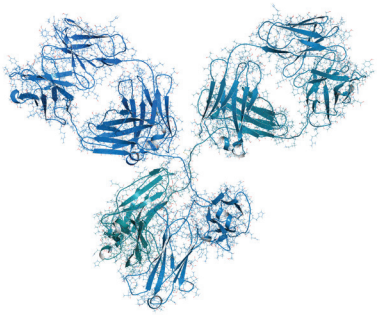

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Shaping IR Spectroscopy into a Powerful Tool for Biopharma Characterizations

DIPANWITA BATABYAL, LIBO WANG, JEFFREY ZONDERMAN, AND MATS WIKSTRÖM

ABSTRACT

Microfluidic modulation spectroscopy (MMS) is a novel automated infrared spectroscopic technique with high sensitivity and repeatability. Here, the authors present a series of experimental studies showcasing the performance of MMS in the secondary structure characterization of biopharmaceutical products and compare the MMS results with the conventional Fourier transform infrared data.

The successful development of biopharmaceuticals involves the study of their higher order structure, a critical quality attribute, to ensure a therapeutically active molecule in appropriate formulation conditions (1–3). Robust structural characterization of the biopharmaceutical products is important throughout the development process. For instance, comparability studies are performed to ensure that a manufacturing process change during clinical and commercial development does not have an adverse effect on quality, safety, and efficacy (4–6).

Infrared (IR) spectroscopy is a powerful method for characterizing the secondary structure of proteins (7–14). However, the lack of automation of conventional Fourier transform IR (FTIR), along with relatively high sample concentration requirements, are major limitations with this technology. Far ultraviolet circular dichroism spectroscopy (far-UV CD) is an important alternative for the characterization of secondary structure, but it also has major drawbacks. Measurement is necessarily carried out at low concentrations, typically at 0.5 mg/mL but down as low as 0.1 mg/mL, which can undermine the relevance of the resulting data. The presence of certain excipients in the formulation buffer can also significantly interfere with the measurements. Furthermore, far-UV CD and conventional FTIR have been shown to lack sensitivity in the characterization of biopharmaceuticals proteins (e.g., immunoglobulin G1 [IgG1] and IgG2) (15).

Microfluidic modulation spectroscopy (MMS) represents a novel automated technique that directly addresses the current limitations with both conventional FTIR and far-UV CD by shaping IR spectroscopy into a far more effective analytical tool in biopharmaceutical product characterization (16). This article presents a series of experimental studies showcasing the performance of MMS applied to challenges in the characterization of the secondary structure of biopharmaceutical products including comparisons with conventional FTIR data.

MATERIALS AND METHODS

Conventional FTIR and MMS were used to determine the secondary structure of two biopharmaceutical samples: a monoclonal antibody (mAb) and a bispecific T cell engager (BiTE, a registered trademark of Amgen) (17) molecule (Amgen, Thousand Oaks, US). The BiTE molecule represents a fusion protein, created by linking the variable light and heavy chain corresponding to two antibodies. Polysorbate (PS) 80 was purchased from Fluka (Cat#: 59924-100G-F Lot: BCBC1232).

Conventional FTIR measurements were carried out using a Bruker Vertex 70 spectrometer equipped with an Aquaspec transmission cell that requires manual injection of the sample and reference buffer at room

