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Microfluidic Modulation Spectroscopy (MMS™)

For Protein Therapeutic Drug Analysis

Overview

This application note provides an overview of RedShiftBio's technology performance and capabilities within a new analyzer currently in late stages of development. A prototype analyzer was used to perform measurements of both commercially available proteins and proprietary proteins. Studies demonstrate significant increases in sensitivity, dynamic range, and utility for determination of protein similarity (fingerprinting), quantitation, protein secondary structure, and protein stability and aggregation through thermal and chemical denaturation methods.

Quick Summary

Simple Measurement Workflow

RedShiftBio has developed a novel technique called microfluidic modulation spectroscopy (MMS) which performs rapid and continuous auto-referencing, yielding simple, accurate and robust spectral measurements. The differential nature of the measurements eliminates separate reference measurements and the need for buffer subtraction is minimized.

Extended Concentration Range

Unlike many competitive solutions, the RedShiftBio's MMS analyzer provides direct, label free measurements over a concentration range from 0.1 mg/mL to over 200 mg/mL for secondary structure measurements, and from 0.01 mg/mL to 200 mg/mL for protein quantitation. Competitive approaches are typically limited to about 1 decade and commonly require dilution/concentration. This paper provides supporting data for both structural determination and quantitation over these concentration ranges.

Accurate Secondary Structure Quantitation and Fingerprinting

Secondary structure composition can be accurately measured using existing automated analysis routines. In addition, since the amide I spectral signature is dependent on secondary structure, it can be used to assure protein structure has not changed (similarity). This paper provides supporting data for both conformation and similarity analysis.

Protein Stability and Aggregation

Both chemical and thermal denaturation measurements can be performed over a wide concentration range. Unlike far UV-CD, mid-IR is very sensitive to beta sheet structures and can even distinguish between the beta sheet of native proteins and the intermolecular beta sheet of the aggregated species. In this paper, we show measurements of both chemical and thermal stability as well as aggregation, and contrast the enhanced performance of RedShiftBio's MMS analyzer relative to UV-CD with its more limited capability.

Introduction

RedShiftBio's MMS analyzer combines laser spectroscopy, microfluidics and signal processing to provide a powerful, efficient tool that uses mid-infrared to directly probe the protein backbone and provide information on higher order structure, aggregate formation, protein stability and concentration. Unlike other techniques, proteins can be measured over a broad concentration range with relative ease in a variety of environments without the issues of background fluorescence and light scattering, and complications related to protein size.

The analyzer uses a tunable mid-infrared quantum cascade laser to generate an optical beam, which is focused through a microfluidic transmission cell of an approximately 25 um optical path length. The laser is run in continuous wave mode which produces a very high resolution (< 0.001 cm^{-1} linewidth), low noise beam with very low stray light that minimizes measurement error and expands the range of concentrations that can be directly measured. The sample (protein) solution and a matching water/buffer reference stream are introduced into the transmission cell as a continuous flow. The two streams are rapidly modulated (1-10 Hz) across the laser beam path to alternately measure the reference stream and the sample stream to produce a differential measurement (Microfluidic Modulation Spectroscopy, or MMS). This produces a drift-free, accurate, high sensitivity absorption measurement. After passing through the microfluidic cell, the beam is focused onto a thermo-electrically cooled MCT detector. The entire optical system is sealed and purged with dry air to minimize interference from the strong absorption lines of atmospheric water vapor. Data was acquired and processed using RedShiftBio's data acquisition system and software. All measurements were taken at room ambient temperature (~21°C) with no temperature control of the fluids or measurement cell. A simplified block diagram of the instrument is shown in Figure 1.

Protein's absorption spectra were measured through optically scanning across the amide I band, from approximately 1700 to 1600 cm^{-1} in 4 cm^{-1} increments, to generate the sample absorption spectrum. The amide I band directly measures the strength of the carbonyl band along the protein backbone and is very sensitive to the local environment which reflects the protein sub-structure type and state of aggregation.[1] The data in Figure 2 show the absorption spectra from four commercially available proteins. The spectrum for each protein can be automatically analyzed to provide information including protein concentration and secondary structure. Changes in the adsorption band can also be used to monitor protein stability and aggregation.

