Introduction To The AQS^{3}™pro

The Next Generation In Protein Structure Determination Powered By Microfluidic Modulation Spectroscopy

REDSHIFTbio®

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A Desktop Resource

July 2020, Second Edition

Introduction to the AQS³pro

Dear Reader,

The ability to measure and characterize changes in the secondary structure of proteins is critical to many research applications, especially the formulation and development of biotherapeutics. There is direct evidence that changes in secondary structure result in changes in efficacy and specificity. Conventional techniques do not possess the adequate feature sets for researchers to See change® in their proteins within the required conditions. The need for high sensitivity, a wide dynamic range, a simplified and automated workflow, and high repeatability is significant and, until now, has not been adequately provided. The solution for today's researcher is Microfluidic Modulation Spectroscopy (MMS).

This eBook will introduce you to MMS, a novel next-generation infrared technology with advanced data analytics to facilitate better, faster decision making in biopharmaceutical development from discovery to market. MMS provides direct measurement of previously undetectable changes in protein structural attributes that prove critical to drug product efficacy and quality. In this eBook, applications are presented which demonstrate how it is now possible to detect smaller magnitudes of change in commercially available proteins as well as biopharmaceutical samples. The results are impressive, detailing high sensitivity, a wide dynamic range and simplified background subtraction. All this while measuring aggregation, quantitation, stability, structure, and similarity within a single sample or between samples using an automated platform. The ability to confidently See change is finally here!

If you have any comments, questions, or suggestions for this eBook, please send an email to info@redshiftbio.com and we will be happy to help in any way we can.

Happy reading!

~The RedShiftBio Team

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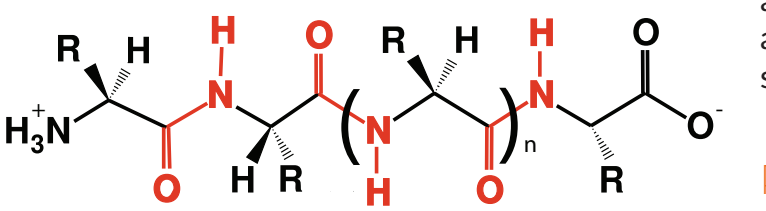
Chapter 1
Introduction to the AQS³pro and Microfluidic Modulation Spectroscopy

The Importance of Protein Secondary Structure in Drug Development

Biologic drug products have an inherent propensity for instability or conformational change which can result in product aggregation leading to a potential for immunogenic responses in patients.[1] The process is triggered by uncontrolled protein-protein interactions which can be a direct consequence of inappropriate formulation of the product, or a result of an external stressor such as temperature, oxidation, or vibration.

The identification of structurally-sound promising drug candidates is becoming increasingly rigorous as the diversity and capabilities of analytical techniques increase. Regulatory bodies are also increasing their demand for comprehensive characterization of both the primary components of a drug product as well as unplanned components and structural changes. Therefore, investigating the effects of manufacturing, shipping, and storage conditions is crucial to understanding and controlling the factors and mechanisms associated with formulation stability and protein aggregation, especially where these factors affect secondary structure and function of the protein.



Protein Structure and the Amide Bond

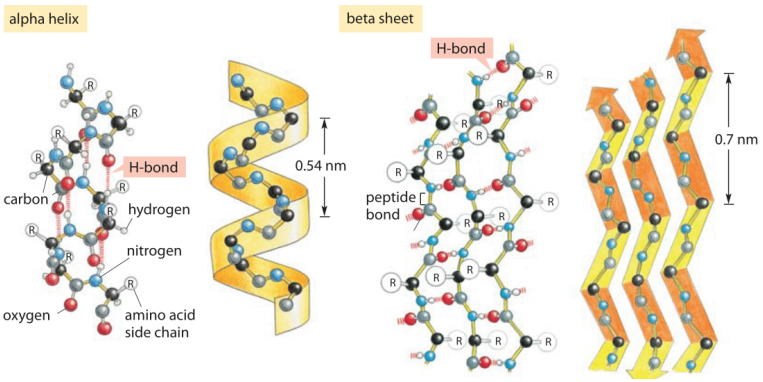


Figure 1: (a) Basic structure of a protein, with amide bond highlighted in red, (b) hydrogen bonding of both alpha-helix and beta-sheet strctures of a protein.

