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Structure Function Relationship: Effect of Changes in Higher Order Structure to Analyte Ligand Binding

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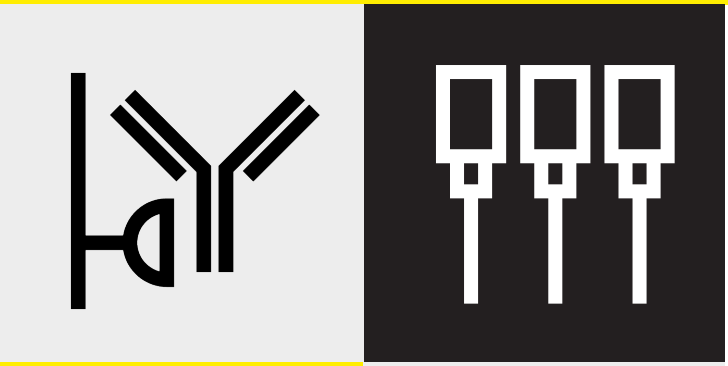

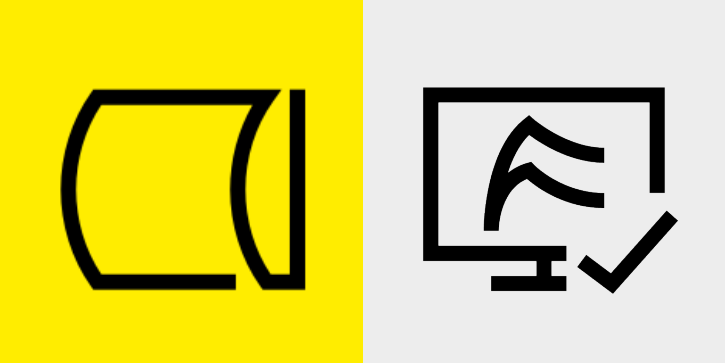

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Introduction

Protein function is often related to the binding of the protein as a receptor to a ligand. The specific ligand binding site on protein is typically a local region on the protein determined by a specific structural conformation. Structure therefore determines function for most biological molecules. The three-dimensional structure of a protein is determined by the tertiary contacts between elements of the secondary structure of the protein that includes α-helices, β-strands/sheets and turns; all of which contribute to the folding of the protein and allow for the formation of the ligand binding active site. These structural elements can however be affected significantly or in subtle ways by excipients or stresses that include salts, buffers, pH and temperature amongst other things.[1] These conditions invariably affect either the local structure of specific sites within the three-dimensional conformation of the protein or the global structure of the protein and can affect the activity of the protein towards its binding partners. Structural and binding analysis studies can also be used to predict how a protein-based drug substance would perform in a given formulation and may be used to determine optimal formulation for the drug substance. Pre-formulation studies aimed at identifying the physicochemical characteristics of a drug candidate may include the evaluation of solvent sol¬ubility, pH stability, size distribution and structural and functional stability with different excipients. Structural stability is typically assessed through biophysical characteristics such as un-folding or mis-folding properties or through the analysis of presence of aggregation while activity may be assessed through affinity characterization or target binding response at different product conditions.

Microfluidic Modulation Spectroscopy (MMS)
Microfluidic Modulation Spectroscopy (MMS) is an automated IR technology that addresses limitations of conventional FTIR by using a **quantum cascade laser** as the **light source** for high sensitivity measurement of protein higher order structures (HOS).[2] The amide-I IR band, which is associated with the C = O vibration of the protein backbone, is highly sensitive to changes in secondary structure and well-correlated with shifts in H-bonding and torsion in the α-helix, β-sheet, and turn structures.[3] With its high sensitivity, MMS can be used to directly monitor aggregation processes by measuring the intermolecular β-sheet structures associated with aggregate formation.[4] RedshiftBio's Aurora system[5], powered by MMS, effectively detects changes in the secondary structure of proteins within a concentration range of 0.25 – 200 mg/mL. This modulation technique facilitates real-time background subtraction, thereby enhancing measurement accuracy and enabling measurements in a wide range of formulation buffers.

