Table 9: MS of RNA and Oligonucleotides: Characterization and Quantitation of PQAs

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Scope:

For small oligonucleotide-based therapeutics, LC-MS methods are widely used for routine purity/impurity analyses in both GMP and non-GMP laboratories including impurity identification and relative quantitation. Additionally, LC-MS/MS is routinely used for sequence confirmation per the request of regulatory agencies.

In recent years, mass spectrometry has become a method of choice for the characterization of large RNA-based therapeutics including mRNA vaccines. New MS tools for the characterization of intact mRNA, the 5´- and 3´- termini including 5´-capping and 3´- polyA tail heterogeneity characterization as well as oligonucleotide mapping for mRNA sequence confirmation have rapidly emerged with COVID-19 mRNA vaccines. The integrity of intact mRNA has been shown to be evaluated by emerging CDMS and mass photometry technologies. Separate LC-MS-based methods focusing on either 5´- capping or 3´- polyA characterization have been used. Given the size (>1000 nucleotides) and complexity of the mRNA biotherapeutics, the development of next-generation MS-based methods will be challenging.

The roles for MS in RNA and oligonucleotides analyses are rapidly expanding to more hybrid qualitative/quantitative applications and, in some cases, only quantitative determinations of oligonucleotide PQAs.

Questions for Discussion:

1. What typical MS analyses are performed for oligonucleotide and mRNA therapeutics?
2. For intact molecule analysis, what are the main challenges and mitigation strategies in sample handling, MS data acquisition, and processing?
3. For oligonucleotide mapping, what are the main challenges and appropriate solutions with sample preparation, LC separation, MS data acquisition, data processing, and reporting?
4. For synthetic oligonucleotides impurity analysis, is the current state of LC/MS technologies adequate for impurity identification?
5. For sequence confirmation, can 100% sequence information be obtained using MS/MS?
6. What are other applications of MS for RNA and oligonucleotide analyses?
7. Are MS vendor or standalone software packages adequate for supporting mRNA and oligonucleotide LC/MS/MS analyses?

Discussion Notes:

1. What typical MS analyses are performed for oligonucleotide and mRNA therapeutics?

    - LCMS (ion pairing reverse phase with amine and HFIP) and CEMS used for purity analysis and identity
    - Use of low-res vs high-res MS: For research and development unanimously high-res, for QC low-res is easier to implement and more frequently used at the moment. Some users are more comfortable with seeing average mass vs monoisotopic. Because the hi-res data is more complex to review, robust software is required

2. For intact molecule analysis, what are the main challenges and mitigation strategies in sample handling, MS data acquisition, and processing?

    Adducts are a significant issue, many mitigation strategies are used:

    - Acid wash and autoclaving of glassware
    - Use of FEP bottles for sample prep and HPLC analysis
    - Combine MS scans with low and high desolvation energy to assist with removal of possible adducts.
    - Daily methanol wash of system is recommended
    - Adjustment of ion pairing agent concentration and type can help. Legacy method often have an excess of HFIP which can be a considerable source of sodium
    - Excessive heat in the ESI can promote the formation of Na adduct, worth optimizing
    - Mobile phase pH can drift over time due to HFIP evaporating. Daily refresh of mobile phase is a good practice
    - Challenge switching back and forth between oligo and protein mode due to salts and residual ion pairing agent in HPLC

3. For oligonucleotide mapping, what are the main challenges and appropriate solutions with sample preparation, LC separation, MS data acquisition, data processing, and reporting

    - Currently mass mapping not needing for release, NGS or HPLC based assays preferred whenever possible.
    - Mapping is often difficult due to the high degree of degeneracy in the small fragments after digestion.
    - RNA T1 digest is the preferred method currently

4. For synthetic oligonucleotides impurity analysis, is the current state of LC/MS technologies adequate for impurity identification?

    - Challenge with isomeric impurities. Ion mobility can help with shorter oligos, some reported success with 8-10mer

- Deaminated impurities are challenging to separate by LC. Only 1 Da difference makes separating the contribution of FLP and impurity difficult. Ionis published an article about analysis of deaminated species by comparing isotope patterns across LC peak.

5. For sequence confirmation, can 100% sequence information be obtained using MS/MS?

    - Stepping CE energy helps, avoid excessive fragmentation of precursor to optimize ratio of termini fragments vs internal
    - Simple peak picking is not enough for MS2 ("a peak at every position"), a robust deisotoping method and threshold is required
    - On longer oligos, multiple charge states and multiple energies can help maximize coverage. It is useful to overlay theoretical patterns to verify data. Especially, to call adducts vs real impurities

6. What are other applications of MS for RNA and oligonucleotide analyses?

    - Intact mRNA is challenging due to high negative charge enhancing typical issues with adducts and desolvation. HPLC conditions that promote charge reduction and minimal denaturation can help. Mobile phase composition and pH control are key.
    - CDMS also used with some success

7. Are MS vendor or standalone software packages adequate for supporting mRNA and oligonucleotide LC/MS/MS analyses?

    - Vendor software is preferred vs 3rd party for GxP (if not manual analysis), while previously limited several new products became available in 2021/2022
    - For larger oligo deconvolution is very parameter dependent, possible source of variability
    - Lack of standardized nomenclature for oligos, even within a company. This makes maintaining a 'library' of sequences more difficult
    - Current mass mapping tools work for characterization but hard for reporting since data is very complex. Runs are long, 2 to 4h. Harder past 1000nt length. Too many isomeric sequencing

    - Peak picking requires suitable model to get good mass accuracy. Default averagine vs customized building block based on template sequence. This is especially challenging when dealing with conjugated oligos (PEG or proteins)