Assessing the Utility of Circular Dichroism and FTIR Spectroscopy in Monoclonal-Antibody Comparability Studies

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ABSTRACT: Protein characterization is a necessary activity during development, technical transfers, and licensure. One important aspect of protein characterization is higher order structure assessment, which can be accomplished in a variety of ways. Circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopies provide global higher order structure and are routinely used to measure the overall structure for product characterization; however, their use as comparability tools is uncertain because of their insensitivity to local or small structure changes. We use a monoclonal antibody (mAb) to explore the usefulness of CD and FTIR compared with other indirect methods of structure characterization such as size-exclusion and ion-exchange chromatographies (SEC and IEC). A panel of degraded samples of a mAb was generated; their higher order structure evaluated using CD and FTIR and was found to be largely unchanged. However, the SEC and IEC chromatograms of certain degraded samples were found to have measurable changes. Based on these studies, we conclude that the application of CD and FTIR should be reserved for global higher order structure identification or product characterization only. The use of CD or FTIR comparability of mAbs should be carefully evaluated, as comparability can be sensitively determined using indirect methods based on chromatography. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:4459–4466, 2015
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INTRODUCTION

During the development and life cycle of a biologic, process changes and technology transfers are occasionally needed to increase efficiency in the process or supply chain, to meet increased product demand, or to improve manufacturability of the biologic. A comparability package is typically filed with the regulatory authorities to show that the revised manufacturing process yields similar product. Comparability studies, as recommended by ICH Q5E, are carried out to ensure physicochemical and biochemical similarity and is a cornerstone activity to support the filing. Degradation rates of one or more variant quantifying assays, for example, size-exclusion chromatography (SEC) to measure size variants, using samples stored in accelerated conditions, that is, increased temperature and humidity, are analyzed to gain confidence that the stability at the intended storage condition will be comparable. Also, levels of charge and size variants as well as melting temperature measurements by calorimetry, peptide map analysis by mass spectrometry (MS) or binding assays are measured and both quantitatively and qualitatively compared between control and comparator samples. The type of analytical characterization data, and whether non-clinical and clinical studies are included, depends on the nature of the process changes and stage of development.

In addition to the characterization data examples listed above, there is a regulatory expectation to provide information on the higher order structure, that is, secondary structure elements (α-helix, β-sheets) and tertiary structure. For large proteins such as monoclonal antibodies (mAbs), detailed higher order structure-determining techniques can be either too complicated (nuclear magnetic resonance), too slow (X-ray crystallography), have poor resolution (small-angle X-ray scattering), or require probes and/or mutations (fluorescence resonance electron transfer). Hydrogen/deuterium-exchange MS (H/D-exchange MS) has increasingly been used to elucidate higher order structure changes at residue- or peptide-level specificity while requiring a reasonable initial investment and ongoing operation costs. However, the most common techniques remain circular dichroism (CD),[1–4] FTIR,[5–10] and intrinsic fluorescence spectroscopies.[11]

Studies with IgG1 mAbs indicate that changes in higher order structure are sometimes detected by CD and/or FTIR spectroscopy in product samples that underwent extreme stress not typically encountered during routine manufacturing [e.g., incubation at high temperature (90°C)[12,13] and extreme agitation of lyophilized products[14]], whereas in other cases, contrary conclusions were made after significant H_2O_2 oxidation[15] or acid exposure.[16] In this account, we outline and conclude that CD and FTIR are insufficiently sensitive to find measurable higher order structure changes in samples after stressed to a degree that is representative of current manufacturing processes.

Instead, our approach has been to use combinations of analytical tools to assess the conformational properties of mAbs in lieu of the spectroscopic assays. Methods that provide a direct assessment of the primary structure and functional properties of the molecule are employed along with methods that are sensitive and quantitative to detect subtle variations in the

Abbreviations used: mAb, monoclonal antibody; CD, circular dichroism; SEC, size-exclusion chromatography; IEC, ion-exchange chromatography; MES, 2-(N-morpholino)ethanesulfate; CpB, carboxypeptidase B; Na_2EDTA, (ethylenedinitrilo) tetraacetic acid disodium salt.

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properties of the surface of the molecule. The canonical primary structure of mAbs leads to regular and predictable secondary, tertiary, and quaternary structures, which are highly thermodynamically stable relative to other protein types. Therefore, classical biophysical tools (e.g., CD and FTIR spectroscopy) are less sensitive to chemical or physical structural heterogeneity of antibodies because of their large size.

Degraded Samples Panel

Relatively harsh degradation stresses have been shown to alter antibodies in a variety of ways.[12] Therefore, degraded samples of the mAb were generated using worst-case levels of different, representative stresses (see *Materials* section) typically found during manufacturing, processing and storage of the drug substance and drug product.

