RESEARCH PAPER

Comprehensive review of peptide therapeutics, market trends and complete characterization techniques along with aggregation.

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ABSTRACT

Therapeutic peptides are a rapidly expanding class of drug products that occupy a significant place between small molecular drugs and large biologicals. These become essential treatments for multiple ailments, ranging from metabolic disorders to malignancy through being highly effective, precise, and with relatively good safety characteristics. Current review includes overview of the current peptide (synthetic or naturally occurring) sector and its significant economic value. Furthermore, it investigates the sophisticated joint analytical approaches essential for their comprehensive assessment or characterization along with methods to find out aggregation.

Keywords: Peptide therapeutic, Characterization, Peptide aggregation.

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1. Introduction

Peptide therapeutics are engineered using either synthetic peptides or naturally occurring peptides which have been engineered to treat diseases. They are also high potency, selective and safe. They are applied in the treatment of a large variety of diseases such as cancer, diabetes, cardiovascular disorders and infectious diseases among others. The advancements in peptide-based drug discovery have resulted in numerous new therapeutic compounds being developed in the recent past. Besides this growth, safe, stable, effective and convenient formulations to patients are required in order to succeed in clinical translation. Despite the fact that the injectable delivery route has been the primary method of peptide delivery over an extended period, there have been continuous technological advances that helped in addressing most of its limitations that have seen the approval of various parenteral peptide products in the recent years. Scientific studies have been done on numerous alternative delivery pathways oral, pulmonary, transdermal etc. which are non-invasive and guarantee patient compliance and comfort, simultaneously. There are measures made in light of use of new formulation technologies to defend the peptide and its absorption and allowing advantage that could not be realized by using conventional dosage forms[1].

2. Current market trends of Peptide Therapeutics:

The peptide therapeutics market has seen great growth in recent years. In 2024 it was valued at approximately US $45.15 billion and is expected to grow to over US $49.21 billion by 2025 marking a CAGR of 9.0%. That growth has been in large part due to the rise in chronic diseases, growth in our molecular bio knowledge, and wide acceptance of these therapeutics in the oncology field. Looking forward the market is to experience great expansion also; by 2029 we see it to be worth around US $74.98 billion, which is an estimate that has a predicted CAGR of 11.1%. What will drive this project is the very large need for personalized medicines, wider use in immunotherapy, in-roads in peptide delivery tech, new discoveries in peptide targets related to infectious diseases, and a larger focus on rare disease treatments[2].

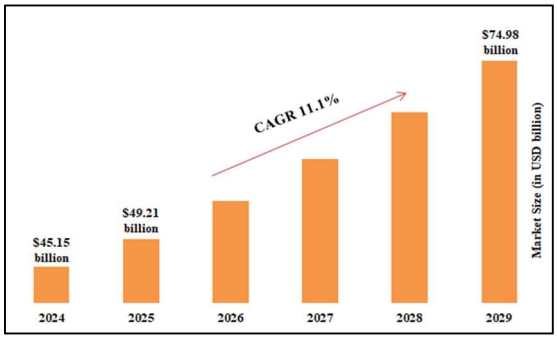

Fig. (1). Peptide therapeutics market size from 2024 to 2029[2]

The generic peptides are being developed more rapidly as the patent on some of the pioneer products is going to be expired soon. Some regulatory authorities like the U.S. Food and Drug Administration (FDA) and the European Medicines

Agency (EMA) have laid down avenues to the approval of generic synthetic peptides with a pressing emphasis on the effectiveness of thorough analytical characterization to prove structural similarity to the Reference Listed Drug (RLD). The use of a multi-faceted analysis is needed to expound on different CQAs, which extends to the primary structure identification and higher-order structure (HOS)[2].

3. Regulatory Framework for Peptide Therapeutics:

U.S. Food & Drug Administration (FDA) has issued specific guidance for generic synthetic peptides referencing recombinant DNA (rDNA) origin peptides. The guidance titled "ANDA's for Certain Highly-Purified Synthetic Peptide Drug Products that Refer to Listed Drugs of rDNA Origin" outlines criteria under which an abbreviated new drug application (ANDA) may be appropriate[3].

Key requirements:

- Demonstration that the active ingredient in the proposed generic synthetic peptide is the "same" as the reference listed drug (RLD).
- For peptide related impurities present in both proposed generic and RLD, the level in the generic should be the same or lower; any new specified peptide-related impurity should be ≤0.5% of drug substance, unless justified otherwise.
- Applicants should apply sensitive, high-resolution analytical methods (e.g., UHPLC-HRMS) to detect and quantify peptide related impurities, often identifying those ≥ 0.10% of drug substance.
- The applicant must justify that any differences in impurity profiles do not increase immunogenicity risk or otherwise affect safety or effectiveness compared to RLD[3].

4. Key Analytical Approaches in Characterization of Peptide Therapeutics:

A Sameness Study or Characterization study is a regulatory requirement intended to prove that the synthetic peptide in a proposed generic formulation is identical in critical quality attributes to the Reference Listed Drug (RLD) of recombinant DNA (rDNA) origin. This study primarily focuses on comparing impurity profiles; particularly peptide-related impurities; that can significantly impact the safety and efficacy of the formulation. FDA considers any alpha amino acid polymer composed of 40 or fewer amino acids to be a peptide rather than a protein. Glucagon, Liraglutide, Nesiritide, Teriparatide, and Teduglutide are all classified as peptides. When an ANDA application is submitted for a synthetic peptide drug product, and the Reference Listed Drug (RLD) is of recombinant deoxyribonucleic acid (rDNA) origin, the ANDA filing must be done under section 505(j); not as an NDA under section 505(b). The FDA also provides guidance to ANDA applicants, recommending an evaluation of whether an ANDA submission formulated with a synthetic peptide is appropriate and whether the API has characteristics similar to that of an rDNA origin peptide[3].

Characterization study/ Sameness study of Peptide therapeutics:

- 4.1. Primary Structure Determination:
  - 4.1.1. Purification of the Peptide
  - 4.1.2. Determination of Amino Acid Composition
  - 4.1.3. Identification of N- and C-Terminal Residues
  - 4.1.4. Cleavage of the Polypeptide Chain into Smaller Fragments
  - 4.1.5. Sequencing of Individual Fragments (The Core Modern Approach)
  - 4.1.6. Overlapping Fragments and Reconstruction of the Full Sequence
- 4.2. Peptide Mapping:
  - 4.2.1. Top-down Proteomics
  - 4.2.2. Bottom-up Proteomics
  - 4.2.3. Middle-down Proteomics
- 4.3. Disulfide (DSB) Bridge Identification:
  - 4.3.1. NMR spectroscopy & X-ray crystallography
  - 4.3.2. Edman degradation & Diagonal paper electrophoresis
  - 4.3.3. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)
  - 4.3.4. Liquid Chromatography (LC) coupled with Tandem Mass Spectrometry (MS)
- 4.4. Secondary Structure Determination:
  - 4.4.1. Far-UV Circular dichroism (CD)
  - 4.4.2. Fourier Transform Infrared (FTIR) spectroscopy
- 4.5. Tertiary Structure Determination:
  - 4.5.1. Near-UV Circular dichroism (CD)
  - 4.5.2. X-ray Crystallography
  - 4.5.3. Fluorescence spectroscopy
- 4.6. Peptide Aggregation (High Molecular Weight Peptides i.e. HMWP):
  - 4.6.1. Size Exclusion Chromatography Multi Angle Light Scattering (SEC MALS)
  - 4.6.2. Sedimentation Velocity Analytical Ultracentrifugation (SV-AUC)
  - 4.6.3. Thioflavin T (ThT) Fluorescence Assay

