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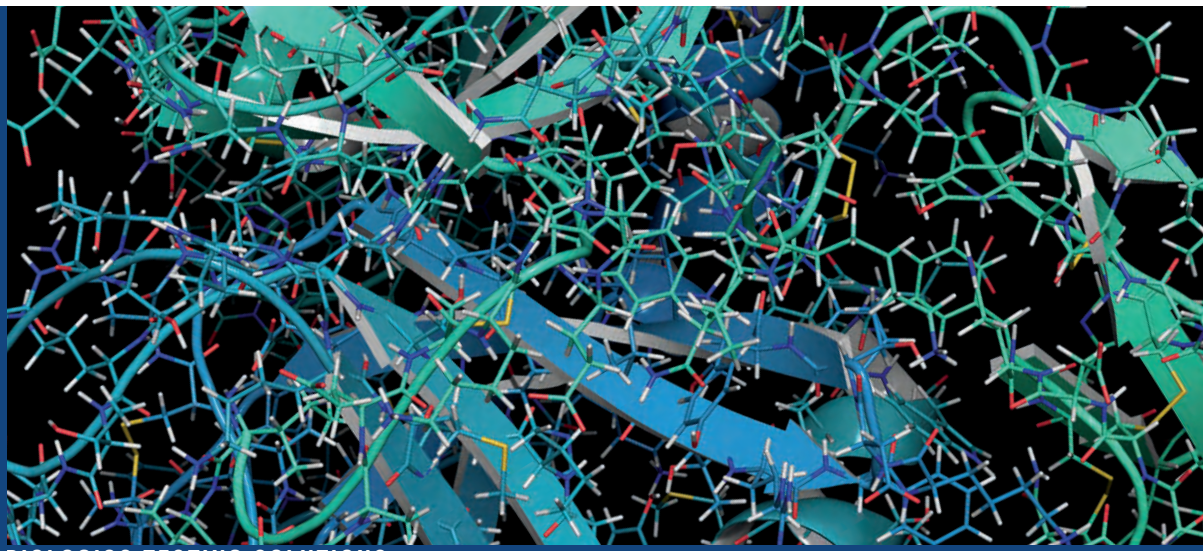
Summary
Secondary and HOS characterizations are essential for complete product characterization, product comparability during manufacturing process changes, and in the development of biosimilar products.



BIOLOGICS TESTING SOLUTIONS

Higher Order Structure Characterization Services

Correct higher order structure (HOS) is critical to ensuring proper functionality, activity, and stability of a biopharmaceutical product. A well-developed panel of methods for HOS characterization is an essential component of a complete product characterization program.

Analytical ultracentrifugation (AUC) separates protein species directly in solution, without the use of a stationary phase such as in size-exclusion chromatography (SEC). The sedimentation rate of the molecule(s) is induced by the centrifugal force, and is monitored continuously by UV absorbance, fluorescence, or interferometry to produce a size distribution profile of the species present within the test sample. Sedimentation velocity analytical ultracentrifugation (SV-AUC) generates sedimentation coefficient values and reports the relative percentages of monomer, multimer, and aggregate species. Information on molecular weight (MW) and hydrodynamic shape are also obtained.

Differential scanning calorimetry (DSC) is conducted by heating the molecule at a constant rate and the detectable changes in heat capacity associated with thermal denaturation are recorded. A single DSC experiment can determine the transition midpoint, T_m, and the enthalpy (ΔH) and heat capacity change (ΔCp) associated with unfolding. DSC is a very useful technique for comparing lots of the same product to ensure lot-to-lot consistency, comparability of product upon manufacturing changes, and to establish biosimilarity.

Circular dichroism (CD) measures differences in the absorption of circularly polarized light arising from structural asymmetry. Far UV scan, 195-250 nm, is dependent on peptide bond alignment/positioning. Ellipticity data in the far UV is de-convoluted to estimate secondary structures such as alpha-helix, beta-sheet, or random coil. Near UV scan, 250-350 nm, measures absorbance of the chromophores of aromatic residues and disulfide bonds. The CD signal within this spectral range provides a fingerprint of the overall tertiary structure of the protein.

