Application Note
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AN-850-0129

Microfluidic Modulation Spectroscopy as a Non-Destructive Structural Characterization Technique: A Case Study Using Bovine Serum Albumin

- ○ 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

Protein structural characterization is crucial at almost all stages of the biological drug development life cycle because structure determines function. In early discovery and development, sample volumes can be scarce, and sample concentration is often limited. In many protein characterization techniques, such as LCMS, DSC, and NMR to name a few, samples are not normally recovered because some important properties, such as the structure of the protein, are permanently altered. The AQS^{3}pro, powered by Microfluidic Modulation Spectroscopy (MMS), is a powerful, mid-infrared (mid-IR) spectroscopy tool that delivers extremely high-quality data and offers significant improvements in sensitivity, dynamic range, and accuracy for protein analysis compared to conventional mid-IR and far-UV CD techniques.[1,2] Additionally, MMS is non-destructive. If MMS is run as one of the first analysis techniques in the characterization workflow, the sample can be recovered post-analysis and be reused by additional biophysical characterization tools, making the MMS analysis essentially free from a sample consumption perspective.

A previous study involving lysozyme in water[3] demonstrated that MMS can be used as a non-destructive technique with a sample yield of 90%. In this subsequent study we use a more complex sample, bovine serum albumin (BSA) in phosphate buffered saline (PBS), to further validate the non-destructive nature of MMS. Our results show that the structures of the BSA samples were almost identical before and after analysis with >99% similarity. Furthermore, we demonstrate that by re-concentrating the post-process diluted sample back to the original volume, the recovery rate is approximately 95% with >99% similarity compared to the original sample. Such a high recovery rate and similarity ensure that the same exact samples can be used for any follow-up orthogonal studies for head-to-head comparisons, or for a full biophysical characterization.

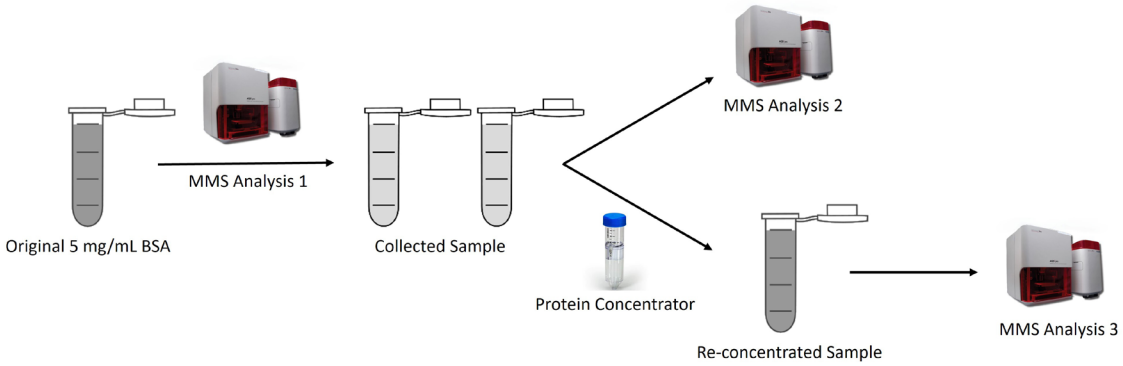

Figure 1. Schematic illustration of the protein recovery and re-concentration workflow for MMS analysis

Methods

Bovine serum albumin (BSA) lyophilized powder (Sigma #5470) was dissolved at 5 mg/mL in 1x phosphate buffered saline (PBS) at pH 7.4. Nine replicates of this sample were run on the AQS^{3}pro at 5 psi backing pressure, 1 Hz modulation, and re-collected from the fraction collector port on the AQS^{3}pro system post-analysis (MMS Analysis 1). Figure 1 shows the workflow of the full protein recovery process. The collected sample from "MMS Analysis 1" was separated into two aliquots. The first aliquot was re-analyzed via MMS (MMS Analysis 2) without any additional pre-treatment. The second aliquot was re-concentrated to the pre-analysis volume with the goal of matching the original BSA concentration using Pierce™ 10k molecular weight cutoff (MWCO) concentrators (re-concentrated sample) prior to re-analysis (MMS Analysis 3).