4.7. Purity/Impurities/Assay of Peptide Therapeutics:
  4.7.1. By Reverse Phase high performance liquid chromatography (RP HPLC)
  4.7.2. By Ion exchange chromatography
  4.7.3. By Ultra High Performance Liquid Chromatography-High Resolution Mass Spectrometry (UPLC-HRMS)
4.8. Physicochemical property of Peptide Therapeutics
4.9. Chiral purity of Peptide Therapeutics
4.10. Forced degradation study along with Mass balance of Peptide Therapeutics
4.11. Bioassays of Peptide Therapeutics
4.12. Immunogenicity in Peptide Therapeutics

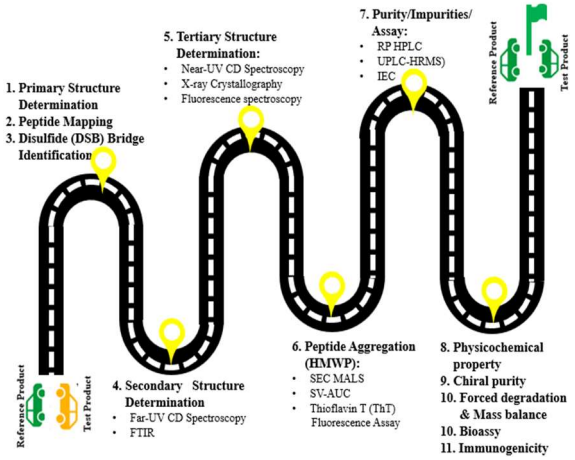

Fig. (2). Characterization study/ Sameness study of Peptide therapeutics

4.1. Primary Structure Determination:

A key step in peptide formulation is determination of primary structure; it is important to determine the exact amino acid sequence of a peptide before any formulation is done.

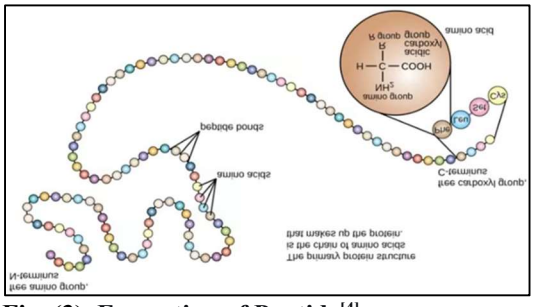

Fig. (3). Formation of Peptide[4]

This is done to ensure that the peptide is who it claims, pure and fit to undergo further pharmaceutical development. The main structure of a peptide defines the exact position of the amino acids in a linear chain and united by the peptide bonds. The identification of such structure is essential in peptide formulation since the biological activity of the peptides, their stability and physicochemical characteristics are always reliant on the sequence of amino acids. Good and precise explanation of the primary structure is used to confirm the identity, quality and consistency in the production of peptide-based drugs[4].

4.1.1. Purification of the Peptide

The peptide of interest will have to be separated to homogeneity in a complex mixture before sequencing can commence. Affinity chromatography, size-exclusion chromatography, and reversed-phase high-performance liquid chromatography (RP-HPLC) are some of the frequently used techniques. This has to be done to eliminate any ambiguous or mixed signals during sequence.

4.1.2. Determination of Amino Acid Composition:

The purified peptide is then subjected to complete hydrolysis which is normally accomplished by the use of 6M HCl at 110°C and a duration of 18-24 hours, which breaks down all peptide bonds. The resultant free amino acids are then isolated, determined and measured. This is traditionally done using an amino acid analyzer**,** which employs ion exchange chromatography followed by post column derivatization (e.g., with ninhydrin). Modern methods often use HPLC with pre column derivatization (e.g., with PITC or OPA).

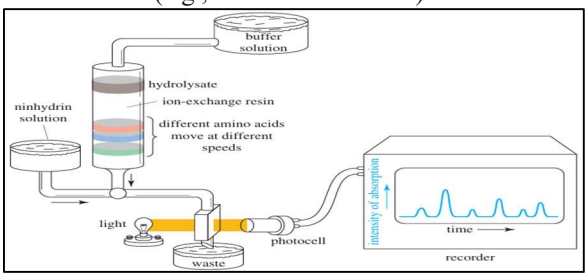

Fig. (4). Basic workflow of Amino acid analyzer[1]

In an amino acid analyzer, the peptide hydrolysate is first separated by passing it through an ion exchange chromatography column. As the components elute, the exiting solution is reacted with ninhydrin, and the resulting colored complexes are monitored by measuring absorbance over time. Each amino acid is then identified based on its characteristic retention time within the column[1].

4.1.3. Identification of N and C Terminal Residues:

- **N-Terminal Analysis (Edman Degradation):** This classical method involves reacting the peptide with phenylisothiocyanate (PITC) under mildly basic conditions. The N terminal amino acid is cleaved as a phenylthiohydantoin (PTH) derivative and identified by

chromatography. The major strength is that the rest of the peptide chain is not destroyed and hence the process can be repeated in a series fashion. Although mostly replaced with whole proteins, it is applicable to short peptides or N-terminal integrity.

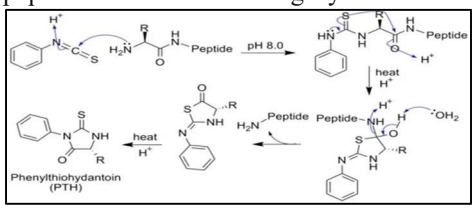

Fig. (5). The Principle of Edman Degradation[4]

- **Sanger method:** The Sanger method is a less used alternative of Edman degradation in determining the N terminal amino acid. Under this method, the peptide is initially reacted with 2, 4 dinitrofluorobenzene (DNFB), Sanger reagent, and then allowed to be hydrolyzed with 6M HCl. The resultant product of this procedure is the liberation of the 2, 4 dinitrophenyl (DNP) derivative of the N terminal amino acid that can be separated and identified. But the Edman procedure along with the Sanger method, are less reliable when using longer polypeptide chains, due to incomplete reactions and side reactions that generally disrupt precise identification.
- **C-Terminal Analysis:** Methods for C terminal analysis are less robust. Enzymatic methods using carboxypeptidases, which cleave amino acids one at a time from the C terminus, can be used but require careful time course analysis.

4.1.4. Cleavage of the Polypeptide Chain into Smaller Fragments

For peptides longer than 50 residues, direct sequencing becomes impractical. The polypeptide is cleaved into smaller, manageable fragments using specific reagents or enzymes.

- **Chemical Cleavage:** Cyanogen bromide (CNBr) cleaves specifically at the C terminal side of methionine residues.
- **Enzymatic Cleavage:** Proteases like trypsin (cleaves C-terminal to Lys and Arg), chymotrypsin (cleaves C terminal to Phe, Trp, Tyr), and Glu C (cleaves C terminal to Glu) are widely used. The resulting mixture of peptide fragments is then separated, typically by RP HPLC.

4.1.5. Sequencing of Individual Fragments (The Core Modern Approach):

Currently, tandem mass spectrometry (MS/MS) is the major technique for sequencing peptide fragments through Ionization by Electrospray Ionization (ESI), Mass measurement and fragmentation followed by Sequence Interpretation.

4.1.6. Overlapping Fragments and Reconstruction of the Full Sequence:

Moreover, to determine the true sequence of the fragments, the same procedure in last two steps i.e. in Steps 4 and 5 is repeated with a different agent of cleavage (e.g. when trypsin was used, trypsin was followed by chymotrypsin or CNBr). This produces a second overlapping peptide fragment set. The entire primary structure can thus be recreated by finding regions of overlapping sequence between the two sets, in a manner like assembling a puzzle.

