

Roundtable Session 1 – Table 7 – MS of RNA and Oligonucleotides: Characterization and Quantitation of CQAs

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Abstract:

The continued growth of nucleic acid based therapeutics requires analytical tools capable of comprehensive characterization of critical quality attributes (CQAs) such as sequence identity, purity, capping efficiency and poly(A) tail length. More emphasis is being placed on stringent characterization of impurities related to the safety and efficacy of mRNA, sgRNA, siRNA and other nucleic acid therapeutics. These therapies offer a unique analytical challenge with some sequences containing more than 10,000 bases comprised of the four canonical nucleobases. In addition, there is growing interest in modified nucleobases which can affect potency and pharmacokinetics. This interactive session will discuss mass spectrometry and the role of this important and powerful analytical technique in the characterization of nucleic acid drug substance and product. Every attendee is encouraged to share their experiences in nucleic acid mass spectrometry.

Discussion Questions:

1. What level of sequence confirmation should be expected to confirm identity of an oligo? What about mRNA? Can LCMS provide value over other sequencing techniques such as sanger, nanopore, etc? What are the latest enzymes used to get the highest coverage?
2. Can LCMS be used to improve impurity/purity methods for mRNA, pDNA, sgRNA? What are the typical degradation pathways for mRNA?
3. What are the latest regulatory requirements for impurity characterization in nucleic acid DS/DP?
4. Can new technologies such as CDMS provide any usefulness for nucleic acid characterization? (for example, secondary structure of sgRNA or pDNA?)
5. What are the current pain points of RNA (or more generally nucleic acid) LCMS?

Notes:

CASSS Oligonucleotide Roundtable Summary

The roundtable focused on the growing role of mass spectrometry in the characterization of nucleic acid therapeutics, including oligonucleotides, mRNA, gRNA, sgRNA, and plasmid DNA. A recurring theme throughout the discussion was that mass spectrometry is increasingly being used to answer questions that sequencing technologies alone cannot address.

Instrument Configuration Remains a Practical Challenge

Laboratories take different approaches to positive and negative ionization modes. Some maintain dedicated systems for each polarity, while others switch between modes. Many groups follow an

"if it works, don't touch it" philosophy, as returning to positive mode after running negative-mode ion-pairing and buffers leads to difficulties in passing positive-mode calibrations.

Sequence Confirmation Goes Beyond Sequence Alone

While Sanger, nanopore, and NGS remain valuable sequencing tools, LC-MS provides unique advantages for identifying and localizing modifications, confirming cap structures, and characterizing degradation products. This is particularly important for heavily modified molecules such as gRNA.

Achieving high sequence coverage from large mRNA remains challenging. Although software tools continue to improve, the enzymatic toolbox is still limited. The question on fragment length for confident identification was raised as few bases long digest have too many possible assignments but while no consensus was reached overlap in fragment would increase confidence in assignment.

Cap Analysis and Modified RNA

5' cap characterization was widely viewed as a major strength of LC-MS. A practical challenge with capping standards was noted, as commercially available technologies such as CleanCap typically achieve >95% capping efficiency, however it is difficult to evaluate and quantify uncapped species due to the lack of a standard. As a result, laboratories would need to generate their own uncapped reference materials for method development and assay qualification, although preparing such standards can be technically challenging and commercial sources are not widely known. Current workflows often use RNase H together with a complementary biotinylated DNA probe to selectively enrich 5' fragments to measure capping efficiency. While effective, this approach requires custom probe design and optimization for each molecule. RNase 4 was highlighted as a potentially more universal alternative for cap analysis, although challenges remain in method development and the reliable characterization of the resulting 5' fragments.

Higher-Order Structure Is an Emerging Gap

Hairpins, duplexes, and other folded nucleic acid structures were identified as analytical gaps. Native MS and ion mobility mass spectrometry (IM-MS) were highlighted as promising approaches for characterizing these higher-order structures, which may become increasingly important CQAs in the future.

Practical Approaches to Common Analytical Challenges

Despite advances in instrumentation, many routine analytical challenges remain. Participants discussed ongoing issues with sample adsorption, metal adduct formation, and quantitative assay robustness. Practical recommendations included using plastic rather than glass consumables and evaluating alternative ion-pairing reagents such as DBA, which may reduce adduct formation relative to conventional TEA-based methods.

Impurities and Critical Quality Attributes

As nucleic acid therapeutics become more complex, impurity characterization becomes increasingly important. Participants mentioned base-conversion impurities, modification-related degradants, and agreed that degradation pathways for many modified nucleotides remain poorly understood and represent an important area for future research. Phosphorothioate stereochemistry was also recognized as an unresolved area. Although every phosphorothioate linkage introduces a chiral center, routine analytical characterization of stereochemistry remains not addressed.

Compared to oligonucleotide and mRNA therapeutics, pDNA generated relatively little discussion, as no participants appeared to be using MS for pDNA characterization. Current workflows remain HPLC-UV based, focusing on attributes such as quantity, linearization, and isoform distribution (supercoiled, open circular, and linear species).

AOC Characterization

AOCs were discussed as an emerging modality with unique analytical challenges. It was noted that DAR is typically kept as low as possible due to the high negative charge of the oligonucleotide payload. A workflow presented by Waters at CASSS that digests siRNA nucleobases while retaining the linker using oligonucleoside mix (NEB) was highlighted, enabling conjugation-site identification on the antibody using standard peptide mapping approaches.

Poly(A) Tail Analysis

Poly(A) tail characterization was viewed as one of the clearest success stories for LC-MS. In addition to average tail length, MS can provide tail-length distributions and heterogeneity information that are difficult to obtain using traditional methods.

Growing Interest in CDMS

Charge Detection Mass Spectrometry (CDMS) used for AAV characterization generated interest for the characterization of large mRNA molecules, RNA therapeutics, lipid nanoparticles (LNPs), and molecular aggregates and is viewed as a promising technology for the next generation of nucleic acid therapeutics.

Overall Takeaway

The discussion highlighted a shift from simple sequence confirmation toward deeper characterization of modifications, capping efficiency, degradation products, impurities, and higher-order structure. While sequencing technologies remain essential, LC-MS is uniquely positioned to provide the structural information needed to support increasingly complex nucleic acid therapeutics. Key opportunities remain in expanding the enzymatic toolbox, understanding degradation pathways, and advancing technologies such as Native MS, IM-MS, and CDMS.