Application Note
DEC 2023
AN-850-0140

Pushing boundaries in protein structure-function analysis: How MMS exceeds CD spectroscopy at in-situ detection and characterization of structural changes in proteins

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

- ✓ Aggregation
- ✓ Quantitation
- ✓ Structure
- ⬢ Stability
- ✓ Similarity

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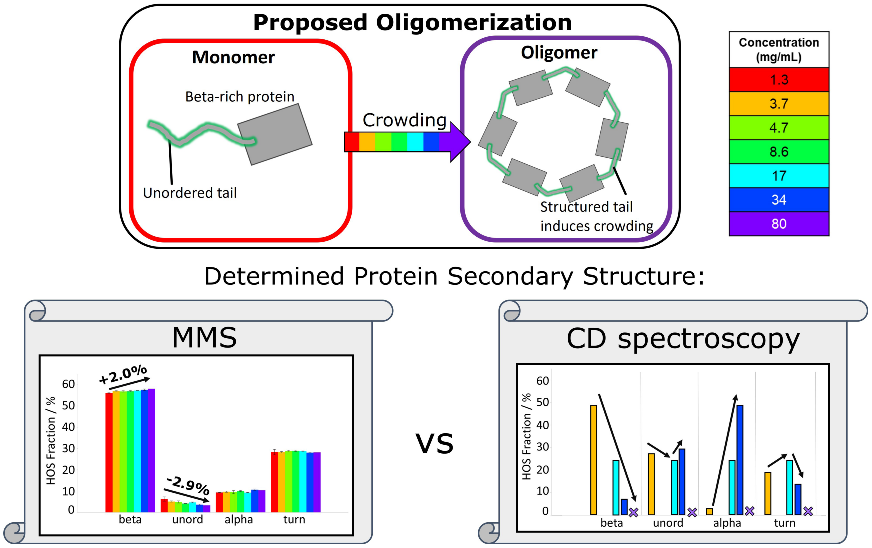
Abstract

The interplay between protein structure and function is well recognized but often scarcely understood. This molecular-level understanding is required not only for studying cellular processes, but also for developing drug agents suitable for specific biological targets. However, characterization of protein higher order structures (HOS) is challenging due to its complexity in the three-dimensional arrangement and the connection between their local and global structural organization. The gold-standard for protein HOS analysis is x-ray crystallography and NMR spectroscopy as they provide high resolution three-dimensional information on protein structure. These techniques are not simple though, requiring immense analytical effort, and are limited in buffer compatibility and low in throughput. Instead, secondary structure can also be used as a reporter for local and global structural differences in proteins. In this study, we compare two secondary structure analytical techniques, Microfluidic Modulation Spectroscopy (MMS) and Circular Dichroism (CD) spectroscopy on the analysis of a beta-sheet rich protein. Since this protein is known to oligomerize upon crowding, we particularly focus on identifying potential structural changes at increasing concentrations.



Introduction

Microfluidic Modulation Spectroscopy has proven to be a robust and sensitive tool for secondary structure analysis.[1] Another analytical method often used to determine secondary structure is Circular Dichroism (CD) spectroscopy. In this application note, we employ a biologically relevant protein example to study the performance of MMS versus CD for secondary structural analysis. The biological function of the investigated protein, from here on referred to as "protein X", is known to vary with changes in concentration. While it acts as a monomer in a diluted state (~1 mg/mL), it oligomerizes and functions collectively at higher concentrations (~80 mg/mL). Protein X is further known to be rich in beta-sheets and has an unordered tail which is proposed to take a crucial role in the reorganization along the oligomerization mechanism. In this work, we measured different concentrations of protein X between 0.8 and 80 mg/mL, using MMS and CD spectroscopy and compared the secondary structural analysis provided by the two methods.

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Introduction, continued

Previous studies showed that MMS is the most sensitive secondary structure technique to detect subtle structural changes (down to 0.76%).[1] In addition, the signals of various secondary structural motifs are well-separated across the probed spectral range and appear on the same order of magnitude. In contrast, CD spectroscopy is known to provide reliable structural characterization for helix-rich proteins but struggles with determining other structural content, i.e. beta-sheets, unordered and turn structures. This is caused by two effects: 1.) CD signals for different structural motifs overlap and have positive and negative components which may even cancel each other out, making deconvolution difficult. 2.) The alpha-helical signal is substantially stronger than those from the other structural motifs which may further distort the structural deconvolution. As an example, Figure 1 compares MMS with CD spectra for a helical protein (BSA, dashed lines) and a beta-sheet-rich protein (IgG1, solid lines).

