Characterization of Intact mRNA Using Ion Pair-Reversed Phase-Time of Flight-MS, Size Exclusion Chromatography-Multi Angle Light Scattering, and Charge Detection Mass Spectrometry

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Summary

- There is an increased need to analyze intact mRNA analytes such as those found in vaccines
- Chromatography-based IP-RP-TOF-MS and SEC-MALS techniques were used to characterize three separate mRNA samples and compared to CDMS analysis
- Results from the three analytical techniques show consistency in the determination of intact mass of the individual samples
- CDMS measurements did not use chromatographic separation and required little method development time

Introduction

There is a pressing need to analyze intact mRNA that has been accelerated by the recent development of mRNA-based vaccines in response to the COVID-19 pandemic. mRNA production by enzymatic *in vitro* transcription followed by enzymatic or chemical capping can produce a variety of reaction products. Unwanted products and contaminants such as double-stranded RNA (dsRNA), truncated RNA fragments, and heterogeneity in the poly(A) tail, and degradation products can be better characterized by examining intact mass distributions. Herein, we demonstrate intact mass analysis of mRNA using a variety of conventional techniques with chromatographic separation: size exclusion chromatography coupled to multiangle light scattering and ion pair-reversed-phase chromatography coupled to a time of flight mass analyzer (SEC-MALS & IP-RP-TOF-MS). Additionally, we analyze mRNA by Charge Detection Mass Spectrometry (CDMS) and compare these results to those obtained by the more conventional methods.

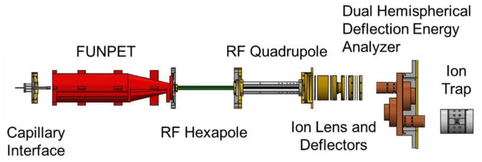

Figure 1. Schematic of CDMS instrument used for intact mRNA analysis.

Experimental

Materials

EPO mRNA (human erythropoietin, 858 nucleotides), Cas9 mRNA (*Streptococcus pyogenes* SF370 Cas9, 4521 nucleotides), and FLuc mRNA (firefly luciferase protein, 1929 nucleotides) were obtained from TriLink Biotechnologies (San Diego, CA)

IP-RP-TOF-MS

- Mobile phases: (A) 60 mM HFIP, 8 mM diisopropyl-ethylamine (DIPEA) in deionized water; (B) 4.5 mM HFIP, 3 mM DIPEA in acetonitrile
- Column: ACQUITY HSS T3™ C18, 1.0 x 150 mm, 1.8 µm
- Gradient: 10-50% B over 4 min, 50-85% B over 0.1 min, hold at 85% B for 0.8 min, 85-10% B over 0.1 min and hold at 10% B for 3 min
- Flow rate: 100 µL/min
- Detectors: TUV @ 260 nm and Synapt XS QTOF MS™ (equipped with H-Class Bio UPLC™)
- Injection volume: 1 µL
- MS parameters: Negative ESI-MS mode; Mass Range: 1000-4000 Da; Cone Voltage 100 V; Acquisition Mode: MS scan, Sensitivity Mode, 1 sec scans

Size Exclusion Chromatography-MALS

- Mobile phase: 20 mM Tris/1 M tetramethylammonium chloride (TMAC), pH 7.4
- Column: Waters BEH450 SEC™, 7.8 x 300 mm, 3.5 mm bead diameter with 450 A pores
- Flow rate: 0.4 mL/min
- Detectors: TUV™ @ 260 nm and a MALS detector (HELEOS)
- Injection volume: 10 µL

CDMS

- Samples were analyzed on a CDMS prototype instrument at Megadalton Solutions (Bloomfield, IN), shown in Figure 1
- Sample prep: 20 µL of each mRNA sample were buffer exchanged with micro-biospin columns (Bio-Rad) into 200 mM ammonium acetate
- Ionization: static nano electrospray using a Triversa Nanomate (Advion, Ithaca, NY), positive ion mode
- CDMS trapping time: 100 msec, triggered trapping
- Ion energy: 100 eV/z
- Quad RF: 450 kHz 200 Vpp
- RNase-free water and supplies were used to minimize degradation