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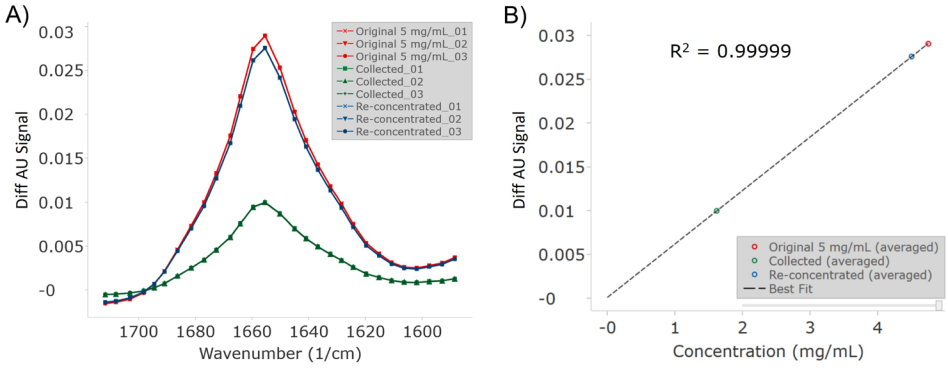
Results

I. Raw Differential Absorbance and Quantitation

The Raw Differential Absorbance spectra of the three samples, the original BSA at 5 mg/mL, the collected BSA sample, and the re-concentrated BSA sample, are shown in Figure 2A . Note that only the first 3 out of 9 replicates of the original sample are shown for consistency with the other two samples. The spectra clearly show differences in peak intensities, indicating different concentrations of these samples. One of the benefits of MMS is its ability to measure protein concentration. Using the ***delta*** software, the peak intensities were plotted against the calculated concentrations of the three samples (Figure 2B). The calculated concentrations are reported in Figure 2C. The collected sample at 1.62 mg/mL is approximately a 3-fold dilution of the original sample calculated at 4.75 mg/mL.



C)

Sample	Diff AU Peak Intensity	Calculated Concentration (mg/mL)
Original 5 mg/mL	0.0290	4.75
Collected	0.0100	1.62
Re-concentrated	0.0275	4.50

Figure 2: A) Raw Differential Spectra of original BSA sample at 5 mg/mL, collected BSA sample, and re-concentrated BSA sample, B) Concentration curve based on the Differential Absorbance (averaged) for the original 5 mg/mL, collected, and re-concentrated samples. C) Table showing the calculated concentrations of the samples.

This is expected as the instrument modulates the sample with its reference buffer through the flow cell to allow for a real-time buffer subtraction, hence resulting in dilution of the of the collected sample. The re-concentrated sample measured at 4.5 mg/mL is 94.7% of the original concentration. Thus, the recovery yield is 94.7% given that the volumes of the original and re-concentrated samples are the same. The loss of 5.3% of the materials is due to the efficiency of the concentrator and this yield is within the specifications of the Pierce™ Protein Concentrators (>90% recovery).

II. Absolute Absorbance and Second Derivative

The raw differential spectra were interpolated and normalized for protein concentration to obtain the absolute absorbance spectra shown in Figure 3A . There are three different samples and nine replicate spectra (three replicates per sample) in Figure 3A, but they are completely overlayed and no spectral difference can be visually observed. To further highlight the spectral details, the second derivative plots of the absolute absorbance spectra are shown in Figure 3B. Some subtle spectral differences can be seen in the tail regions of the plot in Figure 3B, but the tail regions are less significant with respect to a protein's secondary structure. However, the peak region at 1656 cm^{-1} of these spectra is still indistinguishable. Our results demonstrate that the secondary structures of the collected and the re-concentrated BSA samples are almost identical to the original 5 mg/mL BSA sample.