- Protein secondary structure is formed by hydrogen bonds between the backbone amino acids, specifically the amino hydrogen and carbonyl oxygen groups, resulting in repeated structural motifs such as alpha-helix and beta-sheet.
- The structural motifs form regular dihedral angles of the amide bond. Infrared Spectroscopy can be used to examine absorption bands of the amide bond relative to changes in the angle.
- IR measures the three vibrational modes observable from the amide bond: Amide I, II and III. These spectra and can subsequently be used to determine the secondary structure content of a protein.
- The Amide I band (1700-1600 cm-1) is specifically associated with the C=O stretch and is well correlated to shifts in hydrogen bonding, dipole-dipole interactions and geometric orientations of the bond. Amide II and III bands are associated with the C-N stretch and rotation, respectively.
- The relative amounts of secondary structure moities can be determined by comparing measured Amide I spectra with reference library data for the assessment of chemical and thermal stability, aggregation, and biosimilarity.

A Comparison of Traditional Techniques

Though the potential of vibrational spectroscopy in protein characterization is widely recognized, current commercial technologies do not adequately fulfill the requirements for measurement within biotherapeutic development, formulation, and manufacture particularly in relation to measurable concentration range, and sample compatibility amongst other issues.

- **Differential scanning calorimetry** (DSC) is routinely used as an early screen for thermal stability, providing assessment of melting points (T_m) of a protein; however, little structural data can be obtained.
- **Size Exclusion Chromatography** (SEC) is used to detect aggregation but has little facility to provide structural data outside of oligomeric composition.
- **Fourier Transform Infrared Spectroscopy** (FTIR) and **Raman Spectroscopy** are routinely used for structural analysis of proteins with both methods providing structural composition data. Both have limitations with sample concentration as well as additional challenges including background drift, poor sensitivity, and a requirement for close temperature control. These drawbacks limit their use in biopharmaceutical development.
- **Circular Dichroism Spectroscopy** (CD) is an established tool for structural analysis at dilute concentrations, but it is unsuitable for measurement of concentrated samples. In addition, CD is not compatible with many of the common buffers and excipients used for biopharmaceutical development.

