SLIM: A Gateway to the Adoption of High-Resolution Ion Mobility for Biotherapeutic Peptide Mapping

The demand for protein therapeutics, such as recombinant human proteins, monoclonal antibodies (mAbs), and fusion proteins, has grown significantly over the past decade. Often developed to treat previously unmet clinical needs, these biotherapeutics require increasingly advanced analytical technology for detection, identification, quantitation, and quality control (QC)/monitoring of molecular attributes. Some recombinant therapeutics require post-translational modifications (PTMs) such as glycosylation for clinical efficacy, but erroneous PTMs can occur within the mammalian host expression systems that are typically used to produce these therapeutics.

In this context, PTMs are known as product-related impurities and can be broadly categorised as either enzymatic or chemical modifications. Enzymatic modifications most commonly include glycosylation, disulfide bond formation, and proteolytic cleavage of the protein backbone. Chemical modifications are often generated during downstream processing, formulation, and storage, and typically include oxidation, deamidation, glycation, and pyroglutamate formation. These impurities can impact the biological activity, half-life, and immunogenicity of protein therapeutic products, so must be characterised, controlled, and monitored throughout the development process to safeguard the drug's stability, efficacy, and safety.

To achieve consistency in production, quality by design (QbD) is becoming a widely accepted strategy within the industry with the goal of enhancing pharmaceutical manufacture through design and control of processes.[1] QbD systematically establishes the critical quality attributes (CQA) of a drug product, which are identified and closely monitored for efficient QC. A CQA is defined by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q8 (R2) as a "physical, chemical, biological or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality."[2]

The assays and instruments used to analyse CQAs are expected to be precise, reproducible, and robust, with commonly orthogonal techniques carried out to complement and provide confidence in results. Fast analytical methods that can produce large amounts of data have become vital to speed up bioprocessing timelines and the decision-making process.[3]

While conventional analytics such as chromatography and electrophoresis methods are important for early development and characterisation of therapeutic proteins, their sensitivity and specificity can be limited. Orthogonal mass spectrometry (MS) techniques are now recognised as an important tool, particularly for identifying and characterising PTM as CQAs in the biotechnology development pipeline. MS-based assays, such as peptide mapping, provide a means to directly measure PTMs at the peptide and individual residue level. However, the analytical complexity of peptide mapping has, until now, limited its uptake in bioprocess monitoring and PTM characterisation.

Enhancing MS-based Peptide Mapping

MS-based assays are providing ever-richer detail and a more granular perspective on PTM sites and are often coupled with liquid chromatography (LC) separations to overcome the challenge of ionisation variability, suppression, and difficulties in resolving isoforms with small mass differences. However, traditional LC separations have relatively low specificity and require long gradients to identify all components of the protein digest and resolve critical modifications for relative quantitation.

Routine implementation of downstream peptide mapping workflows using LC-MS has proven difficult due to the typical gradient times in the order of 90 minutes, with some as long as 190 minutes.[4] In addition, considerable analytical expertise is required to interpret the data. The separation often requires these long analytical gradients to resolve critical near-isobaric or isobaric PTMs for quantitation.[5]

Incorporating ion mobility (IM) as an orthogonal separation that relies on peptide structure can reduce reliance on the LC separation by providing an additional differentiation filter to resolve isobaric peptides, reducing ambiguity in identification through mobility-aligned fragmentation. Established IM techniques suffer from limited resolution, meaning the condensed-phase separation is still necessary for relative quantitation of PTMs.

In the IM separation, ions traverse a defined path through an inert buffer gas, such as nitrogen or helium, under the influence of an electric field. The arrival time of an ion at the end of the separation path increases with the ion's collisional cross-section (CCS), a value that represents the rotationally averaged cross-sectional area of the ion in the gas phase.[6] CCS values provide a fourth dimension of separation in addition to retention time, mass-to-charge (m/z) ratio, and spectral peak intensity, and have proven advantageous for complex separations and targeted peptide mapping workflows.[7,8]

Resolving power in IM typically scales with the square root of the separation path length, making the resolving power of IM separations with longer path lengths higher than that with shorter distances. Therefore, the path length of traditional IM technology (usually < 1m) can limit the power available to resolve critical isomeric species and necessitates long chromatographic methods for PTM quantitation.

