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Review Article

A review of sample preparation for purification of microRNAs and analysis by mass spectrometry methods

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ABSTRACT

MicroRNAs (miRNAs) play an important role in regulation of different bioprocesses, including multiple diseases, such as cancer, neurodegenerative and immune-related disorders. Analysis of miRNA biomarkers in biological fluids requires accurate, sensitive, reproducible, and multiplexed methods. This review covers miRNA purification and measurement, which are the core of these analytical methods, and critically affect the output of biomarker research studies. With regard to miRNA measurement, the typical bioanalytical methods (e.g. reverse transcription polymerase chain reaction, RT-PCR), which have been extensively reviewed elsewhere, have been excluded to focus on less conventional methods based on mass spectrometry (MS). This review provides a broad overview of liquid-phase and solid-phase extraction purification methods for miRNA clean-up and enrichment and a critical insight into direct and indirect MS-based methods to disclose the true potential of MS in the field.

1. Introduction

MicroRNAs (miRNAs) are single-stranded non-coding RNAs (ncRNAs) of 19–23 nucleotides in length that have an important role in the physiological and pathological regulation of several bioprocesses. MiRNAs control gene regulation by targeting complementary messenger RNAs (mRNAs), preventing their translation or promoting their degradation, as schematized in Fig. 1 [1]. They are found at low concentrations in tissues and biological fluids (e. g. blood, saliva, urine, and cerebrospinal fluid) in a stable and resistant form to RNase activity, pH and temperature. MiRNAs are stabilized and protected from degradation due to association with RNA-binding proteins like Argonaute 2 (Ago2) protein or high-density lipoproteins, as well as by encapsulation inside extracellular vesicles, such as exosomes [2–4]. As specific dysregulation of miRNAs has been observed in different diseases, like cancer, neurodegenerative and immune-related disorders, extracellular circulating miRNAs have a great potential as biomarkers to diagnose, monitor or predict disease evolution, as well as to improve therapeutic treatments [5–8].

Accurate, sensitive, reproducible, and multiplexed miRNA analysis is crucial for biomarker discovery in biological fluids. In biomarker discovery, appropriate standardized clinical protocols must be applied, covering sample type, collection, inter-individual variability, lifestyle, disease condition, comorbidities, etc [9–12]. In addition, it is also widely recognized the challenge posed by the analytical method itself that mainly comprise miRNA purification and measurement.

Indeed, an accurate miRNA detection requires an appropriate purification and enrichment due to their low concentration in body fluids, which present complex matrices with potential interfering components, like proteins. Furthermore, separating short RNAs from longer RNAs is also problematic [12–14]. Nowadays, numerous ready-to-use kits for miRNAs isolation are commercially available [11,14–16], but there is still interest in enhancing miRNA yield and purity by introducing novel extraction methods, as well as in simplifying or automating purification procedures to decrease human handling and increase analytical throughput.

Regarding measurement of miRNAs, a wide range of bioanalytical methods have been reported. The gold standard is reverse transcription polymerase chain reaction (RT-PCR), which is a targeted indirect method that requires retrotranscription of miRNA into complementary DNA (cDNA) before PCR amplification for sensitivity enhancement and fluorescence detection [13,14,17–19]. Several other methodologies have also been applied and recently reviewed elsewhere [18,19], including those based in next generation sequencing (NGS). In general,

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these bioanalytical methods are very sensitive and selective, and they can be adapted to be quantitative or to detect certain post-transcriptional modifications (e.g. 5′-end phosphorylation and dephosphorylation or nucleotide trimming and additions), which have been reported to affect miRNA stability and activity [20]. However, they are time consuming and require high qualified expertise, specific bioinformatics software, as well as specific ligation adaptors, gene libraries or primers [13,14,17–19]. As an alternative, methods based on mass spectrometry (MS) (i.e. electrospray ionization mass spectrometry or matrix-assisted laser desorption/ionization mass spectrometry, ESI-MS or MALDI-MS, respectively) or hyphenated ESI-MS techniques (i.e. liquid chromatography and capillary electrophoresis coupled to ESI-MS, LC-MS and CE-MS, respectively) allow the direct, multiplexed detection and quantitation of miRNAs through molecular mass measurements, as well as the characterization of their post-transcriptional modifications [20–28]. In addition, MS-based methods are better in quantification, easier to normalize and less prone to bias than other bioanalytical methods [29]. However, miRNAs are difficult to analyze by MS because of their structural similarity and relatively large size (relative molecular mass, M_r ~ 7,000–7,500), with predisposition to low ionization efficiency, as well as to complex mass spectrum and extensive formation of alkali metal adducts, especially in ESI [25]. All these issues negatively affect concentration sensitivity, which is inherently lower than in the above-mentioned bioanalytical methods. Therefore, different strategies are necessary to enhance limits of detection (LODs) to reach the low miRNA concentrations in biological samples. All these issues probably explain the current limited popularity of MS-based methods for miRNA analysis and invites to review the topic for the first time to disclose the true potential of this alternative and unique analytical approach.

MiRNA purification is covered in the first part of this review, while the second part is focused on less conventional miRNA measurement methods based on mass spectrometry (MS).

