Dealing with the Challenges of Post Translational Modifications (PTMs).

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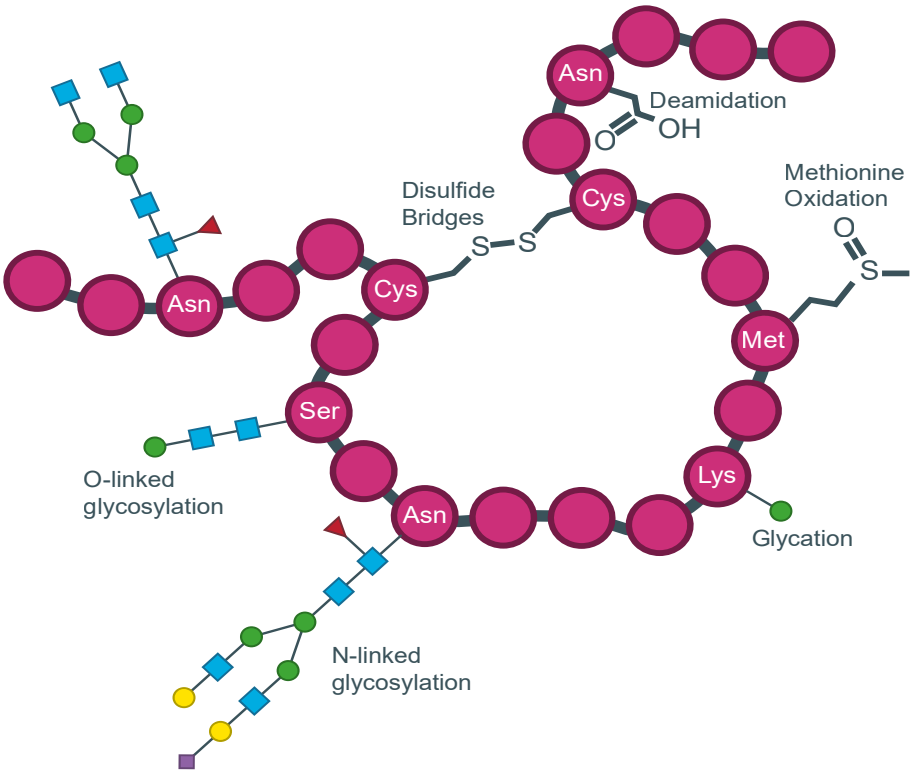
Introduction

During the structural analysis of recombinant protein biopharmaceutical products, a significant challenge is posed by the issue of Post Translational Modifications (PTMs). This is a very large area of investigation due to the number of PTMs that have been identified through the study of protein structures over the course of many years. The requirement to assess PTMs cannot be ignored as this is part of the expectation for structural characterization as required by the ICH Q6B guidelines(1). However, not all PTMs that have been identified need to be investigated for every protein as many are very specific to a certain protein, cell type or cellular process. Nonetheless, analysing PTMs gives rise to many challenges, not the least of which is "what post translational modifications am I even looking for?".

What are Post Translational Modifications?

A PTM is defined as any chemical modification that occurs on the protein chain following translation. So "PTM" is an all-encompassing term for what could happen to a protein on its journey though the cell and the extracellular environment it finds itself in, be that culture media, purification process environments or drug product formulation media. PTMs of one form or another are therefore widely found on biopharmaceutical products. They can be part of the natural cellular machinery -modifications which are found on many proteins such as glycosylation and disulfide bridge formation would fall into this category (2, 3). Glycosylation, the attachment of carbohydrate to the protein, is arguably one of the most important PTMs as it could potentially affect efficacy and safety and, in some cases, result in immunogenicity.

Figure 1: Stylised representation of a protein chain showing some examples of Post Translational Modifications and the amino acids on which they are found.



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Furthermore, the non-templated addition of sugars greatly adds to the heterogeneity of the biologic medicine.

Other natural PTMs of the protein can also occur, but these may be restricted to a particular class of molecule (e.g. Immunoglobulins) or even a specific molecule itself. Examples of these are proteolytic cleavage e.g. for activation of an immature form of a protein or through the action of naturally occurring proteases and modification of the N- and C-termini such as the formation of pyroglutamate residues (as found at the N-terminus of some Immunoglobulin light or heavy chains). Some PTMs are actually the result of controlled chemical modifications of the protein chains such as PEGylation (the introduction of a polyethylene glycol moiety to site within the protein). There are many other examples of PTMs that have been identified and a limited selection of the most commonly encountered types are shown in Figure 2 and summarized in Table 1.

