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Comparison of quantitative spectral similarity analysis methods for protein higher-order structure confirmation

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ABSTRACT

Optical and vibrational spectroscopic techniques are important tools for evaluating secondary and tertiary structures of proteins. These spectroscopic techniques are routinely applied in biopharmaceutical development to elucidate structural characteristics of protein products, to evaluate the impact of processing and storage conditions on product quality, and to assess comparability of a protein product before and after manufacturing changes. Conventionally, the degree of similarity between two spectra has been determined visually. In addition to requiring a significant amount of analyst training and experience, visual inspection of spectra is inherently subjective, and any determination of comparability based on visual analysis of spectra is therefore arbitrary. Here, we discuss a general methodology for evaluating the suitability of numerical methods to calculate spectral similarity, and then we apply the methodology to compare four quantitative spectral similarity methods: the correlation coefficient, area of spectral overlap, derivative correlation algorithm, and spectral difference methods. While the most effective spectral similarity method may depend on the particular application, all four approaches are superior to visual evaluation, and each is suitable for assessing the degree of similarity between spectra.

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Optical and vibrational spectroscopic techniques are important tools for elucidating structural characteristics of proteins in solution [1–7]. They are routinely used in academic and industrial settings for studying protein higher-order structure [1–6,8–10]. In the biopharmaceutical industry, Fourier transform infrared (FT-IR) spectroscopy, near-ultraviolet circular dichroism (near-UV CD) spectroscopy, and other spectroscopic methods are used to characterize the higher-order structure of therapeutic proteins and to verify that the properly folded protein structure has been preserved following changes to a manufacturing process [8–10].

It is common practice to visually assess the degree of similarity between spectra obtained in these studies, resulting in arbitrary conclusions of comparability (or lack thereof). In most cases, differences between spectra are not readily apparent, and the analyst assumes that spectral differences must be visible to be significant. Alternatively, when spectral differences are visible, most analysts assume those differences signify that the structure of the protein has changed. Thus, when a visual assessment of spectral similarity is employed, the prevention of both false failures (declaring that spectra are not comparable when they are) and missed faults (declaring that spectra are comparable when they are not) depends on a somewhat tenuous assumption: namely, that a trained human eye is capable of detecting differences between spectra caused by changes to the higher-order structure of the protein. Furthermore, assuming the human eye is equal to such a task, significant training and experience are required to remove analyst bias such that spectra may be consistently and correctly interpreted. Even then, removal of all subjectivity is difficult, and the end result may be irreproducible.

To mitigate potential human errors, two improvements are required: first, a method must be developed to quantify the degree of spectral similarity objectively, and second, an understanding of the precision and sensitivity of the method is necessary to confirm when a measured difference is significant. In other words, normal variation inherent in any measurement must be quantified to reduce the likelihood of false failures. Conversely, subtle spectral changes that are difficult even for expert spectroscopists to detect visually may signify meaningful changes in the protein structure. Failure to identify these changes leads to higher incidence of missed faults. Both of these problems can be mitigated using numerical approaches to evaluate spectral similarity.

Unfortunately, few approaches for numerically comparing spectra have been proposed. Two common and relatively straightforward techniques for spectral comparison are Prestrelski's

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correlation coefficient approach [11] and Kendrick's area of spectral overlap method [12]. In previous studies [9,10], the utility of numerical comparisons of spectra was established while qualifying the FT-IR and CD methods for product characterization and comparability assessments during process and formulation development. For these studies, we used a derivative correlation algorithm (OMNIC QC Compare [13]) to determine the degree of similarity between spectra. This algorithm uses a scaling function to differentiate between very similar spectra [13].

Alternative approaches have been proposed by D'Antonio et al. [8] and Bierau et al. [14]. D'Antonio and colleagues focused on the analysis of FT-IR data using four numerical comparison methods: the spectral correlation coefficient, the area of overlap, the derivative correlation algorithm, and a modified area of overlap method. The authors showed that by modifying the area of overlap by squaring the signal at each wave number to increase the dynamic range, greater emphasis was given to regions of the spectrum with more intense signals. They concluded that all four algorithms were able to detect statistically significant differences between samples that should differ, and furthermore, results from all four methods were consistent with visual assessments by expert spectroscopists. In Bierau's approach, confidence intervals are calculated around each point of the spectrum to create maximum and minimum spectra that define a range about an average spectrum. At each point, this range defines the criteria for spectral similarity, and measurements that fall within the range are deemed comparable. Although this approach provides objective criteria to evaluate spectral similarity, a determination of comparability is still subjective if some points along the spectrum fall outside (while other points lie within) the bounds.

