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Intact MicroRNA Analysis Using High Resolution Mass Spectrometry

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Abstract

MicroRNAs (miRNAs) are small single-stranded non-coding RNAs that post-transcriptionally regulate gene expression, and play key roles in the regulation of a variety of cellular processes and in disease. New tools to analyze miRNAs will add understanding of the physiological origins and biological functions of this class of molecules. In this study we investigate the utility of high resolution mass spectrometry for the analysis of miRNAs through proof-of-concept experiments. We demonstrate the ability of mass spectrometry to resolve and separate miRNAs and corresponding 3' variants in mixtures. The mass accuracy of the monoisotopic deprotonated peaks from various miRNAs is in the low ppm range. We compare fragmentation of miRNA by collision-induced dissociation (CID) and by higher-energy collisional dissociation (HCD) which yields similar sequence coverage from both methods but additional fragmentation by HCD versus CID. We measure the linear dynamic range, limit of detection, and limit of quantitation of miRNA loaded onto a C18 column. Lastly we explore the use of data dependent acquisition of MS/MS spectra of miRNA during online LC-MS and demonstrate that multiple charge states can be fragmented, yielding nearly full sequence coverage of miRNA on a chromatographic time scale. We conclude that high resolution mass spectrometry allows the separation and measurement of miRNAs in mixtures and a standard LC-MS setup can be adapted for online analysis of these molecules.

Introduction

MicroRNAs (miRNAs) are short, approximately 22 nucleotides in length, non-coding RNAs derived from 60–110 nucleotide RNA precursor structures. MiRNAs post-transcriptionally regulate gene expression of various genes involved in diverse cellular processes, such as development, differentiation, proliferation, apoptosis, and metabolism [1]. Recently, it has been demonstrated that miRNAs play an important role in oncogene regulation (e.g. tumor

suppression), and these small molecules are thought to contain potential diagnostic and prognostic information [2]. There are estimated to be approximately 1000 miRNAs encoded in the human genome and each single miRNA may regulate multiple mRNAs, having a substantial effect on gene expression [3].

Despite significant efforts to study this class of molecules, much remains unknown about their physiological origins and precise biological functions. Several technologies are currently available for miRNA expression profiling, such as quantitative reverse transcription polymerase chain reaction (qRT-PCR), hybridization based methods (microarrays), and high-throughput sequencing (RNA-seq), each with tradeoffs in sensitivity, linear dynamic range, and cost [3]. These technologies have significantly contributed to identification of new miRNAs, elucidated information about miRNA expression patterns, and have increased our understanding of miRNA biological functions. However, due to their small size and the lack of poly(A) tail in their sequence, it can be challenging to accurately detect and quantify this class of molecules. In addition, miRNAs that belong to the same family may differ from each other by only one nucleotide, making it even more difficult to selectively measure them. Furthermore, these technologies are unable to comprehensively detect post-transcriptional modifications present on miRNAs. Relatively little is known about post-transcriptional modifications of miRNAs in human samples, however, it has been shown that some modifications such as nucleotide additions have functional consequences in plants and animals [4–6]. Mass spectrometry is well suited to aid in identification of post-transcriptional modifications, thereby contributing to biological understanding of these molecules.

Over the last several decades, significant effort has been devoted to separation, isolation, and purification of oligonucleotides from biological samples, based on gel electrophoresis, high-performance liquid chromatography (HPLC), and most recently ion mobility [7–9]. Although these technologies have high separation capabilities, they are limited in qualitative analysis abilities and are typically not sufficient for absolute quantification and identification of oligonucleotides [7]. Mass spectrometry (MS) has been previously utilized for the analysis of nucleic acids and oligonucleotides [10–12], particularly with model systems and synthetic oligonucleotides. The majority of mass spectrometry-based RNA studies used enzymatic or chemical digestion of longer RNA species prior to analysis by mass spectrometry [13]. Although analysis of miRNAs by mass spectrometry has been reported [14], previous studies have used alternative approaches to the experimental setup described here, for example direct infusion methods on a Q-TOF mass spectrometer [15] or affinity capture of a specific miRNA of interest [16].