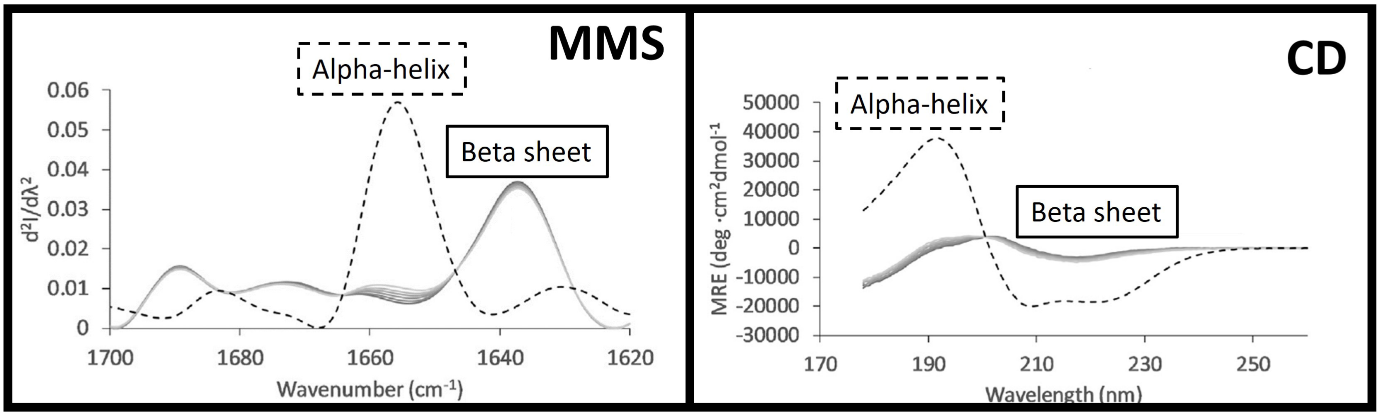

Figure 1: MMS (left) and CD (right) spectra of BSA (dashed lines) and IgG1 (solid lines), highlighting the overlapping features and differing intensities for alpha-helix and beta-sheet structures in CD and compared to the more well-resolved peaks in MMS. This figure was adapted from Kendrick et al.[1]

Methods

For this study, a concentration series of protein X was prepared in PBS yielding concentrations between 0.8 and 80 mg/mL. The samples were measured and analyzed with a first-generation RedShiftBio MMS production system equipped with sweep scan capability (RedShiftBio, Boxborough, MA, US). For background subtraction, a chemically identical buffer was loaded pairwise with each sample onto a 96-well plate. The instrument was run at a modulation frequency of 1 Hz and with a microfluidic transmission cell of 23.5 µm optical pathlength. The differential absorbance spectra of the sample against its buffer reference were measured across the amide I band (1,714 – 1,590 cm^{-1}). For each spectrum, triplicate measurements were collected and averaged. The data were analyzed on the delta analysis software.

Selected samples from this dilution series were additionally measured on a Chirascan CS/PCS CD spectrometer with a 0.01 mm quartz cuvette, particularly designed for a broader concentration range as described in Ioannou et al.[2] Triplicate measurements were recorded with 10 co-adds each, between 200 and 360 nm. CD data were calculated as mean residue ellipticity and fitted with the CDpro[3] routine for secondary structure analysis.

Both methods were qualified by performing secondary structure analysis of a lysozyme sample, a predominantly alpha-helical protein. For that, MMS, and CD spectra of 1 mg/mL hen egg white lysozyme (HEWL) in water were collected and analyzed.

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Results and Analysis

I. Method qualification: MMS and CD analysis of lysozyme

Before studying the oligomerization of protein X, we used 1 mg/mL lysozyme dissolved in water as a much simpler and well-characterized example to validate the employed MMS and CD methods for structural characterization. The higher order structure (HOS) analysis based on the MMS and CD experiments are presented in Figure 2, next to crystallographic data from the protein database file 1DPX[4] which we use as control.