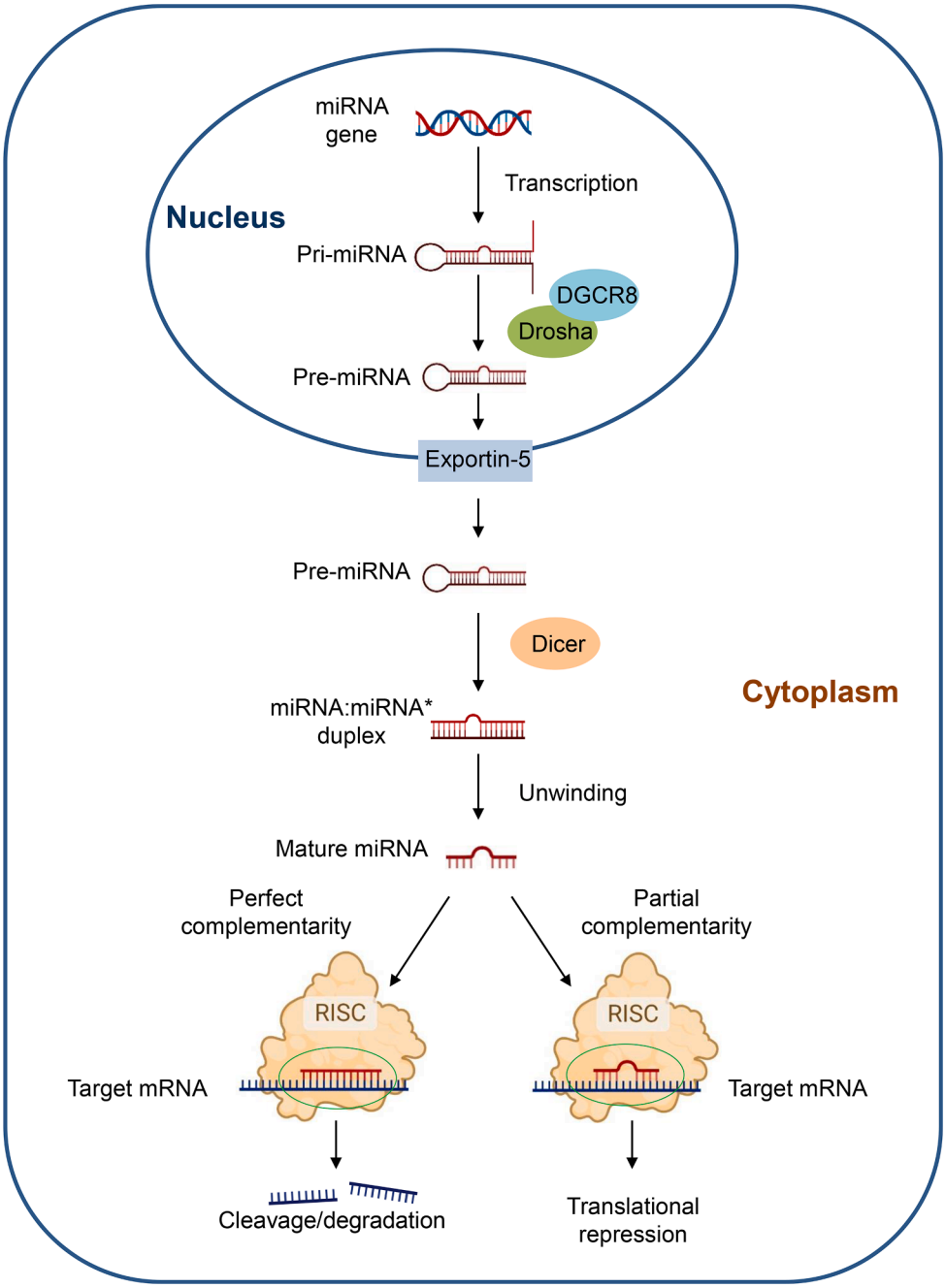

Fig. 1. Overview of miRNA biogenesis pathway. MiRNA gene is transcribed inside the nucleus into pri-miRNA, which is processed into pre-miRNA by microprocessor complex subunit DGCR8 and RNase III enzyme Drosha. Pre-miRNA is exported by protein Exportin-5 from the nucleus to the cytoplasm, to be processed into miRNA:miRNA*duplex by RNase III enzyme Dicer. One strand of the duplex is loaded onto Argonaute 2 (Ago2) protein to form RNA-induced silencing complex (RISC). Once miRNA hybridizes with target mRNA, induces mRNA degradation or translational repression. (Created with BioRender.com).

2. MiRNA purification and enrichment

Nowadays, there are many methods for purification and enrichment of miRNAs, which are in general isolated as a very small fraction of total RNA. The first sample pretreatment steps typically include chaotropic agents, detergents, organic solvents and salts to promote cell lysis, protein and DNA removal, and RNA release [30]. In addition, different preliminary steps are very often included for certain samples to improve miRNAs recoveries. In the case of human blood samples, white blood cells or platelet count, and red blood cells hemolysis have been shown to affect miRNA measurements [9–12,15,31,32]. Several authors have demonstrated that reliability of the measurements and miRNA recoveries can be improved by using platelet-poor plasma samples obtained after centrifugation [15,31,32]. In addition, many authors suggest adding proteinase K (PK) to the lysis buffer to increase miRNA release from proteins and recoveries from plasma samples [33,34], while others propose isolating extracellular vesicles, especially exosomes, before tackling miRNA extraction [35–39]. These preliminary steps are followed by miRNA extraction, which can be regarded as the core of the sample pretreatment procedure. The extraction methods can be classified into two main strategies: liquid-phase and solid-phase extraction methods. Many of these sample pretreatment procedures have been developed into commercial kits to ease miRNA measurement by sequencing-based, amplification-based or hybridization-based bioanalytical methods, and no attention is given to the use of non-volatile compounds (i.e. buffers and salts, detergents, chaotropic agents, etc.), which are incompatible with MS detection. In case necessary, an additional desalting step by, for example, filtration through molecular mass cut-off centrifugal filters ensures an appropriate miRNA detection by MS [24,25]. After miRNA isolation, the amount, quality and integrity are often roughly estimated, for example measuring the absorbance ratio at 260 and 280 nm by UV spectrophotometry [40], before the final analysis.

2.1. Liquid-phase extraction

From the late 1980s, the gold standard methods for RNA isolation have been based on liquid-phase extraction [41–43]. These methods use organic solvents and salts to isolate total RNA, including miRNAs, from small sample volumes (i.e. typical working volumes < 2 mL). The acid guanidinium thiocyanate-phenol-chloroform (AGPC) single-step method developed by Chomczynski and Sacchi in 1987 is the most popular [42,43], due to its simplicity, efficiency and low-cost. In AGPC method, a combination of 4 M guanidinium thiocyanate, phenol and chloroform is added to lyse cells and denature proteins (e.g. ribonucleases, RNases) and maintain RNA in the aqueous phase, followed by phase-separation and RNA precipitation by adding isopropanol (or ethanol) [42,43]. The chaotropic agent guanidinium thiocyanate is a very effective protein denaturant able to deactivate RNases. Similarly, phenol is commonly used for nucleic acid deproteinization since it induces protein folding changes and deactivation. After protein denaturation, partitioning between the aqueous-chloroform phases occurs and RNA remains soluble in the upper acidic aqueous-phase after centrifugation. Salt and buffer concentrations, but especially the acidic pH (pH 4), are critical to separate RNA from DNA and proteins [43]. To enhance alcohol precipitation, several types of carriers can be used (e.g. glycogen, linear acrylamide and yeast transfer RNA (tRNA)). As they are insoluble in alcohols, the precipitated carriers trap RNA molecules increasing recoveries [32–34,44,45], which can be maximized extending the incubation times for pellet formation (e.g. 1 h at −80 °C) [44]. The AGPC method can be applied to a wide variety of human, plant, yeast and bacterium samples, including fluids, tissues and cultured cells. Furthermore, different ready-to-use commercial reagents are available at a reasonable price that ease the applicability and minimize reagent consumption and hazardous residues generation. This is the case of TRIzol™ reagent (Thermo Fisher Scientific Inc.) [46], Tri reagent® (Molecular Research Center Inc.) [47] or QIAzol reagent® (Qiagen Inc.) [48], that have been widespread applied, alone or incorporated for sample pretreatment into different solid-phase extraction kits for miRNA extraction (Table 1) [49–60]. In addition, different modifications have been proposed to improve recoveries, from including variations in the reagent composition (e.g. RNAzol®, Molecular Research Center Inc., no chloroform-induced phase separation is necessary [51,61]) or additional extraction and washing steps with chloroform or alcohol [62] to more specific variations for certain samples [41,63]. For example, adipose tissues, which have a high RNase activity, can be treated with a reducing agent (e.g. 2-mercaptoethanol) to break protein disulfide bonds, which are essential for RNase activity, hence maximizing denaturation by guanidinium thiocyanate [41]. In some cases, the RNAs extracted by guanidinium lysis can be contaminated with polysaccharides and proteoglycans that may disrupt RNA solubilization after precipitation with alcohol, however, adding salts (i.e. NaCl, disodium citrate) may help in reducing contamination [41]. Similarly, a modification named miRICH has also been described to enrich small RNAs from cells. In the miRICH method, an over-drying for 1 h is applied to the RNA pellet after skipping the final washing step with ethanol. The authors describe that the over-drying process prevents solubilization of large RNAs, while enriching small RNAs, including miRNAs. Avoiding the ethanol-washing step was critical to keep the aggregation of large RNA with salts and enhance miRNA recoveries. This improved method performed similar to other solid-phase extraction kits optimized to maximize large RNA depletion and miRNA recoveries [63].

The phenol-chloroform-isoamyl alcohol (PCA) method is another commonly applied single-step liquid-phase extraction method for purification of total RNA, including miRNAs [41,64]. A mixture of phenol: chloroform: isoamyl alcohol (25:24:1 v/v/v) is used, where isoamyl alcohol prevents foaming and facilitates phase separation while reinforcing enzyme deactivation. The operation is similar as in AGPC, including the critical importance of pH and the final precipitation with alcohol. In general, it is widely accepted that the combination of chaotropic disruption and organic solvent extraction in AGPC method allows a more efficient cell lysis and protein denaturation and a higher total yield than in PCA method. However, the presence of a large amount of guanidinium thiocyanate in the extracted miRNAs by the AGPC method can be more detrimental and difficult to clean-up before subsequent analysis, especially if performed by MS [25].

Liquid-phase extraction methods have an excellent performance and many advantages for miRNA purification, and some authors have demonstrated almost 100 times higher extraction yields than solid-phase extraction methods [62]. However, they also have inherent disadvantages related to limited selectivity for small RNAs, incomplete phase separation, extensive sample handling, use of hazardous reagents and difficult automation. There have been some attempts to miniaturize and automate liquid-phase extraction methods for high-throughput miRNA purification, but these approaches are far from being widely applicable yet. A. V. Linares et al. reported on a new centrifugo-pneumatic microhomogenizer chip to efficiently extract miRNAs with Tri reagent® from whole blood [65]. Turbulent conditions were created in a microfluidic disk with different compartments by pneumatically assisted pumping to improve lysis efficiency and miRNA extraction. The chip was designed from a polymeric material called Zeonor®, which was resistant to the components of Tri reagent®. In another study, O. Behrmann et al. presented a microfluidic chip system for the thermoelectric lysis without hazardous reagents of cells before native gel-electrophoresis of the released miRNAs [66].