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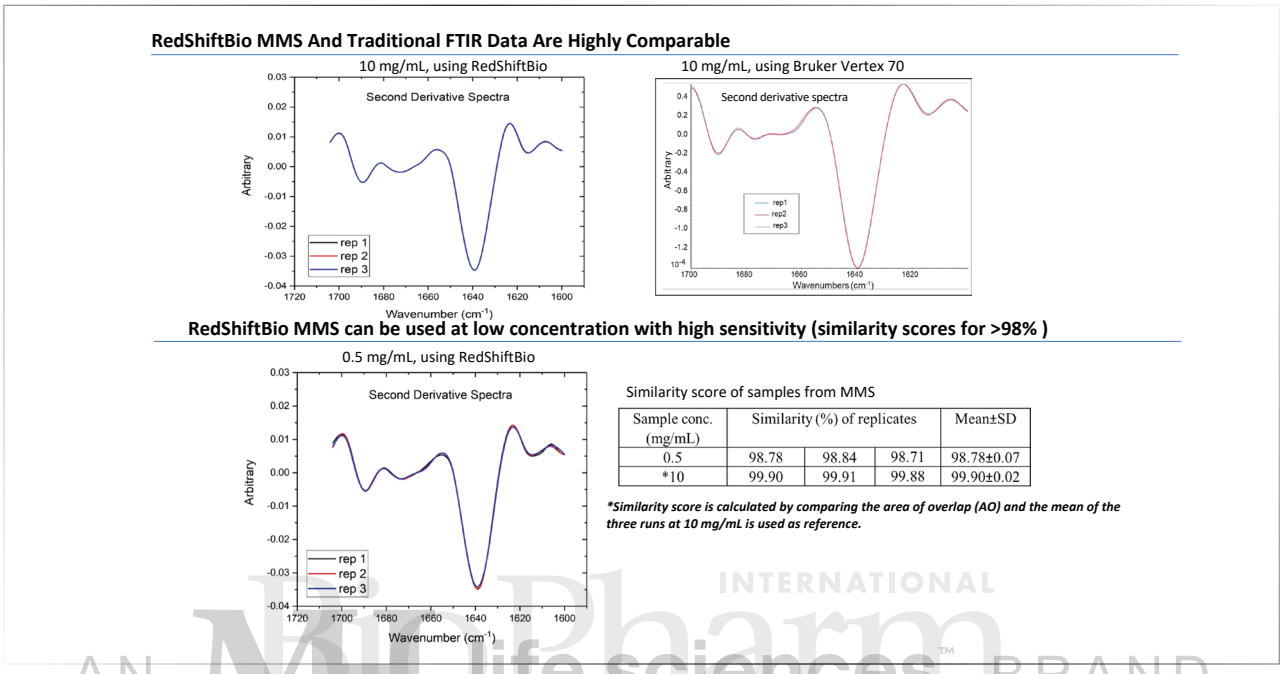
Figure 1. Conventional Fourier transform infrared (FTIR) and microfluidic modulation spectroscopy (MMS) measurements for a monoclonal antibody are highly comparable at a concentration of 10mg/mL. Unlike conventional FTIR, MMS can also measure with high sensitivity at a concentration of 0.5 mg/mL. Acceptable quality data (similarity score >95%) is not possible at 0.5 mg/mL using conventional FTIR.



temperature. The reference spectra for buffer blank were subtracted from the protein spectra according to previously established criteria (18). Spectral similarity was quantitatively determined using the Thermo OMNIC software quality control (QC) compare function.

MMS measurements were conducted at ambient temperature using an AQS3pro system (RedShiftBio, Burlington MA, US) with multi-sample automation. A microfluidic transmission cell of approximately 24 µm pathlength was used. Streams of protein samples and reference buffers were introduced into the flow cell alternatively at a back pressure of 5 psi and a modulation rate of 1 Hz. Simultaneous modulation of the sample and an appropriate buffer enabled a real-time subtraction of the buffer background and allowed the differential absorbance measurement. Thirty-one discrete wavenumbers across the amide I band from 1590 cm^{-1} to 1714 cm^{-1} were scanned, and the differential absorbance spectra of samples were collected. Triplicate measurements were carried out for each sample. The data were analyzed using the software AQS^3 delta analytics package to produce the final spectra and analysis results. For each experiment, interpolate differential absorbance spectra, absolute absorbance spectra, and the second derivative spectra were obtained, and the similarity score was calculated using the area of overlap in the amide I band region (1700 cm^{-1}–1600 cm^{-1}).

