Effective Structural Characterization Strategies for Peptides

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Abstract

The use of peptides as therapeutics can be traced back to the first use of Insulin to treat a diabetes patient in the early 1920s. Today, the US Food and Drug Administration (FDA) considers all biopolymers under 40 amino acids to be peptides. This means that insulin, consisting of 51 amino acids, is now considered a biologic.

Peptide therapeutics are in a unique position in that they share benefits attributed to both small molecules and biologics. Their smaller size compared to biologics means that they are more easily absorbed, yet their larger size compared to small molecule drugs means that they have better target specificity.

Efficient peptide characterization is critical to developing an effective and safe peptide therapeutic and this is recognized by both the FDA (1) and EMA (2). Peptide products can have complex shapes that are highly dependent on the correct amino acid sequence. For this reason, peptides often present unique and difficult structural characterization challenges. Applicable and informative characterization methods are required to solve these challenges, along with detailed understanding of the structure and physicochemical properties of the molecule.

Introduction to Peptide Characterization

Thorough characterization and comparability studies, alongside any reference standard or innovator as required, will allow a detailed understanding of the structure and function of a peptide product and provide a strong foundation for regulatory submissions.

This white paper concentrates on characterization of peptide structure. The functionality of a peptide can be affected by structural changes to the backbone of the molecule, for example, disulfide bridging or nature of an amino acid isomer (D vs L form). Considering these complexities, it is important to plan effective structural characterization studies using up-to-date analytical methods and instrumentation to obtain quality data that will accelerate a peptide through development and towards regulatory submission.

Attributes to Characterize

There are well established guidelines for characterizing the structure of peptides, such as the ICH Q6A and Q6B guidelines. The primary, secondary, tertiary and higher order structure (as appropriate) of the peptide should be characterized using a comprehensive range of analytical techniques (see Table 1).

Not all the below analyses will be applicable to every peptide product and methods will vary depending on the sequence of the molecule. For example, Linaclotide is only 14 amino acids long but contains three disulfide bridges, resulting in a tightly bound structure which presents fragmentation challenges for LC-MS elucidation of the disulfide bridge structure.

Structural	Physicochemical and Biophysical
Primary amino acid sequencing (LC-MS/MS, Edman)	Intact molecular weight (LC-MS)
Peptide mapping (LC-MS/MS)	Charge isoform analysis (icIEF, IEX)
Analysis of PTMs, including but not limited to oxidation and deamidation	Chromatographic profiling (RP-HPLC)
Amino Acid Analysis and Peptide Content (Extinction Coefficient (EC) determination for larger peptides)	Higher order structure analysis (CD, FT-IR, IF, 1D and 2D NMR)
N and C terminal sequencing (LC-MS/MS, Edman)	Melting temperature (μ-DSC)
Disulfide bridge analysis (LC-MS/MS)	Particle size (DLS)
Glycosylation, including monosaccharide analysis, N and O-linked glycan population screening and site occupancy for glycopeptides	Aggregation analysis (SEC-MALS, SV-AUC)

Identity and Sequence

The key analyses used to provide an orthogonal confirmation of the identity of the peptide, including intactness, sequence, glycosylation (for glycopeptides) and Post-Translational Modifications (PTMs), are:

- Amino Acid Analysis
- Intact molecular weight (LC-MS)
- Primary amino acid sequencing (LC-MS/MS, Edman)
- Peptide mapping (LC-MS/MS)

Amino Acid Analysis (AAA) is essential for understanding the amino acid composition of the peptide. Consistent results across different batches also demonstrate control over a manufacturing process. The data obtained can also be used to define the peptide content for a lyophilized product or peptide concentration for a product in solution.

Specific isomers of amino acids may also be present in the peptide sequence. D-amino acids may be specifically included in the sequence of chemically synthesized peptides to modify some aspect of their peptidic properties, such as increased resistance to proteolysis. In this case, investigations should be performed to confirm the presence of D amino acids. This is carried out using on-line LC-MS analysis of amino acids that have been initially released by hydrolysis of the peptide and derivatized. Multiple reaction monitoring is carried out as part of the LC-MS analysis to identify the isomers of the amino acids.

Intact molecular weight analysis demonstrates the overall mass of the peptide is as expected or can serve to quickly highlight a failure in the manufacturing process. Masses observed will also give evidence for the presence of PTMs which may, or may not, be as expected. The use of on-line LC-MS, with its high mass accuracy, allows very small mass differences such as is seen with deamidation (1Da) to be readily detected.

During peptide mapping, the peptide product is digested and these digestion products are analyzed by mass spectrometry. All digestion products should be assigned based on the theoretical masses of the peptides that are produced as a result of the digestion. Detection of these expected peptides serves to support that the amino acid sequence of the product is as expected, although the peptide mapping falls short of generating data to confirm the full primary amino acid sequence.

In cases where there is no theoretical sequence or it is a regulatory requirement to confirm the amino acid positions unambiguously (e.g. during biosimilar development), protein sequencing or de novo primary amino acid sequencing should be performed.