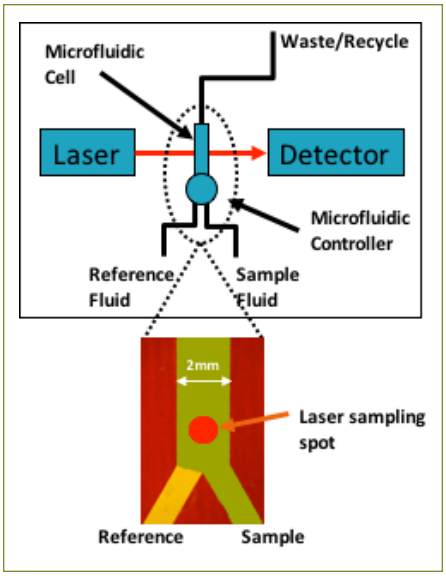

Figure 1. Simplified block diagram of the protein analyzer shows the tunable laser which probes the protein solution through a microfluidic cell. The microfluidic cell rapidly alternates between sample and reference (buffer) streams to continuously refresh the instrument referencing to dramatically improve measurement precision, accuracy, and signal-to-noise.

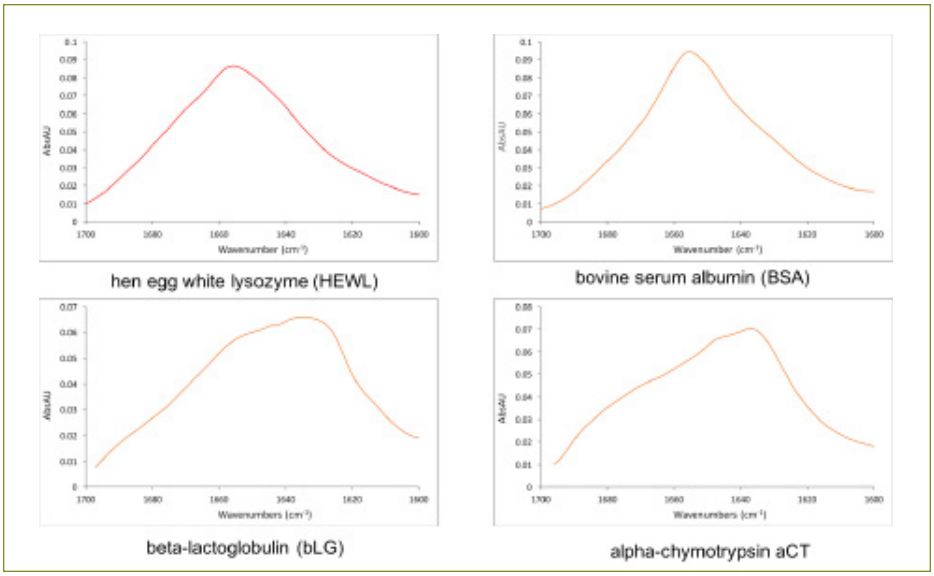

Figure 2. Representative measurements of commercially available proteins made with RedShiftBio's MMS analyzer at 10 mg/mL. The laser scans the amide I band and directly probes the protein backbone. The shape of the band reveals the protein sub-structure making it a powerful tool for protein characterization.