Biolayer Interferometry (BLI)
Biolayer Interferometry (BLI) is an optical analytical technique that utilizes the changing interference pattern of white light shown over a reflective biosensor surface with an immobilized ligand and an interacting analyte in solution. The binding between the ligand and the analyte produces an increase in optical thickness on the tip of the biosensor that can be measured as a wavelength shift from the reference surface and is a direct measure of the change in thickness of the biological layer as a result of the binding between the pre-immobilized molecule on the biosensor surface and the sample in solution. Differences in wavelength shift upon binding for the same analyte sample at different excipient conditions may be used to infer differences in the structural state of the analyte.

In this short article, we investigated the intersection between protein concentration and functionality, as measured by Biolayer Interferometry (BLI), and the secondary structure of the same protein, as characterized by Microfluidic Modulation Spectroscopy (MMS) using the Aurora system. This study uses two examples to showcase the promise of combining the two technologies as potential tools for an in-depth understanding of the effect of different excipients on the structural changes that may occur in a given analyte during formulation development and their potential impact on the functional behavior of the analyte.

Case Studies

This document delineates several study proposals, including the evaluation of binding activity as a function of analyte formulation and the assessment of hydrophobic surface binding for aggregated molecules. These studies underscore the capabilities of both technologies in detecting subtle variations in kinetics and protein structures. Specifically, the study goals include: first, evaluating hydrophobic surface binding as a method to quantify the level of aggregated molecules present; and second, investigating binding activity as a function of analyte formulation across different buffering systems. Accurate background subtraction is imperative for both technologies, necessitating precise buffer matching. Minor variations in buffer concentrations can lead to unreliable data, underscoring the critical importance of meticulous preparation. This aspect is essential for ensuring the reliability of results, particularly in comparative studies.

