Table 8: New Developments in MS Analysis of N- and O-glycans

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Scope:

Glycosylation is one of the most common post-translational modifications (PTMs) of a biotherapeutic, and is often a critical quality attribute (CQA) due to its a considerable impact on the efficacy and safety of biopharmaceuticals. Identification and characterization of O-linked glycans and, more commonly, released N-glycans are important stages of biotherapeutic development. Mass spectrometry is commonly employed in early stage development activity and increasingly in downstream monitoring and QC. Releasing N-glycans is typically achieved via enzymatic methods, and often provides the best quantification results when using fluorescent labelling methodology, however, critical glycan information data is only available when using MS-based approaches. There is currently no commercially available universal O-glycan release enzyme. Therefore released O-glycan analysis typically requires more detailed chemical treatment, of which there are a few - if any - standardized procedures.

Questions for Discussion:

1. Labelled versus un-labelled analysis – what are your approaches for establishing consistency?
2. How to optimize O-glycan release methodologies or O-glycopeptides approaches for MS detection?
3. Is simultaneous (one-pot) analysis desirable or overly complex?
4. How are software advances enabling more efficient analysis? What new tools in characterizations of released glycans are advantageous?
5. Going from characterization to monitoring – what challenges need to be overcome?
6. Is released-glycan analysis by MS in QC realistic? What needs to be done to make this routine?
7. Are companies avoiding commercializing highly O-glycosylated molecules due to analytical challenges? If methodologies for analysis improve, do we think more of these type of molecules will be considered?

Discussion Notes:

1. Key Analytical Challenges from the attendees how to improve the O-glycan analysis (new analytical solutions?)
    a. O-Glycopeptides with multiple glycosylation sites is challenging to characterize. Changes in o-glycosylation are observed from different lot/batch of samples. Also, monitor the fully glycosylated vs. partial glycosylated proteins. CID, HCD and ETD are the common MS tools for linkage site ID. No one at the round table has tried ECD fragmentation, but thinks it has potential for improving linkage site analysis. A reasonable amount of people using OpeRATOR from Genovis but was not suitable for closely spaced o-glycosylation.
    b. O-glycopeptides with sialic acid have poor reversed phase chromatographic performance (smeared peaks). Most people at the table used sialidase to first remove the sialic acids prior to RPLC/MS analysis. One attendee noted that common Sialidases on the market cannot completely remote the sialic acid. Method development or better enzyme are needed. One of the attendees suggested to use TFA to hydrolyze sialic acid.
    c. Beta elimination is still needed for released O-glycan profiling.
    d. View on N-glycan analysis: Do people still use release glycan or switched to map N-glycans at the peptide level?
        i. HILIC/FLR is still the gold standard for released N-glycan assay due to FLR sensitivity and good separation provided by HILIC LC. MS detection of the released N-glycans is not as sensitive compare to FLR detection.
        ii. There are a couple fast FLR tagging reagents on the market shows excellent MS sensitivities)
    e. One pot analysis is desirable: getting N- and O-glycans profile using one preparation. Protein →proteolysis → peptides → PNGase F → for N-glycans release → beta elimination with either NaOH or NH4OH → for O-glycans → permethylation reaction for MS detection. The problem with this workflow is seeing either over or under permethylation. Also, NH4OH yields lower efficiency than using NaOH in the beta elimination step.

    f. Sample Throughput in pharma company:
        i. N-glycan analysis: around 100 samples/week
        ii. O-glycan analysis: around 6 samples/week