Higher Order Structure

Vibrational spectroscopy has long been recognized as a powerful tool in the study of protein and peptide substructure.[2] The amide I band (1700 – 1600 cm^{-1}) probes the C=O stretch vibration of the peptide linkages which constitute the backbone structure of the protein.[3] The differing pattern of hydrogen bonding, dipole-dipole interactions, and the geometric orientations in the α-helices, β-sheets, turns, and random coil structures induce different absorption features in the amide I band that are well correlated with these second order structures.[4] An analysis of the absorption spectrum can be used to quantitatively determine the relative amounts of these substructures[5] which can then be used as a powerful probe for protein characterization, chemical and thermal stability,[6] and protein aggregation.[7] Despite the power of the analytical technique, measurement capabilities are typically limited to concentrations above about 10 mg/mL for Fourier Transform Infrared Spectroscopy (FTIR) and 30 mg/mL for Raman.[8] Ultraviolet Circular Dichroism (UV-CD), currently one of the more prevalent tools for secondary structure analysis, is relatively insensitive to beta sheet formation and has difficulty detecting the very important intermolecular beta-sheet structures which form during aggregation.[8] While UV-CD operates at a lower concentration range than FTIR (typically ~ 0.2 – 2 mg/mL versus ~ 10 – 200 mg/mL),[9] it is not capable of directly measuring the higher concentration ranges typically encountered in formulation. The RedShiftBio's analyzer, however, is capable of measuring protein structure over a very wide dynamic range, from 0.1 mg/mL to over 200 mg/mL. This avoids the need for sample preparation steps such as dilution or pre-concentration which may introduce variability across samples, thus requiring sample replicates and multiple measurements. Perhaps even more importantly, the analyzer enables measurement of the protein at the actual concentration of interest, whether in discovery or formulation. These are capabilities not found in today's protein measurement tools.

The calculation of protein secondary structure for alpha-chymotrypsin was performed on proteins using an automated fitting method of analysis. Figure 3 shows the analysis of alpha-chymotrypsin with good reproducibility over a concentration range from about 0.1 to 10 mg/mL. These results demonstrate good accuracy, agreeing with FTIR methods as well as published values from X-ray and UV-CD.

Figure 4 demonstrates the repeatability of RedShiftBio's analyzer, with multiple measurements of lysozyme (HEWL) taken over a one-month period, with secondary structure results exhibiting a standard deviation of about 1%.

While most technologies are restricted to perform protein analysis at concentration ranges of about one order of magnitude, measurements taken using RedShiftBio's MMS analyzer can be performed at concentration ranges spanning more than three orders of magnitude. Figure 5 shows an example of the analysis from measurements of bovine serum albumin at concentrations ranging from 0.1 to 200 mg/mL resulting in five secondary structure components useful in protein fingerprinting.

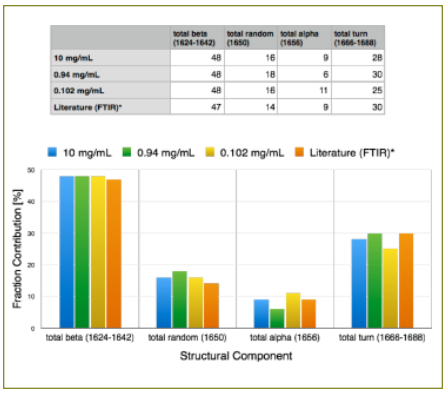

Figure 3. Secondary structure for alpha-chymotrypsin measured over 2 orders of magnitude of concentration (0.1 to 10 mg/mL). Results agree with conventional FTIR results (likely Dong paper, 20 or 30mg/mL).

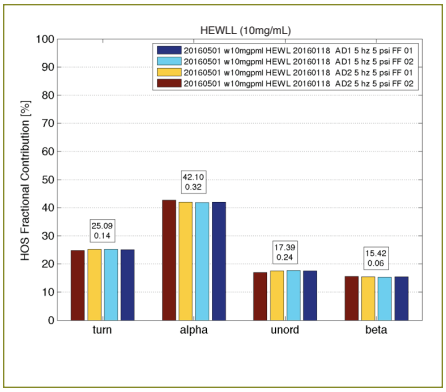

Figure 4. Secondary structure for hen egg white lysozyme (HEWL) from 7 separate measurements of 10 mg/mL samples, taken over one month, showing standard deviation of about 1%.

