Application Note
APR 2022
AN-850-0123

Higher Order Structure Determination of a Highly Pigmented Protein 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

Protein structural characterization is crucial to drug development where the higher order structure of a protein strongly affects its function. It is critical, therefore, to ensure a protein retains its native structure under conditions of study and application. It is also important to understand whether concentration-dependent behavior exists and potentially affects the accuracy of higher order structure characterization. Traditional solution-based analytical techniques are often hindered by various properties of protein samples, including opacity relative to water. For instance, the presence of pigmented proteins resulting in high UV-Vis absorbance can interfere with common spectroscopic techniques such as DLS,[1] CD,[2] and UV-Vis,[3] just as interfering absorbance in other regions of the electromagnetic spectrum can create limitations for different spectroscopic tools. The ability to overcome these physical characteristics is important to be able to fully characterize a biomolecule.

The AQS³®pro, powered by Microfluidic Modulation Spectroscopy (MMS) and developed by RedShiftBio, is a powerful infrared (IR) spectroscopy tool for protein structural analysis that delivers extremely high-quality data even for non-ideal samples as well as those in strongly-absorbing buffers.[4] This technology offers significant improvement in sensitivity, dynamic range, and accuracy for the determination of protein higher order structure compared to conventional mid-IR and far-UV CD techniques.[5,6] The analyzer utilizes a tunable Quantum Cascade Laser (QCL) to generate an optical signal over 1000x brighter than the conventional blackbody sources used in FTIR spectroscopy. Additionally, the sample solution and a matched reference buffer stream are automatically introduced into a microfluidic flow cell, and the two fluid streams are rapidly modulated across the laser beam path to produce nearly drift-free background compensated measurements that enable extremely accurate measurements to be made robustly in a very wide range of conditions.

In this study, MMS was used to accurately determine the higher order structure of hemoglobin, a highly colored protein in solution, by interrogating IR spectra in the Amide I band region. The hemoglobin molecule is primarily composed of alpha-helices, with some turn and unordered structures and very few beta-sheets. Protein spectra were automatically acquired using a 24-well plate format and processed using the AQS³delta software and Data Analysis package. Results were obtained from a range of hemoglobin concentrations with varying amounts of pigmentation demonstrating that the strong color intensity of the samples did not affect or interfere with spectral quality or the resulting higher order analysis of the protein. In addition, the secondary structure composition as determined by MMS was highly consistent with the results obtained from X-ray crystallography, the most widely used technique for resolving the 3-dimensional protein structure, and AlphaFold, the most accurate AI system for protein structural prediction.[7,8]

Methods

Hemoglobin powder from bovine blood (Sigma-Aldrich #H2500) was dissolved in HPLC water to create a concentration series of 0.1, 0.5, 1, 2, 5, and 10 mg/mL with a measured pH of approximately 6.2 with no further adjustment. Samples were analyzed in triplicate at room temperature using the AQS³pro at a modulation rate of 1 Hz and backing pressure of 5 psi. The secondary structure components of the prepared hemoglobin solutions were determined using AQS³delta Data Analysis software. The secondary structure components of bovine hemoglobin obtained from X-ray crystallography (PDB: 2QSS)[9] and AlphaFold were calculated using STRIDE[10] and compared with results obtained using MMS and FTIR[11]. Figure 1 shows the prepared concentration series of hemoglobin in water in the 24-well plate prior to MMS analysis.