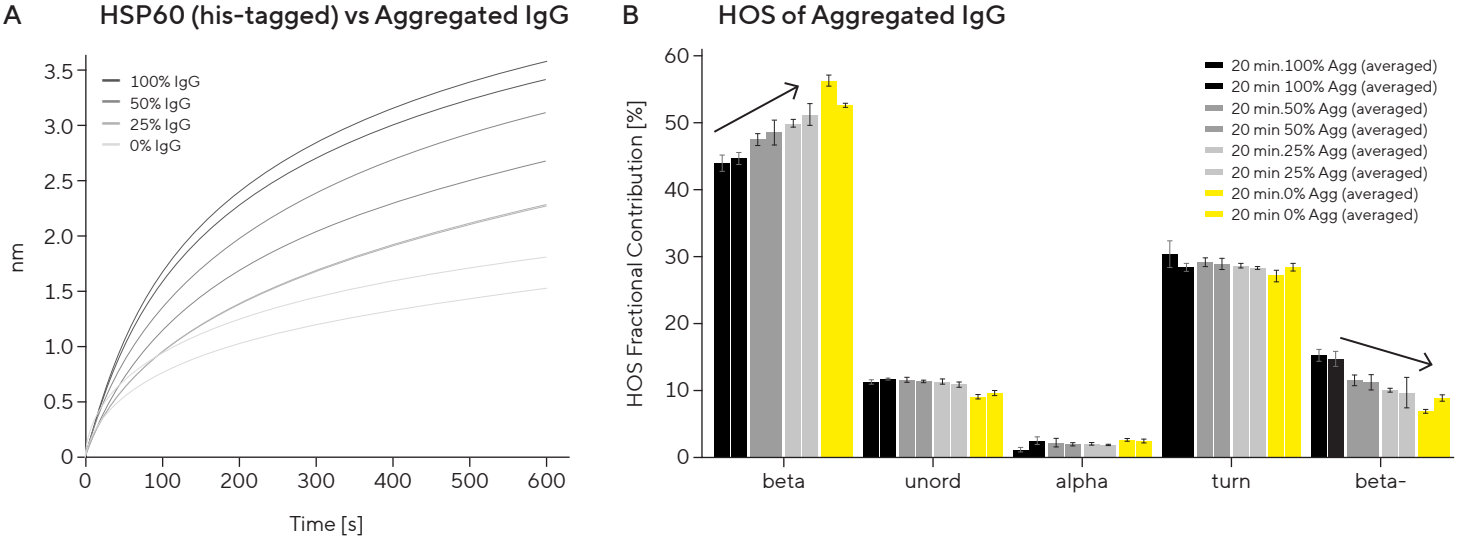
Results and Discussions

Detecting Presence of mAb Aggregates

HSP60 is a molecular chaperone utilized in the study of protein folding and aggregation. In this study, His-tagged HSP60 (5 µg/mL) was immobilized onto Ni-NTA biosensors. Subsequently, varying percentages of aggregated Immunoglobulin G (IgG) were captured as the analyte. The aggregated IgG samples were prepared in 1x PBS in-house and were heated at 70 °C for 20 minutes and then mixed with an equivalent concentration of unheated IgG to achieve final aggregates percentages of 0%, 25%, 50%, and 100%. It is hypothesized that higher percentages of aggregation will result in increased binding to the HSP60 on the biosensor as well as elevated amount of aggregated beta-sheet structure. The experimental protocol involved initially analyzing the material using the Octet® R8 Biolayer Interferometry (BLI) system, followed by immediate analysis of the samples using the Aurora Microfluidic Modulation Spectroscopy (MMS) system. The results of these experiments are presented in Figure 1.

The BLI data (Figure 1A) with 1mg/mL of different aggregated IgG shows higher binding responses, indicative of a higher degree of exposed hydrophobic surfaces, as the percent of aggregated protein increases. The same material was analyzed using in the Aurora instrument (Figure 1B), with 1x PBS employed for background correction. The x-axis labels "beta" denotes the percentage of native beta-sheets within the protein structure, while "beta-" indicates the percentage of aggregated beta-sheets. The results demonstrate that samples with lower aggregation levels (yellow bars) exhibit a higher proportion of native beta-sheets compared to samples with higher aggregation levels (black bars). Conversely, the "beta-" response shows that higher aggregation levels correspond to an increased presence of aggregated beta-sheets. No changes were observed to other secondary structures such as alpha-helices, unordered or turns.

Figure 1: *Aggregation as a Measurement of Binding Relative to Secondard Structures*



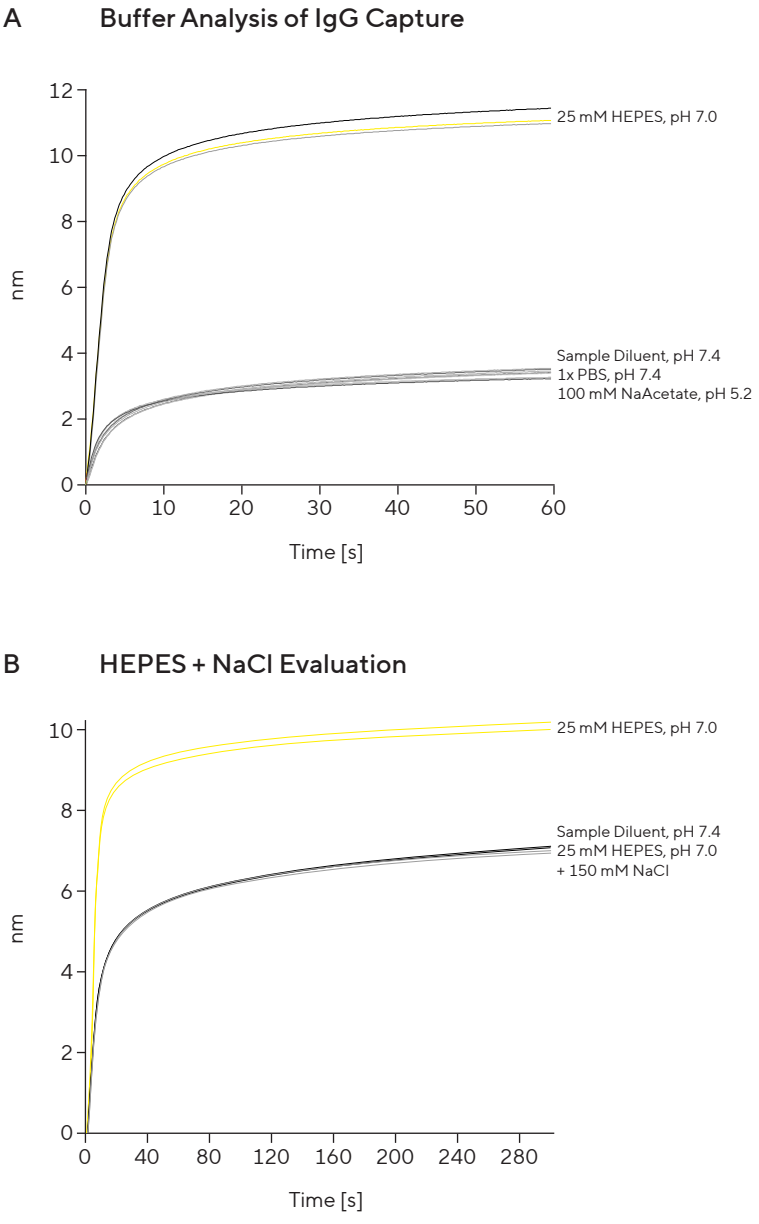
Note. **(A)** Aggregated IgG binding response to HSP60 immobilized NiNTA biosensors. **(B)** Aggregated IgG samples higher order structure (HOS) detection on the Aurora system.