2.2. Solid-phase extraction

Nowadays, solid-phase extraction methods are an excellent alternative to liquid-phase extraction methods for miRNA isolation [67], because they allow a fast and efficient purification, while preventing many of the issues of traditional methods and obtaining comparable or

Table 1
Commercial kits for miRNA solid-phase extraction from different sample types.

Solid-phase extraction	Manufacturer[a]	Kit name	Sample pretreatment	Sorbent	Sample	Reference
Columns or similar devices	Qiagen	MiRNeasy kits	AGPC	Silica	Animal tissues, cultured cells, serum, and plasma	[49]
	Agilent	Absolutely RNA miRNA Kit	AGPC	Silica	Tissues and cultured cells	[50]
	CD Genomics	CD universal microRNA isolation kit	Guanidium salts (chloroform free)	Silica	Animal tissues, plant tissues, cultured cells, and insect samples	[51]
	Zymo research	Direct-zol™ RNA miniprep Plus	AGPC	Silica	Tissues, cells, serum, plasma, and blood	[52]
	TransGen Biotech	EasyPure® miRNA Kit	AGPC	Silica	Tissues, cells, fresh blood, and virus	[53]
	Thermo Fisher Scientific. Invitrogen	PureLink™ miRNA isolation kit	AGPC	Silica	Bacteria, yeast, plant, mammalian cells, tissues, and virus	[54]
		MirVana miRNA isolation kit	AGPC	Glass fiber	Plant and animal tissue, cultured cells, bacteria, yeast, viral particles and enzyme reactions.	[55]
	Omega Bio-Tek Inc	E.Z.N.A ® micro RNA kit	AGPC	Glass fiber	Cultured eukaryotic cells or bacteria, animal, plant, and fungal tissues	[56]
	Roche	High pure miRNA isolation kit	Guanidinium salts	Glass fiber	Animal and plant tissues cultured cells, and formalin-fixed paraffin-embedded tissues (FFPE)	[57]
	Norgen Biotek	MicroRNA purification kit	Guanidium salts (phenol/chloroform free)	Silicon carbide	Tissue samples, cultured animal cells, bacterial cells, plants, and blood	[58]
	Lexogen	TraPR small RNA isolation kit	PCA	Silica	Any organism, tissue, or cell type	[64]
Dispersive	Promega	Maxwell® RSC miRNA plasma and serum kit	Guanidinium salts (phenol/chloroform free)	Cellulose magnetic beads	Plasma, serum, and previously isolated exosomes.	[59]
	Thermo Fisher Scientific. Applied Biosystems	TaqMan miRNA ABC purification kit - Human panel A and panel B	Guanidinium salts (phenol/chloroform free)	Antisense oligonucleotide magnetic beads	Blood, serum, plasma, FFPE tissues, solid tissues, cultured cells, saliva, and urine	[60]

[a] In general, manufacturers sell variants of the different kits under different brands, with slight modifications that are intended to purify different sample types or for other purposes (e.g. reducing sample volumes or automation). We have included only several kits from the same manufacturer if they declare a different sorbent.

higher recoveries in certain applications. Solid-phase extraction methods are based on using an appropriate solid sorbent to retain miRNAs from small sample volumes (typically smaller than in liquid-phase extraction methods). A small amount of sorbent particles, fibers or a membrane are typically packed or mounted into spin columns, filters or similar centrifugal devices, to speed up the operation in the different extraction steps (i.e. sorbent conditioning, sample loading/binding, washing and analyte elution) (Table 1) [49–58,64]. As an alternative, dispersive solid-phase extraction with magnetic sorbent particles, which also simplify the operation, is also applied, but to a lesser extent because a larger sample volume is typically required [59,60,68,69]. Again, in all cases, total RNA must be appropriately released first, using so-called "lysis buffers", which contain the typical chaotropic agents, salts and organic solvents applied in liquid-phase extraction methods [49–60,64]. Retention of miRNAs is in general achieved at high salt concentrations, specific pH, and using ethanol or isopropanol to reduce sample polarity, followed by a washing step to remove remaining contaminants before eluting with a low ionic strength solution.

The principle of purification by solid-phase extraction methods is the high affinity of RNA molecules, including miRNAs, towards the sorbent. Several sorbent types are available, including silicon dioxide (i.e. silica), glass fiber, silicon carbide (SiC), cellulose, titanium dioxide (TiO_2), graphene oxide (GO), hydrophilic-lipophilic balance (HLB) or ion exchange. Most of the available commercial kits for miRNA extraction are based on particle or membrane silica sorbents due to the excellent extraction efficiency [49–54], which some authors have demonstrated to be superior to liquid-phase extraction methods [67,70]. As a variant of conventional silica sorbents for column- or filter-based solid-phase extraction, magnetic silica particle sorbents for dispersive solid-phase extraction [59,60,68,69] and several glass fiber filters are also available [55–57]. The high affinity of nucleic acids towards silica sorbents or related materials (e.g. glass, diatomaceous earth, etc.) is well-known [30,71,72]. Retention on silica sorbents is based on hydrogen bonds formation, but mainly on electrostatic interactions through cation bridges, where cations (e.g. sodium ions) act as a bridge between the negatively charged phosphate miRNA backbone and the negatively charged silanol groups on the sorbent surface (Fig. 2) [30]. Retention can be further enhanced by divalent cations like Ca^{2+}, low dielectric constant solvents (e.g. ethanol or isopropanol), and chaotropic agents, such as guanidinium thiocyanate. Ethanol is also typically added in the final steps of miRNA purification with these sorbents, and the lower solubility in ethanol of large RNAs compared to small RNAs can be used to enhance miRNA separation and recoveries [55]. The use of carriers, such as glycogen, in combination with ethanol has been also described to enhance miRNA recoveries with solid-phase extraction methods and

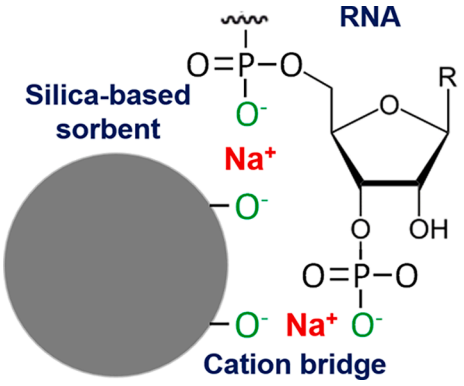

Fig. 2. Schematic representation of the interaction between phosphate backbone of RNA molecules, including miRNAs, and silica-based sorbents.

silica-based sorbents [31,34,54]. Nevertheless, silica-based sorbents inherently favor retention of large RNA molecules and are less efficient for smaller RNA molecules (<200 nucleotides, nt), including miRNAs [58,72–74]. As a consequence, a novel sorbent based on SiC has been also incorporated into commercial kits, which seem not to exhibit size-bias (Table 1) [58,72,73]. Regarding non-silicon-based sorbents, excepting cellulose magnetic particles [59] (Table 1), most of them have been prepared in-house for proof-of-concept applications. This is the case of TiO_2 nanofibers which have been described to extract miRNAs from biological samples [75], because TiO_2 strongly interacts with miRNA phosphate groups, as with phosphorylated or glycosylated peptides and proteins [76]. Adjusting the binding and elution conditions it is possible to selectively retain short RNAs (<500 nt), including miRNAs. The authors claim that interaction of miRNAs with TiO_2 is stronger than with silica, and this could be interpreted by the direct electrostatic interaction between the negatively charged RNA and the positively charged TiO_2 at acidic pH, with no need for salt bridge formation. GO aminated silica coated magnetic beads have also been demonstrated to extract total RNAs (including miRNAs) with improved results compared to conventional liquid-phase extraction methods [77]. Nucleic acids readily bind to GO through Van der Waals forces, p-p stacking and hydrogen bonds. Other more conventional chromatographic sorbents, such as HLB and ion exchange have also been explored. S. Studzińska et al. described the use of HLB cartridges for an efficient protein removal and twofold preconcentration prior to LC-MS analysis of serum miRNAs after PCA and chloroform liquid-phase extraction [78]. This polymeric sorbent is made from hydrophilic *N*-vinylpyrrolidone and lipophilic divinylbenze monomers, hence allowing reversed-phase retention with a neutral polar "hook" for enhanced interaction with polar analytes, such as miRNAs [79].