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Methods, continued

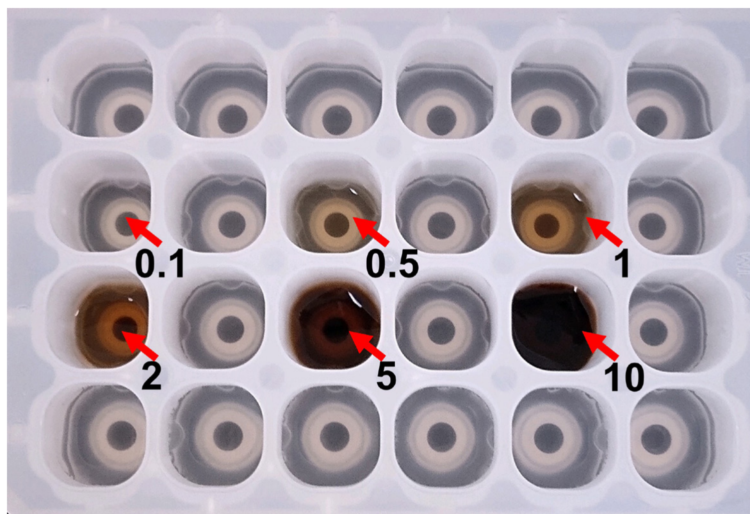

Figure 1: Hemoglobin sample pairs with hemoglobin (left well) next to its water reference (right well) for each concentration of the 0.1 to 10 mg/mL series highlighting the gradually increasing color intensity.

Results

I. Raw Differential Absorbance and Quantitation: The raw differential absorbance (DiffAU) spectra from the 0.1 - 10 mg/mL concentration series for hemoglobin is shown below in Figure 2A. The corresponding quantitation plot of diffAU versus concentration is shown in Figure 2B. A highly linear relationship with $R^2 > 0.9999$ was demonstrated across the series due to the high sensitivity of MMS and the real-time buffer subtraction permitting quantitation of the hemoglobin concentrations as low as 0.1 mg/mL. For the purpose of further analysis at higher concentrations, only the hemoglobin samples ranging from 0.5 – 10 mg/mL are processed going forward.

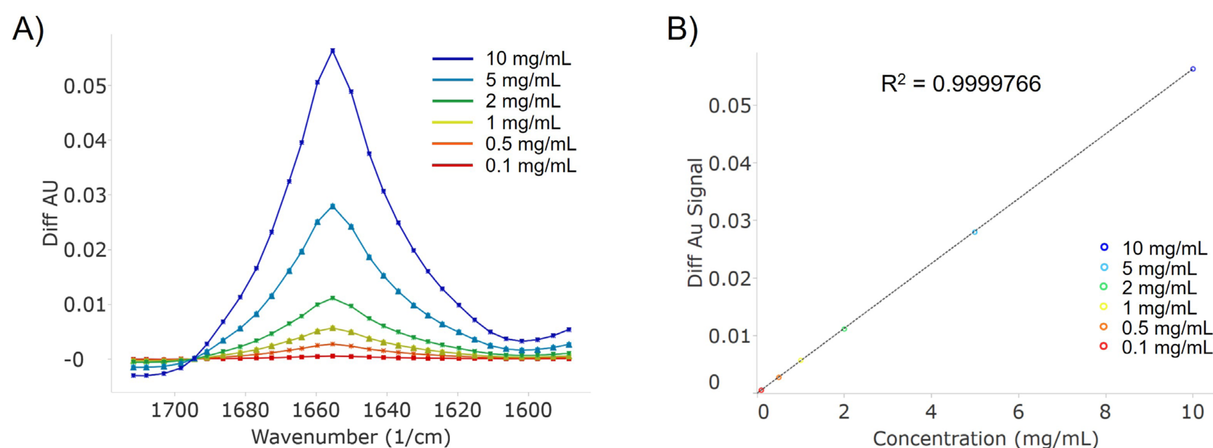

Figure 2: A) DiffAU for six concentrations of hemoglobin in water. B) Quantitation plot of hemoglobin at concentrations ranging from 0.1 to 10 mg/mL.

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Results, continued

II. Absolute Absorbance, Second Derivative, and Similarity: The normalized absolute absorbance spectra, the second derivative spectra, and the similarity plots for the hemoglobin samples at concentrations of 0.5 - 10 mg/mL are shown in Figures 3A, 3B, and 3C, respectively. The highly overlayed spectra in all three plots indicate that the MMS measurements were not affected by the intense colors of the samples, especially at the higher concentrations, and that the structure of hemoglobin was not changed across the concentration series.

