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'Ultra' Biotherapeutic Characterisation

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With the current market value for biopharmaceuticals standing at more than $150 billion (1), the need for higher throughput, more in-depth characterisation techniques for biologics is accentuated. Can recent technological advances in UHPLC meet the practical and regulatory requirements of the biotherapeutic development chain?

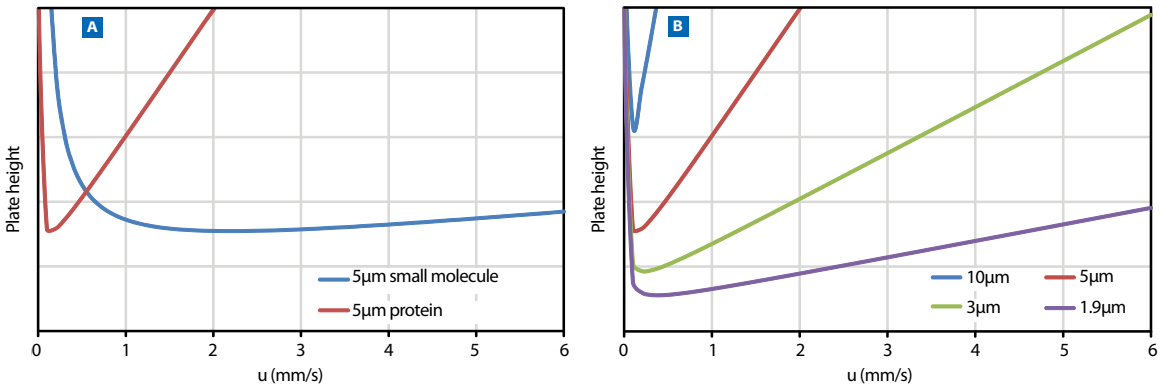

Figure 1: **A:** Theoretical comparison between van Deemter curve for two analytes with 100 x molecular weight difference for the same column. **B:** Comparison of optimal flow change with particle sizes for a protein

Ultra-high performance liquid chromatography (UHPLC) is beneficial for the analysis of small molecules in the pharmaceutical arena, with the ability to simultaneously increase resolution, sensitivity and sample throughput. This potential for improved separation speed, resolution and sensitivity is also a major advancement for the characterisation of large molecules, including peptides and proteins. In response to the rising ageing population and increased prevalence of chronic diseases such as cancer, diabetes, cardiovascular and autoimmune diseases, the protein therapeutic market is expanding rapidly.

Keywords

UHPLC
Characterisation bottleneck
Advanced column chemistries
Separation technologies

Biotherapeutic Market

The range of therapeutics is broader than ever before, and this is set to explode in the coming decade with the advent of biosimilars, antibody-drug conjugates (ADCs) and biobetters. Biologics are composed of sugars, proteins and nucleic acids, and include living entities such as cells and tissues (2).

Monoclonal antibodies (mAbs) are heterogeneous molecules that harbour a number of potential sites of modification/variation. Up to 10,000 variants of mAb can exist in an end formulation. A thorough understanding of the product 'fingerprint' is the crux of the characterisation bottleneck.

An arsenal of chromatographic techniques and column chemistries has been developed to address biologic heterogeneity. Compounding this ever-expanding matrix is the range of biocompatible UHPLC systems – these offer testing for the presence of protein aggregates, fragments, charged variants and conformational changes in shorter timescales than ever before. In the heavily regulated environment of a biopharmaceutical production facility, ensuring compliance with regulatory standards is also vitally important. The underlying principles of UHPLC for proteins, baseline

system requirements and the types of separations required for full characterisation in a regulated environment will therefore be explored.

The Diffusion Quandary

Since chromatography is a diffusion-dictated process, and proteins have low diffusion constants, the adoption of UHPLC for protein analysis has been slower than for small molecules.

