Application Note
MAY 2022
AN-850-0124

Using MMS to Measure Buffer-Induced Structural Changes in an Alpha-helix Rich Enzyme

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

- ⬡ Aggregation
- ⬡ Quantitiation
- ✓ Structure
- ⬡ Stability
- ✓ Similarity

www.redshiftbio.com

Introduction

Formulation buffer components play an important role in the efficacy and safety of biotherapeutics by optimizing activity and improving stability during manufacture and storage. The effect of various buffer conditions on proteins has been widely studied using methodologies that include binding assays, particle sizers, computational simulations, and so forth. While these techniques do provide insight into activity and aggregation, buffer-induced changes in protein structure have not been studied to their full potential.

The ability to directly measure buffer-induced changes in protein structure and folding is an area of interest that is gaining visibility due to improvements in technology and its relevance because structure is directly linked to activity and stability. Secondary structure is a key quality attribute that should be rationally characterized throughout biopharmaceutical development including formulation screening and manufacturing, especially when ideal behavior of a biotherapeutic is well known. However, analyzing protein secondary structure is not straightforward due to limitations of traditional analytical tools, including challenges in the sensitivity of the measurements and the difficulty in easily gathering robust, reliable data in the formulation conditions of interest.

Microfluidic Modulation Spectroscopy (MMS), a novel mid-IR spectroscopy technology developed by RedShift BioAnalytics, Inc., bridges the structural characterization gap by offering ultra-sensitive secondary structure measurements directly in formulation conditions with no interference by aqueous excipients and common stabilizing additives. Confident measurements are readily achieved with the combination of a high-power quantum cascade laser (QCL) coupled with a microfluidic flow cell that enables real-time modulation between sample and reference, generating fully automated, background subtracted spectra and minimizing human error. MMS has demonstrated much greater sensitivity and repeatability than traditional techniques including Fourier-Transform Infrared (FTIR) and Circular Dichroism (CD) spectroscopy[1] and therefore, it succeeds in providing important structural information and quantifying changes in structure where it used to be difficult to obtain.

In this study, MMS was used to characterize buffer-induced structural differences of lysozyme, a well-characterized alpha-helix rich protein, in water and three common formulation buffers: Phosphate Buffer (PB), Phosphate Buffered Saline (PBS), and Tris buffer. Absorption spectra in the Amide I region were automatically collected and processed to calculate higher order structure (HOS) percentages and the overall structural similarities between samples in all prepared conditions. The results showed the enzyme exhibited various degrees of structural change within these different buffers, and that these changes could be quantified to inform buffer selection decisions to support ideal activity.

Methods

Hen Egg White Lysozyme (HEWL) (Sigma #L6876) was prepared at 10 mg/mL in HPLC-grade water and three buffers: 10 mM PB pH 7, 1x PBS pH 7.4, and 10 mM Tris Buffer pH 8. Except for the preparation in PB pH 7 that was analyzed in duplicate, all samples were analyzed in triplicate at room temperature using the AQS^{3}pro first generation MMS platform at a modulation rate of 1 Hz and a backing pressure of 5 psi. The secondary structure components of the prepared protein solutions were determined using the delta Data Analysis feature. All spectra were normalized for concentration and cell path length prior to generation of the Absolute Absorbance, Second Derivative, Delta, and HOS results.

REDSHIFTBio®
131 Middlesex Turnpike | Burlington, MA | 01803 | info@redshiftbio.com

Application Note
MAY 2022

Results

I. Absolute Absorbance, Second Derivative, and Delta: An overlay of the normalized absolute absorbance spectra of HEWL in water, PB, PBS, and Tris buffers is shown in Figure 1 (A). Measurable differences are observed between all buffer conditions as indicated by the poor alignment of the curves in several regions.