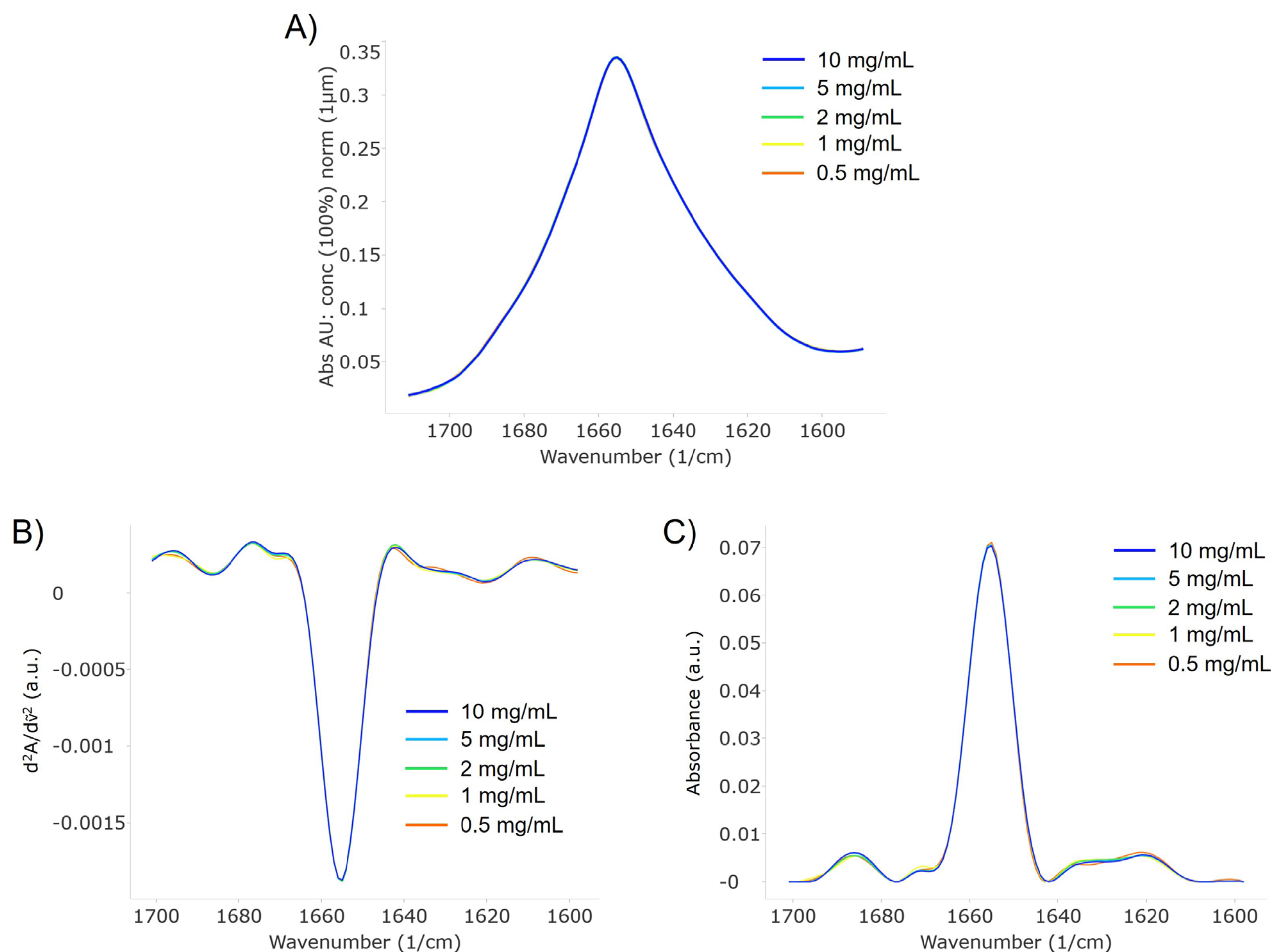

Figure 3: A) Absolute absorbance (normalized) and overlaid spectra of hemoglobin samples from 0.5 - 10 mg/mL. B) Second derivative of the absolute absorbance and C) Similarity plot by area of overlap.

III. Higher Order Structure: Higher order structure (HOS) analysis of the hemoglobin concentration samples and a direct comparison of the 10 mg/mL hemoglobin sample to results obtained from other spectroscopic techniques is shown in Figure 4A and 4B, respectively. The secondary structure motifs measured using MMS in Figure 4A, including β-sheet, random coil (unord), α-helix, and turn, were calculated by Gaussian curve fitting using the same similarity plots shown in Figure 3C. The data confirms that the secondary structure of hemoglobin remained highly conserved over the analyzed concentration range.