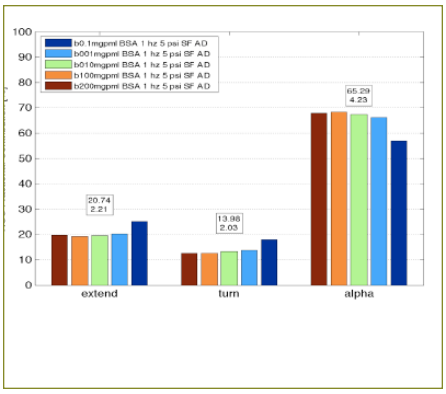

Figure 5. RedShiftBio system measurements of protein secondary structure of Bovine Serum Albumin over a range of concentration from below 0.1 mg/mL to 200 mg/mL showing good analysis results over three orders of magnitude in concentration.

Stability and Aggregation

Since the RedShiftBio analyzer can directly measure protein secondary structure, it is a powerful tool for monitoring and understanding the mechanisms of protein stability and aggregation. As proteins are subjected to stress and the protein begins to change from it's native state, details of the process can be easily followed. IR measurements are particularly sensitive to beta sheet structures, which dominate in protein anti-body based drugs. In addition, it is one of the only techniques which can directly monitor the formation of aggregates due to its ability to measure intermolecular beta sheet structures. Below we illustrate the use of the analyzer for two common types of stability studies, thermal and chemical.

Thermal Stability

A high beta sheet content protein at 1 mg/mL was incubated at an elevated temperature for differing periods of time. The protein type and detailed experimental conditions are not provided due to the proprietary nature of the customer provided samples. The protein series was measured using the MMS analyzer and the second derivative spectra were overlaid and plotted to enhance the spectral changes. The data shown in Figure 6 clearly show the loss of intramolecular beta sheet content as a function of incubation time. Simultaneously, the amount of intermolecular beta sheet structure increases, which is associated with the formation of aggregates. Changes in other regions reflect the state of the protein sub-structure and provide additional details of the denaturation process.

As the protein was incubated, it was noted an insoluble aggregate formed and settled to the bottom of the sample tubes. In this study, only the supernatant fraction was decanted and measured. As a result, the overall concentration of soluble protein decreases at longer incubation times. This is illustrated in Figure 7, which shows the amide I absorbance decrease to less then half of its initial value which corresponds to less then 0.5 mg/mL of protein in solution. Changes in the spectral shape of the amide I band are also clearly evident in the absorption data.

Chemical Stability

Chemical stress studies are another method of studying protein stability. Alcohols are well known to denature the native state of proteins and also tend to stabilize the alpha-helical conformation in unfolded proteins and peptides.[10] In this study a relatively high concentration of beta lactoglobulin was formulated in phosphate buffer at pH 7.4 at 0, 20, 40 and 60% isopropyl alcohol (IPA) concentration. The IPA/protein series were then measured by both Far UV-CD and MMS to track the structural changes. In the UV-CD data, shown in Figure 8, a clear increase in the alpha helix structure occurs while a general decrease in beta sheet occurs.

In contrast, the RedShiftBio data of Figure 9 shows not only an increase in the alpha helix form at higher IPA concentration, it also shows a dramatic and clear shift in the beta sheet type as noted by the shift of the band from ~1630 cm^{-1} to 1620 $cm,^{-1}$ again indicating the formation of intermolecular beta sheet, something the CD does not readily show. Not only does RedShiftBio's MMS analyzer provide greater insight into the denaturation process but it operates over a much wider range of concentrations.

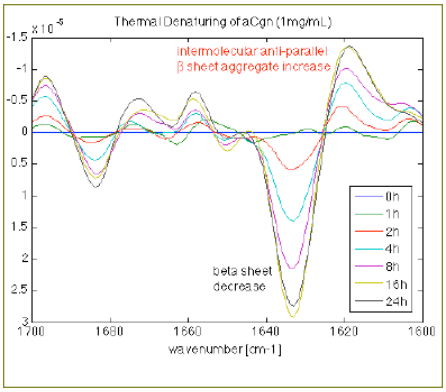

Figure 6. Incubation of a 1 mg/mL high beta sheet containing protein incubated at elevated temperature from 0 to 24 hours. As the incubation time increases the (intramolecular) beta sheet content decreases and the intermolecular beta sheet increases, indicative of aggregate formation.