As well as the PTMs that are a natural part of protein processing, there are others that are the result of chemical modifications that take place during the protein production process, purification scheme or as a result of product formulation and storage. Since proteins, by their very nature, contain amino acids with different chemical side chains, it follows that a variety of PTMs can be produced through different chemical reactions during these stages of drug production and at least some of these PTMs will be frequently encountered as a result of side chain propensities for particular chemical reactions. The most commonly found PTMs that fall into this category are oxidation of the side chain of methionine (and tryptophan) and the deamidation of asparagine and glutamine. These PTMs may form over a period of time as a result of prolonged exposure of the drug product to a particular chemical environment and thus can potentially build up in a product during storage. This formation of certain PTMs can be exacerbated by stress conditions such as the action of heat or light on the formulated sample.

So, a "PTM" is effectively any modification that occurs on a protein and these modifications can happen during biosynthesis, drug product purification, shelf storage lifetime or as the result of directed chemical modifications of the drug during the manufacturing process (e.g.

Table 1: Examples of commonly encountered PTMs.

PTM	Amino acid(s) involved	Nature of the modification
N-glycosylation	Asparagine	Oligosaccharide attachment as part of biosynthesis of a glycoprotein
O-glycosylation	Serine, Threonine	Oligosaccharide attachment as part of biosynthesis of a glycoprotein
Disulfide bridges	Cysteine	Thiol cross-linking between two Cysteine residues. Note that disulfides and thioether linkages can also be found at low levels as a result of incorrect formation of the bridges
PyroGlutamination	Glutamine, Glutamic acid (only N-terminal)	Cyclisation of the N-terminus
Lysine removal	Lysine at the C-terminus of mAb heavy chains	Proteolytic cleavage of the C-terminal Lysine residue
Oxidation	Methionine, Tryptophan	Oxidation of the side chains of these amino acids as a result of exposure to an oxidizing chemical environment. Different degrees of oxidation can take place on the Methionine side chain sulfur atom producing sulfoxide (one oxygen atom added) and sulfone (two oxygen atoms added) forms.
Deamidation	Asparagine, Glutamine	Conversion of Asparagine to Aspartic acid (Glutamine to Glutamic acid) as a result of the chemical environment
Glycation	Lysine	Non-enzymatic modification of the side chain amine of Lysine residues with Glucose or other Hexose sugars if present in sufficient concentrations in the protein buffer.
PEGylation	Cysteine, N-terminus or side chain primary amines (i.e. Lysine)	Attachment of a polyethylene glycol unit of usually between 5 and 20 kDa to the protein via specific chemistry
Phosphorylation	Tyrosine, Serine, Threonine	Specific modification of the hydroxy side chain of certain residues within the structure of the protein
Iodination	Tyrosine	Attachment of Iodine notably in the thyroid hormone thyroglobulin to produce the thyroid hormones

PEGylation). Importantly, these modifications cannot be predicted from the gene sequence and must be determined experimentally. Knowing that there are many PTMs that exist and can be formed at all stages of drug production how does one go about assessing a product for these modifications?