A simplified workflow that would have worked with a novel peptide would have been:

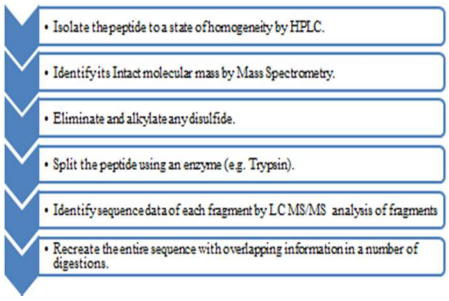

Fig. (6). Workflow Primary Structure Determination

4.2. Peptide Mapping:

Peptide mapping is a crucial form of analytical process that is applied in order to validate or determine the primary structure of a peptide, its amino acid sequence, and any chemical modifications. Under this technique, molecular masses of peptide fragments are determined and compared with theoretical molecular masses to determine structural accuracy. In less technical language, peptide mapping entails the deliberate digestion of a therapeutic protein or peptide in an aqueous environment followed by the separation and subsequent analysis of the fragments to support the existence of a primary and in certain instances, higher order structure of the molecule. This is a widely accepted method that assesses the structural critical quality attributes (CQAs) of biotherapeutic products as a gold standard. This allows to confirm the identity of the drug substance by giving the specifications of the amino acid sequence. Besides, peptide mapping provides site specific understanding of post translational modulations (PTMs), such as degradation associated alterations of oxidation, deamidation, and isomerization that must be cautiously observed during the stages of development and quality control. The mass

spectrometric techniques are used to analyze and characterize proteins through the use of mass spectrometry. This involves the establishment of protein levels, complex protein mixtures profiling, molecular interactions mapping and post translational modification detection. This is popularly referred to as mass spectrometry based proteomics. Mass spectrometric studies of proteomics typically take one of three approaches, which are Top down, Middle down, or Bottom up approaches, each distinguished by the procession and analysis of proteins. Top down proteomics refers to the mass spectrometry analysis of intact (that is, undigested) proteins. In contrast, bottom up proteomics focuses on examining peptides generated through enzymatic digestion of proteins. It is the most widely applied proteomic strategy and relies on a combination of mass spectrometry and high performance liquid chromatography to identify proteins within complex biological samples. The middle down approach serves as an intermediate method between top down and bottom up techniques, in which relatively large peptide fragments rather than full proteins or short peptides are subjected to mass spectrometric analysis[5].

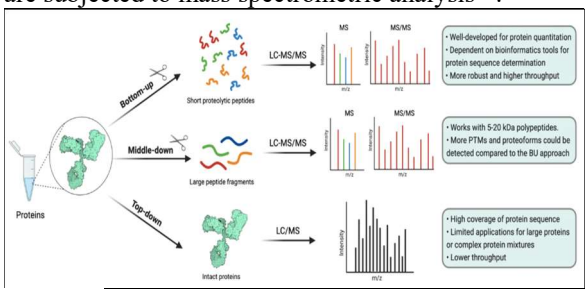

Fig. (7). Three approaches of Mass Spectrometry Based Proteomics[6]

Peptide mapping commonly utilizes a bottom-up mass spectrometry strategy, where scientists examine peptides produced by enzymatically digesting a purified protein or a mixture of proteins. The general process involves breaking the protein into smaller fragments using specific enzymes, separating these fragments through liquid chromatography, and then analyzing them by mass spectrometry. This workflow generates a characteristic fingerprint consisting of distinct peptide ions that represent the protein under investigation. The observed peptide masses or spectra are then matched with predicted values derived from known protein sequences in proteomic databases. Unlike de novo sequencing which determines protein sequences directly without prior knowledge peptide mapping relies on existing sequence databases to confirm or identify the protein of interest[5].

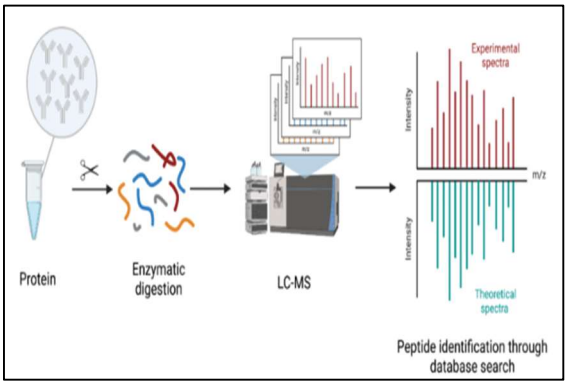

Fig. (8). Basic workflow of Peptide mapping[5]

In regulatory terms, the chapter United States Pharmacopeia (USP) defines peptide mapping as a **chemical identification test** for biotechnology-derived articles, meaning it is used to confirm identity of a biologic product[7].

Why is peptide mapping used in peptide/protein formulations?

In the context of peptide or protein-based drug formulations (therapeutics, biologics, etc.), peptide mapping plays several important roles:

- **Confirmation of identity**: It verifies that the amino-acid sequence of the product corresponds to the expected sequence (or reference/innovator molecule) .
- **Structural integrity/modifications**: It allows detection of post-translational modifications (PTMs) or chemical modifications (e.g., deamidation, oxidation, truncation) that may occur during production, formulation, storage or degradation.
- **Comparability/Consistency**: It is used to compare different lots, to evaluate biosimilarity (when developing a biosimilar), or to monitor changes after manufacturing process changes.
- **Stability indication**: Changes in the peptide map (e.g., appearance/disappearance of peptide peaks) can act as a stability-indicating assay, signaling structural alteration[8] [9].

4.3. Disulfide Bridge Identification:

Disulfide bridges commonly written as S–S bonds are covalent linkages formed between the sulfur atoms of two cysteine residues within a peptide. Cysteine contains a thiol (-SH) group on its side chain, and when two cysteine residues are positioned close to each other, oxidative conditions can promote the formation of a disulfide bond. Such linkages are necessary when keeping the three dimensional structure of peptides since they assist in stabilizing correct folding and enhance structural integrity in general. The presence of several disulfide bonds in many therapeutic peptides has led to the

establishment of disulfide connectivity and the identification of any disulfide bond forms becoming a significant aspect of peptide characterization. The quality of disulfide pairing is linked directly to the safety, functionality, and potency of peptides based drugs, so these bonds are an important quality feature that needs to be observed attentively during the development of the product. Despite their importance, analyzing disulfide bonds remains challenging due to the structural complexity of peptide and protein molecules[10] [11].

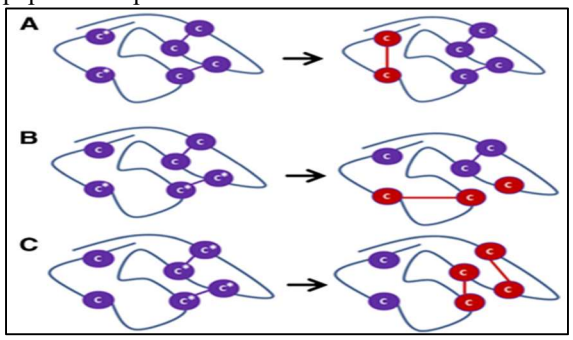

Fig. (9). The different possible routes for formation of nonnative disulfide bonds during sample preparation[11].

In above fig. Cysteine residues shown in purple represent the native disulfide-bonded configuration, while those marked with asterisks indicate modified cysteines that adopt nonnative (red) conformations.

A. Interaction occurring between two free cysteine residues.
B. Reaction taking place between a free cysteine and another cysteine that is already part of an existing disulfide bond.
C. Reaction involving cysteine residues that were previously linked through a disulfide bond.

Analytical methods for disulfide bond characterization:

4.3.1. NMR spectroscopy & X-ray crystallography:

Although both techniques can reveal molecular-level details of disulfide bond arrangements, they generally demand large quantities of highly purified material. Due to this requirement and their inherently low throughput, these methods are not commonly employed for routine disulfide bond mapping[10] [11].

4.3.2. Edman degradation & Diagonal paper electrophoresis:

These two approaches were the primary techniques used for disulfide bond mapping during the early 1960s. Edman degradation, combined with mass spectrometry, continued to be applied to a limited extent even into the late 2000s, although its use had become infrequent by then[10] [11].

4.3.3. Gel electrophoresis (SDS-PAGE):

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) is commonly used to evaluate the migration behavior of peptides within a gel matrix. When performed under reducing conditions, the disulfide bonds within the peptides are cleaved, causing the reduced forms to migrate differently compared to their non-reduced counterparts[10].