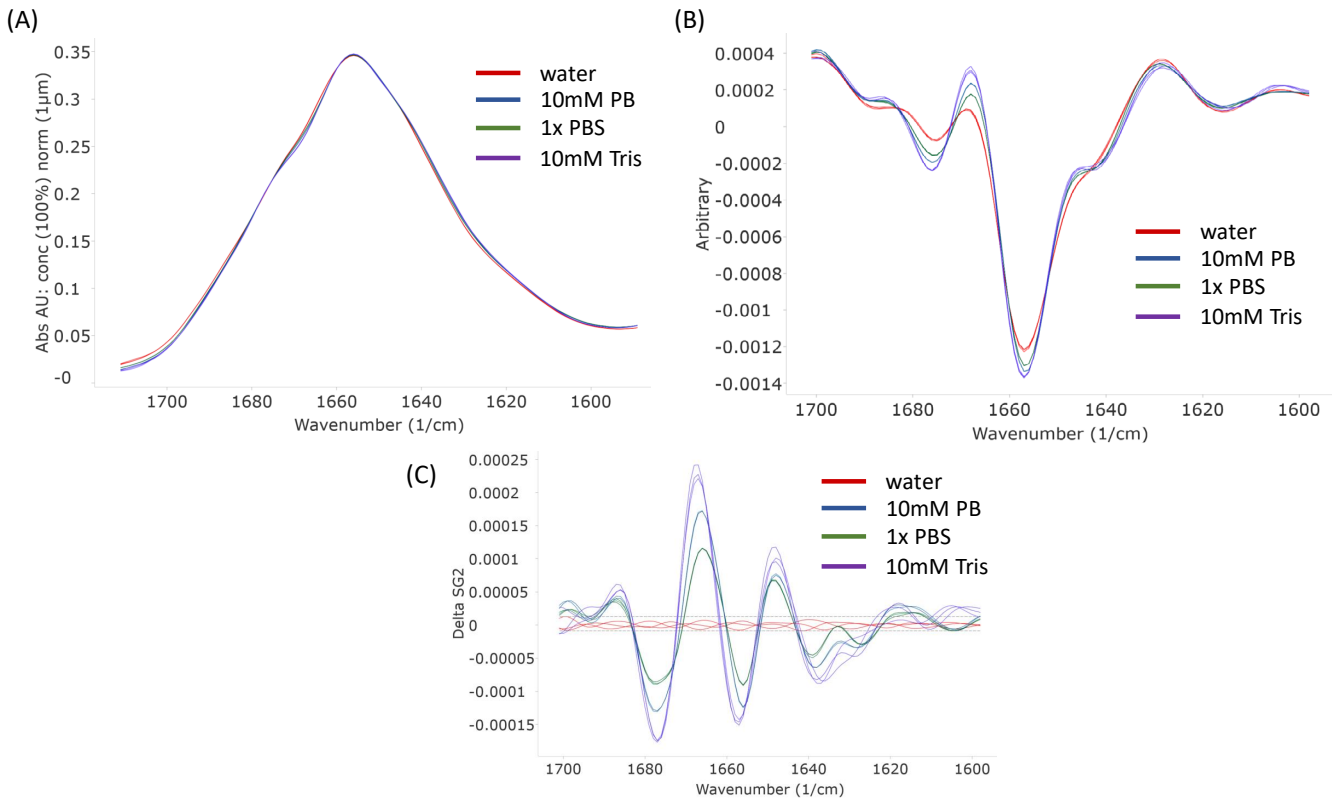

Figure 1. (A) Overlay of Absolute Absorbance spectra, (B) Overlay of Second Derivative of the absolute absorbance spectra, and (C) Delta plot comparing second derivative spectra for 10 mg/mL HEWL in water (reference) to PB, PBS, and Tris buffers.

To magnify the spectral discrepancies, an overlay of the second derivatives was generated and is shown in Figure 1 (B). Significant buffer-induced differences can be identified in the 1654-1658 cm^{-1} alpha-helix region and 1665-1690 cm^{-1} turn region. Rating these differences relative to HEWL in water, the buffer order of decreasing change is Tris > PB > PBS > water.

The delta plot shown in Figure 1 (C) further highlights the spectral differences observed in the second derivative overlay. For reference, the spectra contained between the horizontal dotted lines show the variation among the HEWL replicates in water. In comparison, the spectra collected in the three buffers fall outside these lines and represent significant differences relative to the water reference. For all three plot types, the greatest measured difference between buffers occurred between water and Tris buffer.

Application Note
MAY 2022

Results, continued

II. Similarity by AO and WSD: Area of Overlap (AO) and Weighted spectral difference (WSD) were used to compare overall structural similarity for HEWL in water versus the three buffers. Figure 2 (A) shows the similarity plot as determined by AO and Figure 2 (B) shows the weighted spectral difference (WSD) calculated from the second derivatives. The resulting percent similarity values using both methods are listed in Table 1.