The small size (~7 kDa) of miRNAs makes this class of molecules ideally suited for intact mass spectrometry analysis using electrospray ionization. It has been shown that direct infusion of oligonucleotides to MS allows determination of the molecular weight of oligonucleotides with a high mass accuracy [17]. However, more complex mixtures or oligonucleotides with similar compositions but different sequences require additional attention.

In this study we describe proof-of-concept experiments to identify and characterize miRNAs in mixtures. We describe identification and characterization of synthetic miRNAs on a chromatographic time scale using a reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with a high resolution LTQ-Orbitrap-Velos mass spectrometer. In contrast to PCR and genomic sequencing techniques, this approach has the potential to allow us to systematically study miRNA modifications and identify miRNAs that are modified in tissue. In an effort to optimize this method we used a mixture of synthetic miRNAs. Synthetic miRNAs are well suited as a model system for method development because relatively pure ample quantities are readily available. The synthetic miRNAs were used to optimize chromatographic separation and ionization conditions for MS/MS analysis. Our results show that this method is robust with respect to linear dynamic range, and able to resolve miRNAs with high mass accuracy.

Methods

Materials

HPLC grade water, methanol, acetonitrile, imidazole, piperidine, and triethylamine were obtained from ThermoFisher Scientific (Waltham, MA). 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) was obtained from Sigma Aldrich (St. Louis, MO). The synthetic oligonucleotides miR-141 canonical, miR-141-3'A, miR-141-3'U, miR-200a canonical, miR-200a 3'A, and miR-200a 3'U were synthesized with 5' phosphate groups and HPLC purified by Integrated DNA Technologies (Coralville, IA). The remaining miRNAs, miR-125a-5p 3'U, miR-125a-5p 3'G, miR-191 canonical, miR-191 3'U, miR-24 3'U, miR-199a-3p 3'U, miR-205, and miR-15a, were synthesized with 5' phosphate groups and desalted by Qiagen (Valencia, CA). The sequences for the miRNAs are as follows: miR-125a-5p 3' U (uccugagaccuuuaaccugugau), miR-125a-5p 3' G (uccugagaccuuuaaccugugag), miR-191 canonical (caacggaaucacaaaagcagcug), miR-191 3' U (caacggaaucacaaaagcagcugu), miR-24 3' U (uggcucagucagcaggaacagu), miR-199a-3p 3' U (acaguagucugcacauugguuau), miR-200a canonical (uaacacugucugguaacgaugu), miR-200a 3'U (uaacacugucugguaacgauguu), miR-200a 3'A (uaacacugucugguaacgaugua), miR-141 canonical (uaacacugucugguaaagaugg), miR-141 3'U (uaacacugucugguaaagauggu), miR-141 3'A (uaacacugucugguaaagaugga), miR-205 (uccuauuuccaccggagucug), miR-15a (uagcagcacauaagguuugug).

Static Nano-Electrospray Conditions

Ten μ M solutions of miRNAs were made in 40/40/20 acetonitrile/water/isopropanol containing 20 μ M piperidine and 20 μ M imidazole. The miRNAs solutions were introduced to a LTQ-Orbitrap Velos using static nano-electrospray ionization. The miRNAs were ionized in negative-ion mode due to the negatively charged phosphate backbone. The ionization voltage was set at -1.8 kV, source temperature at 150 °C, normalized collision energy of 35%, isolation width of 2.0, and both MS1 and MS/MS spectra were collected from 400–2000 m/z at a resolution of 60,000 with the Orbitrap mass analyzer. The LTQ-Orbitrap-Velos is capable of routinely providing high resolution and mass accuracy for intact miRNA and MS/MS measurements. MS/MS analysis of miRNA was performed to determine sequence coverage.