RESULTS

Case 1: testing instrument sensitivity

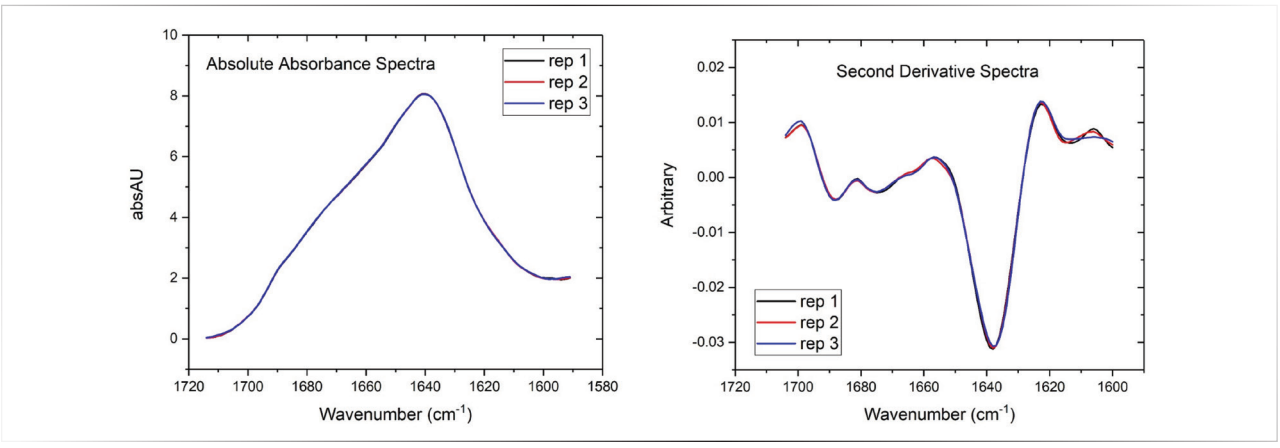
This section discusses testing instrument sensitivity at different protein concentrations and with different modalities.

To compare the relative sensitivity of MMS and conventional FTIR, mAb samples were analyzed using the two methods at concentrations of 0.5 mg/mL and 10 mg/mL in acetate buffer. At 10-mg/mL concentration, the data from MMS matched well with the conventional FTIR. Both techniques showed high repeatability quantified by high spectral similarity scores (> 99% for both, see **Figure 1**). The spectral similarity in the conventional FTIR was calculated using the QC compare function from the OMNIC software. The spectral similarity from MMS data was calculated by comparing the area of overlap of each sample replicate to the mean area of overlap of all three replicates. In general, there was very good agreement in similarity scores between the two methods. The MMS data were further analyzed using the QC compare function from OMNIC, and the results showed consistency between the similarity scores obtained by the two approaches.

At lower concentrations of the mAb (0.5 mg/mL), acceptable quality data (> 95% similarity score between the replicates) were not obtained by conventional FTIR, whereas the MMS data showed high repeatability at low concentrations, indicated by the high similarity scores (> 98%) between the three replicate runs. The similarity score in MMS is calculated by comparing the area of overlap (AO) of the replicates using mean AO of the three replicates as reference.

ALL FIGURES COURTESY OF THE AUTHORS.

Figure 2. Microfluidic modulation spectroscopy data for the BiTE sample show excellent repeatability at low protein concentration (1mg/mL). Panel on the left shows overlaid absolute spectra, panel on the right shows overlaid second derivative spectra.