Results

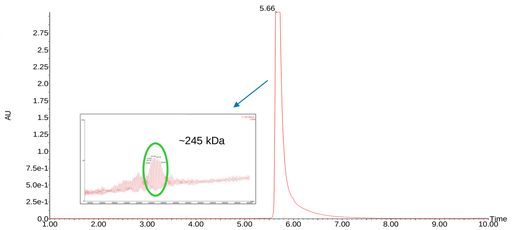

Figure 2. Ion Pair-Reversed Phase TOF-MS data for EPO mRNA. LC-UV chromatogram (260 nm) from the IP-RP separation of EPO mRNA. The EPO mRNA eluted in a single peak with an approximate mass of 245 kDa (insert: deconvoluted mass spectrum from the Synapt XS TOF MS from 10 µg sample injection). This mass is consistent with fragmented EPO mRNA (as seen in Figure 3). MS data was only observed at a loading amount of 10 µg, which saturated the UV detector.

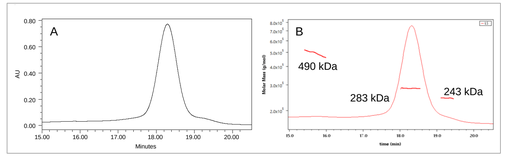

Figure 3. Absorbance and Multi-Angle Light Scattering data for EPO mRNA. Panel A shows A260 for SEC separated EPO mRNA with one large peak apparent. Based on MALS data (panel B) the main SEC peak has an approximate mass of 283 kDa, which is consistent with the expected mass. Larger and smaller variants were observed with masses of 243 kDa (mRNA fragment) and 490 kDa (potentially dimer of the fragment).

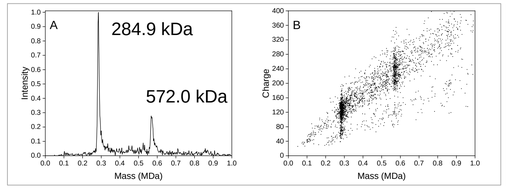

Figure 4. Charge Detection Mass Spectrometry data for EPO mRNA. Panel A is the mass histogram showing mass vs intensity. The peak at 284.9 kDa is consistent with the expected mass of EPO mRNA (283 kDa). A peak at 572 kDa suggests the presence of a dimer of EPO mRNA. Panel B plots the Charge (z) vs Mass (MDa) for the observed ions. This shows the broad distribution of charges each analyte carries during trapping and detection in the CDMS instrument.

Note: mRNA is sensitive to RNase enzymes and special attention should be paid to using RNase inhibitors and/or RNase-free reagents and supplies.

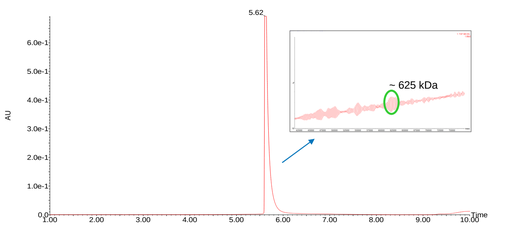

Figure 5. Ion Pair-Reversed Phase TOF-MS data for FLuc mRNA. LC-UV chromatogram (260 nm) from the IP-RP separation of FLuc mRNA. A single peak with an approximate mass of 625 kDa was observed (insert: deconvoluted mass spectrum from the Synapt XS TOF MS from 10 µg sample injection). This mass is a bit higher than the expected mass of Fluc mRNA. MS data was only observed at a loading amount of 10 µg and it would be difficult to determine the correct signal in the deconvoluted mass spectrum if the expected MW was not known.