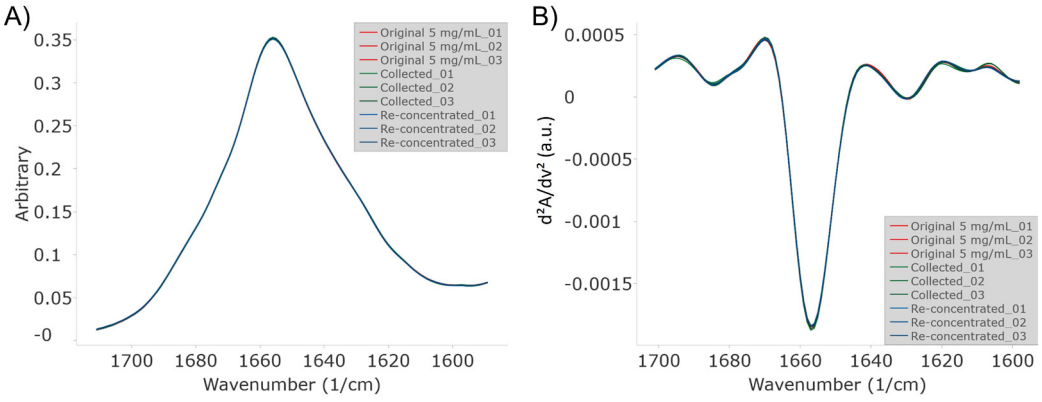


Figure 3. A) Absolute spectra (normalized) of original BSA sample at 5 mg/mL, collected BSA sample, and re-concentrated BSA sample. B) Second derivative plots of the absolute spectra from 3A highlighting the small features in the spectra.

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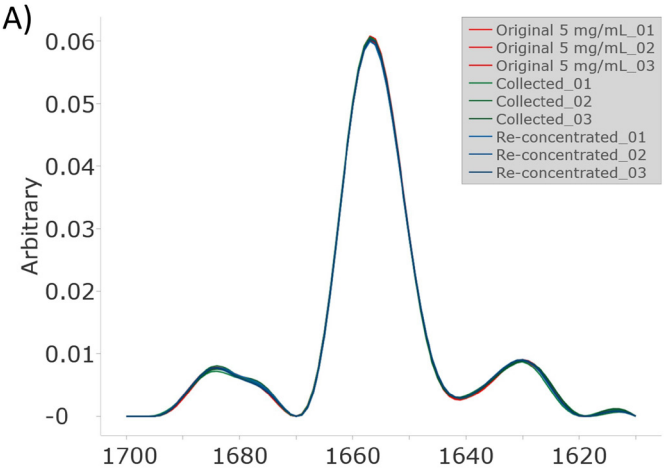
Results, continued

III. Similarity by Area of Overlap

To quantitatively measure the similarity between the samples, the area of overlap is calculated using the similarity plots (Figure 4A). The similarity plots are the inverted and baseline-corrected second derivative plots. Figure 4B shows the calculated repeatability and sample-to-sample similarity for the three samples. For the calculation of repeatability, each replicate's spectrum was compared to the averaged spectrum of the three replicates for the same sample. For sample-to-sample similarity, the averaged spectra of the collected and re-concentration samples were compared to the average of the original 5 mg/mL sample. Both repeatability and sample-to-sample similarity are over 99%, indicating that the spectral variation of both the collected and the re-concentrated samples compared to the original sample is less than 1%. Thus the structures of the analyzed sample are highly conserved and statistically unchanged from the original sample.