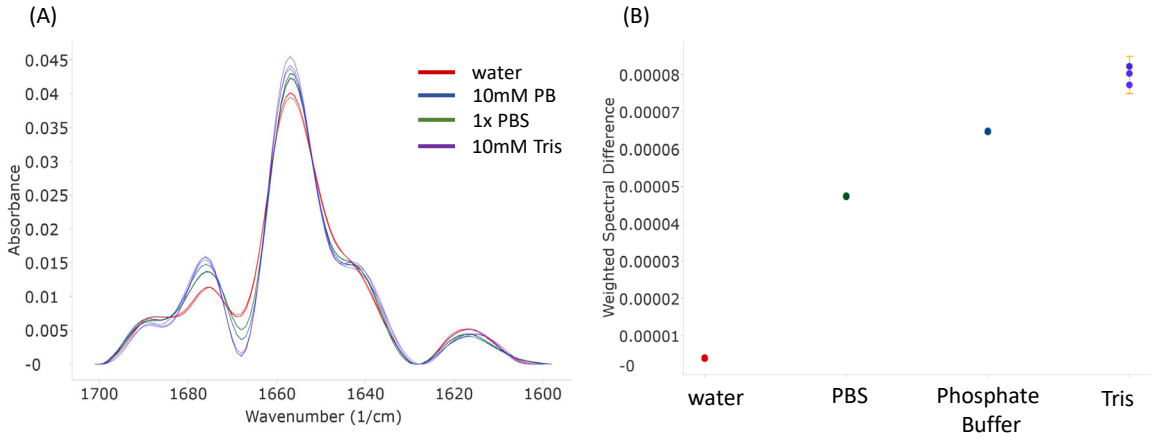

Figure 2. (A) Similarity plot by Area of Overlap (AO) and (B) Weighted Spectral Difference (WSD). Both plots are compared to HEWL in water as a reference.

Table 1: Percent Similarity by Area of Overlap (AO) and Weighted Spectral Difference (WSD) for 10 mg/mL HEWL in water, Tris, PB and PBS.

10 mg/mL HEWL prepared in	Percent Similarity by AO		Similarity by WSD	
	Among Replicates	vs HEWL in water	Among Replicates ($x10^{-6}$)	vs HEWL in water ($x10^{-6}$)
Water	99.84 ± 0.09	100	3.97 ± 0.15	0
Tris, 10 mM pH 8	99.39 ± 0.12	92.61 ± 0.47	8.23 ± 0.81	79.9 ± 2.5
PB, 10 mM pH 7	99.85 ± 0.00	94.23 ± 0.06	1.30 ± 0.00	64.8 ± 0.2
PBS, 1x pH 7.4	99.85 ± 0.04	95.78 ± 0.06	1.07 ± 0.32	47.4 ± 0.2

By AO, the MMS data collected for HEWL demonstrated sample repeatability of greater than 99.4% between replicates for all samples tested. Both AO and WSD gave similar results, with HEWL prepared in Tris being the most dissimilar from HEWL in water, followed by PB, with HEWL prepared in PBS having the most similar structure to that of HEWL in water. These results also agree with the results previously discussed in Figure 1 (B). Both similarity methods are able to distinguish structural differences for HEWL in the 4 different formulations tested.

Application Note
MAY 2022

Results, continued

III. Higher Order Structure (HOS): For the HEWL samples analyzed in water and the three buffers, the percent HOS motif composition was determined by Gaussian curve fitting from the AO similarity plot shown earlier in Figure 2 (A) and is presented as an HOS bar graph for all replicates shown in Figure 3 relative to the HEWL control in water.

Within the HOS data, there is a noticeable increase in the amount of alpha-helical content compared HEWL in water across the buffers in the following order of PBS < PB < Tris. For the turn motif, there is a decrease in similarity for the buffers in the order of Tris > PB > PBS. The changes in HOS are consistent with the similarity results shown previously in results sections I and II across the three buffers relative to HEWL in water.