LC-MS/MS Equipment and Conditions

Nano-LC-MS/MS analysis was performed using an Eksigent nanoLC 2D system (Dublin, CA) interfaced with an LTQ-Velos-Orbitrap mass spectrometer (ThermoFisher Scientific) coupled with a CaptiveSpray source (Michrom BioResources, Auburn, CA). The composition of solvent A was 400 mM HFIP in water pH 7.0 and solvent B was 400 mM HFIP in 50% methanol, the pH of the mobile phases was adjusted by using triethylamine. The synthetic oligonucleotides were injected using a CTC (Leap Technologies, Carrboro, NC) autosampler onto a 150×0.1 mm Chromolith® CapRod® (Merck KGaA, Darmstadt, Germany) capillary monolithic column, consisting of macropores providing a very high porosity making possible to operate at higher flow-rate than typical nano-flow columns (1–3 $\mu\text{L}/\text{min}$). Sample was loaded onto the column at a flow rate of 1 $\mu\text{L}/\text{min}$ for 30 min using (98% solvent A). Sample was eluted from the analytical column using gradient of 1 $\mu\text{L}/\text{min}$ 2% solvent B to 50% solvent B over 10 min, followed by 50% solvent B and held at isocratic 50% solvent B for 10 min, and then from 50% solvent B to 65% solvent B over 5 min, and then held at isocratic at 65% solvent B for 10 min. Mass spectrometry conditions were as follows: ion transfer tube temperature: 150 °C; spray voltage: -1.8 kV; normalized collision energy: 35% for both resonance collision-induced dissociation (CID) in the ion trap and higher-energy collisional dissociation (HCD) in the HCD cell, activation time: 10 ms for CID and 30 ms for HCD [18]. Data dependent MS/MS analysis was carried out using MS acquisition software (Xcalibur 2.1, ThermoFisher Scientific) with CID performed on the top ten ions and MS/MS products analyzed by the Orbitrap mass analyzer at 30,000 resolution, isolation width 2.0, exclusion mass width ± 2.0 , repeat count 1, repeat duration 30 s, exclusion width size 400, and exclusion duration 30 s.

Results and Discussion

The short (~22 nucleotides) size of miRNAs makes this class of molecules particularly well suited for intact analysis by mass spectrometry in the conventional m/z range of <2000. To evaluate the utility of high resolution mass spectrometry for analyzing miRNAs in complex mixtures, six miRNAs (miR-200a canonical, miR-200a 3' A, miR-200a 3' U, miR-141 canonical, miR-141 3' A, and miR-141 3' U) were mixed at equal concentrations (10 μM) and directly infused by nano-electrospray ionization into the mass spectrometer. The mass spectrum of this mixture is shown in Figure 1a. The majority of the miRNA signal falls over the 600–1300 m/z range, where a charge state distribution of -6 to -11 is observed, with the most abundant charge state of -9 for all species. A closer examination of the -9 charge state distribution in Figure 1b shows that each of the six miRNAs can be resolved. For each miRNAs the peaks from most abundant to least abundant are: a single sodiated adduct $[\text{M} + \text{Na} - 9\text{H}]^{9-}$, a double sodiated adduct $[\text{M} + 2\text{Na} - 9\text{H}]^{9-}$, the fully deprotonated peak with no adducts $[\text{M} - 9\text{H}]^{9-}$, and a triply sodiated adduct $[\text{M} + 3\text{Na} - 9\text{H}]^{9-}$. In these experiments, piperidine and imidazole were added to the nano-electrospray solutions to reduce the number and extent of sodium adducts and to increase the signal from the deprotonated peak [19]. Peak assignments were further confirmed by infusing and acquiring mass spectra for each of the miRNAs separately. Figure 1c shows the resolution of the $[\text{M} - 10\text{H}]^{10-}$, $[\text{M} - 9\text{H}]^{9-}$, and $[\text{M} - 8\text{H}]^{8-}$ ions for miR-200a. With the Orbitrap set to 60,000 resolution, the

monoisotopic peaks for each of the observed charge states were 1.2 ppm for $[M-10H]^{10-}$, 0.9 ppm for $[M-9H]^{9-}$, 0.6 ppm for $[M-8H]^{8-}$, and 0.3 ppm for $[M-7H]^{7-}$.

