Application Note
DEC 2025
AN-850-0153

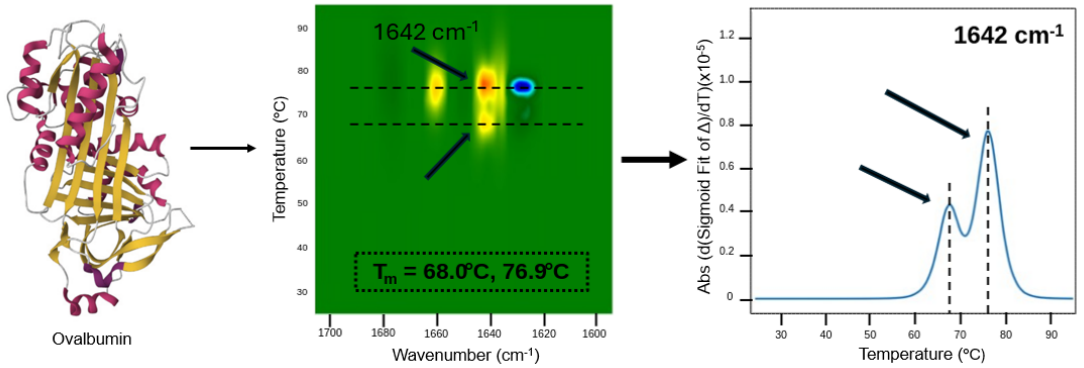
Ovalbumin pH formulation study and multiple domain melting T_m deconvolution using MMS

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

- ✓ Aggregation
- ⬢ Quantitiation
- ✓ Structure
- ✓ Stability
- ✓ Similarity

Abstract

Identifying thermal stability of a protein is an essential part of therapeutic biologic development and formulation to ensure long shelf lives. However, existing techniques that measure thermal stability, such as Differential Scanning Calorimetry (DSC) and Differential Scanning Fluorimetry (DSF), lack information on structural changes associated with melting temperature (T_m). This is especially vexing when multi-domain proteins have multiple, discrete T_m values. In this study, we present Microfluidic Modulation Spectroscopy (MMS) as a versatile alternative that enables simultaneous structure and stability measurements. Using MMS, we identify the optimal pH formulation for ovalbumin, making note of multiple structure-specific melting temperatures. Furthermore, we demonstrate the high sensitivity of MMS by detecting slight differences in protein structure after freeze-thaw stress.



TOC Figure: (Left) X-ray crystal structure of ovalbumin (PDB ID: 1OVA) shows the mixture of alpha-helices and beta-sheets making up the protein structure. (Middle) MMS 2D melt map indicates two discrete melting temperatures with multiple wavenumbers involved, indicating different structures are melting at different times. (Right) Taking a vertical slice of the melt map at 1642 cm^{-1}, each T_m can be clearly defined.

Introduction

Between 2020 and 2024, 74% of Complete Response Letters (CRLs) issued by the FDA cited quality and manufacturing issues, including formulation errors and oversights.[1] These rejections can lead to significant delays in drug development. A critical quality attribute in formulation, storage, and shelf life is the higher-order structure (HOS) of therapeutic proteins. During the submission of an Investigational New Drug (IND) application, the FDA, and other regulatory bodies, require detailed characterization of protein HOS, including assessments of stability and the identification of conditions under which aggregation occurs, such as during freeze/thaw cycles.[2]

One common approach to evaluating protein stability is by measuring the melting temperature (T_m), the temperature at which 50% of the protein population is unfolded. As temperature increases, non-covalent interactions stabilizing the protein begin to degrade, leading to unfolding. Therefore, a higher T_m indicates a more stable molecule.

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Introduction, continued

While techniques are available for measuring T_m, such as Differential Scanning Calorimetry (DSC) and Differential Scanning Fluorimetry (DSF), they provide limited structural context beyond the melting event itself.[3] Common therapeutics, such as monoclonal antibodies (mAbs), often have multiple domains that result in multiple T_m values. Identifying individual domain stability can provide useful information on where to focus formulation efforts, resulting in saved time and resources.

Here , we present Microfluidic Modulation Spectroscopy (MMS) as a powerful alternative that enables HOS- specific measurement of thermal stability. MMS is a mid-IR-based spectroscopic technique well suited for studying both protein and RNA structure. By automatically referencing between sample and buffer, MMS can detect structural changes, determine T_m, and quantify alterations in biomolecules at levels as low as 0.76%.[4] This enables precise observation of how specific structural components respond to various formulation conditions. For this study, we utilize ovalbumin as a model protein due to its extensive past characterization, mixture of HOS, and high solubility across a wide pH range, making it an ideal candidate for rigorous MMS testing.