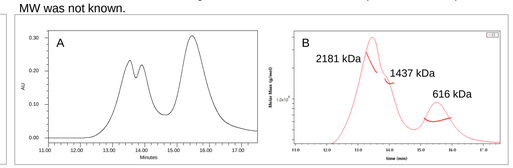

Figure 6. Absorbance and Multi-Angle Light Scattering data for FLuc mRNA. Panel A shows A260 for SEC separated FLuc mRNA with several peaks apparent. Based on MALS data (panel B) the three peaks have approximate masses of 2181, 1437, and 616 kDa. The expected mass is 533 kDa. The main peak in Panel A is likely the FLuc mRNA product, and the mass is similar to that found with IP-RP-TOF-MS (Figure 5).

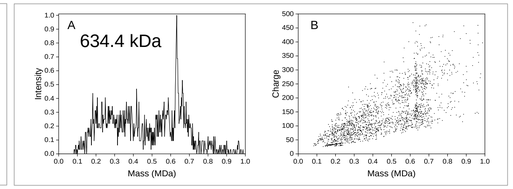

Figure 7. Charge Detection Mass Spectrometry data for FLuc mRNA. Panel A shows a peak at 634 kDa, close to the mass of FLuc mRNA determined by SEC-MALS (Figure 5) and IP-RP-TOF-MS (Figure 6), but far off from the expected mass of 533 kDa. In addition, there is evidence of lower MW signal, indicating potential degradation.

With all 3 analytical techniques providing similar results that are significantly higher than the expected MW, we are looking into potential reasons for this mass error (modifications to the mRNA).

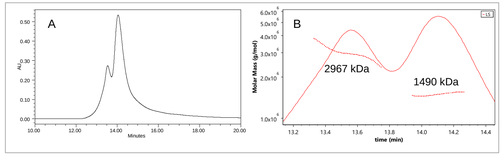

Figure 8. Absorbance and Multi-Angle Light Scattering data for Cas 9 mRNA. Panel A shows A260 for SEC separated FLuc mRNA with two peaks apparent. Based on MALS data (panel B) the two peaks have approximate masses of 1490 and 2967 kDa, agreeing with the expected mass of the Cas 9 mRNA monomer and dimer, respectively. It is not clear if the dimer is formed during exposure to SEC mobile phase or is already in dimer form in the sample.

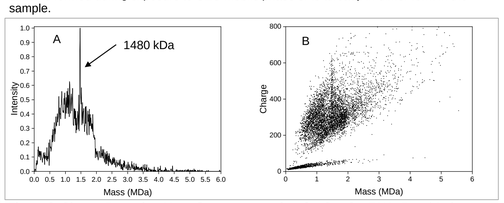

Figure 9. Charge Detection Mass Spectrometry data for Cas 9 mRNA. Panel A shows a peak at 1480 kDa, close to the mass of FLuc mRNA determined by SEC-MALS (Figure 8) and similar to the expected mass of 1493 kDa. IP-RP-TOF-MS was not able to generate intact data for Cas 9 mRNA due to its large size and the mass range limitations of the TOF-MS.

Mass (kDa)	EPO	FLuc	Cas 9
Expected Mass	283	533	1493
IP-RP-TOF-MS	245	625	Out of mass range
SEC-MALS	283	616	1490
CDMS Mass	285	634	1480

Table 1. Comparison of results from the 3 analytical techniques capable of measuring intact mRNA.

Conclusions

- CDMS and SEC-MALS techniques were successful in determining the MW for three different intact mRNA samples
- Results from all the FLuc mRNA measurements indicate a potential mass addition occurring to the mRNA molecule, however this requires further investigation
- IP-RP-TOF-MS may require gradient optimization and is limited with regard to detector saturation (with UV) and an upper mass limit that many intact mRNAs may exceed
- SEC-MALS provided mass results similar to those expected, but may have challenges with samples
- Compared to the LC-based techniques, CDMS requires little sample and no method optimization