The extraction selectivity with all these sorbents is inherently limited by the type of interactions governing retention. Several improved strategies have been described based on particle sorbents with immobilized anti-miRNA oligonucleotides (in general single-stranded complementary DNA (cDNA) or its fragments). In this case, miRNA extraction relies on the selective hybridization of the target miRNA to the complementary oligonucleotides. A commercial kit based on magnetic particles with anti-miRNA oligonucleotides for dispersive solid-phase extraction of miRNAs is available for extraction of two different panels of 377 microRNAs from biological samples, including extracellular vesicles [60,80]. M. Konno et al. used magnetic beads with complementary oligonucleotides followed by C18 solid-phase extraction for preconcentration and clean-up of several target miRNAs, to study their distinct methylation levels in gastrointestinal cancers [20]. It has also been demonstrated that a similar approach with a streptavidin sorbent can be used to retain biotinylated complementary antisense oligonucleotides

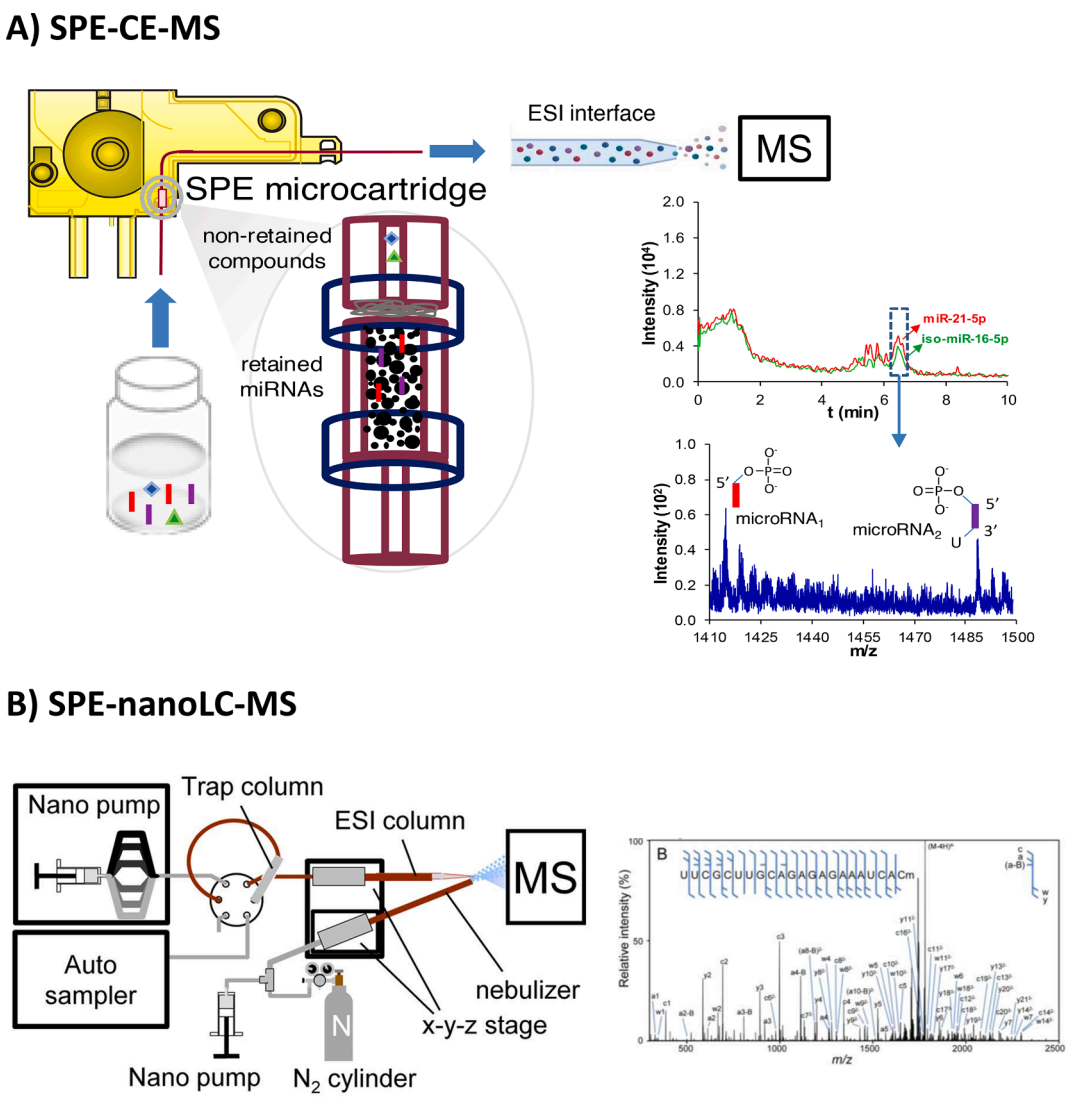

Fig. 3. Schematic representations for A) on-line solid-phase extraction-capillary electrophoresis-mass spectrometry (SPE-CE-MS) (reproduced with permission from [25]) and B) on-line solid-phase extraction nanoliquid chromatography-mass spectrometry (SPE-nanoLC-MS) (reproduced with permission from [82]).

against specific miRNAs [26,81].

In recent years, commercial kits based on solid-phase extraction methods for miRNA purification have become very popular, and some companies have launched dedicated instruments to automate the extraction process with their kits [59]. As an alternative to these instruments for off-line automation, several authors have described fully integrated systems that further minimize sample handling during extraction, separation and detection [25,82]. In a recent study, on-line solid-phase extraction capillary electrophoresis-mass spectrometry (SPE-CE-MS) with a SiC sorbent was demonstrated for the analysis of serum miRNAs at concentrations down to 10 nM (Fig. 3-A) [25]. Alternatively, H. Nakayama et al. reached subfemtomole LODs by on-line solid phase extraction nanoLC-tandem MS (SPE-nanoLC-MS/MS) with a C18 sorbent for the analysis of miRNAs in tumor cell extracts (Fig. 3-B) (there is no enough information in the original paper to calculate the LODs in molarity units) [82].

3. Mass spectrometry detection

Nowadays, there are several ESI-MS, hyphenated ESI-MS (i.e. LC-MS and CE-MS) and MALDI-MS methods for the direct or indirect multiplexed detection, structural characterization and quantitation of miRNAs (Table 2). Hyphenated ESI-MS techniques are recommended over direct MS techniques, due to their higher potential for the analysis of complex samples, allowing high-resolution separations, preventing ion suppression, and preconcentrating separated compounds as sharp and narrow peaks. In general, measurements are preferably performed in negative ion mode, due to the enhanced sensitivity for oligonucleotides, which are polyanions at very low pH due to the ionized phosphate groups. However, as can be observed in Fig. 4, formation of Na^+ and K^+ adducts must be prevented for an optimum performance, especially in ESI-MS (Fig. 4-A). In this section, these MS-based methods are reviewed, as well as the different preconcentration, nucleic acid amplification, and sensitivity enhancement strategies proposed to decrease the LODs that are necessary to reach the low miRNA concentrations in biological samples.

3.1. Direct detection

These methods allow the targeted or untargeted measurement of miRNA by direct detection of the miRNA molecules using hyphenated ESI-MS techniques (i.e. LC-MS and CE-MS), ESI-MS or MALDI-MS (Table 2).

3.1.1. ESI-MS and hyphenated ESI-MS

Several ion-pair reversed-phase LC-MS methods have been described for the direct analysis of oligonucleotides, including miRNA [83]. It is well-known that the presence of strong organic bases such as

Table 2
Mass spectrometry (MS) methods for the direct and indirect detection of miRNAs.