Analytical Challenges of Post Translational Modifications

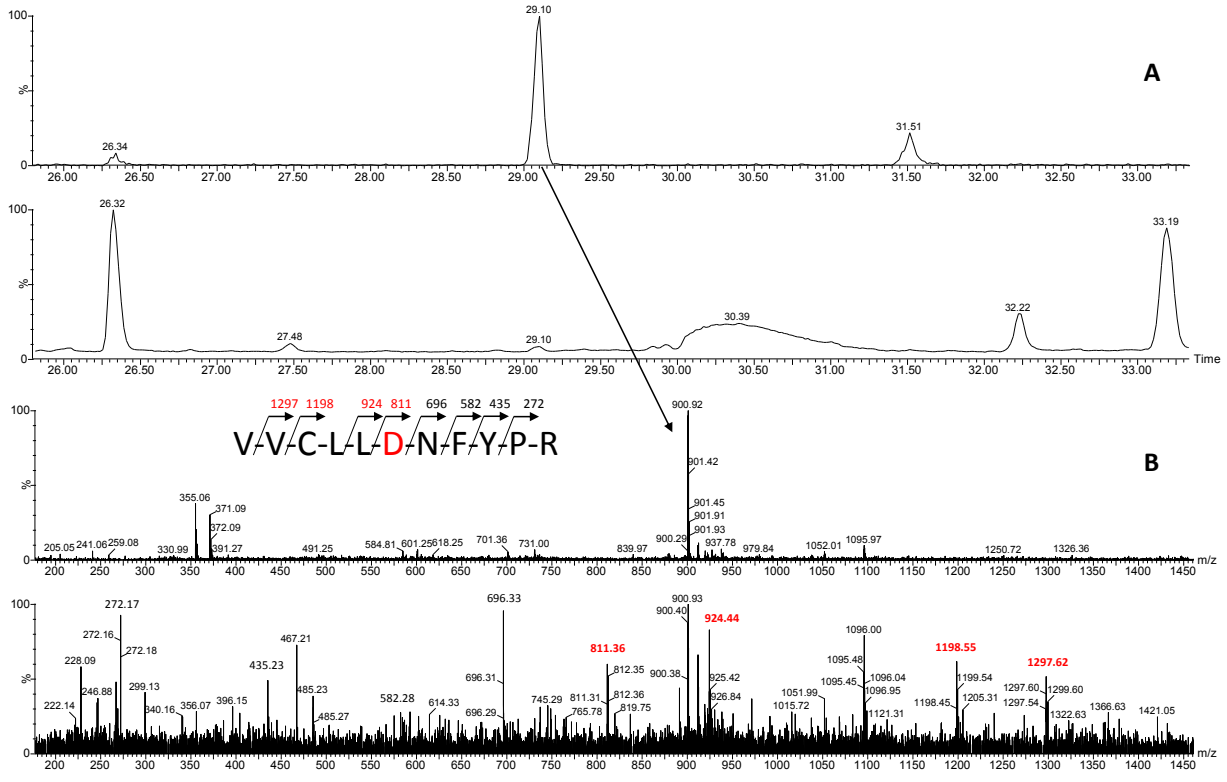
The most effective means of PTM analysis by far is mass spectrometry. A combination of intact mass analysis and, critically, peptide mapping will give significant information on both the nature and location of PTMs within any given product.

Intact mass analysis as a first step allows the determination of the overall mass(es) of the molecule, which is necessary for identifying what the resultant overall mass or mass distribution of the product is, including any PTMs that may be present. The mass observed will be derived from the overall protein chain (the sequence and therefore mass of which can be predicted from the DNA sequence used) along with any subsequent post translational processing of that chain. Intact mass of course is unlikely to allow a full determination of the PTM profile as the mass observed is the product of the sum total of all PTMs present.

Peptide mapping is a very powerful technique for assessing PTMs as the proteolytic cleavage of a protein into smaller peptides results in separation of PTMs and easier subsequent analysis. In a peptide mapping study, the sample is digested with a protease which cleaves the protein at defined sites (e.g. trypsin or endoproteinase GluC). The resultant peptides are separated by liquid chromatography and analysed by on-line mass spectrometry. Using a Q-TOF type of mass spectrometer for this work allows not only the intact mass of the peptide to be determined but also, through the use of the real-time higher energy fragmentation channel, data can be obtained giving confirmatory sequence ions from that peptide. For PTM assessment, peptide mapping in this way is very informative since not only can the peptide bearing the PTM be identified based on the intact peptide mass but, depending on the extent to which the PTM is present, it may be possible to generate fragment ions that not only confirm the identity of the modified peptide but also give the location of the PTM within the peptide. Two examples of this are shown in Figure 2 and Figure 3, which represent the location of the site of deamidation within a peptide and the identification of the pyroGlu containing N-terminal peptide from an antibody heavy chain respectively.

Since modification of a peptide will virtually always

Figure 2: On-line LC/ES-MS analysis of a tryptic digest of a protein. A: The upper two panels show a section of the total ion chromatogram and the extracted ion chromatogram for a deamidated peptide of interest. B: The lower two panels show the low energy mass spectrum demonstrating the presence of the deamidated peptide and the higher energy MSe data showing the fragmentation of that peptide. Signals observed are not only consistent with the peptide, confirming its detection but also demonstrate that deamidation has occurred exclusively on the first of the two adjacent asparagine residues.