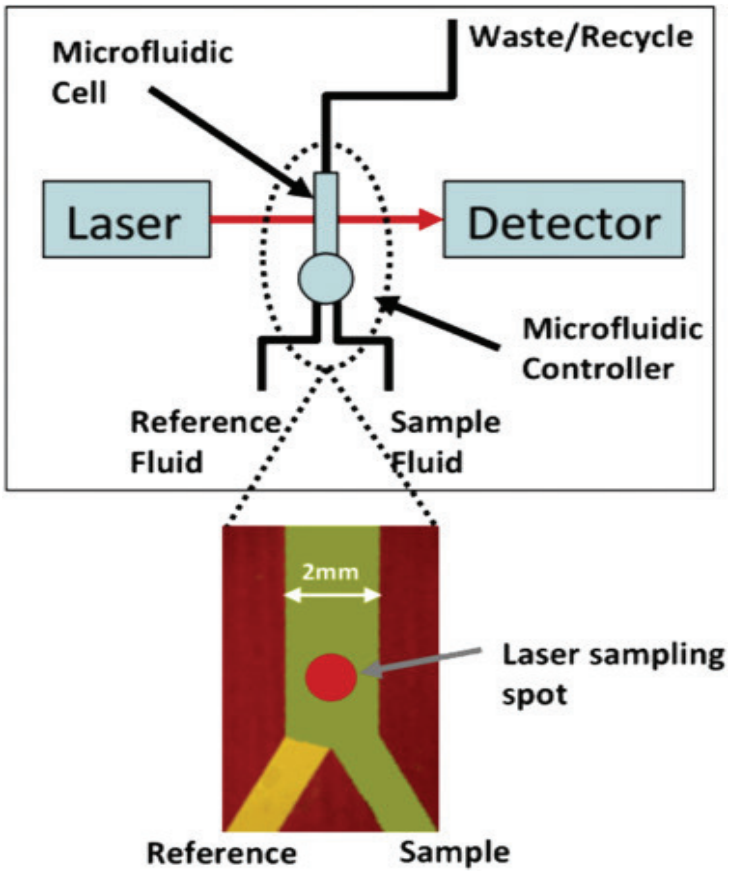


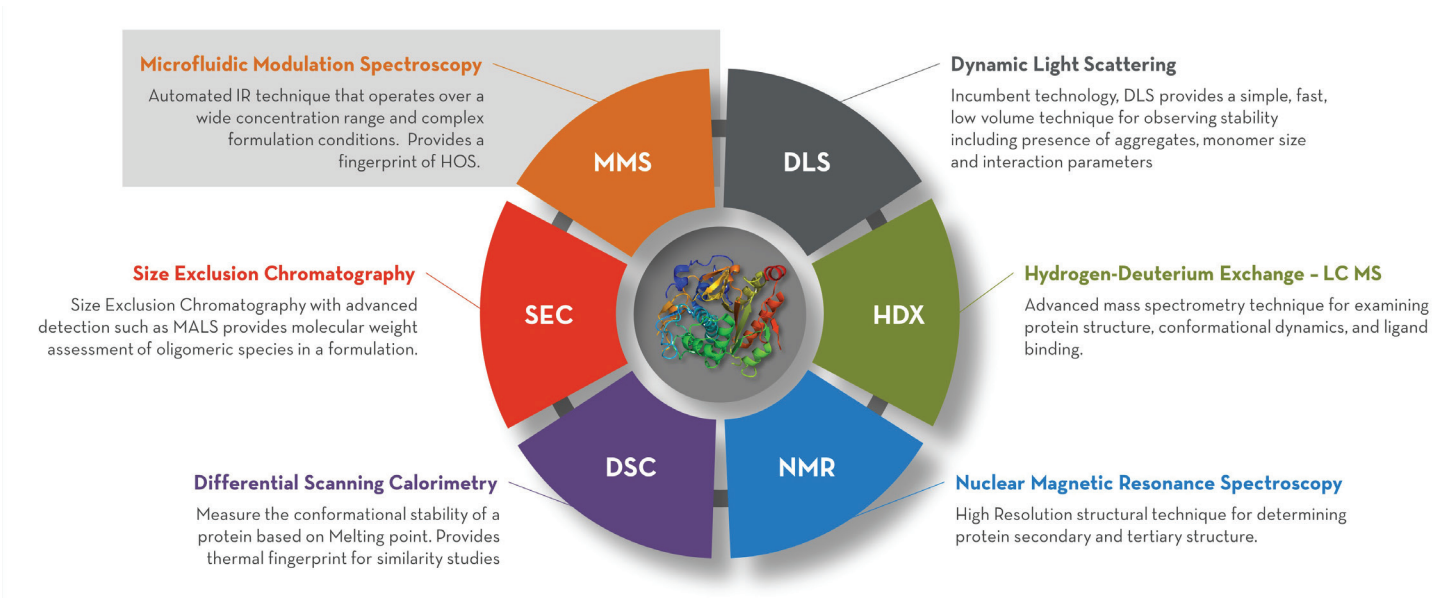
Figure 2: General schematic of a microfluidic modulation spectroscopy flow cell and microfluidics showing sample and reference solution entry points and enlarged diagram of a flow cell.

What is MMS

Microfluidic Modulation Spectroscopy (MMS) is a novel spectroscopic tool which directly addresses the limitations of current technologies by facilitating direct, label-free analysis of protein secondary structure over a wide concentration range (0.1 to 200 mg/mL) in complex formulations without the need for buffer exchange or dilution. MMS employs a tunable mid-infrared quantum cascade laser that is 1000 times brighter than conventional FTIR. In addition, MMS enables the measurement of currently undetectable changes in protein structure.[2]

Now with the power of the AQS^{3}pro MMS system, the sample (protein-in-buffer) solution and a matching buffer reference stream are introduced into the transmission cell under continuous flow and then rapidly modulated (1–5 Hz) across the laser beam path. This produces nearly drift-free, background compensated, differential absorbance scans of the Amide I band (1700-1600 cm^{-1}) and allows changes in HOS to be easily measured and compared.