Detection type	Separation technique	MS technique	Post-transcriptional modifications	Targeted/ Untargeted	Multiplexed	Amplification	LOD	Samples	Reference
Direct detection	Ion-pair RP-LC	ESI-MS	Yes	Untargeted	Yes	No	10 μM	–	[84]
	Ion-pair RP-nanoLC	ESI-MS/MS	Yes	Untargeted	Yes	No	1 nM	–	[22]
	–	ESI-MS/MS	Yes	Untargeted	Yes	No	<100 nM	–	[21]
	Ion pair RP-UHPLC	ESI-MS/MS	Yes	Untargeted	Yes	No	8–13 nM	Human serum	[78]
	SPE-ion pair RP-nanoLC	ESI-MS/MS	Yes	Untargeted	Yes	No	Subfemtomole level[a]	HeLa cells	[82]
	Ion pair RP-UHPLC	ESI-MS/MS	Yes	Targeted	Yes	No	<75 pM	Human plasma	[26]
	CapZIC-HILIC	ESI-MS	Yes	Untargeted	Yes	No	1 μM	Human serum	[27]
	CE	ESI-MS	Yes	Untargeted	Yes	No	5 μM	Human serum	[27]
	Sample stacking-CE	ESI-MS	Yes	Untargeted	Yes	No	5 nM	Human serum	[24]
	SPE-CE	ESI-MS	Yes	Untargeted	Yes	No	10 nM	Human serum	[25]
	–	MALDI-MS	Yes	Targeted	Yes	No	–	Human tissue	[20]
	–	MALDI-MS	Yes	Targeted	Yes	No	–	–	[23]
	–	MALDI-MS	Yes	Untargeted	Yes	No	5 nM	Human serum	[28]
Indirect detection	–	MALDI-MS	No	Targeted	Yes	Yes	pM level	Human cells	[94]
	–	ESI-MS/MS	No	Targeted	Yes	Yes	41–95 pM	Human cells	[95]
	HILIC-UHPLC	ESI-MS	No	Targeted	No	Yes	50 amol[a]	Human cells	[96]
	LC (no details are given)	ESI-MS/MS	No	Targeted	No	Yes	60 fM	Human cells, fetal bovine serum and urine	[97]
	–	ESI-MS/MS	No	Targeted	Yes	Yes	0.25 pM	Mouse cells	[98]
		Matrix free MALDI-MS	No	Targeted	No	No	fM level	Human cells	[99]
	–	Laser ablation ICP-MS	No	Targeted	Yes	No	Femtomole level (on membrane)[a]	–	[100]
	RP-LC	ESI-MS/MS	No	Targeted	No	No	<pM level	Human cells and tissue	[101–103]

[a] There is no enough information in the original paper to calculate the LODs in molarity units.

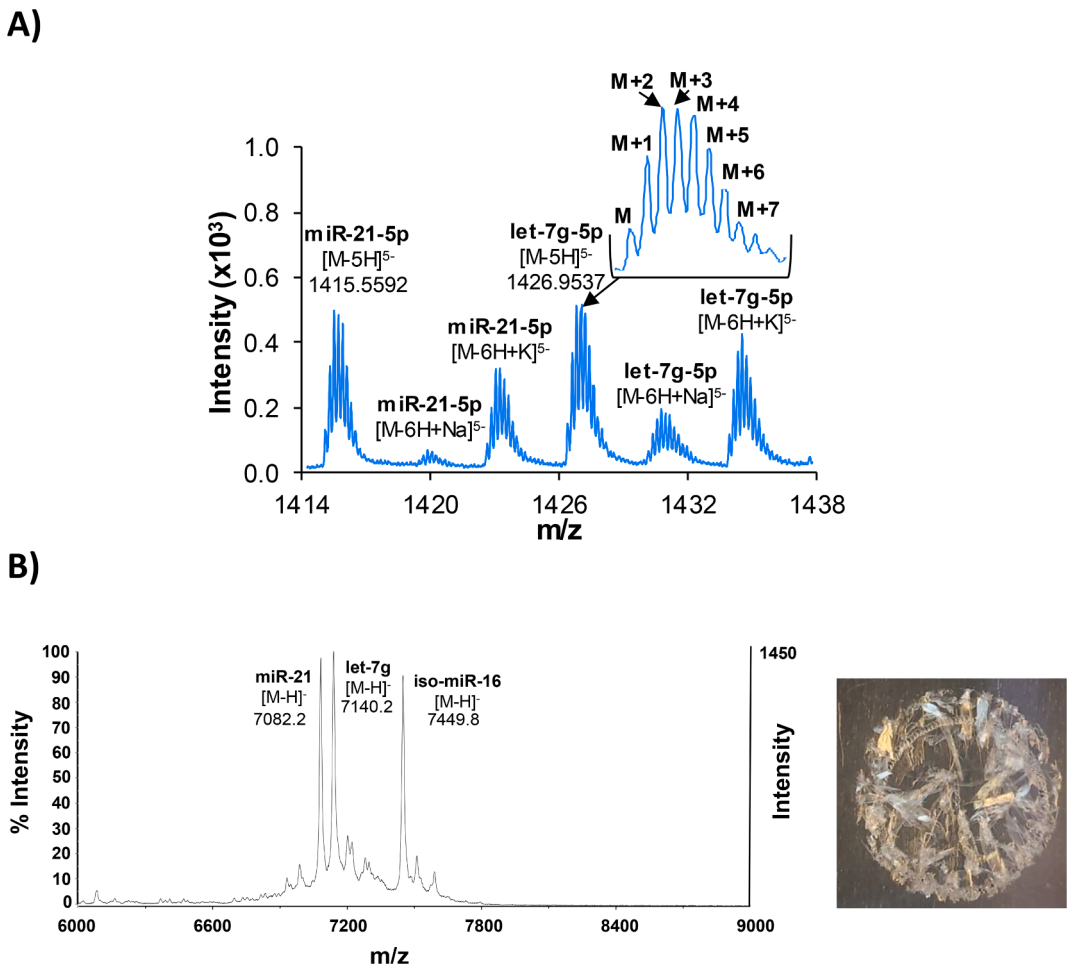

Fig. 4. A) Electrospray ionization (ESI) mass spectrum for a miRNA standard mixture (miR-21-5p, let-7g-5p) (reproduced with permission from [25]) and B) matrix-assisted laser desorption/ionization (MALDI) mass spectrum for a miRNA standard mixture (miR-21-5p, let-7g-5p, isomiR-16) with ATT-PYR ionic matrix and an image of the spot under the microscope (reproduced with permission from [28]).

triethylamine (TEA) or its acetate salt in the typical acetonitrile-water mobile phases, significantly suppresses alkali ions adduct formation through a displacement mechanism, while maintaining acceptable sensitivity and good separations. In order to improve mobile phase volatility and sensitivity, A. Apffel et al. developed an improved LC-MS method to analyze oligonucleotides of up to 75 bases in length using a C18 column and an acetonitrile-water mobile phase with 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), TEA and methanol, adjusted to pH 7.0 [84]. M. Kullolli et al. used similar conditions for characterizing 3′-terminal modifications and sequencing synthetic miRNAs with minimum sample and reagent requirements by nanoLC-MS/MS. The better ionization efficiency in nanoLC-MS/MS with a reversed-phase macroporous monolithic column at a 1 μl/min flow rate allowed LODs until 1 nM [22]. However, Y. Izumi et al. showed that HFIP-TEA acetonitrile-water solvents produced carry over and contamination in the MS instrument and ion suppression for the characterization of synthetic miRNAs by ESI-MS/MS, hence they proposed as a replacement dibutylamine acetate (DBAA) [21]. Alternatively, S. Studzińska et al. proposed *N*,*N*-dimethylbutylamine (DMBA) HFIP-methanol-water mobile phases for the separation and characterization of synthetic miRNAs by ultra high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS), reporting LODs of around 10 nM. Under the optimized conditions, this method was able to quantify miRNAs at around 50 nM in spiked serum samples, which were previously pretreated by the PCA liquid-liquid extraction method followed by solid-phase extraction with HLB cartridges [78]. H. Nakayama et al. showed that the LODs previously presented for the analysis of miRNAs could be improved until 100 times incorporating in an instrumental set-up operated with valves a C18 mini trap column for on-line preconcentration after injection of a large volume of sample before nanoLC-MS/MS (Fig. 3-B) [82]. In this case, it was used an acetonitrile-water mobile phase with triethylammonium acetate (TEAA) and methanol, adjusted to pH 7.0, at 100 nL/min to achieve the best separations. Furthermore, they described an application adapted from the analysis of RNA [82,85], for the annotation and automated database search of miRNA MS/MS spectra (Fig. 3-B).