A BiTE molecule sample was analyzed at 1.0 mg/mL to further assess the sensitivity of MMS at concentrations lower than those accessible with conventional FTIR, with a different modality. As shown in **Figure 2** (left panel), the absolute absorbance spectra generated through triplicate measurements are almost indistinguishable, indicating high repeatability of the MMS measurements. The second derivative spectra of the three replicates (**Figure 2**, right panel) overlay very well, indicating the high consistency between measurements further quantified by comparing the similarity score. Overall the second derivative spectrum exhibits a strong β-sheet peak at around 1639 cm^{-1} together with a β-sheet peak at 1689 cm^{-1}. The similarity score of three replicates for the BiTE molecule are all > 99% (**Table I**), indicating high repeatability between the runs. The contributions from the different secondary structure elements (referred to as the higher order structure analysis, or HOS analysis) were further determined using Gaussian peak assignment from known correlations with absorption at specific wavenumbers within the amide I band (19). As shown in **Table II** the BiTE molecule antibody consists predominantly of β-sheets (58.67 ± 0.80%) along with some contributions from β-turns (31.38 ± 0.48%).

Case 2: buffer excipient

This section assesses the impact of the buffer excipient on secondary structure.

To investigate the impact of buffer composition and different excipients, an MMS test of a mAb sample (5 mg/mL) was carried out and analyzed in three different buffers—Buffer A, B, and C—with the same base composition but with different amounts of polysorbate (PS) 80 (0.01%, 0.05%, and 0.1% w/v, respectively).

As shown in **Figure 3**, the absolute absorbance spectra and the second derivative spectra of the three replicates are very closely matched, suggesting that different amounts of PS 80 have no effect on the secondary structure of the mAb. Similarity scores of the three replicates in all three buffers are > 99% (**Table III**), indicating high repeatability. In **Table IV,** the HOS analysis shows that the secondary structure of the mAb consists predominantly of β-sheets (approximately 61.5%), with β-turns at approximately 29%. When compared to the BiTE molecule HOS analysis (**Table II**), there is a relative higher proportion of β-sheet content and a lower proportion of β-turns in the secondary structure analysis of the mAb samples.

Table I. Similarity scores of the BiTE sample measured by microfluidic modulation spectroscopy. SD is standard deviation.

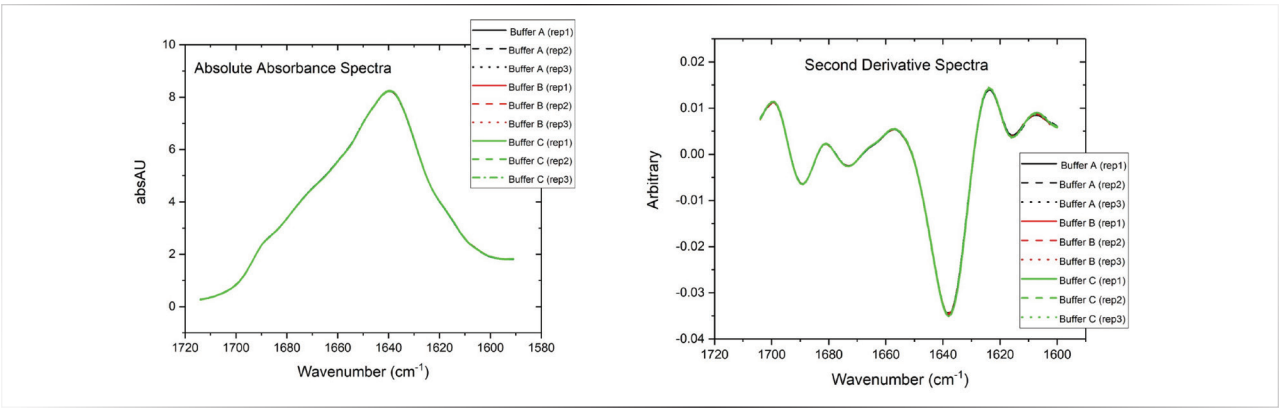
Sample conc. (mg/mL)	Similarity (%) of replicates			Mean±SD
1.0*	99.01	99.20	98.97	99.06±0.12

*Similarity score is calculated by comparing the area of overlap (AO), and the mean AO of the three replicates is used as the reference.