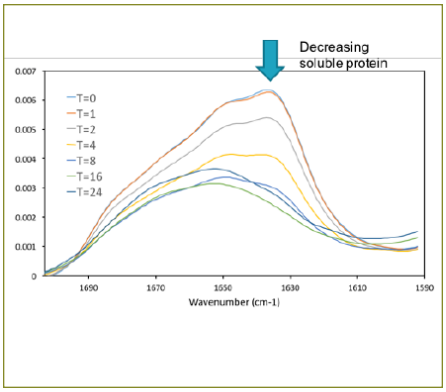

Figure 7. As the protein was denatured insoluble aggregate formed and precipitated out of solution. As only the supernatant of the sample was measured the intensity of the amide I band decreases, which is a direct indicator of soluble protein concentration.

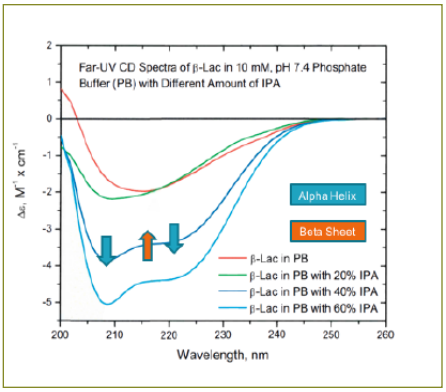

Figure 8. Far UV-CD studies of the chemical denaturation of beta lactoglobulin in IPA show increasing alpha helix and decreasing beta sheet.

Protein Similarity

Protein similarity is a quantitative approach for detecting small changes in protein secondary structure by analyzing and comparing the amide I band spectra between proteins. As the amide I band is very sensitive to changes in protein secondary structure as discussed previously, the ability to measure small differences in the spectra can be a powerful tool in monitoring the biosimilarity of a protein.[11, 12] A number of algorithms have been proposed for this comparison, including the correlation coefficient and the area of overlap.[11, 12] For the purposes of this paper, the area of overlap method was used to calculate protein similarity. These results can be compared to published results using other methods to assess the sensitivity of the MMS method relative to more traditional methods such as FTIR or UV-CD.

Figure 10 shows the overlaid spectra of BSA at four concentration levels (0.1, 1.0, 10 and 200 mg/mL) as acquired by the MMS analyzer. In the middle concentration range, similarity exceeds 97% with a drop off at the high and low ends of the range

For comparison, FTIR values found in the literature[12] show a mean similarity of 86.37% +/- 7.98% at a single concentration of 10 mg/mL for HEWL. Using FTIR, protein similarity values at the 97% level could only be obtained at a concentration of 50 mg/mL. Thus, RedShiftBio MMS analyzer achieves better similarity with less deviation over a concentration range that far exceeds the measurement capability of FTIR, while also addressing the limitations of UV-CD at higher concentrations that may require additional workflow and dilutions.

Quantitation

Protein quantitation is critically important in biochemistry research and development labs in applications ranging from enzymatic studies to providing data for biopharmaceutical lot release. Direct assay measurements include UV and visible absorption measurements relative to a standard using extinction coefficients or indirect measurements using dye base assays such as BCA, Lowry, and Bradford assays. No one approach is universal due to the specific limitations of each approach, including aromatic residue dependency, chemical interferences in dye-based assays and the limited dynamic range of the spectroscopic tool.[13] One of the biggest limitations of these measurements comes from the spectroscopic tools themselves. Conventional spectrometers have limited linearity primarily due to stray light and the instrument slit width (resolution) as well as detector linearity. As such, the sample absorbance is targeted to a very limited dynamic range, typically between .1 and 1.5 au.[14] This limited range forces scientists to adjust either the sample concentration or the cell path length to acquire accurate protein quantitation. Either alternative can be time consuming and problematic in its effect on the measurement.

Infrared absorption spectroscopy can be an effective tool for direct, label free protein quantitation. It provides an advantage over UV/VIS methods as sample absorption bands in the infrared are much narrower and are not dependent on aromatic residues.