MMS is an Essential Part of Every Biophysical Toolkit



Microfluidic Modulation spectroscopy is a novel tool that can strengthen a bioanalytical tool kit by providing critical insight through all phases of protein-based drug development. This technique enhances current toolkits with the ability to monitor 5 key protein characteristics including aggregation content, concentration, structure, stability, and similarity. MMS provides the following benefits:

We believe [MMS] can play a significant role in the protein characterization technology toolkit, overcoming shortcomings in existing techniques: in our case the quality of the results obtained, the small sample volumes, the ability to quickly probe low concentrations of amyloid proteins in solution under 'physiological' conditions. While there are many other interesting and important applications of this technology, RedShiftBio's MMS platform fills a void in the amyloid field and is helping to shed light on the fundamental conformational changes associated with disease."

Professor of Bioengineering

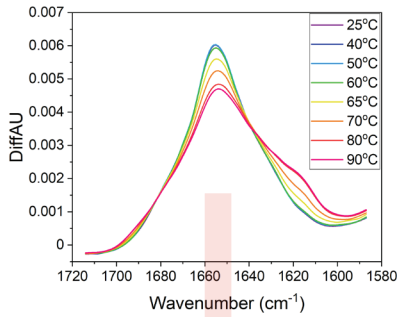
- A reduction in time and cost through automation (24/96 well formats).
- Improvement in product quality with measurements of previously undetectable changes in protein structure.
- De-risking of downstream product failures through earlier up-stream monitoring.
- Generation of quality data for confident decision-making in order to accelerate development timelines.

AQS³delta Software Workflow

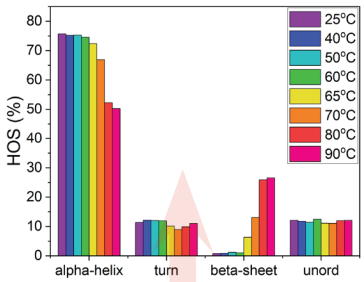
The AQS³pro is powered by AQS³delta control software, providing easy access to multiple measurements and the ability to See change in protein structure.

Below is an example data analysis showing the results of a thermally stressed BSA sample at 1.0 mg/mL

Differential Absorbance



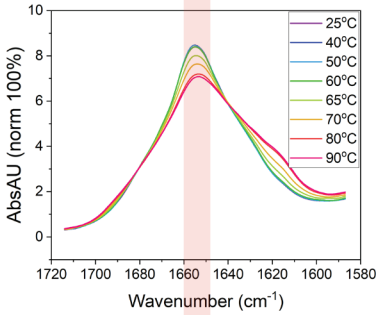
Higher Order Structure



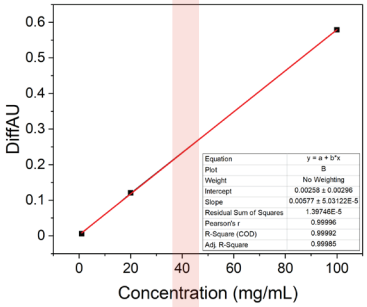
AQS³pro

MICROFLUIDIC MODULATION SPECTROSCOPY

Absolute Absorbance

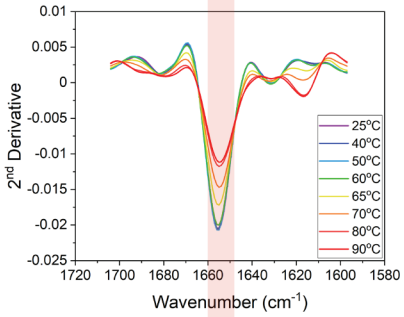


Quantitation

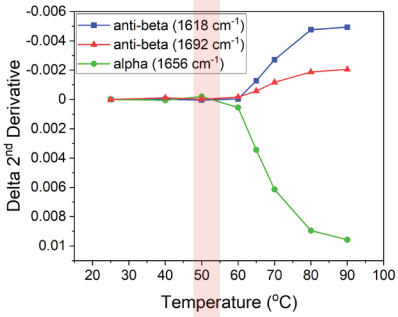


Analyze similarity with the Area of Overlap tool, or take advantage of the wide dnamic range for quantitation. Here is a look at the data flow analysis for BSA to see how the AQS3delta software processes and presents data to maximize the value of each measurement.

