Table 10: Successful Implementation of Top/Middle-Down MS/MS for Characterization

Facilitator: Peilu Liu, *Pfizer, Inc., Andover, MA USA*
Scribe: Krisztina Radi, *Thermo Fisher Scientific, Hemel Hempstead, United Kingdom*

Scope:
Mass spectrometry provides critical information for the characterization and identification of proteins. Top down analysis is increasingly being preferred over conventional peptide based and sub-fragment based approaches of protein characterization, since the protein is introduced into the mass spectrometer in its "native" state. However, there are several challenges associated with top down analysis such as protein separation, resolution, fragmentation techniques, sensitivity and data complexity. This roundtable aims to discuss the applications, challenges and recent advances in Intact/Top Down Analysis.

Questions for Discussion:

1. What kind of instruments and fragmentation techniques are most widely used? (Do you consider MS1 only experiments as top-down approaches)
2. What are the traditional and novel applications of Top- and Middle-Down analysis in BioPharma Characterization? What are the current challenges?
3. What are the current technological/instrumental needs to be met for Top/Middle-Down analysis to be more widely used?
4. What are the current software needs to be met for Top/Middle-Down analysis to be more widely
5. Can Top-Down approach be used for quantitation (of modifications)?

Discussion Notes:

1. **What kind of instruments and fragmentation techniques are most widely used? (Do you consider MS1 only experiments as top-down approaches)**
    - Top-down is considered an analytical approach which includes fragmentation, not just MS1. MS1 only attendees consider calling intact and subunit analysis. Top-down is less frequently used in BioPharma, for discovery mostly, and for development using intact MS more.
    - Instrumentation used and considered for top-down: FTICR with different fragmentation options – ECD, CID, UVPD.
    - CID and ECD (on FTICR instruments only) used most often for characterization work. Orbitraps have ETD and EThCD, and a recent ToF instrument has EAD (not widely used for top-down yet).
    - EAD, ETD and ECD is the preferred way. No head to head comparison currently published between instruments.

2. **What are the traditional and novel applications of Top- and Middle-Down analysis in BioPharma Characterization? What are the current challenges?**

   Not that strong uptake of top/middle down approaches yet, intact MS is preferred and peptide mapping. Challenges are both seen as instrumental and software. The sequence coverage is not high enough, half of the sequence not covered and the PTM is in the not covered region.

   - Leaning towards - it is more an instrument limitation why it is not more widely used.
   - In development no sample or time limitations – so peptide mapping is better for PTM localization, than achieving only partial sequence coverage.
   - Sequence coverage depends on the fragmentation options and potential to combine them.
   - Peptide mapping is used to pinpoint the point mutation if observed at intact level.
   - Benefit of top-down would be overcoming the risk of introducing artefacts as no digestion is applied.
   - Top-down is challenging for big proteins, middle-down would be more useful with online sample preparation to produce the 25 kDa subunits for a mAb for example.
   - Gene Therapies- virus capsid proteins have a good size for top-down approach to be more useful. Virus proteins are highly similar, so sequencing the highly similar terminus would be useful.
   - Intact MS hard to see deamidation – and that would be extremely useful information to see through top-down
   - Data analysis is also a bottleneck – many instruments are available, but data processing is also a challenge. Vendor neutral software is preferred as support can focus on the customer needs.

3. **What are the current technological/instrumental needs to be met for Top/Middle-Down analysis to be more widely used?**

   - Instruments providing fragmentation techniques allowing for higher sequence coverage. ETD gives more fragments and higher efficiency. Increasing interest for EAD technology. Further development and experiments to test and compare different techniques is needed, analysing standards and more complex samples.
   - UVPD may provide increased sequence coverage, but if the instrument is not used, it is time and effort to get it up to speed and provide good data. Realigning the lazer for UVPD needs to be performed by an engineer, which is a challenge. Data complexity also increases.

4. **What are the current software needs to be met for Top/Middle-Down analysis to be more widely used?**

   - Xtract Algorithm used to deconvolute the data and use Prosight Light or Prosight PC to assign fragmentation. Some commercially available software may be investigated. Protein Metrics vendor neutral software have good deconvolution algorithms.

- Is it necessary to deconvolute the data and to assure there are no artefacts which would influence the assignments?
- Challenges with top-down – some false positives which sometimes requires one to look at data manually and confirm there are no false positives.
- PMI software can reconstruct the data (but not deconvoluting the data, but using peptide mapping algorithms, like very large peptides), can compare experimental to theoretical to help assignments.
- Above mentioned UVPD increases the data complexity and improved software or specific software.
- Software is especially needed for new modalities like Gene Therapies
    - would be a good example to show benefits of top-down approaches because of the high similarity of the proteins
    - and to identify and quantify the sequence variations

5. **Can Top-Down approach be used for quantitation (of modifications)?**
    - Currently used for characterization
    - Synthetic peptide analysis – sometimes used it for quantitation against UV.
    - Both MS1 and MS2 used for quantification – also use signature MS2 for coeluting impurities. For deamidation hard to use MS1 only, so XIC is used.
    - There may be potential to use top-down data for relative quantification but currently not actively pursued.

If had one wish, what would it be:

1. As data analysis is a bottleneck – many instruments, but data being the challenge. One vendor neutral software is preferred, that allows support to be focused on the customer needs.

Additional comment – charge heterogeneity follow up investigations like presented as part of the conference program: analysis with iCIEF-MS and next step could be to perform top-down