In this application note, we demonstrate the capabilities of the AuroraTX in evaluation of protein stability, identifying multiple pH-dependent and secondary structure-specific melting temperatures. Furthermore, we identify freeze stress-induced HOS changes, giving insight into optimal formulation conditions.

Methods

Ovalbumin preparation:
Ovalbumin from Sigma (A2512-1G) was prepared at 1 mg/mL in 10 mM citrate buffer at pH 4.2, 10 mM acetate buffer at pH 5.0, HPLC water at pH 6.5, 10 mM phosphate buffer at pH 7.6, and 10 mM glycine buffer at pH 8.5.

Freeze-thaw cycles:
Ovalbumin in 10 mM citrate buffer (pH 4.2) and 10 mM phosphate buffer (pH 7.6) were frozen at -20°C for 18 hours, then allowed to thaw at room temperature for 6 hours. This process was repeated for a total of three freeze-thaw cycles.

Thermal Ramping Methods:
All samples were run with full automation on a 96-well plate using the AuroraTX. Temperature was ramped from 25°C-95°C at a rate of 1°C/min. Samples were maintained at room temperature on the plate, while the flow cell was ramped from 25°C-95°C.

Results

Figure 1 displays the effect of pH on the stability of ovalbumin. Figures 1A–E present a 2D thermal heatmap (top) and a similarity plot (bottom). Ovalbumin was found to be least stable at pH 4.2, with a T_m of 65.5°C, and most stable at pH 8.5, with a T_m of 77.2°C. Although stability was highest at pH 8.5, only slight increases in T_m were observed beyond pH 6.5. These results are consistent with previous findings that ovalbumin is most stable between pH 6–10, with a reported T_m of approximately 78°C.[5]

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Results, continued

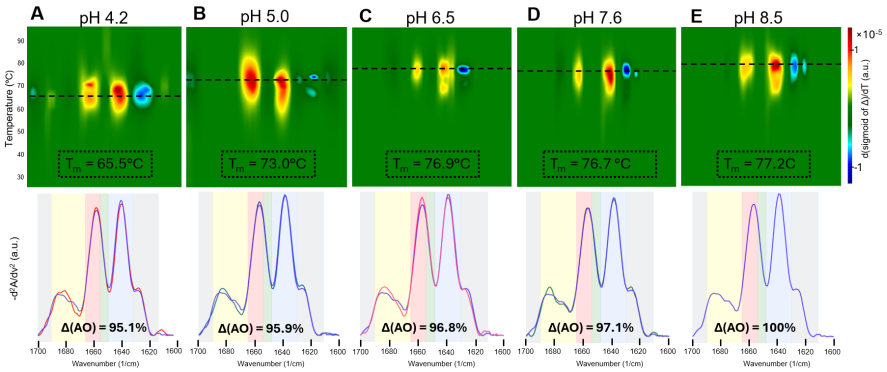
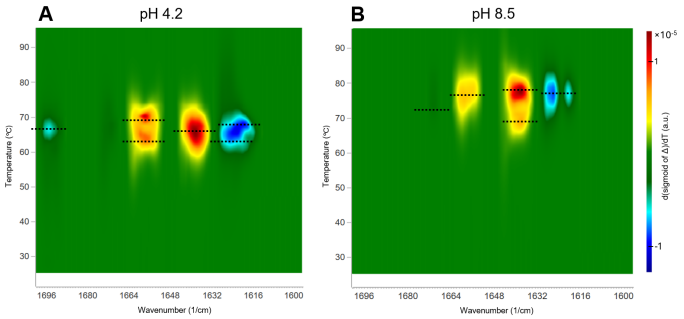


Figure 1: Thermal stability of ovalbumin increases with pH. Thermal heat maps are shown from 25°C to 95°C. The heat maps show the first derivative of the difference spectra at each temperature compared to the room temperature spectra where hot spots (warm colors) indicate structural loss and cold spots show a structural gain. Displayed T_ms are taken at 1640 cm^{-1}. Similarity plots were generated by inverting and baseline-subtracting the second derivative of the 25°C absolute absorbance spectra. Similarity percentages were calculated based on the area of overlap between each pH condition and pH 8.5.

In addition to increased melting temperature, similarity percentage also increased with pH. This suggests that differences in secondary structure exist between thermally stable and unstable forms of ovalbumin. The similarity overlap plots provide a direct comparison of secondary structure profiles across pH conditions. When compared to the spectrum of ovalbumin at pH 8.5, notable differences were observed in the turn region (1690-1665 cm^{-1}), alpha-helix region (1665-1654 cm^{-1}), and beta-sheet region (1648-1630 cm^{-1}).