More sophisticated structure quantification techniques have been the subject of extensive study [15,16]. Although quantifying the amounts of various structural elements within proteins is quite useful for protein characterization purposes, this application of spectroscopy is less valuable when the intent is to compare the overall similarity of two spectra. In addition, these methods often require solution conditions that are not compatible with formulated drug product. Therefore, our objective is to evaluate the suitability of quantitative approaches to determine the degree of similarity between spectra obtained from formulated samples. We present a relatively simple method for quantifying the difference between two spectra, which we refer to as spectral difference, and then we systematically compare four numerical spectral similarity methods: the correlation coefficient, area of overlap, derivative correlation algorithm, and spectral difference methods. Finally, we propose a general framework for evaluating any spectral similarity or difference method, including a set of criteria by which to compare the advantages and disadvantages of each method. In this way, analysts can select the method that is most suitable for their particular application and sample characteristics.

Materials and methods

Materials and sample preparation

Six manufacturing lots of a monoclonal antibody (mAb1) were measured six times each by FT-IR and near-UV CD across multiple days, yielding 36 total spectra by each technique. This experiment was specifically designed to estimate measurement repeatability, normal variation across lots, and the variability of measurements from day to day. Furthermore, to induce spectral changes, samples of mAb 1 were modified by pH adjustment from 5.2 to 2.5, by exposure to elevated temperatures (80 to 85 °C), and by addition of 6 M guanidine HCl (Gdn) to intentionally change the higher-order structure of the protein.

Samples of a second monoclonal antibody (mAb 2) were also measured by FT-IR and near-UV CD after inducing structural changes to the protein. Specifically, samples were modified by pH adjustment from 5.0 to 3.0 and by addition of 6 M Gdn. Then, the samples were measured by FT-IR and near-UV CD spectroscopies. For both mAb 1 and mAb 2, a properly folded reference standard was measured every day, and all spectral similarity results were calculated with respect to the reference standard spectrum obtained on the same day.

Fourier transform infrared spectroscopy

FT-IR spectroscopy measurements were obtained on a Bruker Vertex 70 Fourier transform infrared spectrometer (Bruker Optics, Ettlingen, Germany) using an MCT (HgCdTe) detector and a flow through AquaSpec measurement cell. The detector was cooled with liquid nitrogen for 30 min prior to measurements, and the instrument was continuously purged with ultrapure nitrogen. The sample cell was flushed with buffer solution and deionized water between measurements. Spectra were collected with a resolution of 4 cm^{-1} and an aperture setting of 6 mm, and 128 scans were averaged to obtain the final spectrum. Spectral processing, including water vapor and buffer subtraction, normalization, and differentiation, was performed using OPUS 6.5 software.

Near-ultraviolet circular dichroism spectroscopy

Near-UV CD spectroscopy measurements were obtained using J-710 and J-715 spectropolarimeters (Jasco, Inc., Easton, MD, USA). Spectra were collected with a data spacing of either 0.1 or 0.5 nm, a bandwidth of 1 nm, and a scan speed of either 10 or 20 nm per minute. All spectra were corrected for the contribution from the solution, and each spectrum was an average of 10 scans. The final spectra were converted to mean residue ellipticity (MRE).

Simulation of spectra by mathematical blending

A mAb 2 reference standard spectrum was mathematically blended with spectra obtained from structurally altered mAb 2 to create simulated spectra with varying degrees of similarity to the native spectrum. Specifically, two blends were created mathematically: (1) the native spectrum was mixed in varying ratios with a spectrum obtained from a sample with reduced pH, and (2) the native spectrum was mixed in varying ratios with a spectrum obtained from a 6 M Gdn sample. Mathematically blended spectra were generated for both FT-IR and near-UV CD spectroscopy.

Simulation of spectra as a sinusoidal wave

To simulate the effects of phase shifts and amplitude changes on spectral similarity determinations, sine waves were generated according to Eqs. (1) and (2),

$$y_1 = c \sin x, \tag{1}$$

$$y_2 = a \sin\,(x + b), \tag{2}$$

where y_1 and y_2 are the reference and sample sine waves, respectively. Constants a and b were systematically varied to evaluate the impact of amplitude and phase shifts, respectively, on the response of each of the spectral similarity approaches. The amplitude, c, of the reference sine wave was held constant at a value of 1 for all sine wave comparisons.

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