4.3.4. Liquid chromatography (LC) coupled with tandem mass spectrometry (MS):

Bottom-up mass spectrometry is the most commonly employed strategy for analyzing disulfide bonds in peptides. This approach is favored because numerous proteolytic enzymes are available to break large biomolecules into smaller peptide fragments that still retain their native disulfide linkages, making them easier to study. In addition, the method benefits from multiple gentle ionization options, diverse and complementary fragmentation techniques, and the capability to integrate liquid chromatography with mass spectrometry to separate enzymatic digests prior to analysis[10].

4.3.5. Sample preparation for bottom-up mass spectrometric disulfide bond analysis:

Proper sample preparation is essential for conducting bottom up mass spectrometric analysis of disulfide bonds. Typically, two general strategies are used: non-reduced (intact) analysis and intact/reduced comparative analysis. In the non reduced workflow which is the most commonly applied approach free cysteine residues are first alkylated, followed by deglycosylation if required, and then enzymatic digestion of the protein is performed without reducing the disulfide bonds. The resulting disulfide-linked peptide fragments are then examined to determine the pattern of disulfide connectivity. In the intact/reduced method, two parallel sets of digested samples are generated: one processed under non-reducing conditions (as described above) and another prepared after reducing the disulfide bonds. Disulfide linkages are deduced by comparing the liquid chromatography (LC) peptide maps of the reduced and non-reduced samples, allowing identification of peptides that originally contained disulfide bonds.

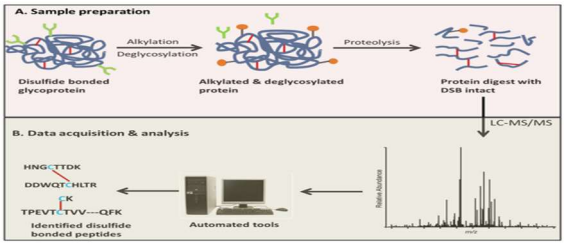

Fig. (10). Disulfide bond (DSB) analysis workflow[11]

(A) In non-reduced protein digestion workflows for disulfide bond (DSB) analysis, proper sample preparation is essential. Alkylating free cysteine residues is a key step, as it prevents disulfide bond rearrangement or shuffling during processing[11].
(B) DSB assignment from Liquid chromatography Tandem mass spectrometry (LC-MS/MS) data.
To sum up, Disulfide bridges (DSBs) play a crucial role in structural stability of biopharmaceuticals, purified proteins and numerous cyclic peptide therapeutics that depend on such covalent bridges. The formation of disulfide bonds in the wrong way a phenomenon referred to as DSB scrambling of resulting molecules can cause misfolding of the resulting molecules thus resulting in the loss of biological activity, enhanced immunogenic properties, and increased aggregations. Therefore, proper characterization of disulfide bond is a requisite in protein confirmation[12].

4.4. Secondary Structure Determination:

The arrangement of amino acids forming a primary structure of a peptide is not rigid. Instead, it is also highly flexible because of the nature of the bonds between the amino acids. As the length of the chain increases, hydrogen bonds may occur between the carbonyl and amide functional groups of the peptide backbone (not counting the side chains). These interactions lead to the folding of some parts of the chain into regular shapes of helices and sheets. These secondary, local structural motifs are the secondary structure of a peptide[13].

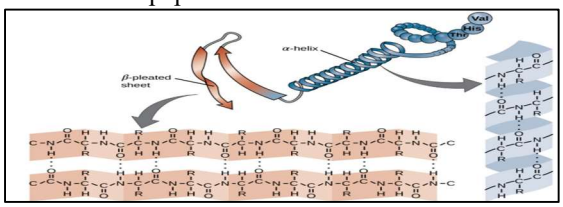

Fig. (11). The secondary structure of a peptide may be an α-helix or a β-pleated sheet, or both[13]

4.4.1. Circular dichroism (CD) Spectroscopy:

Circular dichroism (CD) spectroscopy is a highly effective analytical method for examining the secondary structural features of peptides, including α-helices, β-sheets, and random coil conformations. CD is the most commonly applied technique for this purpose, as different segments of a peptide can adopt distinct structural motifs. In the far-UV region (approximately 185–240 nm), each type of secondary structure produces a characteristic pattern in the CD spectrum. As illustrated in the figure below, the variation in ellipticity across this wavelength range allows for the assessment and quantification of the different secondary structure elements present within a peptide[14] [15].

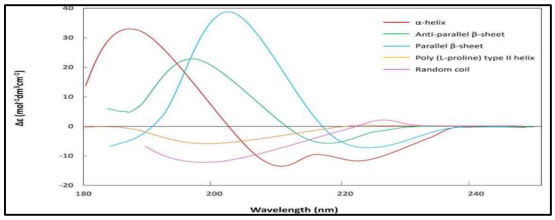

Fig. (12). Circular Dichroism Chromatograms - Secondary Structures of Peptides[14] [15].

4.4.1.1. The Principles of CD Spectroscopy:

Circular dichroism (CD) spectroscopy works by measuring the difference in absorption between left- and right-circularly polarized light by chiral molecules. When a peptide sample is illuminated with plane-polarized light, its optically active groups such as peptide bonds, aromatic residues, and disulfide linkages interact with the light in an unequal manner. This unequal absorption produces a distinctive CD spectrum that reflects the chiral characteristics of the molecule[16].

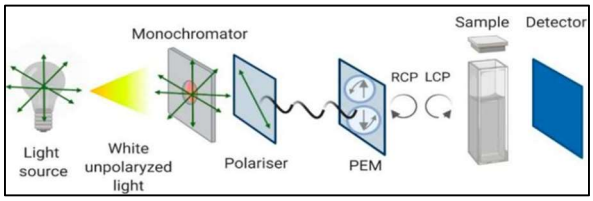

Fig. (13). Schematic Representation of CD Principles[16]

CD measurements are generally interpreted across two primary wavelength regions, with each region providing unique information about different structural features of peptides: Out of which Far-UV CD Spectroscopy is primarily used for secondary structure of peptides while Near-UV CD Spectroscopy for tertiary structure of peptides.

4.4.1.2. Far UV CD Spectroscopy (190 to 250 nm):

The spectral region is mostly applied to determine the secondary structure of peptides since it is used at this spectral range is representative of the repetitive folds including α helices, β sheets and random coils. Each of these folds generate a unique profile of CD. As an illustration, Alpha helical structures normally have strong negative ellipticity at near 222nm and 208nm with positive peak at near 190nm. Through the analysis of these typical indicators, the relative richness of the various secondary elements of structure are measurable, and they give informative information of peptide folding and general structural stability. The solvent should also be carefully chosen since it should maintain the peptide natural or desired structure and is transparent in the far-UV region. It is usually preferred to use buffer systems when analyzing peptides that are under physiological or natural conditions. Proper adjustment of sample

concentration and cuvette path length is important to maintain an appropriate absorbance window and achieve a good signal-to-noise ratio. Typical far-UV CD measurements use peptide concentrations between 0.1 and 1 mg/mL, with shorter path lengths (e.g.,1mm) enabling the use of higher concentrations, and longer path lengths (e.g.,5mm) suitable for more dilute samples. Additionally, instrument settings such as scanning rate, number of spectral accumulations, and nitrogen purge efficiency play a significant role in ensuring high-quality, reproducible CD data[16] [17]. An ammonium camphor sulfonic acid standard is generally used to test system suitability. A circularly polarized light beam passes through the sample, and depending on its polarity, the peptide will absorb left or right polarized light, generating a characteristic CD spectrum[18].

4.4.1.3. Data Interpretation:

The raw data file is processed through specialized software (e.g., CDNN), which provides a tabulated format for different components of the secondary structure; such as alpha helix, beta sheet, beta turn, and random coil; expressed in percentages. If the secondary structure values from the generic batch differ significantly from those of the RLD, it may indicate altered folding, leading to a loss or change in the peptide's pharmacological activity[18].