Table II. Higher order structure (HOS) contents (%) of the BiTE molecule sample determined by microfluidic modulation spectroscopy.

Sample conc. (mg/mL)	HOS% (mean±SD) of replicates			
	Beta	Turn	Unordered	Alpha
1.0	58.67 ± 0.80	31.38 ± 0.48	7.64 ± 0.78	2.32 ± 0.36

Figure 3. Microfluidic modulation spectroscopy data of monoclonal antibody samples with different amounts of polysorbate 80 (PS 80) in buffer. Buffer A: 0.01% (w/v) PS 80, Buffer B: 0.05 % (w/v) PS 80 and Buffer C: 0.1 % (w/v) polysorbate 80. The left panel shows the overlaid absolute spectra and the right panel shows the overlaid second derivative spectra.



Case 3: higher protein concentrations

This section discusses test consistency, precision, and accuracy of MMS at higher protein concentrations.

In this experiment, the same mAb sample that was used in the case 2 study was analyzed by MMS at concentrations of 50 mg/mL and 100 mg/mL in the base buffer without any PS 80. Tests were performed on different days, and the resulting data were compared to check for consistency and precision of MMS measurements.

The similarity scores are shown in **Table V** and were calculated using mean AO of a 50 mg/mL sample as reference. Overall, > 99% similarity was observed at all protein concentrations (ranging from 5 mg/mL to 100 mg/mL) indicating high repeatability of the MMS measurements. The high consistency of measurement is retained even though measurements are made over multiple days. The data further confirmed that neither the protein concentration nor the buffer excipient PS 80 impacts the secondary structure of the mAb. Further HOS analysis giving the secondary structure component also reflects the high consistency that was observed in the similarity score data (data not shown).

DISCUSSION

The criticality of HOS makes measurement essential throughout biopharmaceutical development. Robust formulation and process development relies on measuring the impact of concentration—of different buffers and of processing conditions as a drug candidate proceeds toward commercialization—with the different types of therapeutic molecules that are increasingly part of the new drug pipeline. All analytical techniques have strengths and limitations when assessed against this informational need. For example, unlike conventional FTIR or near-UV CD, MMS is not a general-purpose platform at this time. MMS has been optimized

Table III. Similarity scores of the monoclonal antibody sample in different buffers measured by microfluidic modulation spectroscopy. SD is standard deviation.

Samples @ 5 mg/mL	Similarity (%) of replicates			Mean±SD
In Buffer A*	99.62	99.79	99.76	99.72 ± 0.09
In Buffer B	99.76	99.64	99.52	99.64 ± 0.12
In Buffer C	99.66	99.55	99.69	99.63 ± 0.07

*Similarity score is calculated by comparing the area of overlap (AO), and the mean AO of sample replicates in Buffer A is used as the reference. Buffers A, B, and C contain 0.01%, 0.05%, and 0.1% (w/v) polysorbate 80, respectively, in the same base buffer.

Table IV. Higher order structure (HOS) contents (%) of the monoclonal antibody sample in different buffers determined by microfluidic modulation spectroscopy. SD is standard deviation.

Samples in	HOS% (Mean±SD) of replicates			
	Beta-sheets	Beta-turn	Unordered	Alpha-helix
Buffer A	61.41±0.09	29.40 ± 0.11	6.90 ± 0.01	2.29 ± 0.03
Buffer B	61.67±0.15	29.22 ± 0.06	6.87 ± 0.10	2.24 ± 0.10
Buffer C	61.63±0.13	29.29 ± 0.07	6.80 ± 0.10	2.27 ± 0.15

Table V. Similarity scores of a monoclonal antibody sample analyzed in different buffers and at different concentrations. PS 80 is polysorbate 80. SD is standard deviation.