Faster, More Detailed Analyses

To overcome this challenge, researchers have developed a high-resolution ion mobility (HRIM) technique based on SLIM (Structures for Lossless Ion Manipulations) that can achieve 10 times higher resolution compared with traditional IM methods.

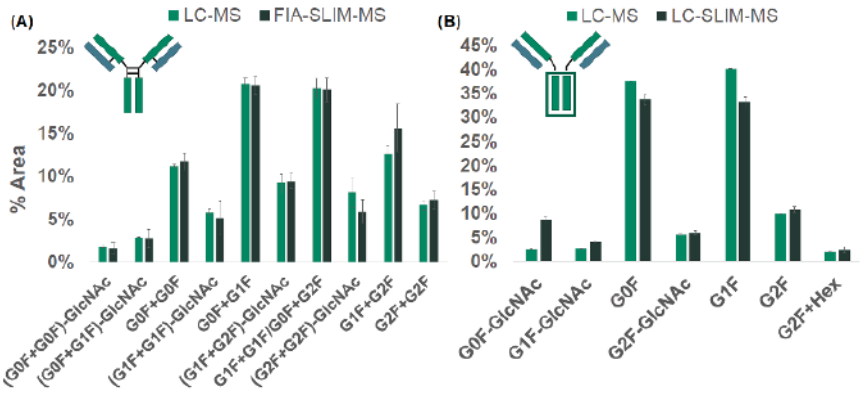

Figure 1 – Glycoform quantitation on intact NIST RM 8671 using (A) a two-minute flow injection method on SLIM-MS (13m SLIM device, MOBILion Systems, 6545XT QToF MS, Agilent) and a five-minute LC-MS method, and (B) an eight-minute integrated LC-SLIM-MS method.[14]

SLIM – originally developed in the laboratory of Dr. Richard D. Smith at Pacific Northwest National Laboratory – transmits ions along a path between two parallel printed circuit boards (PCBs) with negligible ion loss, with a throughput of minutes, rather than hours. The electric fields that propel the ions also prevent them from striking surfaces while moving, therefore preventing any losses along their way. Ions are able to turn around the corners of SLIM's serpentine path, removing the limitations set by linear paths and achieving previously impossible separations. SLIM has recently incorporated a multiple pass path for further enhanced separation of isomeric species.[9–11]

Similarly to other IM technologies, in HRIM separations, an ion's migration time is determined by its mass-to-charge ratio (m/z) and its size and shape. As ions are driven along the separation path, the collision with an inert buffer gas slows them down to a degree proportional to their size. The serpentine path shape of the PCBs allows unprecedented ion path lengths to fit into a benchtop device (e.g., 13m SLIM device, MOBILion Systems), leading to resolutions high enough to separate the most challenging isomeric structures and collect data in the order of milliseconds to seconds. Compared with LC, HRIM set-up is relatively straightforward as there is no need to optimise columns, flow rates, or liquid components.

Enhancing Peptide Mapping Workflows

Using SLIM-based HRIM coupled to a quadrupole time-of-flight (QToF) mass spectrometer, researchers can now achieve faster chromatographic separations for peptide mapping workflows. In a recent study, a NISTmAb was characterised by LC-HRIM-MS and LC-HRIM-MS with collision induced dissociation (HRIM-CID-MS) using only a 20-minute gradient – between 3–4.5x faster than traditional peptide mapping gradients.[5]

LC-HRIM-MS experiments achieved a sequence coverage of 96.5%, with LC-HRIM-CID-MS experiments providing additional confidence in sequence determination. HRIM-MS resolved critical oxidations, deamidations, and isomerisations that coelute with their native counterparts in the chromatographic dimension, demonstrating the ability of SLIM-based HRIM to maintain PTM quantitation capabilities and add isomeric resolution of critical coeluting PTMs.