This procedure allowed the untargeted detection of miRNA in HeLa cell extracts, which were previously pretreated using a glass fiber solid-phase extraction miRNA isolation kit followed by reversed-phase LC with a polystyrene/divinylbenzene column. As an alternative to pre-concentration by on-line solid-phase extraction, B. Basiri et al. proposed dispersive solid-phase extraction with streptavidin magnetic sorbent particles conjugated with biotinylated complimentary oligonucleotides before UHPLC-MS/MS [26]. Using a dibutylamine (DBA) HFIP-methanol-water mobile phase at 0.15 mL/min, limits of quantification of 75 pM (0.5 ng/mL) for miR-451 were achieved, which were enough to detect it in human plasma samples.

Indeed, ion-pair reversed-phase LC-MS with strong organic bases, such as TEA, DMBA and DBA, or its acetate salts, is not very popular between MS practitioners, including those focused on RNA analysis [86], because of the potential drawbacks indicated before. As an alternative, zwitterionic-hydrophilic interaction capillary liquid chromatography-mass spectrometry (CapZIC-HILIC-MS) has been

recently investigated [27]. Nowadays, HILIC is regarded as a good option for the separation of oligonucleotides [83]. In ZIC-HILIC, a zwitterionic sulfoalkylbetaine or phosphorylcholine polar stationary phase is used in combination with similar mobile phases to those in reversed-phase LC, namely acetonitrile:water with ammonium acetate at pH 7.0. With a phosphorylcholine stationary phase [27], separations of miRNAs, which are very similar in size and structure, were only acceptable, hence there is still a lot to be done regarding resolving power, but also about repeatability, column durability and detection sensitivity (LODs were 1 μM) [27]. Regarding repeatability and column durability, CE-MS is another good alternative that performs better than CapZIC-HILIC-MS in miRNA analysis [27]. However, miRNAs are very difficult to separate by CE-MS and differentiation typically relies on MS detection [24,25,27]. In CE-MS, separation conditions are necessarily selected to be compatible with on-line MS detection and miRNAs migrate according to their different charge-to-hydrodynamic radius ratios. LODs in CE-MS for miRNA analysis, which are around 5 times higher than by CapZIC-HILIC-MS [27], can be improved combining on-line preconcentration by electrophoretic sample stacking or solid-phase extraction to detect miRNAs in human serum until 5 nM [24,25]. In sample stacking CE-MS, preconcentration depends on the injected sample volume, which is limited by the total volume of the separation capillary, and on the physicochemical properties of the analyte and sample matrix components, especially the electrical charge [24]. On-line solid-phase extraction-capillary electrophoresis mass spectrometry (SPE-CE-MS) has a greater versatility. In the most widely applied SPE-CE configuration, which is typically operated without valves, a micro-cartridge with an appropriate sorbent to selectively retain the target analyte (e.g. SiC sorbent for miRNAs [25]) is integrated near the entrance of the separation capillary. Therefore, SPE-CE-MS allows injecting a larger sample volume than sample stacking CE-MS and is less prone to poor performance due to slight differences in sample matrix. Anyway, the LOD values that have been currently demonstrated for miRNA by sample stacking CE-MS and SPE-CE-MS (i.e. 5 nM and 10 nM, respectively) are higher than the values reported by M. Kullolli et al. [22] and H. Nakayama et al. [82] by ion-pair reversed-phase nanoLC-MS using TEA or TEAA and HFIP as mobile phase additives. This improved sensitivity could be related to the enhanced ionization efficiency of nanoESI in nanoLC-MS/MS [22,82] compared to microESI in CE-MS with a sheathflow interface [24,25], which also promote analyte dilution, as well as with the specific performance of the different mass spectrometers. In the future, the use of improved CE-MS interfaces (e.g. sheathless nanoESI interfaces), mass spectrometers and novel high-extraction capacity and selective sorbents may place on-line SPE-CE-MS as the best alternative to ion-pair reversed-phase nanoLC-MS approaches for miRNA direct and sensitive analysis, as no MS-contaminant additives in the mobile phases or complex instrumental set-ups with valves for on-line solid-phase extraction are required.

3.1.2. MALDI-MS techniques

MALDI-MS has been extensively applied to the direct analysis of oligonucleotides [87–93]. However, applications for miRNAs are scarce, due to the still limited sensitivity, although better than by ESI-MS, performance for quantification, and capacity to deal with complex samples, as well as because the on-line coupling with high-performance separation techniques is not straightforward [20,23,28]. However, MALDI-MS is advantageous from the perspective of simplicity, speed, low reagent and sample consumption and easy mass spectrum interpretation. In the typical experiments, a matrix solution is prepared from a solid organic compound, mixed with the sample solution and the mixture crystallized before applying the laser energy under the high vacuum conditions inside the mass spectrometer [20,23]. Matrix compounds, such as 3-hydroxypicolinic acid (3-HPA), contain a chromophore that is able to absorb the applied laser energy to promote miRNA ionization. In addition, Wambua et al. showed that at certain concentrations, 3-HPA acidity facilitate sequencing of synthetic miRNAs from the fragments obtained after partial hydrolysis produced during sample-matrix preparation, without further sample pretreatment or performing MS/MS experiments [23]. However, these conventional matrices are prone to produce non-homogeneous spots (i.e. hot-spots), lower mass spectrum quality, and reproducibility, if sample-matrix crystallization procedure is not appropriately and carefully performed. As an alternative, ionic matrices (IMs) have been recently described for the analysis of miRNAs [28]. A solid organic salt was formed by mixing equimolar amounts of 6-aza-2-thiothymine (ATT) and pyridine (PYR), and ammonium citrate dibasic was added as signal enhancer. ATT-PYR IM provided LODs slightly lower than conventional matrices for the studied miRNAs (up to 5 nM), with an excellent spot-to-spot repeatability due to the improved homogeneity of the spots (Fig. 4-B). The established method was also applied to the analysis of miRNAs in spiked serum samples after using a silica solid-phase extraction commercial kit. However, current LODs of MALDI-MS for direct analysis of miRNAs are far from the concentration levels of endogenous miRNAs in biological fluids. To the best of our knowledge, only M. Konno et al. have demonstrated that MALDI-MS can be used with conventional matrices to study distinct methylation levels of miRNAs in gastrointestinal cancers, if appropriate purification and enrichment are achieved using magnetic beads with complimentary oligonucleotides followed by C18 solid-phase extraction [20].

3.2. Indirect detection

These methods allow high-sensitivity targeted measurement of miRNAs by indirect detection of the miRNA molecules through detecting specifically related reporter molecules by hyphenated ESI-MS techniques (e.g. LC-MS), ESI-MS or MALDI-MS (Table 2). The surrogate process is typically used for purification and sensitivity enhancement, through nucleic acid amplification or non-nucleic acid amplification techniques. A major drawback of indirect detection is the impossibility of unequivocally confirming the identity of miRNA or obtaining detailed structural information about their sequences and post-transcriptional modifications. Moreover, it requires complex experimental processes that need special primers, enzymes and other reagents.

3.2.1. Nucleic acid amplification techniques

Nowadays, the most sensitive MS-based methods for miRNA analysis include some sort of nucleic acid amplification process. In the most typical nucleic acid amplification approach, the measured miRNAs are reverse-transcribed into single-stranded complementary DNA (cDNA), which is then amplified. S. Kim et al. developed a method that combined stem-loop reverse transcription primers, competitive polymerase chain reaction (cPCR), solid-phase capture-single base extension (SPC-SBE) and MALDI-MS for the multiplexed detection and quantification of miRNAs (Fig. 5) [94]. Stem-loop RT primers with an overhang of 6 nucleotides that annealed to the 3́-end of the miRNA were used for reverse transcription. The multiplexed cDNA amplification happened with competitors of known concentrations. The competitors shared the same sequences with the cDNAs excepting for one base. SBE reaction for cDNA and competitor amplicons was done using a library of SBE primers that annealed next to the altered base site, followed by an extension with a biotinylated dideoxynucleotide triphosphate (biotin-ddNTPs). Then the biotinylated amplicons were selectively extracted with a streptavidin-based sorbent, and eluted before MALDI-MS analysis with 3-HPA and ammonium citrate as matrix solution. In contrast to RT-PCR, this method provided the possibility of multiplexed detection of miRNAs, while allowing quantification and LODs at the picomolar level in cell extracts obtained using TRIzol™ [94].