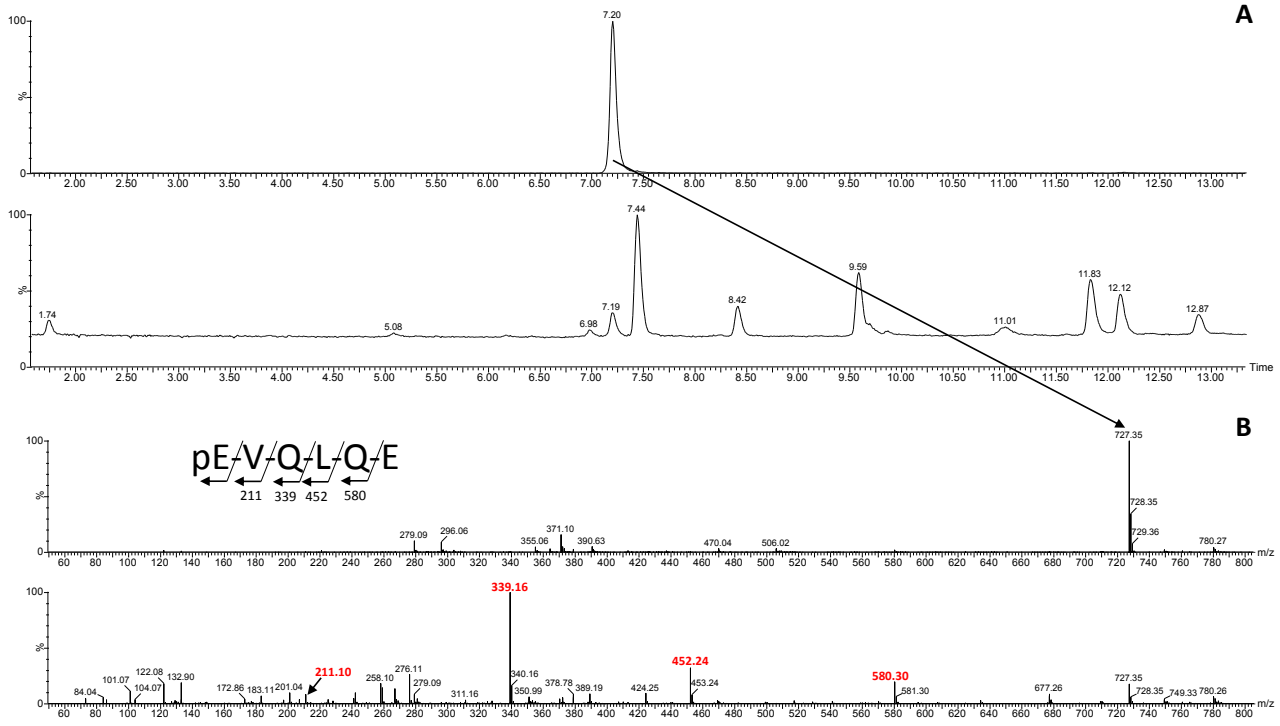


Figure 3: On-line LC/ES-MS analysis of an endoproteinase Glu-C digest of a monoclonal antibody. A: The upper two panels show a section of the total ion chromatogram and the extracted ion chromatogram for the predicted N-terminal heavy chain peptide if modified by pyroGlu (pE) at the N-terminal. B: The lower two panels show the low energy mass spectrum demonstrating the presence of the peptide and the higher energy MSe data showing the fragmentation of that peptide. Signals observed are consistent with the peptide bearing a pyroGlu residue at the N-terminus.