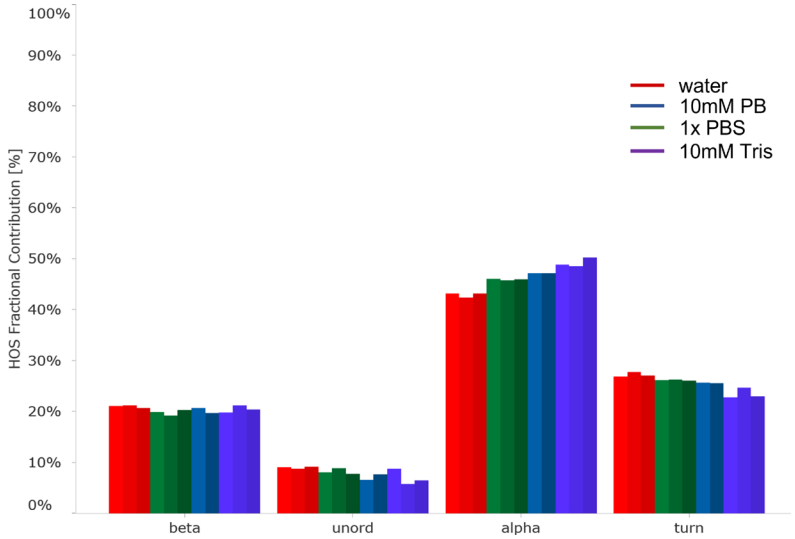

Figure 3. Higher Order Structure (HOS) plot of HEWL in different buffers.

For comparison, the calculated percent HOS values per motif are listed in Table 2 relative to the same values reported using FTIR[2]. Note, the FTIR data was collected as 5% (w/v) HEWL in 1% saline, pH 6.5.

Table 2. HEWL secondary structure in different buffers measured by MMS, compared to that measured by FTIR.

As measured in or measured by	Percent HOS motif			
	turn	alpha	unordered	beta
Water	27 ± 0.5	43 ± 0.5	9 ± 0.2	21 ± 0.3
Tris, 10 mM pH 8	23 ± 1	49 ± 0.9	7 ± 1.6	20 ± 0.7
PB, 10 mM pH 7	26 ± 0.1	47 ± 0.2	7 ± 0.8	20 ± 0.7
PBS, 1x pH 7.4	26 ± 0.1	46 ± 0.2	8.2 ± 0.6	20 ± 0.5
FTIR	27	40	14	19

Application Note
MAY 2022

Conclusions

In this study, MMS was used to characterize the differences in HEWL structure in 4 buffer conditions: water, Tris, PB, and PBS. It is documented that the ideal HEWL activity is maintained when it is prepared in Tris buffer, pH 8, and this lends some clarity to the results that were observed for this protein in several buffer systems and value to being able to differentiate structural changes in either direction, towards or away from ideal enzymatic activity.

HEWL clearly showed structural differences across the buffers tested as evidenced by the change in similarity. Compared to water, the overall structure of HEWL changed 4-8% in different buffers in an increasing order of PBS < PB < Tris.

Buffer-induced changes in characteristic peaks were also observed. For HEWL, the presence of buffer promoted an increase in alpha-helix formation compared to water in a descending order of Tris (strongest signals) > PB > PBS > water. The HOS motif percentages were also quantified in different buffer conditions and compared to water, HEWL showed an increase in alpha helix in increasing order of PBS < PB < Tris.

MMS measurements have been shown to be more sensitive than traditional secondary structure analysis techniques with outstanding system repeatability demonstrated in this study. This enables the detection of very small changes that were not previously measurable due to limitations in sensitivity with traditional techniques. MMS also allows direct measurements in the formulation conditions of interest with no need for dilution, buffer exchange, or crystallization.

Thus, the ability to detect very subtle differences in protein structure under different buffer conditions allows for confident decision making when choosing the right buffer components in the drug formulation process. It also shines light on the mechanistic questions regarding buffer influences on protein activity and stability.

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 109, 933–936 (2020).
2. pdb6ABN
3. Data sheet from Sigma for HEWL product #L687.

Primary Author: Maria Ma, Ph.D.
Contributors: Richard Huang, Ph.D.; Valerie A. Ivancic, Ph.D.

Contact

RedShift BioAnalytics, Inc
131 Middlesex Turnpike | Burlington, MA | 01803
T: 781.345.7300 | F: 781.345.7301 | E: info@redshiftbio.com