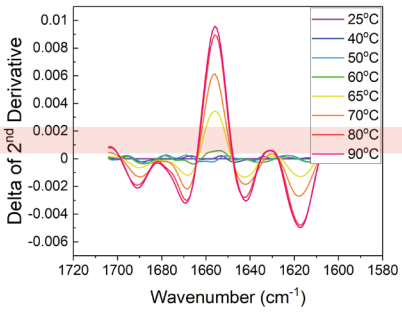
Second Derivative



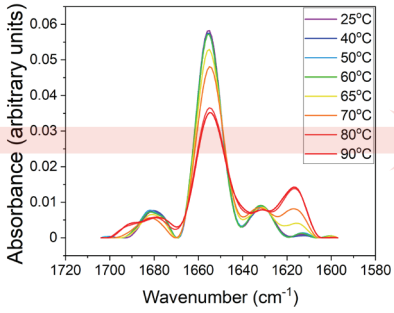
Stability Trend



Delta of Second Derivative



Area of Overlap



Chapter 2
Protein Analysis Using the AQS^{3}pro

Detecting AGGREGATION

Protein aggregation is the process by which proteins undergo self-association to form higher molecular weight components in a biopharmaceutical. This process can be initiated by strong protein-protein interactions resulting in secondary structure unfolding, as seen in the lower pathway in Figure 8. Alternatively, proteins may also aggregate due to stress from oxidation, agitation, or thermal changes – all of which can initiate unfolding. These unfolded proteins are highly prone to self-association which typically leads to the formation of aggregates, as seen in the upper pathway in Figure 8. However aggregates are formed, they are a significant problem in drug development and formulation.

Research has shown that the formation of anti-parallel beta-sheets is a common indicator of aggregation in biopharmaceuticals.[3] The data shown in figure 3 represents the size exclusion chromatogram (3a) and MMS generated Higher Order Structure (HOS) comparison (3b) of an antibody drug conjugate (ADC) exposed to thermal stress, along with the representative control sample. The grey data represents a control sample of the ADC kept at 4 °C while the pink data represents a sample exposed to 70 °C for 20 minutes. The chromatogram in figure 3a clearly shows the transformation of the monomeric species into a higher molecular weight aggregate as evidenced by the reduction in retention time of the sample. The MMS data shown in figure 3b indicates a change in structural content from ~50% parallel beta-sheet to only ~20% parallel beta-sheet, and an increase in anti-parallel beta-sheet from ~18% to ~40% for the 70 °C treated sample, a clear indicator of the presence of aggregate in a sample. Due to its sensitivity, resolution, and ability to work in complex formulation, Microfluidic Modulation Spectroscopy is uniquely able to resolve these structural changes which would not be easily observable