result in mass and hydrophobicity changes to a peptide, a modified peptide will elute at a different position in a chromatogram compared to the native peptide. Furthermore, since PTMs are likely to result in partial modification of the peptide, a mixture of modified and unmodified forms of the peptide will exist in the sample. It is therefore possible in a peptide map to use extracted ion chromatograms (a chromatogram generated based on a search of the data for the predicted mass to charge ratio of the peptide) to search for the modified and unmodified forms of the peptides and determine the peak areas. The relative percentages of the modified and unmodified forms can then be determined from these peak areas. However, there are factors that must be borne in mind when performing this type of PTM assessment. Firstly, the ionization efficiency of the modified and unmodified forms of the peptide may not be the same, meaning the relative signal intensity may not reflect the actual relative abundance. Rather it is a reflection of the amounts of material present and their ability to ionize. This does not in any way negate the assessments of the PTM abundances. For any comparability program, all samples are handled in the same way such that any differences in ionization efficiencies are cancelled out. This means that comparisons can be fairly drawn. Secondly, depending on the nature of the PTM there could be an effect on charge distribution (i.e. the most abundant charge state of the modified peptide may not be the same as the unmodified peptide). It is therefore important to ensure that the most abundant signal in the charge profile envelop is being assessed or XIC measurements are based on a summation of mass:charge values observed. Assessments of peptide mapping data can be performed either through manual interpretation or through the use of software-based data interrogation, such as Waters UNIFI, which often forms part of a general peptide mapping study. Software driven data interrogation is a more rapid means of data interpretation but care must be taken as false assignments can be made (through, for example, incorrect assignment of fragment ions) so a check of the output is essential to filter out false positives.

Whilst mass spectrometry is an incredibly powerful tool for PTM analysis due to its ability to identify their nature and location within a protein chain, it is worth remembering that the use of other, orthogonal techniques can provide supportive data for their assignment. Importantly, multiple Regulatory Authorities expect orthogonal techniques and data to be presented in analytical data packages, as discussed in a previous article (4). There are several techniques that can be used in an orthogonal manner to support the MS based assessment of PTMs. One example is imaged capillary isoelectric focusing (icIEF) which provides a charge-based separation of proteins and can therefore give assessments of charge-based modifications such as variation in mAb C-terminal Lysine and, more generally, deamidation events on

proteins. Ion exchange chromatography could also be used in a similar way to provide orthogonal data for charge-based PTMs. Other PTMs, such as PEGylation, glycosylation and proteolytic processing result in significant mass modification of the basic protein chain. Capillary gel electrophoresis (CE-SDS) can be used to give mass-based separation of species present in a sample to investigate these types of PTMs and support the MS based investigations.

Commonly occurring PTMs

So, given that PTMs are widespread, what types of modification are commonly observed and likely to need assessment irrespective of the type of protein? This is more easily addressed for modifications that are chemically derived, rather than as a result of biosynthetic processing. The most commonly encountered PTMs in a general sense are oxidation and deamidation, since the chemical environments that proteins encounter during the manufacturing, purification process and storage can readily modify susceptible side chains. An assessment of these two PTMs not only provides information on the nature of the sample but the demonstration of low levels of oxidation and deamidation is a good indication that the manufacturing process is well controlled.

In terms of product derived PTMs disulfide bridges and glycosylation are the most common types of PTM encountered. For monoclonal antibodies, N-terminal pyroGlu formation (seen more frequently with glutamine than glutamic acid if present at the N-terminus of the heavy or light chain) and heavy chain C-terminal lysine need to be examined. It is also possible that directed chemical modifications of the drug as part of the production process may result in other unwanted PTMs occurring on the molecule so it is very important to consider PTM assessments as different stages of production, where different PTMs may be created as result of the varying chemical environments to which the protein is exposed.

Conclusion

In summary, an assessment of PTMs needs to be performed in order to fully investigate the structural characteristics of the protein as part of the requirements of the ICH Q6B guidelines. This is true both for the characterization of new products but also for comparability studies of biosimilars, where PTMs need to be assessed between innovator and reference products as part of the comparability exercise. Mass spectrometry is a universally applicable technique in PTM investigations with its ability to obtain precise mass and fragment ion information, allowing detailed investigation of the potentially wide variety of PTMs that can be encountered on proteins.

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Richard obtained his PhD in glycoprotein structural characterization using mass spectrometry from Imperial College of Science, Technology and Medicine. He subsequently spent several years there as a postdoctoral research scientist working in the field of glycoprotein structural characterization with emphasis on glycan elucidation. He moved to GlaxoSmithKline for a short time where he was head of mass spectrometry for the toxicoproteomics and safety assessment group. Richard joined M-Scan Limited (now part of SGS Life Sciences) as a biochemist and became the Team Leader for Carbohydrate Analysis before being appointed Principal Scientist. Richard joined BioPharmaSpec in 2016 as Technical Director for Structural Analysis and is responsible for management of all aspects of carbohydrate and glycoprotein characterization at the primary structure level.