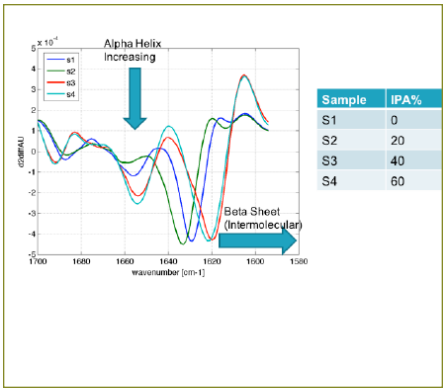

Figure 9. Protein characterization results obtained using RedShiftBio's MMS analyzer not only show the expected increase in alpha helix with higher alcohol concentrations, but also shows a shift in beta sheet to the aggregate form of intermolecular beta sheet.

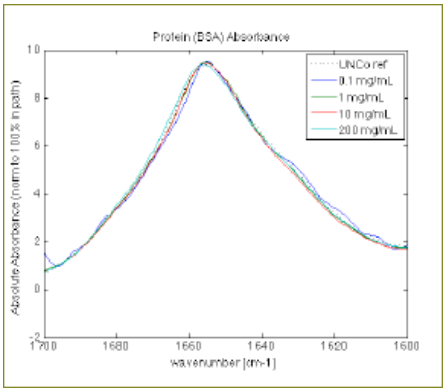

Figure 10. Protein similarity of BSA is shown over the range from 0.1 mg/ml to 200 mg/ml, again demonstrating the viability of the measurement technique to compare protein characteristics across multiple steps of protein development.

As a result, the technique is more selective with less susceptibility to interferences. In addition, since the IR method probes the carbonyl backbone of the protein and is not dependent on a UV chromophore, the variation extinction coefficient is much smaller which can be an advantage in measuring unknown proteins. However, IR spectroscopy has not been routinely used in situ due to its lower sensitivity, added cost, and difficulty of operation (i.e. background subtraction, water vapor interference, and narrow pathlength cells). RedShiftBio's MMS platform overcomes these issues by increasing sensitivity and significantly reducing the errors common to conventional spectroscopy. MMS's high resolution (<0.001 cm^{-1}) and low stray light susceptibility increases the linear concentration range for the measurement by more than 2 orders of magnitude. The differential measurement of microfluidic modulation spectroscopy and direct control over laser power also improves linearity by reducing signal dynamic range and maintaining high detector linearity throughout the measurement range.

Using MMS, one or at most only a few wavelengths need to be measured. Figure 11 shows a plot at ~1656 cm^{-1} for BSA in the range from 0.1 to 200 mg/mL. With a minimum measurable concentration of less than 10 ·g/mL (3 sigma, HEWL) and an upper limit of greater than 200 mg/mL, this offers a significant improvement over conventional absorbance based assays.

Conclusion

RedShiftBio's MMS analyzer provides a fast, simple system for the characterization of proteins over a broad concentration range to simultaneously deliver information on secondary structure, stability, aggregation, similarity, and quantitation. The microfluidic referencing enhances the reproducibility of the method and simplifies the workflow by eliminating separate buffer measurements. The analyzer can be a powerful and versatile tool for direct, label-free characterization of proteins through all phases of biologic drug development, from discovery through formulation and manufacturing.

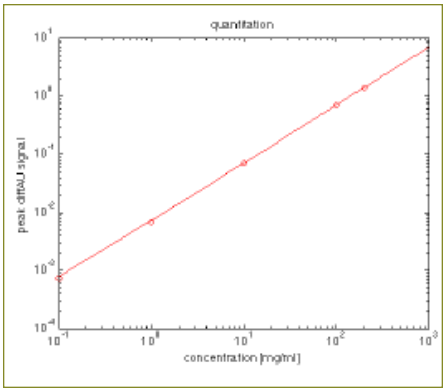

Figure 11. Differential absorbance at ~1656 cm-1 (BSA peak) plotted as a function of concentration at 0.1, 1, 10, and 200 mg/mL.

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