Physicochemical Characterization

Physicochemical characterization analyses center around the physical properties of the peptide including:

- Charge isoform analysis (icIEF, IEX)
- Liquid and spectroscopic profiling (RP-HPLC and UV spectrophotometry)
- Melting temperature (μ-DSC)
- Particle size (DLS)

Charge isoform analysis (icIEF, IEX) and liquid and spectroscopic profiling (RP-HPLC and UV spectrophotometry) will provide hydrophobicity, charge and UV spectrum data, which are also regulatory requirements for peptides.

Biophysical Characterization

Biophysical characterization methods will confirm the higher order structure of the peptide. This will not be applicable for all peptides, since a very small peptide will not have higher order structure.

- Circular Dichroism (CD)
- Fourier Transform InfraRed (FT-IR)
- Intrinsic Fluorescence (IF)
- 1D and 2D NMR

These techniques probe different aspects of secondary and tertiary structure such as levels of alpha helix, beta sheet and information on the local environments of amide bonds and aromatic residues. Figure 1 shows representative near and far- UV CD data used for structural comparability. The data from these analyses provide an orthogonal assessment of the higher order structure of the peptide. NMR analyses can also provide high end structural information for the peptide.

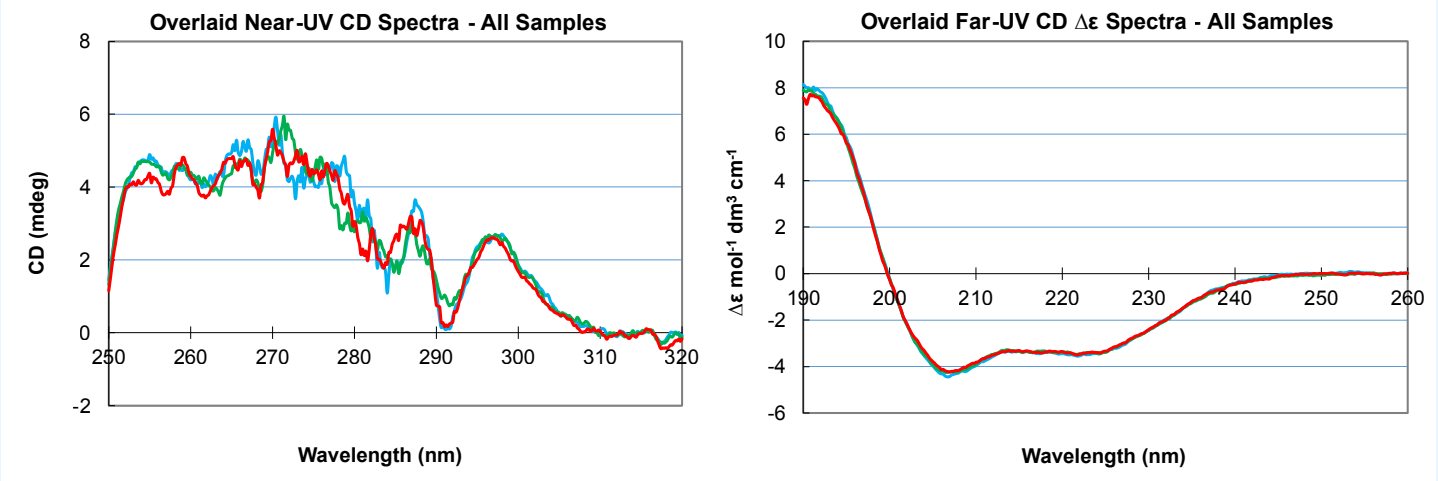

Figure 1. Overlay of Near-UV (left) and Far-UV (right) CD spectra from three lots of an Innovator peptide product

Product and Process Related Impurities and Residuals

Process related impurities include, but are not limited to,:

- cell substrate-derived impurities (e.g. Host Cell Proteins (HCP) and Host Cell DNA),
- cell culture-derived impurities (e.g. inducers, antibiotics, serum), blocking groups (e.g. OPA and FMOC) and
- downstream-derived impurities (e.g. enzymes, inorganic salts, reducing agents, solvents).

For synthetic peptides, amino acid deletions and modifications may be present in the product and these need to be assessed. LC-UV-MS(/MS) is a powerful tool for assessing relative amounts and identifying impurities. Residual metals are also recognized as potential contaminants in both the EMA and FDA guidelines.

Hyphenated chromatographic and mass spectrometric techniques are accepted for the identification, quantitation and monitoring of these impurities down to low ppm/ ppb limits. Monitoring throughout the process and in the final product demonstrates that downstream purification is reducing or removing these components effectively (or enables process changes to be made based on the data obtained).

Conclusion

Growth in the peptide market has been driven by continuing advancements in manufacturing process technologies and the need for more and better therapeutics for chronic diseases associated with ageing. Companies developing peptides must ensure that their analytical strategies include structural characterization studies that will generate high-quality data, tailored to the peptide in development, which can then be used to support regulatory submissions.

References

1. ANDAs for Certain Highly Purified Synthetic Peptide Drug Products That Refer to Listed Drugs of rDNA Origin. Guidance for Industry, May 2021.
2. Guideline on the Development and Manufacture of Synthetic Peptides – Draft. EMA/CHMP/CVMP/QWP/387541/2023, October 2023

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