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Results, continued

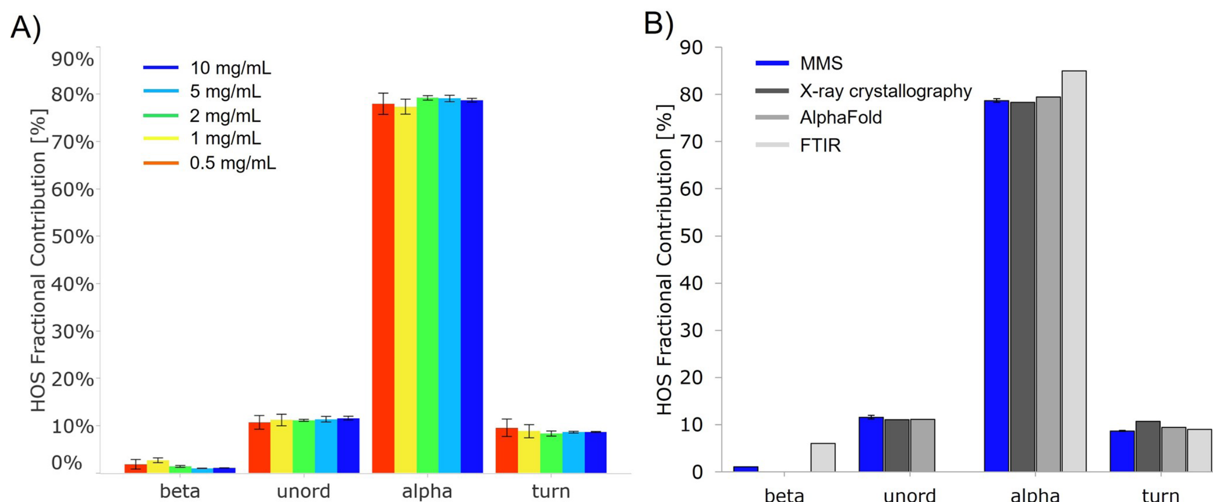

Figure 4 A) Secondary structure compositions of hemoglobin solutions from 0.5 to 10 mg/mL B) Secondary structure composition of hemoglobin (10 mg/mL) compared to 10 mg/mL data obtained from X-ray crystallography, AlphaFold, and FTIR. Error bars show mean percentage ± SD.

To validate the HOS analysis calculated by MMS, results were compared with the data obtained from X-ray crystallography, AlphaFold, and FTIR all at a concentration of 10 mg/mL. It is important to note that because there are currently no methods that can achieve an absolutely accurate determination of protein secondary structure in solution, the X-ray structure of hemoglobin will be used as the reference in the comparison herein. As shown in Figure 4B, the calculated secondary structure components of hemoglobin are highly similar between MMS, X-ray crystallography, AlphaFold, and FTIR. Among these three additional analytical methods, FTIR estimated moderately higher percentages of α-helix and β-sheet components and showed no unordered structures. Overall, MMS results demonstrate that this analytical technique provides more accurate secondary structure information compared to FTIR and is more sensitive to β-sheet structures where X-ray crystallography and AlphaFold did not register any.

Conclusions

Highly pigmented protein samples are difficult to analyze due to spectral interference for common spectroscopic techniques including DLS, CD, and UV-Vis. Hemoglobin, when dissolved in water, produces intensely colored solutions due to the heme group and therefore creates analytical challenges. MMS is a microfluidic spectroscopic technique which successfully subtracts interfering background absorbance in real-time, allowing proteins with interfering characteristics to be structurally analyzed. Despite the large difference in color intensity across the full 0.1 to 10 mg/mL concentration series, the overlaid absolute absorbance spectral results showed strong linearity in quantitation and a stable secondary structure across the concentrations tested. Secondary structure determination using MMS gave highly similar results compared to those obtained from X-ray crystallography and AlphaFold, and proved to be more accurate than FTIR which overestimated the α-helix and β-sheet structures. This study demonstrates that the AQS^{3}pro, powered by MMS technology, can accurately determine the secondary structure of highly colored proteins without spectral interference, closing an analytical gap for pigmented protein solutions.

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