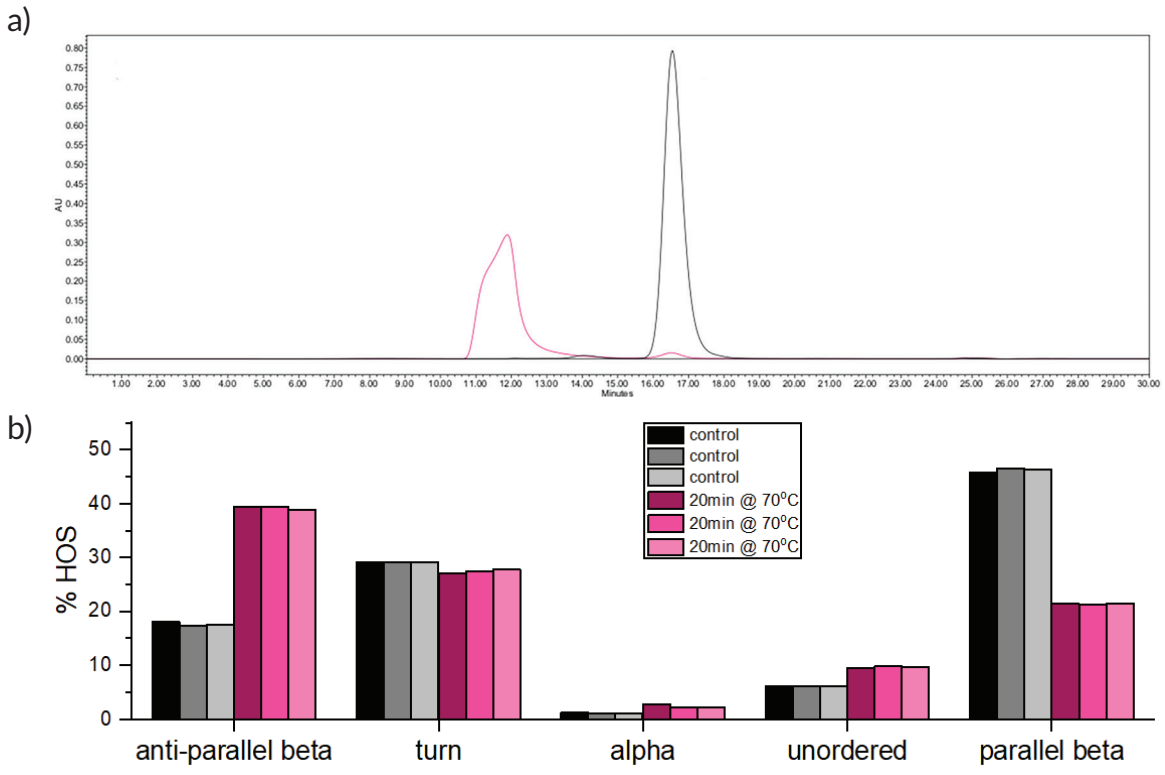


Figure 3: a) chromatogram of ADC sample exposed to 70 °C for 20 min (pink) compared to the control (grey) sample, showing significant fomation of higher molecular weight species. b) HOS summary of ADC sample exposed to 70 °C for 20 min (pink) versus control sample (grey) showing the control sample with approx 45% parallel beta-sheet converting to 21% after thermal stress, and forming approx 40% anti-parallel beta sheet, a strong indicator of aggregation.

QUANTITATION of Protein Concentration

Protein concentration of biologic drug products has traditionally been measured using UV/Vis spectrophotometric techniques. These techniques look at either the absorbance of the protein itself using E_{280} type measurements, or they utilize a colorimetric assay such as a Lowry protein assay to measure total protein in a sample. A significant limitation of these methods is that linearity occurs only within a narrow concentration range, typically from about 0.1 mg/mL to about ~20 mg/mL. As biopharmaceuticals move to higher concentrations throughout the development pipeline, the limitation of these methods cannot be overcome using neat samples of protein and the likely addition of excipients. While strategies exist to address this limitation, including the use of serial dilutions and variable path length cells to improve the dynamic range, each method introduces an additional variable and opportunity for error, resulting in increased statistical uncertainty.

Microfluidic Modulation Spectroscopy is an alternative tool for direct, label-free protein quantitation over a wider concentration range. By using IR absorbance and a multi-datapoint fitting algorithm, MMS allows for high precision concentration measurement over a wider concentration range of 0.01 to greater than 200 mg/mL.

Figure 4 shows measured concentration data of sample series of monoclonal antibody (mAb) over a concentration range of 1 to 150 mg/mL. The antibody is formulated in a Histidine buffer which includes trehalose, methionine, and polysorbate 80. The data acquired using the AQS³pro shows clear linearity across this measurement range with an R^2 value of 0.999.

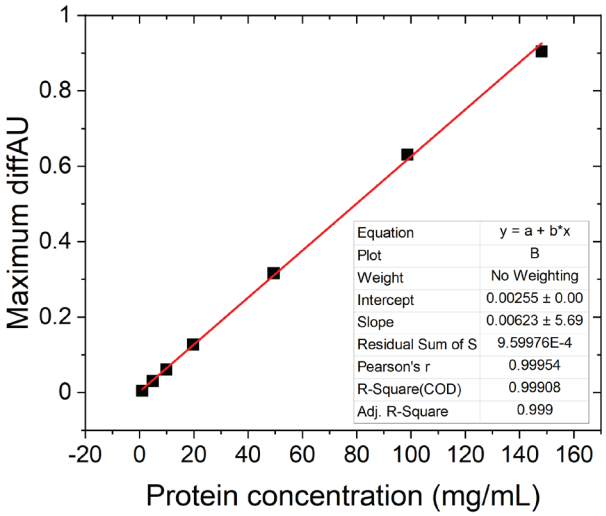


Figure 4: Concentration plot of mAb sample series from 1 to 150mg/mL showing good linearity across the range with R^2 = 0.999.

