Application Note
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Characterization of Three USP Reference Standards using Microfluidic Modulation Spectroscopy

- Biosimilars
- ☑ mAbs
- ADCs
- AAVs
- Ligand Binding
- Protein/Peptide Analysis
- VLPs
- Nucleic Acid
- Fusion Proteins
- Enzyme Analysis

- Aggregation
- ☑ Quantitiation
- ☑ Structure
- Stability
- ☑ Similarity

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Introduction

In biologic drug production, monitoring critical quality attributes (CQAs) is paramount to high-quality products. These CQAs are physical attributes that should be measured and monitored throughout the drug development process to ensure the end product is exactly as designed. Some common CQAs are primary sequence, molecular weight, charge, aggregation, and higher order structure (HOS) variants.[1] CQAs need to have specific passing criteria with clearly defined metrics, therefore, it is necessary to have tools that are validated with known reference standards and can give highly sensitive and quantitative data.

Microfluidic Modulation Spectroscopy (MMS) is a novel technique for measuring HOS that is 30 times more sensitive than traditional techniques like FTIR.[2] The increase in sensitivity comes from the introduction of a light source that is 1,000 times more intense than traditional light sources, and from our innovative microfluidic flow cell that allows real-time background subtraction. These innovations yield a 0.76% limit of quantitation[2] and the ability to analyze samples in complicated formulation buffers without interference,[3] meaning that biologic drug products can be run without changing the formulation conditions to get a sensitive reading of HOS.

In this study, we tested three reference standard monoclonal antibodies (mAbs) from U.S. Pharmacopeia (USP), a trusted supplier of high-quality biomolecular standards for over 200 years. The three mAbs tested are expected to have similar overall structures as they are all the same IgG1 subtype, but they have unique antigen binding regions, so subtle differences in structure are expected. In addition to testing the reference standards neat (10 mg/mL in formulation buffer), we also tested a diluted condition (2 mg/mL in the same formulation buffer) to highlight the ability to test low concentrations.

Furthermore, we also tested the antibodies with and without dialysis, to investigate buffer matching.

Methods

I. Materials:

USP mAb 001 (cat# 1445539), 002 (Cat# 1445547), and 003 (cat# 1445595) were obtained and 1 L of matching buffer was prepared for each mAb according to Table 1 below. Dialysis was performed on half of each sample to determine if dialysis was necessary for sufficient buffer matching. Slide-A-Lyzer cassettes (Pierce®) with a 10 kDa molecular weight cut off were used to facilitate dialysis.

Sample	Buffer components
mAb 001	25 mM citrate, 0.9% sodium chloride, 0.07% PS 80, pH 6.5
mAb 002	0.58% monobasic sodium phosphate monohydrate, 0.12% dibasic sodium phosphate anhydrous, 6% a,a-trehalose dihydrate, 0.04% PS 20, pH 6.2
mAb 003	20 mM histidine HCl, 120 mM sucrose, 0.02% PS 20, pH 6.0

Table 1. mAb 001-003 buffer components

II. Sample Preparation (Dialysis):

Slide-A-Lyzer cassettes should be hydrated in the buffer that they will be submerged in before loading the sample inside, therefore, each mAb has its own cassette and the empty cassettes were allowed to float in the appropriate buffer for approximately one minute before loading the samples. One mL of each mAb was loaded with an 18-gauge syringe into its own Slide-A-Lyzer cassette (Figure 1A) and the filled cassette was left at 4°C overnight with gentle stirring to allow for the most efficient equilibration (Figure 1B). The following day, samples were retrieved from the cassettes with a fresh 18-gauge syringe. All samples were loaded in a 24 well plate in sample and reference buffer pairs for MMS testing.

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Methods, continued

III. MMS Testing:

All samples were run in triplicate on an AQS^{3}pro that was equipped with sweep scanning data collection and a 24-well plate. A backing pressure of 5 psi was used to move the samples into the flow cell where they were modulated at 1 Hz between sample and reference buffer (using the same buffer from the dialysis) for background subtraction. The differential absorbance was measured between 1588-1711 cm^{-1}. Replicates were averaged and all samples were normalized for concentration and interpolated to get the absolute absorbance spectra.