Glycoform Quantitation

Protein glycosylation is a vitally important PTM in the functioning of a range of biological processes, such as molecular recognition, protein trafficking, regulation, inflammation, and abnormalities in protein glycosylation are associated with several disease states such as cancer, inflammatory diseases, and congenial disorders. Careful monitoring of protein glycosylation is also crucial for the development of stable and effective drugs and for comparing biosimilar products to reference drugs, as required by regulatory agencies.[12] Changes in manufacturing process conditions for biologics, such as process optimisation, scale-up production, and site changes, may impact glycosylation patterns of the resulting recombinant antibody.[13]

Glycans have many structural and functional roles and display enormous heterogeneity between glycoforms, so determining the profile of the glycans and glycosylation sites is crucial for producing safe, good quality, consistent therapeutic proteins. Although LC-MS can detect variations in glycoform species, peptide mapping for glycoform quantitation requires long gradients that hinder analysis in fast-paced bioprocessing environments.

Research has recently examined the utility of SLIM-MS and LC-SLIM-MS for faster and more in-depth characterisation of protein therapeutics.[14] Glycoform quantitation on intact NIST RM 8671 was performed using a two-minute flow injection method on SLIM-MS and showed highly similar quantitation to a five-minute LC-MS method (Figure 1A). The study found that integrating SLIM to existing eight-minute subunit LC-MS workflows (LC-SLIM-MS) maintained quantitative capabilities of the method (Figure 1B).

The study also found that SLIM-integrated peptide mapping enables a five-minute analytical gradient compared to traditional 60–90 minutes using LC-MS alone, with the LC-SLIM-MS method maintaining nearly complete sequence coverage. Figure 2 shows coeluting critical quality peptide deamidations could be fully or partially resolved by SLIM separation using the five-minute gradient method.

Future of PTM Monitoring

LC-MS has long been used for sensitive and accurate impurity analysis in the biopharmaceutical industry. However, developments in analytical technology over the last decade have accelerated the characterisation and monitoring of product-related impurities such as PTMs beyond the capabilities of traditional LC-MS workflows. Advances in IM separations and the development of SLIM-enabled HRIM now combine higher resolving power with rapid speed of analysis, without compromising sequence coverage. The resulting increase in

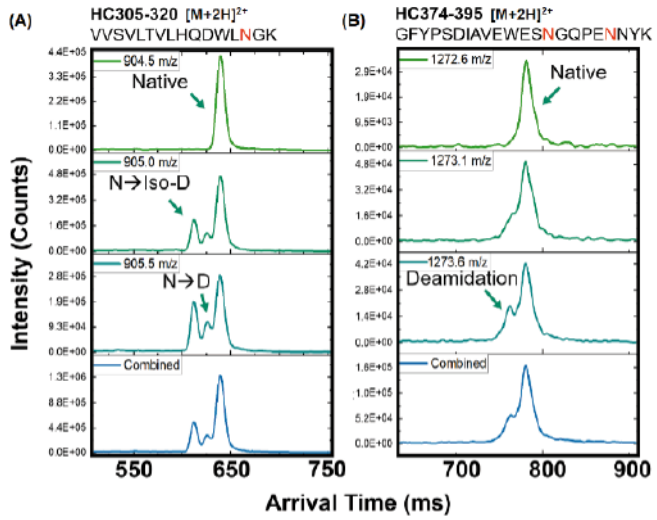

Figure 2 – Coeluting critical quality peptide deamidations are fully or partially resolved by SLIM separation in the five-minute gradient method shown here.

confidence of PTM characterisation and quantification leads to a more robust QbD approach to biopharmaceutical manufacturing and QC, with CQA monitoring that meets regulatory expectations in short time frames.

For more information about SLIM, please visit https://www.mobilionsystems.com/.

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