If two analytes with 100 times' difference in molecular weight on a van Deemter plot are compared under optimal chromatographic conditions, it is revealed that, for the same type of stationary phase, the flow optimum is much lower for proteins. The range in which this performance is achieved is also much narrower (see Figure 1, part A). Fortunately, the trends observed for small molecules with lesser particle sizes is the same for proteins: the range for optimal performance increases as a result of the shorter diffusion pathway (see Figure 1, part B). Therefore, UHPLC enables speed of protein analysis comparable to that of smaller molecules with high-performance liquid chromatography.

The use of smaller particles results in increased pressure requirements – and this is where a specific bioinert UHPLC system finds its niche.

Bio-UHPLC Matrix

In order to accommodate the varied nature of biotherapeutics and the necessity for highly regulated analyses, the technical features of the UHPLC system become more pertinent than for small molecules.

Proteins have a propensity to interact and bind with one another, with the solid phase of the column and with certain metal (notably iron) surfaces they encounter on their journey. This occurs because proteins present a myriad of charged, hydroxyl (-OH), carbonyl (-CO), amide (-NH) and hydrophobic groups on their surface – which provide electrostatic, hydrogen bonding and hydrophobic interactions, leading to adsorption.

If a system were to be designed and built for biopharmaceutical analysis, the following modular features should be considered:

- The pump should be quaternary for maximum flexibility; be able to operate at a high pressure (more than 10,000 psi) for UHPLC column performance to be realised; generate low baseline noise for highest detection sensitivity; have low dispersion and high gradient and flow rate accuracy, reproducibly
- The autosampler should utilise valve materials that are tolerant to high pressure switching – for instance, ceramic rather than metal – and should include sample loop pre-compression to minimise pressure changes (and therefore retention time variation run-run) during sample injection
- The sample manager should be of high capacity for maximum throughput. If shorter methods are leveraged, more samples can be analysed
- The detector options should be applicable to a range of workflows, such as fluorescence detection (FLD) for labelled released glycan analysis; charged aerosol detection for non-labelled released glycan analysis and intact protein analysis; ultraviolet (UV) and mass spectrometry (MS) for intact protein analysis
- The column oven should offer versatility in terms of its capacity, temperature range and stability. Rapid temperature adaptation is also desirable for method development and workflow switching

Multiple complementary techniques are required to characterise biologics, with five key UHPLC analyses for complete characterisation of mAbs:

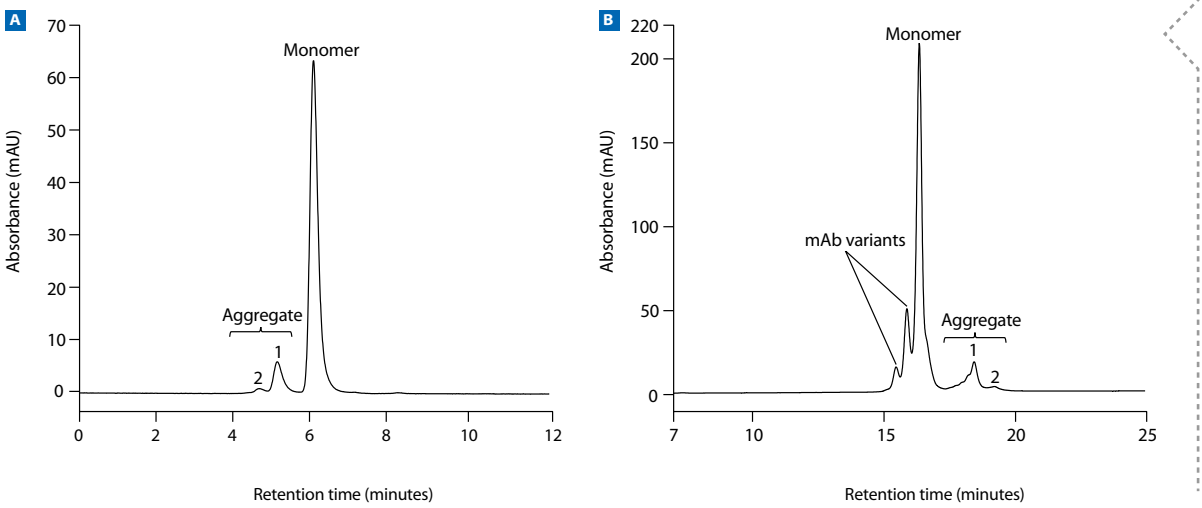

Figure 2:
A: Separation of mAb aggregates using SEC.
B: Separation of mAb aggregates and hydrophilic variants using HIC