The higher order structure of these degraded samples was assessed by CD and FTIR. These results were compared with the SEC and IEC chromatograms of the same sample.

CD and FTIR Spectroscopies

Circular dichroism is an established technique that easily determines the higher order structure of proteins[2,17–19] and mAbs in particular.[3] The secondary structure of a protein is probed in CD using the far-UV wavelength range (190–250 nm) where the peptide bond chromophore is active. In contrast, the near-UV wavelength region (250–340 nm) of CD can detect changes in the relative chiral orientation of internal aromatic residues such as tryptophans (266, ~295–305 nm), tyrosines (265–290 nm), and phenylalanines (~265 nm),[3,20] and can provide some insight on changes in the overall tertiary structure of a protein.

Another technique is FTIR.[5–9] The most useful FTIR signal in protein structure characterization are the Amide I band (~1625–1695 wavenumbers or cm^{-1})[7] and the Amide II band (~1550 cm^{-1}).[7,9]

These bands are often sufficiently broad to result in unresolved peaks, which can be deconvoluted by taking the second derivative of the absorbance spectrum to distinguish the position of multiple peaks.

Size-Exclusion and Ion-Exchange Chromatographies

In contrast to the spectroscopic techniques previously described, SEC utilizes change in apparent size to separate degradants from the monomer peak. In general, SEC is highly dependent on the molecular shape and hydrodynamic radius.

Similar to SEC, ion-exchange chromatography (IEC) generally detects changes in charge due to chemical degradation. However, IEC could also detect structural changes that would result in a change to the exposed charge residues. Both SEC and IEC are well-known techniques and have been extensively utilized to compare samples for regulatory filings, for example, lot-to-lot variability, changes in upstream processes, and technology transfers to different sites.

Although neither SEC nor IEC directly measures the higher order structure of a protein, their relative sensitivity combined with the loose correlation between higher order structure to the general shape as measured by SEC or extent of chemical degradation as measured by IEC,[21] may provide some indirect indication of higher order structure changes. However, other techniques (e.g., peptide mapping by MS, capillary electrophoresis, antigen binding affinity, etc.) not evaluated in this manuscript should be included to provide a more complete evaluation of higher order structure.

MATERIALS

Blank Formulation Buffer

A 20 mM histidine-based buffer at pH 6.0 was used to blank all spectra.

Control and Degraded Samples Panel

Samples were purposefully degraded using specific extreme conditions: acidic (pH 3.2 for 1 day at 40°C), basic (pH 8.5 for 3 days at 40°C), light (1.2 million lux-hours), heat (40°C for 42 days), and oxidation (0.5% tBHP, 24 h, ambient) stress conditions. For those degraded samples that required spiking a reagent (e.g., acidic, basic, and oxidative stresses), the sample was desalted and exchanged back into formulation buffer immediately after the appropriate amount of incubation time. These samples were compared with an unaltered sample or a control sample specific for that experiment (e.g., sample protected from light by aluminum foil).

METHODS

CD Spectroscopy

The various samples were diluted using formulation buffer to ~10 mg/mL. To determine the concentration, the 10 mg/mL samples were diluted to 0.5 mg/mL and the absorbance measured at 280 nm. The 10 mg/mL samples were then placed in 0.01 or 1.0 mm cuvettes for measurements in the far- and near-UV regions, respectively, and the ellipticity was measured at ambient temperature in duplicate. Blank spectra were also obtained with the same CD parameters and subtracted from the sample spectra. The resulting subtracted spectra were subjected to an offset correction by subtracting the same ellipticity value to all wavelengths such that the ellipticity at the longest wavelength equaled zero. The ellipticity of these final spectra were then corrected for the concentration, molecular weight, and cell cuvette pathlength to normalized units of mean residue ellipticity (deg cm^{-2} $dmol^{-1}$ res^{-1}) for far-UV and molar ellipticity (deg cm^{-2} $dmol^{-1}$) for near-UV.

Each sample was prepared in duplicate on different days, and the data were processed as described above. The resulting spectra were averaged and then compared with the reference material.

The displacement chromatography samples that enriched the acidic and basic IEC variants were generated and analyzed as described elsewhere.[22]

FTIR Spectroscopy

Neat samples, with a concentration of ~30 mg/mL for the degraded samples panel, were loaded into a syringe and then slowly injected into the Bruker FTIR cuvette cell. The infrared spectra were acquired using a consistent set of parameters. A blank spectrum was also measured and subtracted from the sample spectrum. All samples were measured in duplicate on the same day; therefore, the assay variability measured does not account for day-to-day variability.

The subtracted spectra were normalized based on concentration differences between samples before being converted to