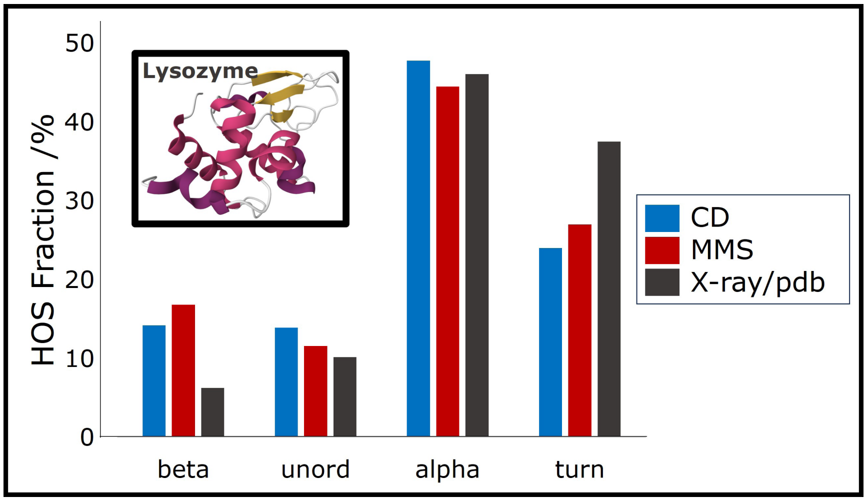

Figure 2: Secondary Structure Analysis based on MMS and CD measurements of lysozyme at 1 mg/mL in water, compared to crystallographic data (pdb identifier: 1DPX).[4]

Overall, MMS and CD analysis both identify about 45% alpha helix, 25% turn structure, 15% beta sheet and 15% unordered structure for the dissolved lysozyme sample. This is mostly in line with the presented crystal structure of lysozyme. Interestingly, MMS and CD both predict slightly less turn structure (~10%) in favor of more beta-sheet content. Consequently, lysozyme seems to be slightly more structured in solution than it is in its crystal structure. Here, MMS and CD analysis are in good agreement with each other demonstrating the suitability of both methods for a simple, helix-rich protein as lysozyme at low concentration (1 mg/mL) dissolved in water. To test the suitability of MMS and CD for a more complex and biologically relevant system, we will use these methods to study the concentration-dependent structure-function relationship of protein X driven by oligomerization. The experimental challenge of this study is that protein X is a beta-rich protein, the buffer background is not water but PBS and the concentration range that needs to be covered is broad (0.8-80 mg/mL).

II. Case study: MMS and CD on the oligomerization mechanism of protein X

In this study, we have recorded MMS and CD spectra of protein X in PBS at different concentrations between 0.8 and 80 mg/mL. For the MMS spectra, we have calculated the overall spectral differences of the higher concentrated samples compared to 0.8 mg/mL by means of Weighted Spectral Difference (WSD). The result is presented in Figure 3, showing the biggest change between about 1 and 20 mg/mL and smaller changes from 20 mg/mL towards higher concentration. The inset of Figure 3 resolves the corresponding spectral differences across the amide I band, showing that the changes predominantly occur in the beta-sheet and unordered range. By trend, the beta-sheet band increases as a function of concentration while the unordered band decreases correspondingly.

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Results and Analysis, continued

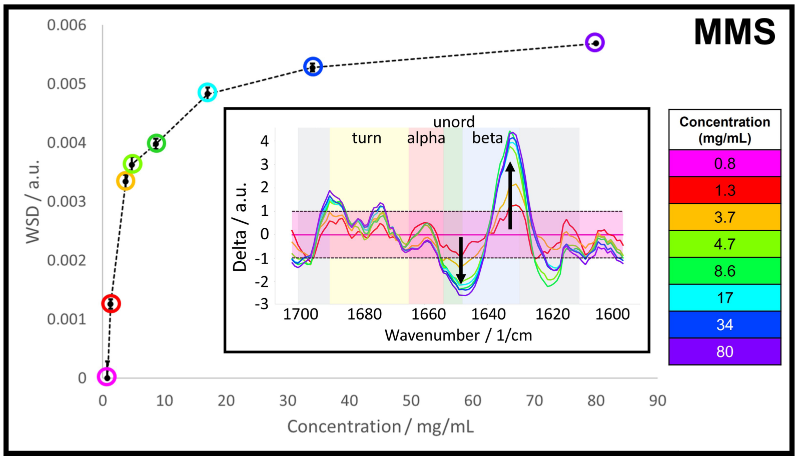

Figure 3: The MMS Weighted Spectral Difference (WSD) across the measured concentration range between 0.8 and 80 mg/mL. Inset: Difference spectra with 0.8 mg/mL as the control spectrum, the background is color-coded according to the typical spectral ranges for the individual structural motifs.