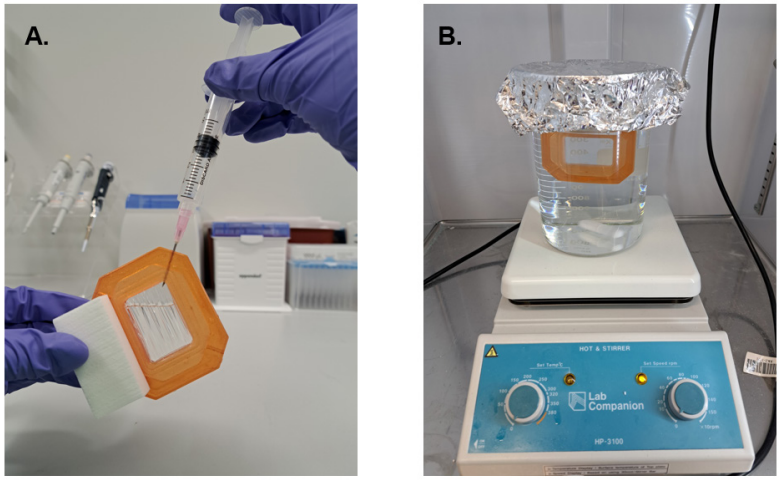

Figure 1. A) Filling the Slide-A-Lyzer cassette with an 18-gauge syringe and B) the overnight dialysis set-up.

Second derivatives were taken to enhance spectral features. This plot was then inverted and baselined to result in the "similarity plot" where the area of overlap is calculated compared to a control to quantitate similarity between samples. Finally, using Gaussian curve fitting, 14 Gaussians were curve-fit and the HOS was calculated based on the identification of different secondary structural motifs across the amide I band.

Results

I. High Precision Data on High-Concentration Samples:

All three mAbs were run against their matching reference buffer. The repeatability for each sample is shown in Table 2 and this represents the variation among triplicate measurements. All repeatability measurements are 99.9% showing high data quality and precision. When comparing each sample to mAb 001, we see a drop in similarity of about 7% and 8%, indicating detectable structural differences between the samples.

Sample	Repeatability (AO%) among triplicates	Similarity (AO%) compared to mAb 001
mAb 001	99.9	100
mAb 002	99.9	92.6
mAb 003	99.9	93.7

Table 2. mAb 001-003 repeatability among triplicate measurements and similarity

The spectra shown in Figure 2A are the plots used to generate the similarities listed in Table 2 and the HOS percentages in the bar graph in Figure 2B. MAb 001 has the highest fractional contribution from beta-sheets and turn structures, while mAb 002 has the most alpha-helix and unordered. These changes in structure are subtle but clearly distinguishable and not surprising considering these are all unique mAbs.

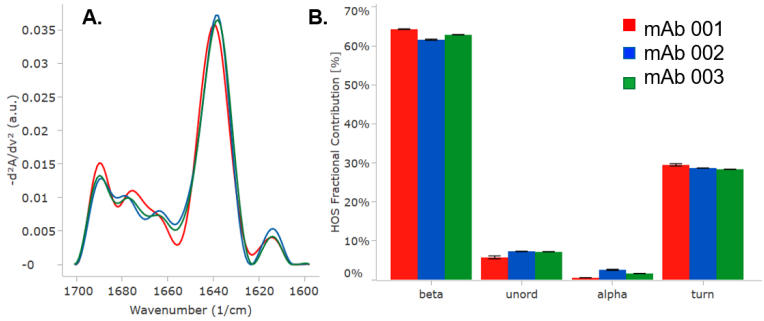

Figure 2. A) Similarity plots for 10 mg/mL samples of mAb 001, 002, and 003 in red, blue, and green, respectively. B) HOS fractional contribution bar graph showing the structural differences between each sample. The error bars represent +/- the standard deviation.

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Results, continued

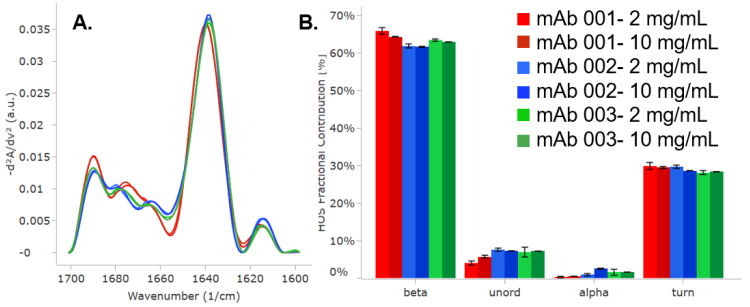
II. High Quality Data Maintained on Low-Concentration Samples:

In order to save material, we also tested 2 mg/mL of each mAb to see if we could have similar data quality at a fraction of the sample requirements. Table 3 shows that the samples maintain >99% repeatability even at 2 mg/mL. When comparing that to the 10 mg/mL samples, each one is above 98%, indicating no structural change occurs by changing the concentration.