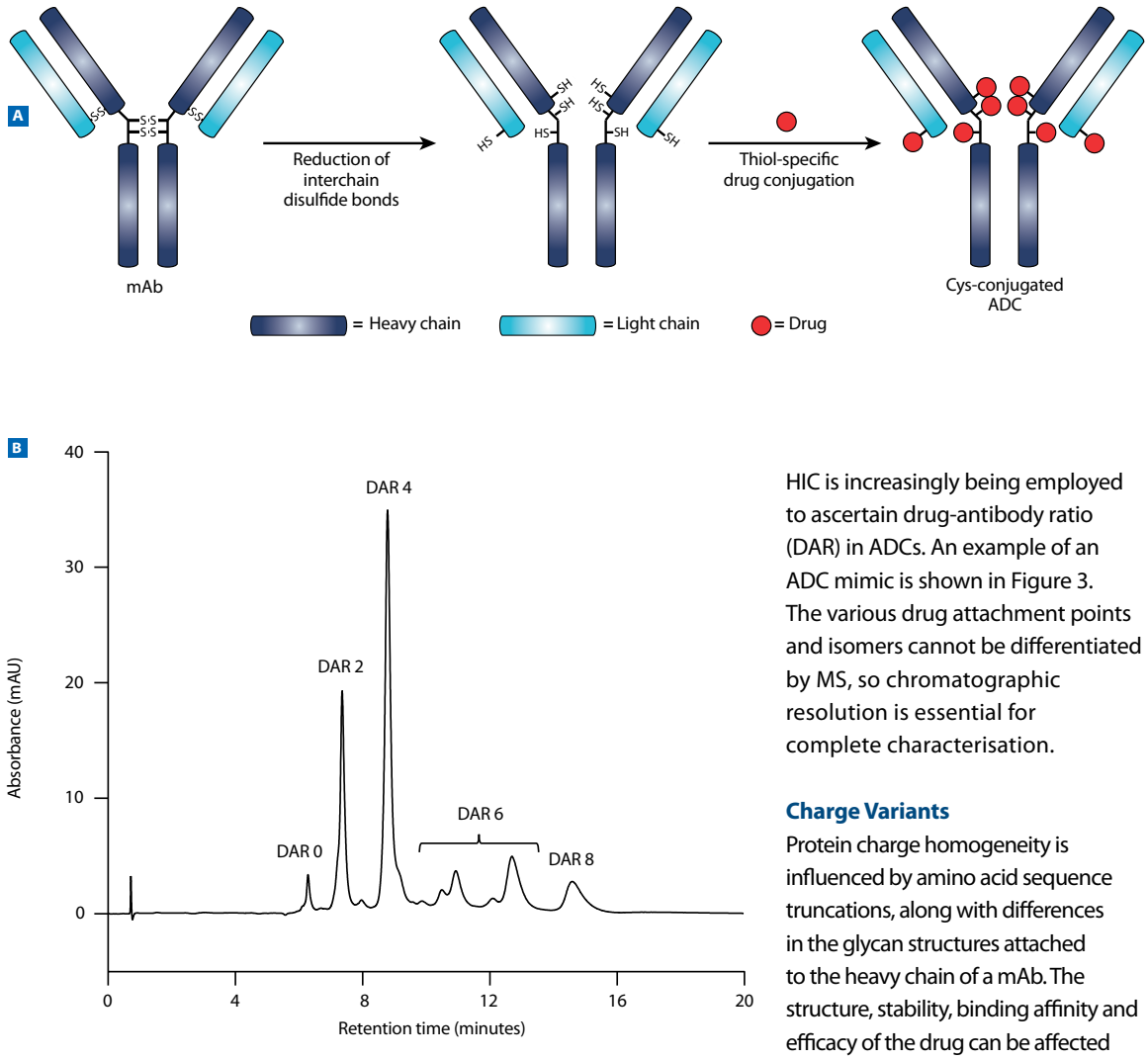

Figure 3: Separation of a cysteine-conjugated ADC mimic. **A:** Schematic representation of conjugation of drug mimic via interchain cysteine residues. **B:** Separation on a HIC-butyl column