C. Shi et al. have alternatively described a method for multiplexed miRNA analysis by nanoESI-MS/MS based on cyclic enzyme amplification with a duplex-specific nuclease (DSN) [95]. In this method, DNA/miRNA heteroduplexes were formed after hybridization of target miRNAs with cDNA probes. Then, DSN selectively cleaved the DNAs of the

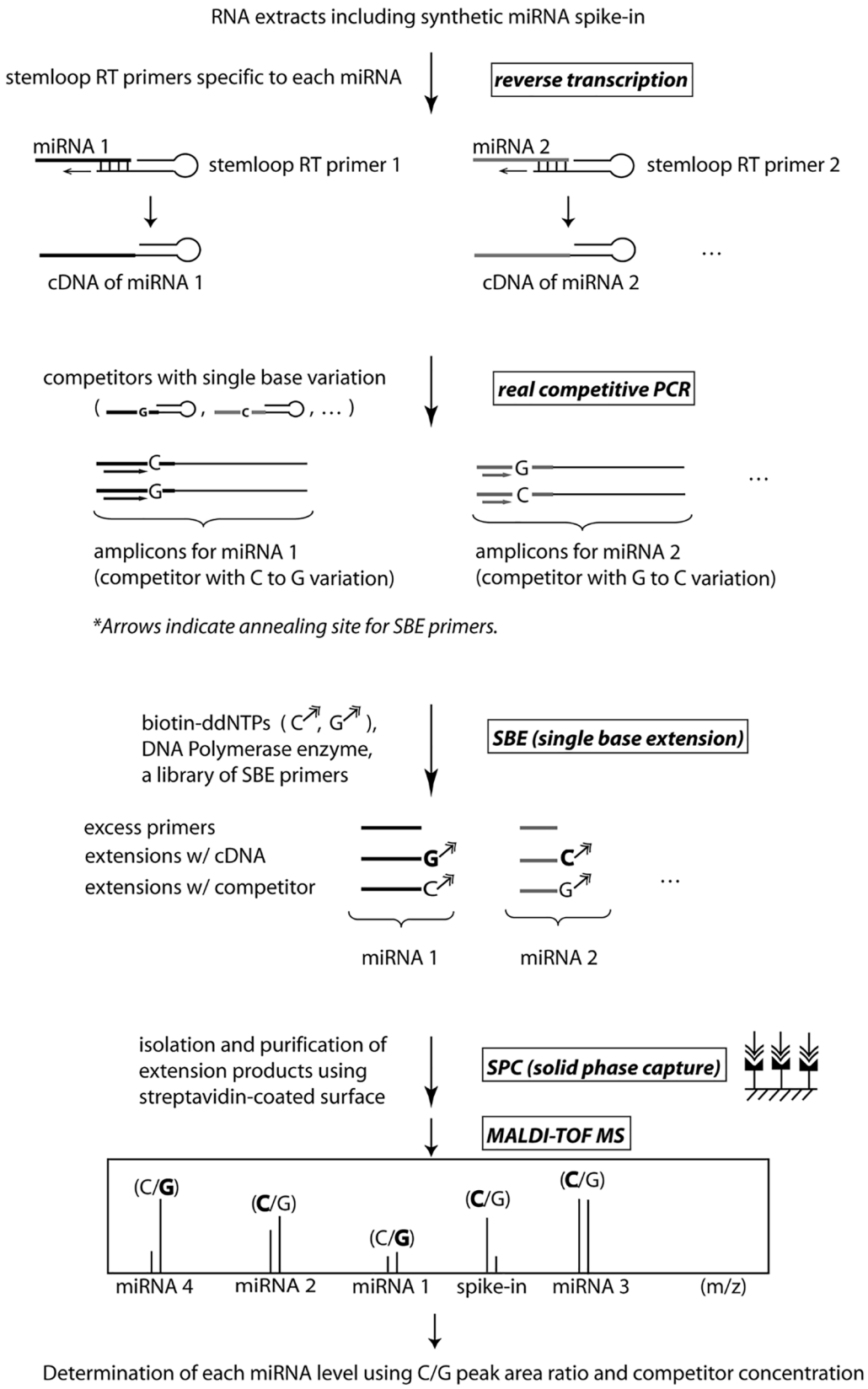

Fig. 5. Schematic representation of a method combining stem-loop reverse transcription primers, competitive polymerase chain reaction (cPCR), solid-phase capture-single base extension (SPC-SBE) and MALDI-MS for the multiplexed detection and quantification of miRNAs (reproduced with permission from [94]).

heteroduplexes into fragments, releasing miRNAs for a new hybridization/cleaving cycle. After completing the amplification, the hydrolyzed fragments of DNA probes were analyzed by nanoESI-MS/MS. The method allowed LODs at the tens of picomolar level and was applied for quantification of miRNAs in several tumor cell culture samples, without needing any sample pretreatment for the isolation of total RNA. However, an advanced calibration model was needed for an accurate quantification.

As an alternative to detection of cDNA or their fragments in nucleic acid amplification methods for miRNA analysis, several authors have proposed nucleobases as molecular reporters. Xiuxiu Li et al. introduced a procedure based on rolling circle amplification (RCA) with DNA padlock probes, which contained sequences for target miRNA recognition, specific capture and adenine (A) tagging. After amplification, A-rich cDNA probes were generated. Then the amplicons were extracted by solid-phase extraction with a selective sorbent against DNA, and completely hydrolyzed by adding acid to release the A molecules, which were used as reporters in HILIC-UHPLC-MS/MS analysis. This indirect method allowed 4,000 times sensitivity enhancement compared to direct MS detection, and was applied to cancer cell extracts pretreated with a solid-phase extraction commercial kit [96]. Xiangtang Li et al. have also presented a method for the targeted analysis of miRNAs at tens of femtomolar levels by LC-MS/MS using as a reporter a different nucleobase (Fig. 6) [97]. The target miRNA was preconcentrated using magnetic beads containing cDNA probes and additional cytosines (C). After amplification with a DSN, fragments of the C-rich cDNA probe were separated from the magnetic beads and completely hydrolyzed, before quantification of C by LC-MS/MS. The method was demonstrated for the analysis of miR-21 in spiked fetal bovine serum and urine, as well as in tumor cell culture samples, without needing any sample pretreatment for the isolation of total RNA. Methods based on nucleic acid amplification and nucleobases as reporter molecules are very sensitive, but applicability in multiplexed analysis has not been demonstrated yet, as a different reporter nucleobase will be needed for each targeted miRNA. A broader range of reporter molecules can be obtained if two-base nucleobase sequences are used, as proposed by X. Li et al. for the analysis of miRNA until picomolar concentrations by nanoESI-MS/MS with a microfluidic platform and DSN amplification [98].

3.2.2. *Non-nucleic acid amplification techniques*

Different methods have been described for analyte enrichment and signal enhancement in indirect detection of miRNAs without involving a prior nucleic acid amplification process.

H. Seo et al. described the use of nanoengineered micro gold shell (μAuS) for the femtomolar detection of miRNAs by matrix-free MALDI-MS (Fig. 7) [99]. The target miRNA was captured using fragments of cDNA immobilized on a gold chip (Fig. 7-A). Then, they were reacted with μAuS presenting a different fragment of cDNA and a large excess of small molecules as what they called “amplification tags” (Am-tags, see detailed structure in Fig. 7-B). After rinsing the chip, Am-tags were used as reporters for high-sensitivity matrix-free MALDI-MS (Fig. 7-A) [99]. T. C. de Bang et al. used also a similar strategy to capture the miRNA with full cDNA labelled with lanthanides for indirect detection of miRNA from *Arabidopsis Thaliana* by northern blotting and laser ablation inductively coupled plasma mass spectrometry (laser ablation ICP-MS) in the low femtomole range [100].