The six miRNAs in Figure 1 are miR-200a canonical (uaacacugucugguaacgaugu) and miR-141 canonical (uaacacugucugguaaagaugg) and two variants of each of these miRNAs with a single nucleotide (U or A) added to the 3' terminus of each canonical sequence (miR-200a 3'A, miR-200a 3'U, miR-141 3'A, and miR141 3'U). The ability to clearly discriminate variants of a single nucleotide at the 3' end is a unique point of leverage for mass spectrometry, as traditional molecular biology approaches (i.e. polymerase chain reaction and next generation RNA sequencing) are cumbersome and/or insensitive to such variations in comparison to mass spectrometry. Here, we demonstrate the ability of high resolution mass spectrometry to resolve each variant of two different miRNA families, and all the sodiated adducts, in a mixture simultaneously without any chromatographic or other separation.

The mass accuracy of the LTQ-Orbitrap Velos for measuring intact miRNA masses was evaluated by static nano-electrospray ionization and infusion of a variety of different miRNAs as shown in Table 1. The mass accuracy measurements were calculated from the theoretical monoisotopic masses of each of the charge states observed ($[M-7H]^{7-}$, $[M-8H]^{8-}$, $[M-9H]^{9-}$, and $[M-10H]^{10-}$) as shown in Table 1. The average of the overall mass accuracy measurements is 1.3 ppm. A vast majority of the masses measured have error less than 3 ppm. The mass accuracy could presumably be further improved with the use of an internal calibrant or lock mass, or by increasing the resolution setting of the Orbitrap.

The ability to obtain miRNA sequence information by mass spectrometry was evaluated for two different dissociation techniques: collision-induced dissociation (CID) and higher-energy collisional dissociation (HCD). Isolated $[M-9H]^{9-}$ ions were subjected to CID and HCD as shown in Figure 2a and 2b respectively. The fragment ions are labeled by a previously established nomenclature for oligonucleotides [20]. The high mass resolution provided by the Orbitrap allows clear charge state determination for each fragment peak. This charge state identity information in combination with the high mass accuracy allows relatively straightforward identification of the fragment ions and base composition determination. In both the CID and HCD spectra, the losses of neutral bases (A, C, and G) are the dominant fragmentation channels and these ions comprise the majority of the product ion signal. With both CID and HCD fragmentation, a charged loss of HPO_3^- is also observed as a major fragmentation product. This charged loss of HPO_3^- is presumably due to the 5' phosphate group present on the miRNAs, as loss of HPO_3^- has not typically been reported in previous studies. Fragmentation along the oligonucleotide backbone yields a combination of c, a, w, and y ions. Both CID and HCD also yield frequent secondary fragmentation resulting in (a-B) ions. The fragmentation mechanisms for RNA anions [21] and for charged and neutral base loss from multiply charged oligonucleotide anions have been previously described [20]. Neutral base loss following backbone cleavage and internal fragmentation could be minimized by lowering the internal energy of fragment ions from the primary backbone cleavage as previously reported [22–23].

The miRNA fragmentation information obtained from between CID and HCD is similar. Complementary ions (c/y, a/w, and (a-B)/w) are observed for a number of cleavage sites, providing additional confidence to the miRNA sequence determination. The sequence coverage obtained from CID and HCD is similar as CID yielded fragmentation at 17/21 backbone sites and HCD yielded fragmentation at 18/21 backbone sites. The one fewer backbone fragment by CID is due to the notable lack of fragment ions between m/z 765–780 in the CID spectrum. This notch is presumably due to the resonance excitation applied during CID and results in a missing cleavage between the C₁₀ and C₁₁ residues in the CID spectrum. This notch is not present in the HCD spectrum and fragmentation between C₁₀ and C₁₁ is observed by the w₁₂⁵⁻ ion.