Based on the MMS spectra, we have employed a Gaussian deconvolution model to calculate the Higher Order Structure (HOS) of protein X at the tested concentrations. Correspondingly, we have interrogated the CDpro fitting routine to predict the HOS from the collected CD spectra.

The results of both HOS predictions are presented in Figure 4. The MMS analysis calculates about 55% beta-sheet, 30% turn, 10% alpha-helix and 5% unordered structure. MMS shows that, with increasing concentration, the beta-sheet fraction increases by 2% and the unordered content decreases by 2.9%. According to the proposed oligomerization mechanism, the unordered tail of protein X drives that process through interaction with nearby protein X monomers while in turn becoming more structured. This notion is supported by the concentration-induced structural changes which are identified here with MMS. By contrast, the CD-based analysis does not report a transient structural change of protein X with increasing concentration. Instead, it predicts dramatic changes of the structural composition, i.e. -40% beta-sheet fraction, +40% helical content, and non-monotonic changes in turn and unordered content. As a consequence, the CD data did not predict the structure of protein X as well as it did for lysozyme, and failed to accurately describe the crowding-induced structural changes of protein X.

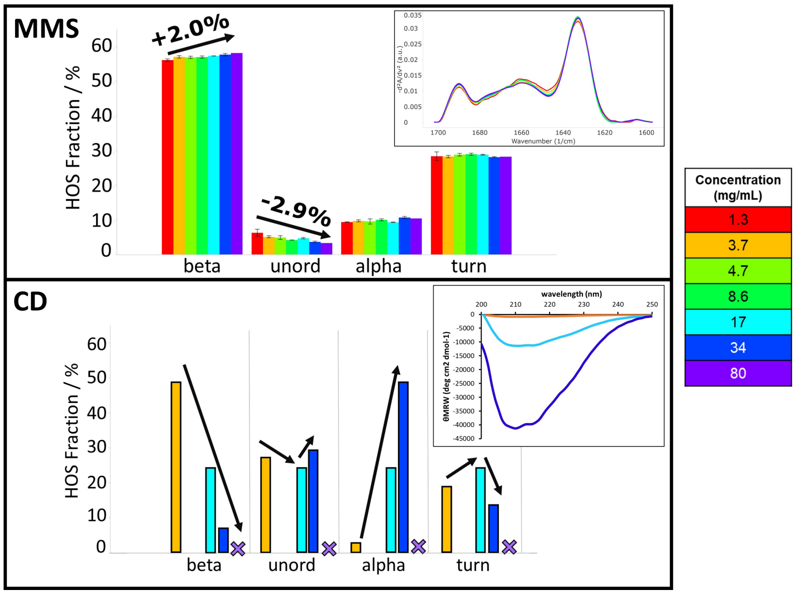

Figure 4: HOS analysis based on MMS (top) and CD (bottom) spectra of protein X in PBS at different concentrations between 1.3 and 80 mg/mL. The MMS and CD spectra are shown each in the corresponding insets in the top right corners. The MMS spectra are presented as the inverted and baselined 2nd derivative spectra, the CD spectra are shown as mean residue ellipticity.

Conclusions

In this work, we have shown that MMS serves as a suitable technique to characterize proteins with different structural profiles, across a broad range of concentrations. This study demonstrated that MMS spectra can be used as a sensitive reporter for subtle structural changes in proteins. The oligomerization of protein X was previously proposed to be driven by local changes in the protein structure, which agrees with the MMS results. CD spectroscopy has performed sufficiently on a helix-rich protein in water and at low concentration like 1 mg/mL. However, on the example of protein X oligomerization, we have highlighted how CD spectroscopy struggles severely to provide realistic HOS predictions for proteins with substantial beta-sheet fractions.

Contributors

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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. *J Pharm Sci* **109**, 933–936 (2020).

2. Ioannou, J. C., Donald, A. M. & Tromp, R. H. Characterising the secondary structure changes occurring in high density systems of BLG dissolved in aqueous pH 3 buffer. *Food Hydrocoll* **46**, 216–225 (2015).

3. Sreerama, N. & Woody, R. W. Estimation of protein secondary structure from circular dichroism spectra: Comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set. *Anal Biochem* **287,** 252–260 (2000).

4. Weiss, M. S., Gottfried, Palm, J. & Hilgenfeld, R. Biological Crystallography Crystallization, structure solution and refinement of hen egg-white lysozyme at pH 8.0 in the presence of MPD. *Acta Crytallographica* **D56,** 952–958 (2000).