNA probes, DIG labels, and the cross-linking reagent had been used previously, the combination of the three techniques improved the LOD. Kim et al. applied this technique to detect low-abundance miRNA from a breast-cancer cell line. Unfortunately, these improved methods still require an initial sample that is too large to be feasible for most diagnostic applications.

Sandwich assays are another form of immobilization technique that takes advantage of two different functional probes: capture and labeled. The capture probe typically includes regions of complementarity to both target and labeled probe. Hybridization of the labeled probe depends on the hybridization status of the target-specific region of the capture probe, through exploitation of the base-stacking phenomenon. In base-stacking, the presence of an adjacent duplex region stabilizes hybridization of a very short nucleotide that otherwise would have been too weak to remain bound. Yang et al. combined the use of a sandwich assay design with gold-nanoparticle labeling, and were able to achieve a 10-fM LOD through signal amplification by silver enhancement [36]. They detected mir122a and mir128 from mouse brain and liver tissue in as little as 2 ng of sample.

Roy et al. further improved the LOD of the sandwich assays by introducing an exonuclease-processing step [37]. In their design, after the capture probe had a chance to hybridize with the target miRNA, exonuclease was added to degrade any unbound capture probe. This decreased the probability of non-specific interactions between capture and labeled probes, and resulted in a lower background signal. Employing the differential interference-contrast method for detection of gold-nanoparticle labels, Roy et al. were able to detect as few as 300 copies of miRNA (1 fM) without using signal amplification.

There are only a few physical separation techniques that do not require miRNA immobilization, because non-hybridized probe and duplexes are difficult to separate based on differences in their inherent physical properties. Chang et al. were the first to use capillary electrophoresis (CE) to separate excess DNA probe from the miRNA-probe duplex. They did not alter the size-to-charge ratios of probe or duplex, thus achieving very little separation, making miRNA quantitation difficult [38].

To overcome this problem, Khan et al. altered the size-to-charge ratio of the excess probe or the miRNA-probe duplex using various separation enhancers [39].

Wegman and Krylov employed CE to separate multiple probe-miRNA duplexes from excess probes using single-strand DNA-binding protein (SSB) [40]. Addition of SSB, which binds to only ssDNA, caused a significant shift in the migration time of all the unbound probes. High separation efficiency of CE allows this technique to be used directly with biological samples as complex as crude cell lysates. Furthermore, the design of this method eliminates the need for calibration curves, significantly reducing sample-analysis time. Direct quantitative analysis of multiple miRNA (DQAMmiR) in this work was achieved through attachment of ''drag tags'' [41,42] to the labeling probes, which result in a controlled shift of electrophoretic mobilities of individual probes, which otherwise would not be separated (Fig. 6). An LOD of 100 pM was achieved through commercially-available CE instrumentation, while a custom-made time-resolved-fluorescence apparatus was used to detect down to 1000 miRNA molecules [43].

Unfortunately, most physical separation techniques possess comparatively poor LODs. To achieve LODs compatible with diagnostics (in the fmol range), specialized instrumentation or signal amplification is required. This can increase analysis cost and assay times. Furthermore, physical separation methods cannot be applicable to measurements in *in vivo* settings. Future improvements to the LODs of physical separation methods will require the incorporation of sensitive detection techniques. Highly-sensitive techniques [e.g., selected reaction monitoring (SRM) MS] can potentially be amalgamated with physical separation methods, similar to how Dodgson et al. combined confocal microscopy and time-resolved fluorescence with CE [43]. The incorporation of these techniques into commercially available instruments could then allow for sufficient sensitivity without the use of signal amplification or specialized instrumentation.

Physical separation between excess probe and miRNA-probe duplexes significantly reduces possibilities of detecting non-specific interactions, giving separation-based methods excellent quantitative accuracy. Similar to other hybridization assays, physical separation methods are capable of 1-nucleotide specificity. Furthermore, the dynamic range of these methods spans up to 4 orders of magnitude, making them well-suited for simultaneous analysis of deregulated miRNAs. Multiplexing a wide range of miRNA targets is possible through either the adoption of a microarray format or the use of powerful separation techniques. Additionally, some of the physical separation techniques do not require as many sample preparation steps, such as RNA

purification, making them especially attractive for use with medical samples.

4. Conclusions

Due to realization that miRNA has a significant role in disease development, there has been a significant focus on developing miRNA-detection techniques. Common methods (e.g., qRT-PCR and microarrays) were great for identifying deregulated miRNA, but their indirect nature limits their value in validating miRNA fingerprints and using them in diagnostics. As mentioned, modifications of the miRNA can cause sequence-specific biases, affecting the quantitation of different miRNA. The optimum method would not require any miRNA-purification steps, detecting miRNA directly from a clinical sample. With current technology, hybridization assays are at the forefront of direct miRNA detection. For miRNA to be used in diagnostics it would require a method to be quantitative, multiplexed, robust, specific, and to have a low LOD and a reasonable cost and assay time.

Electrochemical methods provide great LODs, which make them very attractive for detection of low-abundance miRNA molecules. Unfortunately, they do not satisfy most of the other criteria for being applicable to diagnostics. Several stumbling blocks would have to be resolved before they can become a feasible alternative to other methods.

Both separation and spectral-based methods share similar LODs and rely on specialized equipment or signal-amplification techniques to make them applicable to medical samples. Otherwise, both these types of method perform equally well against dynamic range, specificity, multiplexing ability and ruggedness criteria. However, their major difference lies in their quantitative accuracy, as spectral-based methods suffer from high background signal due to quenching inefficiencies. Physical separation methods thus currently satisfy most of the defined criteria and have the most potential to become applicable to real medical samples in the immediate future. Currently, the only major limitation that hinders their adoption in clinical settings is the lack of commercial equipment with sufficient LODs.

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