Sample Conc.	Buffers	Similarity (%) of replicates			Mean±SD
50 mg/mL*	Base Buffer (no PS 80)	99.45	99.75	99.60	99.60 ± 0.15
100 mg/mL	Base Buffer (no PS 80)	99.37	99.36	99.37	99.37 ± 0.01
5 mg/mL	Buffer A (0.01% PS 80)	99.30	99.31	99.17	99.26 ± 0.08
5 mg/mL	Buffer B (0.05% PS 80)	99.18	99.28	99.06	99.17 ± 0.11
5 mg/mL	Buffer C (0.1% PS 80)	99.36	99.26	99.23	99.28 ± 0.07

*Similarity score is calculated by comparing the area of overlap (AO) and the mean AO of 50 mg/mL sample replicates, which were used as the reference. Buffers differ in the amount of PS 80 in the same base buffer.

for all types of protein- and peptide-based secondary structural analysis, which is of interest in biopharmaceutical development and manufacture, but not other structural features (i.e., tertiary) or molecule types.

In contrast, the application of multiple techniques to characterize HOS, a necessity when combining conventional FTIR and far-UV CD to cover the range of conditions of interest, is inherently problematic. Such an approach can introduce uncertainty, where there is overlap between techniques and discrepancies in the results produced as well as complicating analytical workflows. Any requirement for sample preparation can also undermine data integrity because proteins are labile, changing in response to their local environment. The adoption of techniques that can be applied directly, to a broad range of sample types, is therefore technically advantageous.

The results from this study clearly demonstrate the performance of MMS. A direct comparison with conventional FTIR illustrates a number of ways in which MMS is a superior presentation of IR spectroscopy for this application, while the ability to measure with high sensitivity and precision at high concentrations and in the presence of different buffers highlights the potential of MMS relative to far-UV CD.

The data show that MMS allows the determination of secondary structure over a much wider concentration range than conventional FTIR, thus removing the requirement of either dilute or concentrated samples for measurement. In this study, MMS measurements were successfully made across a concentration range from 0.5 mg/mL to approximately 100 mg/mL. In contrast, conventional FTIR measurements require a minimum concentration of approximately 10 mg/mL to acquire data of acceptable quality.

The capability to measure at low concentrations means that MMS is not limited to studies of mAbs, but can also be applied to other protein therapeutic modalities, such as BiTE molecules, which are typically measured at product concentrations below the minimum required for conventional FTIR. For low-concentration measurements, far-UV CD would typically be the technique of choice, but it can be unreliable for formulations containing chromophores other than those associated with the drug entity, necessitating filtration or dilution of the sample prior to the measurement. The data showing the repeatability of mAb measurements in solutions with different buffer concentrations are helpful in demonstrating the ability of MMS to address this limitation.

Finally, the results show that MMS data are highly repeatable with high precision, unlike conventional FTIR, which routinely exhibits instrument drift. This characteristic is due to the way in which MMS generates differential data via a process of continuous auto-referencing that eliminates the issue of background drift. High repeatability contributes directly to the ability of a technique to detect difference and indicates that MMS will exhibit greater sensitivity to changes in protein structure.

CONCLUSION

MMS is a powerful new technique for the assessment of the secondary structure of proteins. The results presented here show how it enables accurate, highly repeatable characterization across a wider concentration range than conventional FTIR, and measures with high sensitivity with different buffers. These capabilities offer potential to streamline the routine analysis associated with biopharmaceutical development for various protein therapeutic modalities, including mAbs and BiTE molecules.

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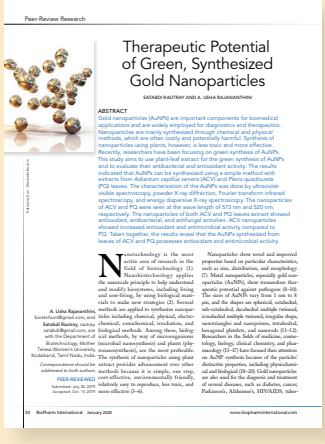
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