A practical consideration for the analysis of miRNA is the ability to automate analysis, preferably on a chromatographic timescale, using a standard LC-MS experimental setup. To assess the limit of detection (LOD), limit of quantitation (LOQ), and linear dynamic range of a typical LC-MS setup, miR-15a was diluted to seven different concentrations: 1 nM, 10 nM, 50 nM, 100 nM, 500 nM, 1000 nM, and 10,000 nM. Five µL of each solution were injected onto the column and blank LC-MS runs were performed to determine the noise level. The dilution curves for miR-15a charge states: [M-11H]¹¹⁻, [M-10H]¹⁰⁻, and [M-9H]⁹⁻ are shown in Figure 3a, 3b, and 3c respectively. The dilution curve for the peak areas of all three charge states summed is shown in Figure 3d. The peak area for the deprotonated charge state distribution is plotted versus the solution concentration injected on column. Regression analysis was performed over the linear portion (1 nM is outside the linear region) of the dilution curves as represented by the lines and R² values. All R² values are equal to or greater than 0.95. From this experimental setup, the limit of detection (signal-to-noise ratio greater than 3) is approximately 36 pg (1 nM, 5 fmol) and limit of quantitation (signal-to-noise ratio greater than 10) is approximately 1.8 ng (50 nM, 253 fmol). The limit of detection and limit of quantitation was consistent for Figures 3a, b, c, and d.

To evaluate the potential feasibility of online LC-MS/MS analysis for miRNA, a mixture of four different miRNAs (miR-205, miR-15a, miR-200A 3'U, and miR-141 3'U) were loaded onto an online HPLC column simultaneously. The monolithic column was chosen for the lower back pressure which allowed flow rates of 1 µL/min, allowing more sensitive miRNA ion detection than traditional (300–400 nL/min) nano-LC flow rates. All four miRNAs eluted between 47.37 and 49.17 minutes (Supplementary Material). The LC-MS setup and HFIP and triethylamine containing buffers significantly reduced the sodium adducts (consistent with previous reports [7]) observed on miRNAs compared to static nano-electrospray. While the static nano-electrospray conditions yielded sodiated miRNAs as the most abundant peaks in the MS1 spectrum and much larger than the deprotonated peaks, the most abundant peaks in the LC-MS eluted miRNAs were the deprotonated ions, with the most abundant sodiated adduct typically approximately 30% as abundant as the deprotonated peak (Supplementary Material).

The LTQ-Orbitrap was set to data dependent acquisition mode to acquire MS/MS data on the top ten ions. Despite the short chromatographic elution time of the miRNAs (less than two minutes), the data dependent acquisition was able to isolate and fragment multiple charge states for each of the four miRNAs. MS/MS was performed on the deprotonated ions

of six charge states of miR-205a ($[M-7H]^{7-}$ to $[M-12H]^{12-}$), seven charge state of miR-15a ($[M-7H]^{7-}$ to $[M-13H]^{13-}$), six charge states of miR-200a 3'U ($[M-7H]^{7-}$ to $[M-12H]^{12-}$), and five charge states of miR-141 3'U ($[M-8H]^{8-}$ to $[M-12H]^{12-}$) (Supplementary Material). This demonstrates the ability of the LC-MS setup to obtain fragmentation information on multiple charge states of multiple miRNA species in a single chromatographic run.

The MS/MS spectra collected during the single chromatographic run of the mixture of the four miRNAs for miR-200A 3'U $[M-11H]^{11-}$, $[M-10H]^{10-}$, $[M-9H]^{9-}$, and $[M-8H]^{8-}$ are shown in Figure 4a, b, c, and d, respectively. In all charge states, the losses of charged and neutral bases are the major fragmentation channels. Loss of HPO_3^- , presumably from the 5' phosphate group, is also frequently observed. The extent of backbone fragmentation differs by charge state with 15/22 backbone cleavages in the fragmentation of $[M-11H]^{11-}$ and $[M-7H]^{7-}$ and 18/22 backbone cleavages in the fragmentation of $[M-9H]^{9-}$ and $[M-8H]^{8-}$. The combination of all four charge states yields almost complete sequence coverage with 21/22 backbone cleavages, with the U_9-C_{10} uncleaved.