A)



B)

Sample	Repeatability	Sample-to-sample Similarity
Original 5 mg/mL	99.63 ± 0.10%	100%*
Collected	99.23 ± 0.14%	99.09 ± 0.06%
Re-concentrated	99.68 ± 0.10%	99.26 ± 0.11%

Figure 4. A) Similarity plot derived from the inverted and baseline subtracted second derivative plots from Figure 3B. B) Table showing the repeatability of each sample and the sample-to-sample similarity using the original 5 mg/mL sample as the reference.

IV. Higher Order Structure

To further validate that there were no structural perturbations after analyzing and re-concentrating the BSA samples, the higher order structure (HOS) composition of each sample was calculated. Four HOS structural motifs, beta sheet, unordered, alpha helix, and turn, were quantitated by Gaussian curve fitting of the similarity plots in Figure 4A. The percent contribution of each structural motif for the three samples is shown as a bar graph in Figure 5. As expected, there were no significant changes in any of the HOS motifs among the original, collected, and re-concentrated BSA samples. Once again, the results in this section indicate that MMS is a non-destructive technique because the protein samples maintained their native structures before and after running through the instrument, and can be re-concentrated for re-analysis without loss of sample.

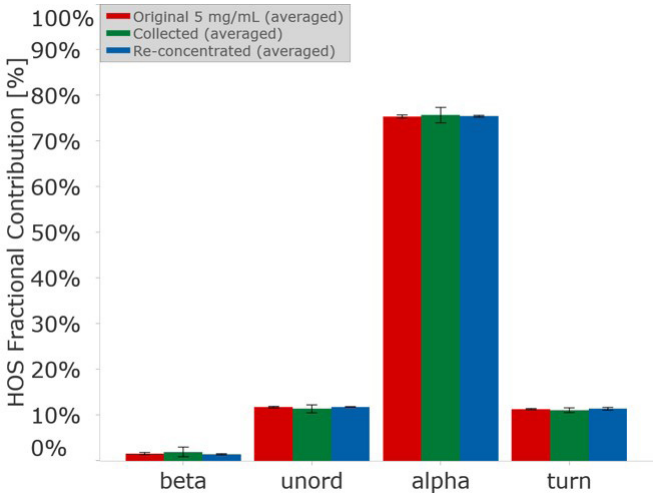


Figure 5. HOS bar graph showing the percentage breakdown of the secondary structure motifs of the original 5 mg/mL BSA sample, the collected sample, and the re-concentrated sample.

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Conclusions

Non-destructive protein characterization techniques are advantageous over destructive techniques such as LCMS and DSC, especially during the early discovery stage of drug development when samples are limited. MMS is a non-destructive, microfluidic, spectroscopic technique, which provides highly reproducible and sensitive measurements for protein secondary structures. We demonstrated in this study that the structure of BSA maintained >99% similarity before and after MMS analysis. Since MMS dilutes the collected samples, we also re-concentrated the collected samples and re-analyzed them on MMS. The recovery rate was approximately 95% and the structural similarity was over 99% compared to the original sample, demonstrating the ability to recover, re-use, and re-analyze samples after MMS analysis. This feature of MMS is particularly beneficial for studies where samples are extremely precious, allowing users to save time and the expense of producing more material.

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References

1) Kendrick, B. S.; Gabrielson, J. P.; Solsberg, C. W.; Ma, E.; Wang, L. Determining Spectroscopic Quantitation Limits for Misfolded Structures. *Journal of Pharmaceutical Sciences* **2020**, 109 (1), 933–936. https://doi.org/10.1016/j.xphs.2019.09.004.

2) Liu, L. L.; Wang, L.; Zonderman, J.; Rouse, J. C.; Kim, H. Y. Automated, High-Throughput Infrared Spectroscopy for Secondary Structure Analysis of Protein Biopharmaceuticals. *Journal of Pharmaceutical Sciences* **2020,** 109 (10), 3223–3230. https://doi.org/10.1016/j.xphs.2020.07.030.

3) Ivancic, V. MMS as A Non-Destructive Secondary Structure Characterization Technique (Application Note: AN-850-0120). Redshift Bioanalytics **2022.**