Aggregates

mAb aggregates may form during protein expression, downstream processing and storage, and can be caused by high concentration, elevated temperature, abrupt change in pH, shear strain, freeze-thaw cycles or surface adsorption. Aggregates can result in an undesired immune response that affects the safety and efficacy of the drug due to incorrect dosage (3).

Monitoring mAb aggregation is important for their production and quality assurance. Size-exclusion chromatography (SEC) has been the method of choice for the detection and quantification of mAb aggregates. Using shorter length columns, the speed of analysis can be reduced to a number of minutes while still retaining enough resolution to separate and quantify the aggregation and fragmentation products (see Figure 2, part A, page 33). The use of both standard hydrophobic interaction chromatography (HIC) and mixed-mode HIC for separation of protein aggregates has also been reported (4,5). The HIC method separates proteins based on hydrophobicity in the native state and can often detect changes in protein structure, as well as aggregates (see Figure 2, part B, page 33). Furthermore, this technique can also be used as a secondary assay to SEC to confirm aggregation, or for the removal of aggregates during purification.

HIC is increasingly being employed to ascertain drug-antibody ratio (DAR) in ADCs. An example of an ADC mimic is shown in Figure 3. The various drug attachment points and isomers cannot be differentiated by MS, so chromatographic resolution is essential for complete characterisation.

Charge Variants

Protein charge homogeneity is influenced by amino acid sequence truncations, along with differences in the glycan structures attached to the heavy chain of a mAb. The structure, stability, binding affinity and efficacy of the drug can be affected as a result of this. Ion exchange (IEX) chromatography is often used to profile mAb charge variants, either by salt gradient or, more recently, pH gradient (6). During pH gradient IEX, the mAb is loaded onto a cation exchange column at a pH below the isoelectric point (pI) of the protein, ensuring that it carries a positive charge. The protein binds to the negatively charged surface of the stationary phase. Using a gradient, the pH is increased over time, effectively reducing the overall positive charge until at or around the pI of the mAb when it elutes from the column.

Intact

Analysis of biologics at an intact level quickly exposes the major variants (glycoforms) of mAb expressed in similar cell lines or conditions,