Conclusions

Here we describe proof-of-concept experiments demonstrating the ability of high resolution mass spectrometry to analyze miRNAs in mixtures. We perform this analysis on a chromatographic timescale. The methods developed here have the potential to be applied to biological samples and complement the information obtained by conventional miRNA analysis methods. For example, high resolution mass spectrometry would offer significant advantages for de novo sequencing and analysis of post-transcriptionally modified miRNAs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Nano-ESI MS of miRNA mixture. a) MS of mixture of six miRNAs (miR-200a canonical, miR-200a 3' A, miR-200a 3'U, miR-141 canonical, miR-141 3'A, and miR-141 3'U), b) Expanded MS view of the -9 charge state ions, and c) the distribution of the most intense charge states observed for miR-200a. *indicates monoisotopic peaks

Figure 2.

Fragmentation of miR-205 [M-9H]⁹⁻. a) MS/MS by CID. b) MS/MS by HCD. A normalized collision energy of 35% was used for both CID and HCD. An activation time of 10 ms was used for CID and 30 ms for HCD. *indicates precursor ion [M-9H]⁹⁻.

Figure 3.

Dilution curves for miR-15a. a) Dilution curve for [M-11H]¹¹⁻, b) dilution curve for [M-10H]¹⁰⁻, c) dilution curve for [M-9H]⁹⁻, and d) sum of peak areas for all three charges. Peak area was extracted from isotopic distribution from monoisotopic peaks. Five μ L of each solution were loaded on column. Power regression analysis was performed on each dilution curve with the R^2 and regression equations listed on each curve.

Figure 4.

MS/MS of miR-200a 3'U from a single LC-MS run of a mixture of four miRNAs (miR-205, miR-15a, miR-200A 3'U, and miR-141 3'U) injected on column. a) CID of $[M-11H]^{11-}$, b) CID of $[M-10H]^{10-}$, c) CID of $[M-9H]^{9-}$, and d) CID of $[M-8H]^{8-}$. *indicates precursor ion m/z.

Table 1

Mass Accuracy Measurements for Various miRNAs.

miRNA	[M-7H] ⁷⁻			[M-8H] ⁸⁻			[M-9H] ⁹⁻			[M-10H] ¹⁰⁻			All Charge States	
	Observed			Observed			Observed			Observed				
	m/z	m/z	ppm	m/z	m/z	ppm	m/z	m/z	ppm	m/z	m/z	ppm	Average ppm	Standard Deviation ppm
miR-125a_5p_3'U	1132.135	1132.134	0.53	990.492	990.493	1.25	880.326	880.326	0.06	792.192	-	-	0.61	0.60
miR-125a_5p_3'G	1137.710	1137.707	1.93	995.370	995.369	0.56	884.661	-	-	796.094	-	-	1.25	0.97
miR-200a_canonical	1010.408	1010.408	0.30	883.981	883.981	0.57	785.649	785.648	0.89	706.983	706.984	1.27	0.76	0.42
miR-141_3'U	1063.130	-	-	930.113	930.116	2.47	826.655	826.657	2.30	743.889	743.892	3.50	2.76	0.65
miR-200a_3'U	1054.126	1054.125	0.76	922.234	922.235	0.65	819.652	819.653	1.59	737.586	737.587	1.63	1.16	0.52
miR-191_canonical	1063.573	1063.569	3.38	930.500	930.498	2.47	826.999	826.997	3.26	744.199	-	-	3.04	0.50
miR-191_3'U	1107.291	-	-	968.753	968.753	0.72	861.002	861.002	0.58	774.801	774.801	0.90	0.74	0.16
miR-24_3'U	1065.134	-	-	931.867	931.866	0.64	828.213	828.214	0.72	745.292	-	-	0.68	0.06
miR-199a_3p_3'U	1054.126	1054.126	0.09	922.234	922.234	0.43	819.652	819.651	0.61	737.586	-	-	0.38	0.26