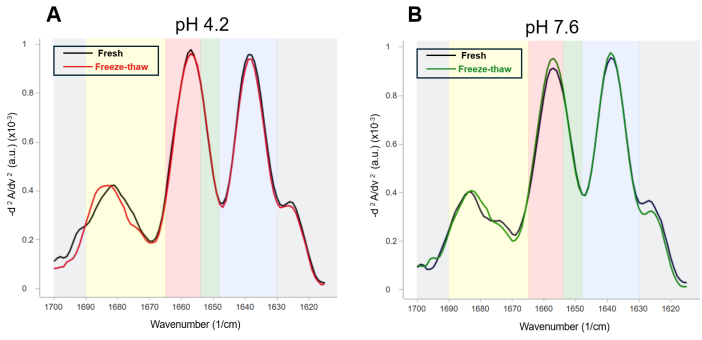
Further analysis revealed pH-dependent, structure-specific melting temperatures. Thermal heatmaps (Figure 2, top) and corresponding tables of secondary structure melting temperatures (Figure 2, bottom) are shown for ovalbumin at pH 4.2 (Figure 2A) and pH 8.5 (Figure 2B). At low pH, earlier melting of all secondary structure motifs was observed, with greater magnitudes of loss in the turn (1695 cm^{-1}) and alpha-helix (1658 cm^{-1}) regions. Additionally, the formation of intermolecular beta-sheets (1620 cm^{-1}), a known marker of protein aggregation,[6] occurred at lower temperature compared to higher pH. At high pH, two distinct melting events were observed for intramolecular beta-sheets (1638 cm^{-1}), and intermolecular beta-sheet formation was the second to last transitions to occur. These results demonstrate the ability of MMS to measure individual structural melting behaviors and indicate greater structural stability at higher pH values.



Structure	T_m (°C)
β-turn (1695 cm^{-1})	66.4
α-helix (1658 cm^{-1})	63.9, 69.9
Intramolecular β-sheet (1639 cm^{-1})	65.6
Intermolecular β-sheet (1620 cm^{-1})	62.4, 68.4

Structure	T_m (°C)
β-turn (1670 cm^{-1})	71.7
α-helix (1660 cm^{-1})	75.4
Intramolecular β-sheet (1638 cm^{-1})	68.5, 77.2
Intermolecular β-sheet (1621 cm^{-1})	76.7

Figure 2: Melting temperature detection of specific secondary structural elements using MMS. Thermal heat map data is from Figure 1. Melting temperatures for structural elements were assigned by fitting multiple sigmoid curves over temperature at each wavenumber. Dashed lines represent reported melting temperatures.



Condition	Similarity (%)	Repeatability (%)
Fresh	100	98.31
Freeze-thaw	95.61	98.56

Condition	Similarity (%)	Repeatability (%)
Fresh	100	98.33
Freeze-thaw	97.45	97.97

Figure 3: Structural similarity after freeze-thaw cycles at different pH values. Similarity spectra show fresh, unfrozen, samples (black line) and three-times frozen and thawed samples (colored line). Similarity was calculated using the area of overlap between freeze-thaw and fresh spectra.

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Results, continued

To further investigate the stabilizing effects of pH, a freeze–thaw stability assay was performed. Ovalbumin was prepared in buffers at pH 4.2 and pH 7.6, subjected to three freeze–thaw cycles, and analyzed via similarity overlap between stressed and fresh samples (Figures 3A–B). Ovalbumin maintained the highest similarity to fresh samples at pH 7.6 (97.45%). At this pH, most differences were observed in the alpha-helix and intermolecular beta-sheet regions, with minor changes in the turn and intramolecular beta-sheet regions. Interestingly, at pH 4.2, a blue shift in the turn peak was observed in the freeze-thawed samples, accounting for most of the spectral difference (Figure 3A). Only slight changes were observed in the alpha-helix and beta-sheet regions. These results support the thermal stability findings and demonstrates MMS as a powerful tool for characterizing protein structural stability, especially proteins with complex melting behavior.

Conclusions

This study demonstrates the effectiveness of MMS, utilizing the AuroraTX platform, for comprehensive protein stability analysis. By simultaneously capturing thermal stability and structural changes, MMS provides deeper insight into how proteins respond to formulation conditions such as pH variations and freeze-thaw stress. Importantly, MMS can detect multiple thermal transitions within multi-domain therapeutic proteins, enabling targeted stabilization of less stable domains. Unlike traditional methods, MMS offers high sensitivity in detecting specific structural shifts and quantifying changes, making it a powerful tool for accelerating formulation development and preserving the structural integrity of therapeutic proteins.

Contributors

Ben Lyon
Valerie Collins, Ph.D.
Scott Gorman, Ph.D.

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