4.4.2. Fourier-transform infrared (FTIR) spectroscopy:

Fourier-transform infrared (FTIR) spectroscopy is a widely used analytical method for assessing the secondary structure of proteins and peptides by examining the amide I region (1600–1700 cm^{-1}). It is mainly due to the C=O stretching vibrations of the peptide backbone, which is highly sensitive to conformational changes and thus, this spectral region is mostly generated. Differences in hydrogen bonding patterns between various secondary structural motifs result in different positions of the amide I band, which can be interpreted to provide structures[19].

Following are the probable characteristic signals exhibited by secondary structure of peptides

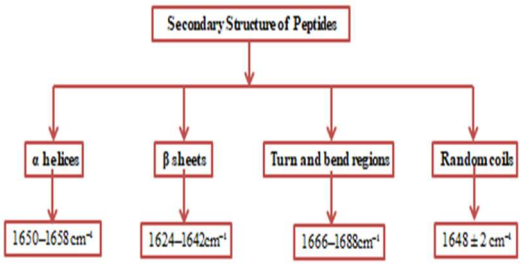

Fig. (14). Characteristic signals exhibited by secondary structure of peptides

4.5. Tertiary Structure Determination:

The tertiary structure of a peptide refers to the overall three dimensional conformation adopted by a single polypeptide chain. This higher-order structure arises from interactions among amino acid residues that may be widely separated in the primary sequence but come into proximity as the chain folds. Multiple noncovalent and covalent forces contribute to the stabilization of tertiary structure. Disulfide bonds formed between the sulfhydryl (SH) groups of cysteine residues provide strong covalent cross-links that enhance structural rigidity. Additional stabilization is achieved through hydrogen bonding, electrostatic (ionic) interactions, and hydrophobic interactions among nonpolar side chains, which drive the peptide into a compact, energetically favorable arrangement. A combination of these interactions shapes out the ultimate three dimensional structure of the peptide which ultimately determines its biological activity, stability and functional specificity. Several intramolecular interactions, such as hydrophobic interactions of nonpolar side chains, ionic (electrostatic) bonds between oppositely charged residues, hydrogen bonds in the backbone shape and covalent disulfide bonds formed between cysteine residues, stabilize the tertiary structure of peptides. A combination of all these forces, forces the peptide chain into its common three-dimensional structure[13].

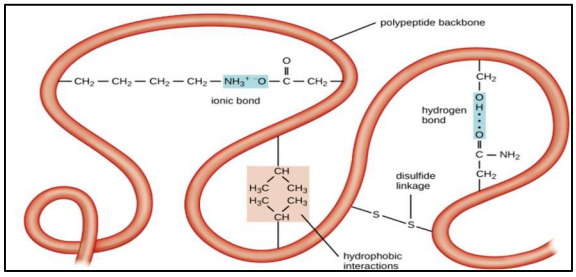

Fig. (15). Tertiary Structure of Peptides[13].

4.5.1. Near UV CD Spectroscopy (250 to 350 nm):

At near UV, CD spectroscopy can be used to give information on the tertiary structure of peptides by studying both the asymmetric environment around aromatic residues like phenylalanine, tyrosine, and tryptophan and disulfide bonds. The signals at this spectral range are due to the differentiated absorption of the circularly polarized light by these chromophores, the spectra are very sensitive to small changes in local conformations. As a result, changes in near UV CD profiles can show changes in peptide folding, tertiary packing, changes in conformational and interactions of these changes with ligands or binding partners, and provide a sensitive diagnostic tool in following higher order structural integrity[16].

4.5.1.1. Overlapping and Data Interpretation:

The CD spectra of the proposed generic and the RLD batches are superimposed and compared graphically. When the tertiary structure values calculated of the generic batch have a significant difference with the RLD, it could signal a change in folding, which causes the loss or alteration of the pharmacological activity of the peptide[18].

4.5.2. X ray Crystallography:

The X ray crystallography is based on the premise that the short wavelength X rays interact with the electron clouds around atoms in a peptide molecule. One or few molecules are too weak to scatter X rays to obtain structural information and crystallization is therefore necessary. Peptides in a crystal are arranged in a very ordered and repetitive structure, which allows them to create coherent scattering of the X rays at the angles that are dependent on the incident beam. The beams of the diffraction are treated as waves and their analysis involves a solution of equations of periodic functions (e.g. Fourier transforms). Although the strength of an individual reflection can be directly determined, sometimes the phase data is lost in the process of data acquisition a phenomenon referred to as the phase problem. To eliminate this, crystals have been commonly made complex with heavy atoms like mercury or gold. The phase information can be obtained by estimating it with the help of techniques such as isomorphous replacement by acquiring another diffraction dataset with the heavy atom derivative and comparing it with the native crystal[20].

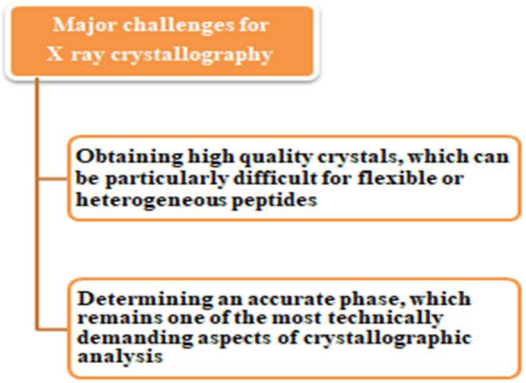

Fig. (16). Major Challenges for X ray crystallography

4.5.3. Fluorescence spectroscopy:

Fluorescence spectroscopy is a very sensitive analysis tool that is largely employed to study peptide conformation, folding, and intermolecular interactions, even at nanomolar concentrations. In this technique, intrinsic fluorescent species (tryptophan, tyrosine and phenylalanine) or externally added fluorophores are excited with incident light of a specific wavelength. These molecules in turn release light of lower energy (longer wavelength) and the properties of the released light are used to give a lot of useful information about structural changes, the microenvironment around fluorophores and dynamic molecular processes of peptide systems. It offers the information on conformational stability, folding and the microenvironment of aromatic amino acid residues which are mainly Tryptophan (Trp), Tyrosine (Tyr) and Phenylalanine (Phe) and in the peptide chain. Fluorescence spectroscopy is a nondestructive, time resolved, analytical methodology that provides the real time observation of the molecular events in peptide systems. Since the measurements can be performed in a short time and can be measured repeatedly, this allows the researcher to trace structural or environmental changes of the sample. Significant fluorescence based keynotes incorporates:

- Changes in fluorescence intensity, which usually indicate ligand binding, conformational changes or changes in the local environment of fluorophores.
- Understanding of the micro environmental polarity, structural dynamics surrounding aromatic residues.
- Rotational diffusion (a measure of conformational flexibility), peptide size and dynamic motion in the system.
- Energy transfer efficiency, which is most commonly, monitored using FRET (Fluorescence Resonance Energy Transfer), which is a distance change reporter between molecular domains or interacting partners over 1 to 10 nm[21].

4.6. Peptide Aggregation: (High Molecular Weight Peptides i.e. HMWP)

The formation of peptide aggregates represents a widespread problem which occurs throughout all development stages of Peptide Therapeutics. Aggregates which were formed during aggregation can be Amorphous aggregates or Fibrillar aggregates or Crystalline aggregates or Oligomers or Protofibrils[22].