Dynamic light scattering (DLS) provides information on the hydrodynamic size and size distribution of particle emulsions and molecules dispersed or dissolved in a liquid. Population size information is expressed as a distribution curve. Dynamic light scattering allows for the measurement of a protein's diffusion coefficient, along with the hydrodynamic size, and an estimation of molecular weight.

EVERY STEP OF THE WAY

Intrinsic tryptophan fluorescence (ITF) assesses the conformational state of a protein. The intrinsic fluorescence of a folded protein is due primarily to tryptophan residues emission, with some small contributions from tyrosine and phenylalanine residues. Tryptophan has a wavelength of maximum absorption of 280 nm and an emission peak that is solvatochromic, ranging from 300 to 350 nm depending on the polarity of the local environment. Hence, ITF is used as a diagnostic for structural characterization of a protein.

Fluorescence polarization anisotropy (FPA) determines binding constants from the interaction of relatively small fluorescently emitting ligands with larger receptor molecules. This technique is based on the use of polarized light to excite a fluorescent molecule, which in turn will emit polarized light; however, the degree of polarization and thus anisotropy of the emitted light is directly dependent on the rotational diffusion of the fluorescent molecule. The measurement of anisotropy, therefore, can be used to generate binding curves upon varying the concentration of ligand with respect to its receptor. Affinity constants can then be calculated from the respective binding curves.

Surface plasmon resonance (SPR) protein binding studies commonly use biacore instrumentation and take on various configurations and formats, including both Protein:Protein and Protein:Drug binding studies. Rank-order screening methods for lead selection, as well as optimization of running conditions to determine K_{on}, K_{off}, and KD values for IND/CMC programs can be developed.

The **apolar fluorescent dye 8-Anilinonaphthalene-1-sulfonic acid (ANS) method** evaluates the surface hydrophobicity of a protein in its native state. This method is a relatively rapid, non-destructive, and simple means of quantitatively assessing the apolar or hydrophobic nature of a protein. The number and relative size of hydrophobic sites on a protein's surface may be influenced by conditions of pH, temperature, and ionic strength. As the molecule unfolds, more hydrophobic regions are exposed. Thus, this method is very sensitive to the conformational state of the protein.

Size-exclusion chromatography with multi-angle laser light scattering (SEC-MALLS) detection is used to separate proteins based on size and to determine the molar mass of the separated proteins. The angular dependence for large species (>200 kDa) may also be used to estimate size and shape factors, such as the protein radius of gyration.

Fourier transform infrared (FT-IR) spectroscopy is a well established experimental technique for the analysis of secondary structure of polypeptides and protein. Analysis of polypeptide and proteins by FT-IR yields a series of characteristic IR absorption bands. Amide I and II bands are the two most significant vibrational bands of the protein backbone. Furthermore, the more sensitive of the two amide bands is the amide I band (1700−1600 cm−1), which corresponds to the C=O stretch vibrations of the peptide linkages. The frequencies of the amide I band components are well correlated with the secondary structural characteristics of proteins.

Higher Order Structure Characterization Methods

Aggregation and Sizing	Secondary/Tertiary Conformation	Thermal Stability/Transitions	Protein Binding Kinetics
Size exclusion chromatography with multi angle laser light scattering (SEC-MALLS) detection	Circular dichroism (CD) spectropolarimetry	CD thermal denaturation study	Surface plasmon resonance (SPR) via biacore
Analytical ultracentrifugation (AUC)	Fourier transform infrared spectroscopy (FT-IR)	Differential scanning calorimetry (DSC)	Fluorescence polarization anisotropy (FPA)
Dynamic light scattering (DLS)	Intrinsic tryptophan fluorescence (ITF) spectroscopy	Intrinsic tryptophan fluorescence (ITF) spectroscopy	
Electrophoresis via SDS PAGE and CE-SDS	Extrinsic fluorescence ANS dye binding	Extrinsic fluorescence ANS dye binding	
	Disulfide linkage mapping via peptide mapping LC-MS/MS		

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