Examining the Effect of pH on mAb Analyte

In the second study, a wide range of human IgG concentrations (mg/mL) were prepared in various buffers at different pH conditions. Biotinylated Protein A was immobilized onto SAX biosensors using Octet® sample diluent buffer, followed by processing in the respective buffers for IgG capture. As in the previous study, these IgG samples were subsequently analyzed on the Aurora system, utilizing their respective buffers for background subtraction.

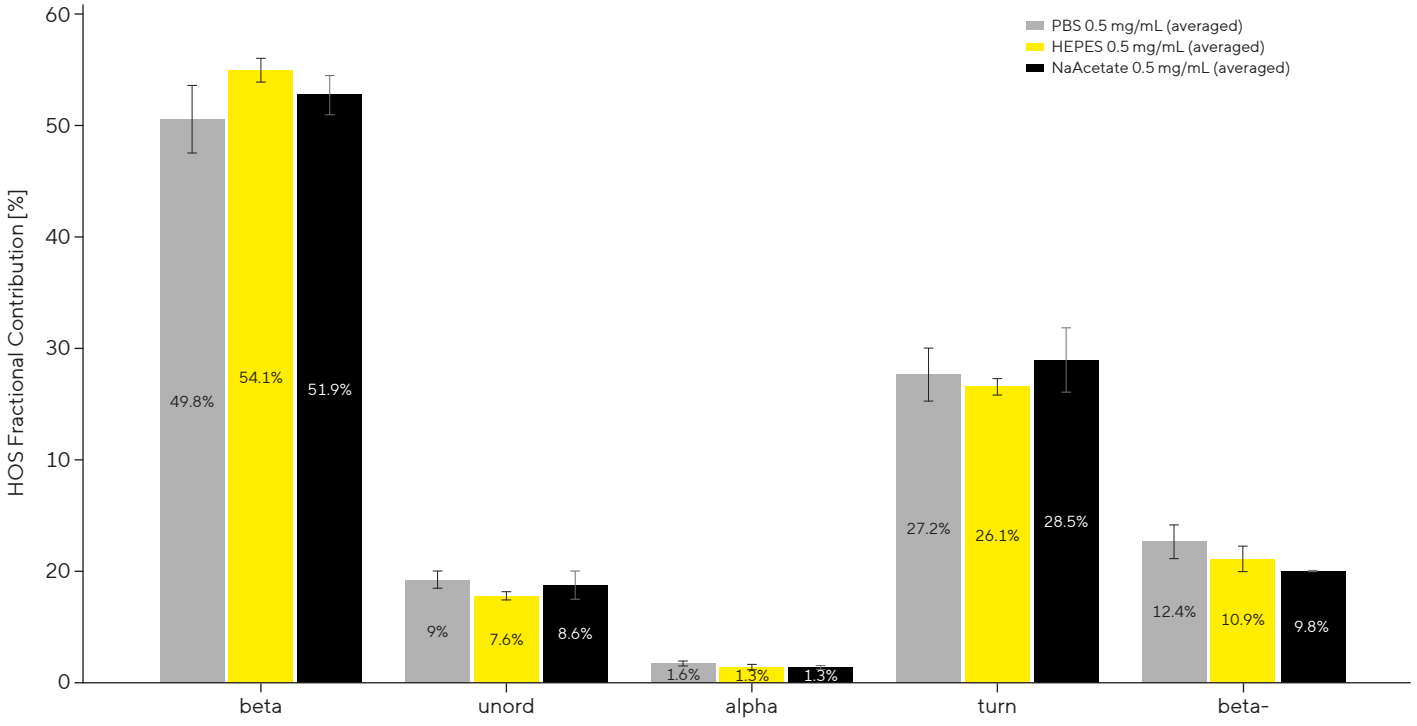
The binding of the IgG samples to Protein A revealed significant differences between the effects of 25 mM HEPES at pH 7.0 and 1x PBS at pH 7.4 (Figure 2A). An examination of the structural data through the Aurora system however indicated no significant change in structures between the IgG samples in the two different buffer systems (Figure 3). A major difference between the two buffer systems was the NaCl which was present in the 1x PBS but not in the HEPES buffer. A repeat experiment with the addition of 150 mM NaCl in the HEPES buffer revealed a diminished binding response of the IgG samples in the presence of NaCl with identical binding to the sample in 1x PBS (Figure 2B) indicating that the earlier observation may have been the result of non-specific binding (NSB) likely due to electrostatic interactions. The secondary structure data on the Aurora (Figure 3) indicates that the relative percentages of native beta-sheets ("beta") remain consistent across different buffers, as evidenced by overlapping error bars.