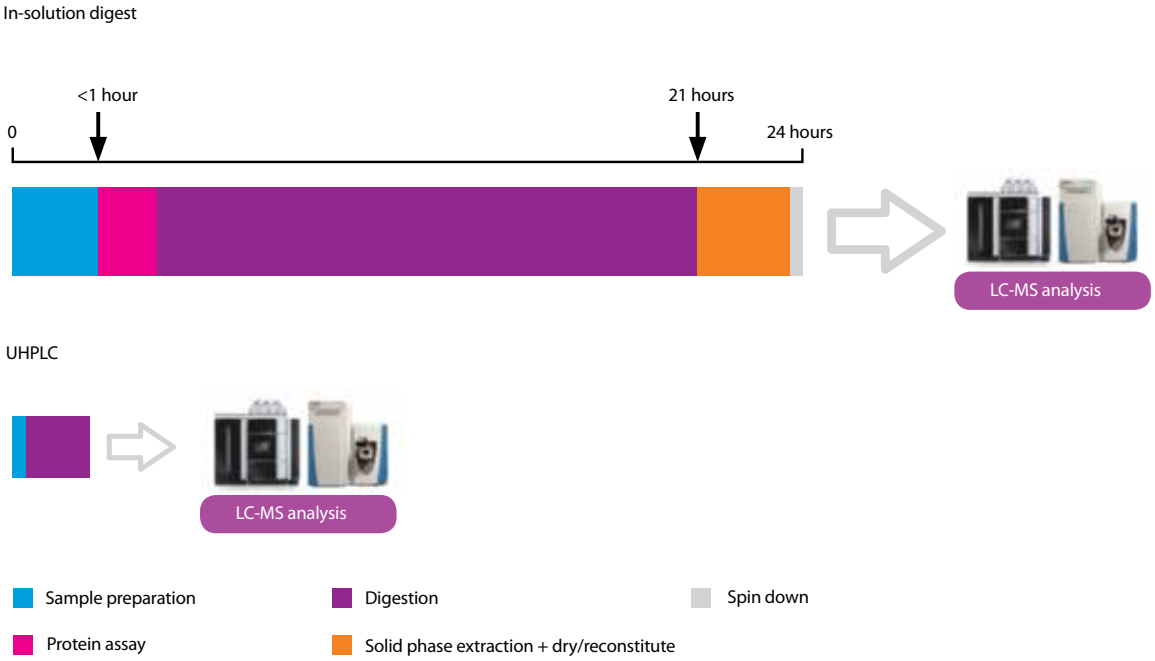

Figure 4: Fast sample digestion workflow comparing in-solution digest with a UHPLC digestion kit

and allows for checking the correct molecular weight of the product. Applying a rapid method for the verification of this intact 'fingerprint' can save precious time and money in the early development stages, and is necessitated right through to post-production quality control.

Reversed phase (RP) protein chromatography has been the domain of the silica C4 column for many years. The use of polymeric columns and larger pore sizes provides UHPLC quality resolution (2 second peak width at half height) of large proteins. Additionally, the carry-over on this column from proteins – unlike silica resins – is minimal. The compatibility of the mobile phases for RP chromatography with MS, and the advent of short (10mm) desalting cartridges, are making rapid intact mass analysis with high-resolution MS an attractive fingerprinting option throughout the development chain.

Peptide Mapping

This process reveals minor variations that would be difficult or impossible to see at the intact level. High-resolution, high-peak capacity peptide maps that detail the entire protein – and ensure 100% coverage – are required. Protein digests are complex, and it is common to have 50 or more peptides to separate and characterise by MS. The column of choice is RP-wide pore silica C18 – of which there are numerous UHPLC versions to pick from. The bottleneck in peptide mapping is undoubtedly the digestion step. Digestion kits employing the necessary protein denaturation and proteolysis with immobilised heat-stable trypsin facilitate digests in minutes, rather than hours (see Figure 4).

Released Glycan Analysis

Glycans are considered an important species in biotherapeutic drug development, as there is strong evidence that bioactivity and efficacy of glycosylated proteins depend on the structure and type of glycans (7).

These compounds are analysed both qualitatively and quantitatively after enzymatic or chemical release from the protein. This approach leads to complex and heterogeneous mixtures of oligosaccharides that require high-efficiency separation techniques prior to characterisation, usually by MS. Characterisation cannot be accomplished by UV detection, since glycans lack chromophores.

However, glycans can be labelled with fluorescent tags, separated by UHPLC and detected with FLD. Even though the tagging and sample cleanup steps are time-consuming, no further sample treatment prior to MS analysis is needed. Consequently, a UHPLC system with an FLD can be coupled directly to an MS. Hydrophilic interaction liquid chromatography (HILIC) is often employed, but more recently, mixed-mode surface chemistries – combining weak anion exchange and RP functionalities – have demonstrated effectiveness.

Future-Proof

Advances in instrument technology and column chemistries have aligned to bring large molecule analysis into the realms of UHPLC. The use of high-resolution chemistries and more efficient

sample preparation accomplishes the goal of higher throughput without increasing the effort required. SEC, HIC, IEX, RP and HILIC peptide mapping, glycan and intact protein analyses have been improved, in order to show highly selective resolution of different types of mAb variants to provide global methodologies.

Overall, the growth of the biopharmaceutical market has instigated true change in the capabilities of protein characterisation. With an increase in the number of drug candidates and the emergence of new drug types such as ADCs, which require additional forms of analysis, there is pressure to develop new high throughput analytics. To meet this new demand, future-proofing the biologic laboratory is going to be necessary for companies to hold true competitive advantage in this fast-paced market.

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