Sample	Repeatability (AO%) of triplicates	Similarity (AO%) compared to the 2 mg/mL samples	Similarity compared to mAb 001
mAb 001 – 2 mg/mL	99	100	100
mAb 001 – 10 mg/mL	99.9	98.7	98.7
mAb 002 – 2 mg/mL	99.3	100	92.6
mAb 002 – 10 mg/mL	99.9	98.9	92.5
mAb 003 – 2 mg/mL	99	100	93.9
mAb 003 – 10 mg/mL	99.9	99	93.7

Table 3. Comparing 2 and 10 mg/mL mAbs 001-003. Repeatability is maintained above 99% and above 98% similarity between the different concentrations of the same mAbs.

Figure 3. A) Similarity plots for 2 mg/mL and 10 mg/mL samples of mAb 001, 002, and 003 in red, blue, and green, respectively and 10 mg/mL samples are the darker shades. B) HOS fractional contribution bar graph showing the structural differences between each sample. Error bars represent +/- the standard deviation.



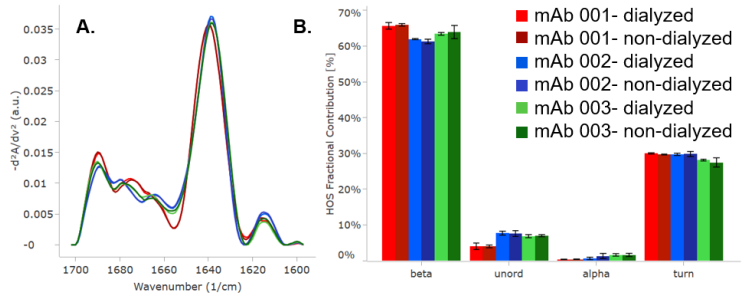
III. Importance of Buffer Matching:

With a technique as sensitive as MMS, the importance of buffer matching becomes paramount. The continuous real-time background subtraction used in MMS means that differences between the sample buffer and the reference buffer will be observed and could lead to interpreting differences in buffer composition as opposed to protein structure. To ensure we were looking at differences in protein structure, we compared the samples that underwent dialysis with the samples that were not dialyzed. Each non-dialyzed sample showed >98% similarity compared to the samples that was dialyzed to show that the buffer prepared was a perfect match to the buffer that the samples came dissolved in. Additionally, these three buffers are highly compatible with MMS and do not show interference.

Sample	% similarity compared to dialyzed
mAb 001 dialyzed	100
mAb 001 non-dialyzed	99
mAb 002 dialyzed	100
mAb 002 non-dialyzed	99.1
mAb 003 dialyzed	100
mAb 003 non-dialyzed	98.9

Table 4. Comparing samples that were dialyzed vs samples run without dialysis show no significant difference.

Figure 4. A) Similarity plots for 2 mg/mL samples dialyzed and not dialyze of mAb 001, 002, and 003 in red, blue, and green, respectively and neat samples are the darker shades. B) HOS fractional contribution bar graph showing the structural consistency whether the samples were dialyzed or not. Error bars represent +/- the standard deviation.



Conclusions

The three USP mAbs tested in this study are highly characterized for primary structure, size, charge, and quaternary structure, however, the characterization is lacking HOS such as secondary structure. Secondary structure is closely linked to protein function and is important to monitor throughout the drug-development process.

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Conclusions, continued

Therefore, it is critically important to have a tool that can monitor and detect small changes in structure, specifically in the native formulation conditions because structure can be affected by the buffer.[4] Other tools used for secondary structure such as CD, struggle with interference from the buffers used in this exercise and would require buffer-exchange into a more compatible buffer, not considering that the buffer can affect the structure.[5,6] In this study, secondary structure for each mAb was tested under neat formulation conditions and at more dilute concentrations to save material, highlighting some of features of testing with MMS. Our results show that each mAb has a unique secondary structure and can be easily quantified using our all-in-one delta analysis software. The distinct changes observed are representative of structural changes that are detectable across the development process. MMS is an automated solution that saves time and provides critical data which would be unattainable with the conventional secondary structure characterization techniques of FTIR and CD. In this study we demonstrated the ability to measure the secondary structure of three different mAbs, in three different complex formulation buffers, and at a high and low mAb concentration. The high reproducibility and ability to see small structural changes make MMS the ideal tool for characterization of monoclonal antibody therapeutics.

Contributor

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