Y. Chen et al. proposed to use peptides as molecular reporters to enhance sensitivity of miRNA MS analysis [101–103]. The best option to maximize signal enhancement is to use cDNA-peptide dendrimer probes containing multiple reporter peptides (until 8 in this case) to be released after tryptic digestion [102,103]. The probe was first hybridized with the target miRNA that was biotinylated and attached to streptavidin agarose. After trypsin digestion, the reporter peptides were released and analyzed by LC-MS/MS. The signal enhancement was approximately until 8 times higher than without using the probes, at the picomolar level, and enough to detect several miRNAs in different tumor cell and tissue samples, after treatment with a ready-to-use liquid-phase extraction commercial reagent [102].

4. Conclusions and perspectives

MiRNA isolation by liquid-phase or solid-phase extraction methods is

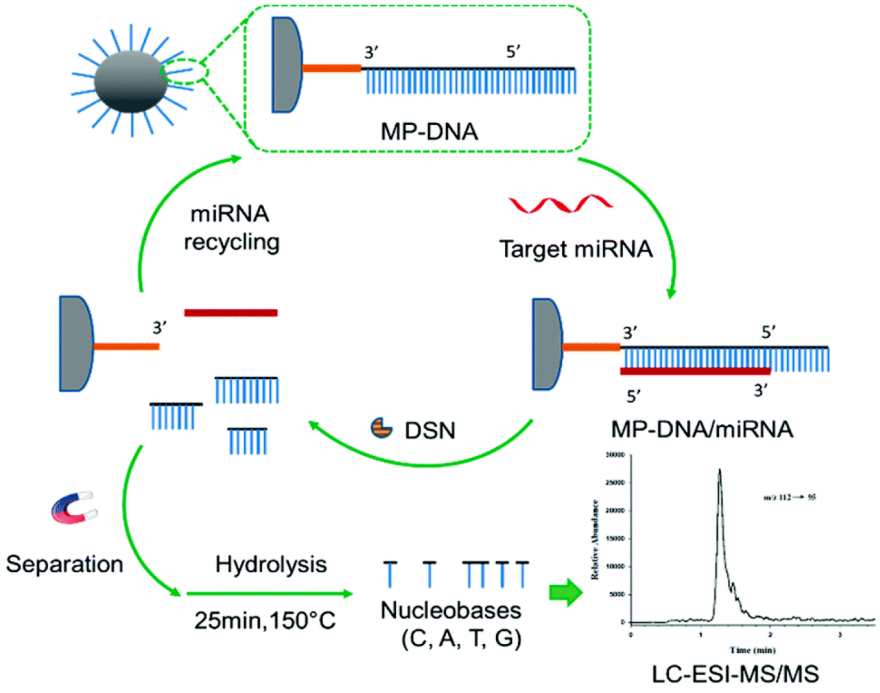

Fig. 6. Schematic representation of a method based on preconcentration using magnetic beads containing cDNA probes and additional cytosines (C), followed by amplification with a duplex-specific nuclease (DSN), separation of the C-rich cDNA probe fragments from the magnetic beads, complete hydrolysis and quantification of C by LC-MS/MS (reproduced with permission from [97]).

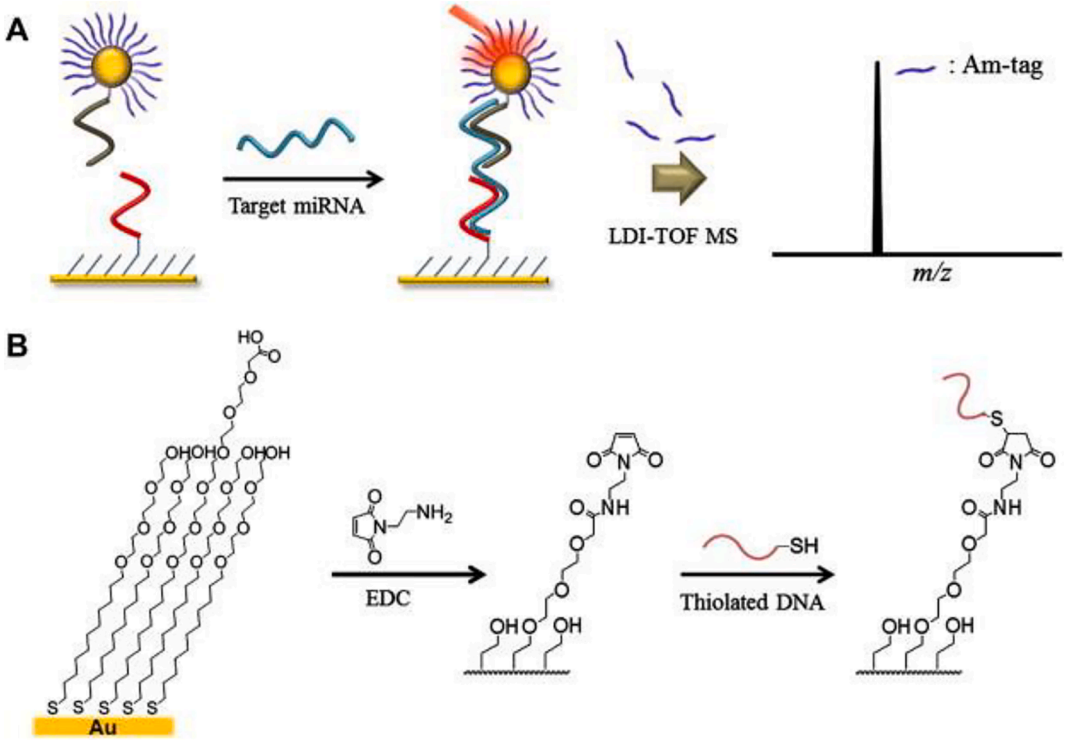

Fig. 7. Schematic representation of a method using a gold chip with fragments of cDNA and micro gold shell (μAuS) with fragments of cDNA and amplification tags (Am-tags). Am-tags were released and measured by matrix-free MALDI-MS (reproduced with permission from [99]).

fundamental for purification and enrichment of miRNAs from biological samples. Nowadays, solid-phase extraction methods commercialized as kits have surpassed in popularity liquid-phase extraction methods, but still these kits require a sample pretreatment to roughly isolate miRNAs using the typical additives (i. e. chaotropic agents, salts, organic solvents, proteases and carriers) applied in liquid-phase extraction methods. Therefore, both extraction methods need to be regarded as necessarily complementary, and they may evolve together to improve miRNA extraction recovery and purity from small sample volumes. The development of large extraction capacity and appropriate selectivity silica and non-silica-based sorbents for targeted and untargeted solid-phase extraction at the microscale may drive progress in the field, as well as the on-line coupling with the analytical methods to reduce sample handling and increase throughput or the standardization of the sample pretreatment workflows for different biological samples to improve reliability of the measurements and fully exploit miRNAs as biomarkers. In the field of MS detection, it will be necessary to pay attention to the use of non-volatile additives for miRNA isolation to avoid the need for additional sample pretreatment. So far, MS has demonstrated a great potential for the analysis of miRNA and especially direct detection may provide unique information compared to other conventional bioanalytical methods, as it allows the multiplexed detection, quantitation, sequencing, and characterization of post-transcriptional modifications of miRNAs. However, further research must be done to decrease the LODs, and solid-phase extraction coupled on-line to microscale separation techniques (e.g. SPE-nanoLC-MS and SPE-CE-MS) may be the key tool to solve this issue while maximizing throughput. Anyway, now the most sensitive MS-based methods for miRNA analysis are indirect methods with nucleic acid amplification. Unfortunately, the simplicity of the measurement and the potential of unequivocally identifying and characterizing the structure of the miRNAs are lost with indirect detection, as well as the multiplexed performance if reporter molecules are used. Therefore, at this moment, only direct MS methods may be regarded as a true complementary alternative to conventional bioanalytical methods, which deserve to be further investigated to exploit its full potential.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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