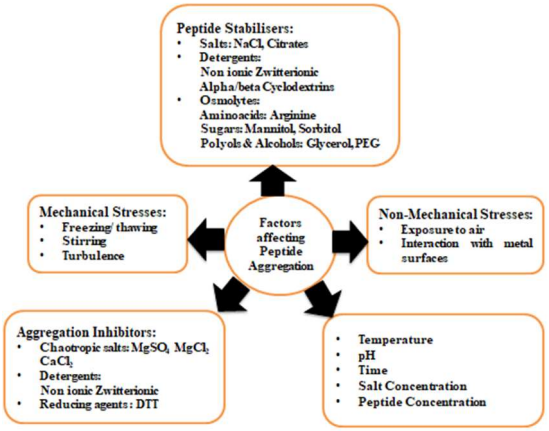

Fig. (17). Factors affecting Peptide Aggregation[23]

Under physiological conditions, newly synthesized peptides normally fold into well-defined three-dimensional structures and may also assemble into functional multimeric complexes. When peptide folding is disrupted, structural changes often promote the formation of small oligomers, which can further elongate into linear aggregates and eventually develop into large, insoluble particles. Such aggregation-driven instability in peptide therapeutics can be triggered by external additives, harsh processing steps, or unfavorable environmental conditions. These instabilities not only compromise product quality but also resemble pathogenic pathways seen in several human disorders. Many neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease are associated with abnormal peptide or protein misfolding and subsequent aggregation[24].

Method to determine Peptide Aggregation:

1. Size based approaches:
    - Size Exclusion Chromatography Multi Angle Light Scattering (SEC MALS)
    - Sedimentation Velocity Analytical Ultracentrifugation (SV-AUC)
2. Thioflavin T (ThT) Fluorescence Assay:

4.6.1. Size Exclusion Chromatography Multi Angle Light Scattering (SEC MALS):

SEC-MALS is an essential technique for peptide scientists who must establish the biophysical properties and solution behavior of peptides intended for biological or biotechnological applications[25].

4.6.1.1. Principles of SEC-MALS:

In summary, SEC separates molecules according to their hydrodynamic size. The column is packed with spherical porous beads of defined pore sizes, allowing molecules to partition based on their ability to diffuse into these pores. Larger molecules are excluded from most of the pore volume and therefore travel more rapidly through the column, eluting earlier with shorter retention times. In contrast, smaller molecules penetrate more pores, follow a longer diffusion path, and elute later. As a result, SEC generates a size-dependent separation in which species elute in the order of decreasing molecular weight. This predictable relationship between size and elution behavior enables the construction of a calibration curve using proteins or polymers with known molecular weights. By plotting log (MW) against the corresponding elution (retention) volumes, a calibration profile—typically modeled as a third-order polynomial is obtained. Within its linear region, the curve provides the highest resolving power and allows more accurate estimation of molecular weight for analytes behaving ideally in the column[26].

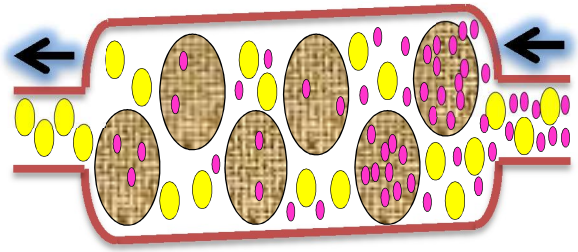

Fig. (18). Separations of molecules in SEC-MALS [27]

In SEC–MALS analysis, the size-exclusion chromatography system is connected to a multi-angle light scattering (MALS) detector along with refractive index (RI) and UV detectors to enable comprehensive characterization of the sample. Together, these detectors provide information on molecular size, absolute molar mass, and concentration across the chromatographic peak. As the separated molecules elute from the SEC column and pass through the MALS detector, they are illuminated by a laser beam. The molecules scatter light in all directions, and the instrument records the intensity of scattered light at multiple fixed angles. Simultaneously, RI and UV detectors measure changes in refractive index and absorbance to determine sample concentration independently of structural assumptions. By combining the multi-angle scattering data with concentration profiles, SEC–MALS yields precise, absolute measurements of molar mass and size for each species within the elution profile[28].

Extended utilization of SEC-MALS has been done by measuring the molecular mass of the separated species to identify whether an oligomer is a dimer, trimer, tetramer, etc.[18]

4.6.2. Sedimentation Velocity Analytical Ultracentrifugation (SV-AUC):

Sedimentation Velocity Analytical Ultracentrifugation (SV-AUC) is a powerful biophysical technique extensively used for peptide characterization, particularly for analyzing size, aggregation, molecular weight, and solution behavior of peptides and proteins under near-native conditions. SV-AUC allows the separation of molecules based on their sedimentation coefficients, providing detailed insights into peptide heterogeneity, aggregation states, and molecular interactions without the artifacts introduced by chromatographic or surface-based methods[29].

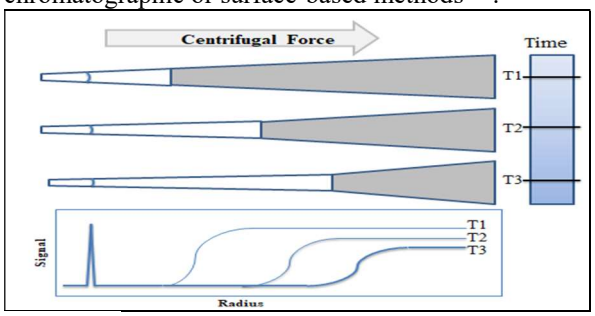

Fig. (19). Schematic presentation of an SV-AUC measurement[29]

In a sedimentation velocity analytical ultracentrifugation (SV-AUC) experiment, the distribution of solute concentration is recorded over time along the radial axis of the sample cell. As the rotor spins, solute molecules sediment toward the bottom of the cell, and this boundary movement is detected by an optical system such as absorbance, interference, or fluorescence optics. During SV-AUC, the rotor speed is sufficiently high to minimize the opposing effect of diffusion; however, some peak broadening still occurs due to back-diffusion along the concentration gradient that forms during sedimentation. Although advanced algorithms such as the SEDFIT c(s) and PCSA (parametrically constrained spectrum analysis) in UltraScan apply mathematical diffusion corrections, reducing back-diffusion experimentally remains critical for achieving high sensitivity and accurate resolution of closely related species[29].

4.6.3. Thioflavin T (ThT) Fluorescence Assay:

Certain extrinsic fluorescent dyes provide valuable insight into peptide folding, unfolding, and aggregation behavior. These dyes are widely used to monitor amyloid fibril formation and to assess the extent of peptide aggregation. When a dye is mixed with a peptide sample, changes in fluorescence intensity or in the emission wavelength such as a blue shift indicating a hydrophobic environment or a red shift indicating a more hydrophilic environment are recorded. Because aggregated or structurally altered peptides interact with dyes differently compared to their native conformations, fluorescence-based measurements enable quantitative evaluation of aggregation processes[24].

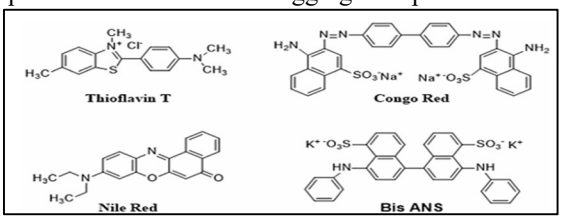

Fig. (20). Structure for extrinsic dyes in detection of Peptide Aggregation[24]

Among the commonly used dyes, Thioflavin T (ThT; 4-(3,6-dimethylbenzothiazol-2-yl)-N,N-dimethylaniline) is the most widely utilized probe for detecting amyloid fibrils. ThT can monitor fibril formation in situ without significantly altering aggregation kinetics. Upon binding to amyloid fibrils, ThT exhibits a substantial fluorescence enhancement, typically showing emission around 482–490 nm when excited at 440–450 nm. Increased fluorescence intensity or a red shift in emission is characteristic of its interaction with β-sheet–rich structures such as amyloid fibrils. Although the detailed mechanism of ThT binding is not fully established, the prevailing model suggests that ThT molecules intercalate into the grooves formed along the amyloid fibril axis. In this bound state, the torsional angle between the benzothiazole and dimethylaminobenzene rings becomes restricted, enhancing fluorescence[24].