Figure 2: *The Effects of Different Buffering Conditions*



Note. (A) The effect of different buffering conditions on the capture of 0.5 mg/mL IgG by Protein A. The sample diluent consists of 1xPBS, supplemented with BSA and detergents. (B) The same experiment demonstrating that the addition of 150 mM NaCl to the 25 mM HEPES buffer results in a binding affinity comparable to that observed with the sample diluent. Further studies were conducted using "naked" biosensors (i.e., without Protein A), which revealed binding in the 25 mM HEPES-only buffer, further indicating that the elevated response is attributable to non-specific binding (data not shown).

Figure 3: *Aggregated IgG binding response vs Higher Order Structure*



Note. The secondary structure analysis of 0.5 mg/mL of IgG in 1x PBS pH 7.4, HEPES pH 7.0, and sodium acetate pH 5.2.

Summary

Traditional technologies for downstream assessment of biological drug candidate stability such as dynamic light scattering (DLS), multiple-angle light scattering (MALS) and circular dichroism (CD), while effective and versatile, are not sensitive enough to detect minor changes in these drug candidates that may impact their function. The Aurora platform, with its higher sensitivity that can be used to measure structural changes at the secondary structure level, is better suited for detecting subtle changes in product structure. A workflow that combines the Octet® BLI technology[6], known for its ease of use, plate-based high through-put capabilities, with the Aurora MMS technology, known for its high sensitivity in structural analysis, should provide downstream bioprocessing and product quality control analysts with better insight into optimal formulations during the final stages of drug development. While this short article showcases only two case studies, the platforms can be used extensively and in a high throughput manner to screen for the effect of multiple excipients on the stability and binding activity of biological molecules.

References

1. Naz H, Shahbaaz M, Bisetty K, Islam A, Ahmad F, Hassan MI. Effect of pH on the structure, function, and stability of human calcium/calmodulin-dependent protein kinase IV: combined spectroscopic and MD simulation studies. Biochem Cell Biol. 2016 Jun;94(3):221-8.
2. Kendrick, B. S.; Gabrielson, J. P.; Solsberg, C. W.; Ma, E.; Wang, L. Determining Spectroscopic Quantitation Limits for Misfolded Structures. J Pharm Sci 2020, 109 (1).
3. Dong, A.; Huang, P.; Caughey, W. S. Protein Secondary Structures in Water from Second-Derivative Amide I Infrared Spectra. Biochemistry 1990, 29, 3303–3308.
4. Ma, T. M.; Huang, R. H.; Collins, V. I.; Wikström, M.; Batabyal, D. Advancement of Microfluidic Modulation Spectroscopy as a Highly Sensitive Protein Characterization Technique for the Detection of Small Structural Changes. Anal Biochem 2023, 683.
5. RedShiftBio Aurora. **https://www.redshiftbio.com/products/aurora**
6. Octet® BLI. **Biolayer Interferometry (BLI) | Sartorius**

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