4.7. Purity/Impurities/Assay of Peptide Therapeutics:

4.7.1. Purity of Peptide Therapeutics:

Reversed phase high performance liquid chromatography (RP HPLC) and Mass spectrometry (MS) are commonly used analytical methods to determine the purity of peptides. These techniques measure the percentage of the peptide by measuring either UV absorbing chemical groups (in HPLC) or ionized molecular species (in MS). Nevertheless, these methods do not include other non peptide impurities like residual water, inorganic salts or counterions, e.g. glacial acetic acid, trifluoroacetic acid (TFA) or sodium ions that could be found in the final product. To have a better estimation of the actual peptide, one can use content amino acid analysis (AAA), which gives it a direct measure of the actual yield of the peptide in terms of composition of hydrolyzed amino acids[30].

4.7.2. Impurities of Peptide Therapeutics:

The impurity profile between chemically synthesized peptides and their rDNA derived counterparts serves as an essential factor to determine if the synthetic version meets ANDA submission requirements. The

presence of peptide related impurities in the product affects both safety and therapeutic outcomes when compared to the reference listed drug. For peptides falling under this regulatory guidance, applicants are generally expected to characterize and identify any peptide-related impurity present at or above 0.10% of the drug substance. In cases where a product presents heightened immunogenicity concerns, regulatory authorities may also require identification of impurities present at levels below this threshold[3]. Impurity profiling is crucial for ensuring the safety, efficacy, and quality of peptide drugs. Impurities whether process-related or degradation-related can arise during manufacturing, formulation, or storage. Factors such as exposure to light, oxygen, moisture, or inappropriate temperature conditions can promote impurity formation. An overview of the impurity types most frequently observed in synthetic peptide formulations is provided below;

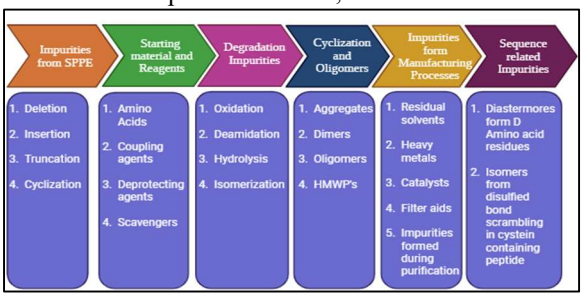

Fig. (21). Source of Impurities in Peptide Therapeutics[19]

4.7.2.1. Truncation:

Truncated peptides are shorter versions of the intended full-length sequence. These impurities commonly arise during solid-phase peptide synthesis when coupling reactions are incomplete or when the elongation process terminates prematurely. Truncation products may also appear during accelerated stability studies of the drug product as a result of peptide degradation. Because they lack one or more amino acid residues, truncated peptides exhibit a lower molecular weight than the desired peptide[19].

4.7.2.2. Deamidation:

Deamidation is one of the most frequently observed chemical modifications in peptides, especially in those containing asparagine (Asn) or glutamine (Gln) residues. During this process, the side-chain amide group (–$CONH_2$) of Asn or Gln is hydrolyzed and converted into a carboxylic acid (–COOH). This transformation leads to a small but detectable mass increase of approximately 0.984 Da, corresponding to the replacement of the –NH_2 group with –OH[19].

4.7.2.3. Oxidation:

Oxidation is also a common degradation route in peptide type drug substances and drug products, and can also significantly change their stability, bioactivity, and safety. Oxidative degradation may be facilitated by several factors enabling them to include:

- Exposure to atmospheric oxygen at time of synthesis, purification, lyophilization or storage.
- Exposure to UV light.
- Trace metal ions or Excipients containing peroxides.
- Elevated temperatures
- Oxidative radicals formed during processing or storage.

The presence of oxidative impurities in therapeutic peptides with oxidable residues including Met or Trp demands the implementation of monitoring and control of the oxidative impurities to guarantee the quality of the products and stable clinical functionality[19].

4.7.2.4. Amino acid insertion:

One of the most common types of impurities that occur during peptide synthesis are insertion impurities, which occur when a peptide is formed with more than one amino acid sequence. Since this will result in a peptide that has a higher molecular weight than what was originally targeted, the presence of such impurities can impact the purity, safety, and efficacy of the drug. Regulatory bodies (both the FDA and EMA) to ensure that peptide therapeutics are appropriately characterized in terms of their impurity profiles, perform extensive testing of the impurities associated with these compound[19].

4.7.2.5. Isomerization:

One specific type of peptide isomerization is the isomerization that occurs at aspartic acid (Asp) where they may undergo isomerization to form isoaspartic acid (isoAsp). Isomerization may also occur secondary to asparagine (Asn) deamidation that forms Asp or isoAsp. Therefore, the peptide can contain many types of stereo isolated products including L-aspartyl, D-aspartyl, L-isoaspartyl, and D-isoaspartyl forms. For precise analysis, tandem mass spectrometry (MS/MS) is widely used to identify and pinpoint the location of isomerized residues within the peptide sequence[19].

4.7.3. Methods to determine the Purity/Impurities/Assay of Peptide Therapeutics:

4.7.3.1. By Reverse Phase high performance liquid chromatography (RP HPLC):

Reversed-phase chromatography (RPC) is widely favored for the separation of impurities in peptide therapeutics due to its reproducibility and versatility. Among reversed-phase columns, C18-bonded silica is the most commonly used packing material and is inherently non-polar. In RPC, the mobile phase typically consists of water mixed with a polar

organic solvent such as acetonitrile or methanol, which elutes compounds based on their polarity. Consequently, more hydrophobic peptides exhibit greater retention on C18 columns. Although C18 is most popular, alternative ligands including C4, C8, RP18, Phenyl, C18-CSH, and C18-BEH—can be employed to achieve optimized selectivity and separation of closely related compounds. Mobile phase pH is a key factor influencing selectivity; therefore, screening across a range (e.g., pH 2–9, within the column's operational limits) is recommended to achieve optimal separation. Mobile phase additives, such as ion-pairing reagents, may further enhance resolution and ensure baseline separation. Gradient elution is commonly used to separate impurities with varying polarities, and employing a slow gradient, higher column temperature, and lower flow rate can improve the separation of co-eluting peaks. Detection is typically performed using UV detectors at 214 nm (peptide bonds) and 280 nm (aromatic amino acids). The developed method should be stability-indicating and suitable for both release testing and stability studies of peptide therapeutics[19] [31].

4.7.3.2. By Ion exchange chromatography:

Ion-exchange chromatography has long been employed for separation of impurities because of presence of either positively or negatively charged amino acids in peptide therapeutics[31].

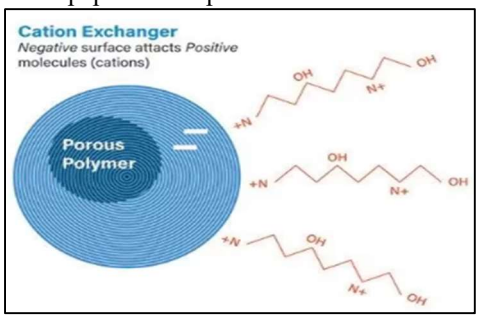

Fig. (22). Attraction of peptide to cation exchange column packing[31]

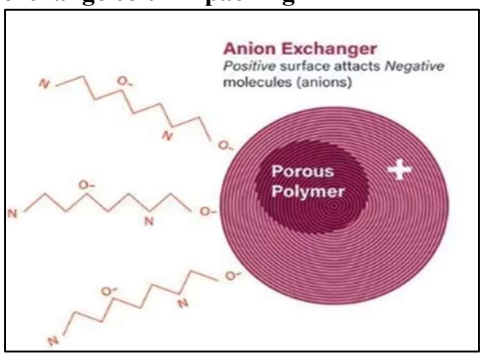

Fig. (23). Attraction of peptide to anion exchange column packing[31]

Ion-exchange chromatography (IEC) relies on charged stationary phases to separate molecules based on their net charge. There are four types of ion-exchangers: weak cation, strong cation, weak anion, and strong anion. Cation-exchange chromatography retains and separates positively charged molecules on a negatively charged surface. Anion-exchange chromatography retains and separates negatively charged molecules on a positively charged surface. Strong ion-exchange resins remain fully ionized over a wide pH range (2–12), while weak ion-exchange resins are charged only within a narrower pH range (approximately 5.5–9.5), allowing selective tuning of retention. In peptide purification, cation-exchange chromatography is more commonly employed than anion-exchange, though the choice depends on the peptide's sequence and charge properties. At pH below 3, the carboxyl groups of peptide side chains are largely neutralized, while the N-terminal amino groups are protonated. This results in positively charged peptides, which are retained by the negatively charged sites on a cation-exchange column[31].

4.7.3.3. By UPLC-HRMS (Ultra High Performance Liquid Chromatography-High Resolution Mass Spectrometry):

The FDA recommends that applicants employ sensitive and high-resolution analytical techniques, such as UHPLC coupled with high resolution mass spectrometry (UHPLC HRMS), to detect and thoroughly characterize peptide-related impurities in a proposed generic synthetic peptide. These analyses should be conducted in comparison with the Reference Listed Drug (RLD) to ensure similarity in quality, purity, and safety[3].

4.8. Physicochemical property of Peptide Therapeutics:

As part of the characterization study, the physicochemical properties of the drug product including pH, density, osmolality, viscosity, and buffer capacity should be carefully evaluated and compared with those of the reference product to ensure similarity in formulation performance and quality[19] [32].

4.9. Chiral purity of Peptide Therapeutics:

Synthetic peptides can display unwanted biological activity, pharmacokinetic properties and toxicity due to the presence of D amino acid residues. It is therefore critical to ensure chiral purity is evaluated during the deamidation reaction. Hydrolyzing amino acids with D protected acid such as DCl or D_2O or HCl d_1 in the presence of deuterated acetic acid will prevent the formation of racemized amino acid residues due to the deuterating chemistry of D

protected acids which converts the α carbon to deuterated carbon and results in a 1 Da mass increase of racemization products. The hydrolyzed amino acids are then derivatized with a chiral reagent, commonly Marfey's reagent (FDAA), producing diastereomeric pairs of D- and L-amino acid derivatives. These derivatives are separated and quantified using reversed-phase HPLC coupled with mass spectrometry (HPLC-MS), enabling the accurate determination of the D- and L-enantiomer ratios present in the original peptide[19] [33] [34].

4.10. Forced degradation study along with Mass balance of Peptide Therapeutics:

Forced degradation studies are conducted to understand the degradation pathways and identify degradation products of a drug substance or product under various stress conditions, including heat, light, oxidation, and hydrolysis. These studies are essential for the development of stability-indicating analytical methods, which are used to monitor degradation during stability studies and throughout the product's shelf-life. In biopharmaceutical comparability studies for example, following process changes forced degradation data are critical for evaluating potential differences in degradation pathways and kinetics between the test and reference products. A comparative forced degradation study is recommended to demonstrate that the analytical purity method is suitable for fully characterizing the impurity profiles of both products[19] [35] [36].

Mass balance assessment correlates the loss of the parent drug with the formation of degradation products under forced degradation conditions. Evaluating mass balance ensures that all major degradation products are adequately detected and quantified, confirming that the analytical method is truly stability indicating. The accuracy of quantification and mass balance can be affected by factors such as co-eluents (or co-occurring peaks), non-chromophoric (invisible to the UV end of an absorbance spectrum, or having no absorbance at the wavelength at which they are measured) species, and differences between response factors; thus, it is essential to optimize the analytical method [19] [37] [38].

4.11. Bioassays:

Bioassays are experimental methods used to assess the biological activity or functional effects of peptides. There are several common bioassay types used including,

Table (1): Types of Bioassay

Types of Bioassay	Description
Cell based Assays	Examine how peptides affect cultured cells (e.g. proliferative activity, signaling pathways), as well as how they exert cytotoxicity on cultured cells.
Organism based Assay	Determine the amount or type of specific peptide activity in a whole organism or animal model.
Enzymatic Assay	Assess the ability of a peptide to modulate the activity of enzymes.
Receptor Binding Assay	Assesses whether a peptide interacts with a specific receptor.
Antimicrobial Activity Assay	Evaluates how effective a peptide is at preventing or inhibiting the growth of microorganisms.

Each of these techniques can provide valuable information about the functional, potency and biological relevance of peptides in a range of different biological systems and targets[19] [39] [40].

4.12. Immunogenicity in Peptide Therapeutics:

A substance's Immunogenicity, whether it is a naturally occurring substance (autologous) or a foreign substance, refers to its ability to provoke (elicits) an immune response by activating either the innate or adaptive immune system. Immunogenicity is usually not an intended outcome of peptide drug use (including potential impurities associated with them) because of the chances that this could have a detrimental impact on their efficacy and safety. Various product and process characteristics are thought to contribute to the development of an immune response to peptide products, including:

1) Aggregation or fibril formation
2) Degradation during storage
3) Impurities introduced as a result of the synthetic production of the API

In order to help facilitate the development and distribution of lower-cost generic drugs, the FDA has created a pathway, known as "Abbreviated New Drug Application" (ANDA), which assists with the implementation of streamlined processes for reviewing generic drug applications while continuing to ensure that products meet established safety and quality standards. The ANDA guidelines specifically require applicants to identify and characterize impurities associated with a product and production process that differ from those of the Reference Listed Drug (RLD) either qualitatively (the kind of impurity) or quantitatively (the amount of the impurity). One of the most important elements in the submission of an ANDA is the assessment of the immunogenicity of the product. Sponsors are expected to demonstrate that the proposed generic peptide exhibits immunological equivalence to the

RLD using orthogonal assessment methods. Common tools and approaches include:

1. **In silico immunogenicity prediction:**
   - ISPRI platform (Immunogenicity Screening and Protein Re-engineering Interface) for comprehensive risk assessment.
   - Incorporates algorithms such as EpiMatrix and JanusMatrix for computational prediction of T cell epitopes.
2. **In vitro assays:**
   - Human Leukocyte Antigen (HLA) binding assays
   - $CD4^+$ T cell proliferation assays
3. **Mass spectrometry-based approaches:**
   - Major Histocompatibility Complex-II (MHC-II)-Associated Peptide Proteomics (MAPPs) for identification and relative quantification of naturally processed and presented MHC-II peptides.

Over the clinical lifetime of certain RLDs, immunogenicity has been observed, reinforcing the ANDA program's primary goal: to ensure that approved generic drugs are no more immunogenic than their corresponding RLDs. Currently, no standardized assay exists for T cell immunogenicity evaluation in generic peptide drug ANDAs. Sponsors are required to provide detailed immunogenicity risk assessment data to allow regulatory reviewers to determine assay adequacy. Typically, at least two orthogonal methods are expected for a robust evaluation [41].

5. Conclusion:

An analytical approach that can provide a thorough and precise structural characterization of the product is necessary for peptide characterization. Whether made synthetically or recombinant, this is essential for all peptide therapies. In addition to being explicit, the legal requirements can be met with cutting-edge equipment and procedures that adhere to scientific principles. As such as the peptide "landscape" evolves, an extensive structural characterization will be required to ensure the products will be safe, effective and compliant with regulatory authorities.

6. Future Prospects:

The further growth of peptide therapeutic agents, multifunctional conjugates, and future technologies of next generation delivery are expected to drive the fast paced expansion of peptide characterization in the future. The analytical technologies must be enhanced in terms of throughput, sensitivity, and accuracy when peptides are altered to more complex structures and peptide drug conjugates. The future field of the peptide characterization will be characterized by a shift in paradigm to integrated, intelligent, high throughput platforms and not only minor modification of existing methods.

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