See the AQS³pro in Action

Visit our website to see a short introductory video to our MMS technology

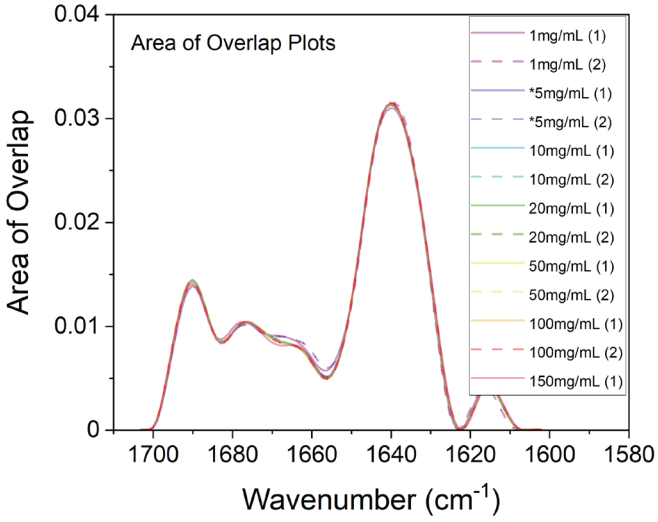
Visit www.redshiftbio.com/videos

Measuring Protein STRUCTURE

Structural comparison of drug products is essential across the entire development pipeline. Critical points for assessing comparability include formulation design, fill-finish and container closure, stability assessment, and process monitoring to name a few. With biosimilar drug products, structural comparisons are used to support the totality of evidence used to demonstrate bioequivalence and comparability. Microfluidic Modulation Spectroscopy is a high sensitivity, high precision tool for structural determination of proteins through examination of the Amide I band (1700-1600 cm^{-1}) of an IR spectrum and therefore a reliable measure of common secondary structure moieties and changes in these structures. The AQS^{3}pro, powered by AQS^{3}delta software, can measure total structural similarity at any point during drug development through the use of spectral overlay and HOS comparisons.

Figure 5a shows an area of overlap plot acquired from a serially diluted monoclonal antibody in Histidine buffer from 1 to 150 mg/mL. A similarity score is calculated based on total area of overlap with a reference spectrum (5 mg/mL). When compared to the reference spectrum, replicates show 99.2-99.8% similarity. Gaussian deconvolution of this data generates the higher order structural composition of each sample as shown in figure 5b. The results show that the structure of this sample is not changing over the dilution series and contains an average of 60.5% (±0.6%) beta-sheet, 28.6% (±0.3%) beta-turn, 9.3% (±0.2%) unordered structure, and 1.5% (±0.1%) alpha-helix content. The high reproducibility >98% of this technique exemplifies MMS as an excellent technology for measuring both similarity and comparability in a biologic formulation.

a)



b)

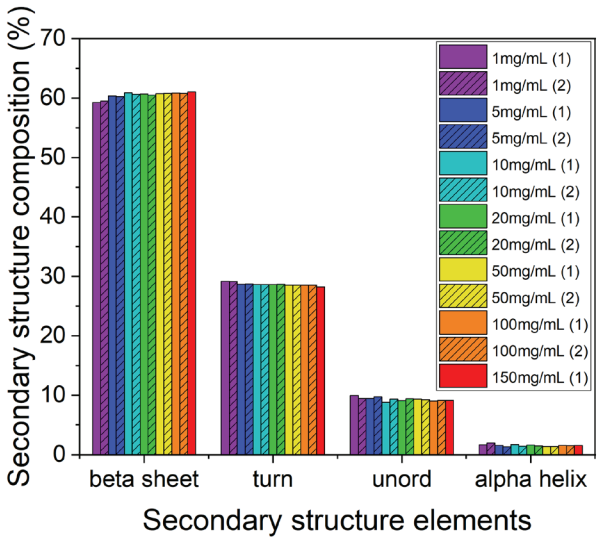


Figure 5: (a) Area of Overlap plot from a serially diluted monoclonal antibody solution (1 - 150 mg/mL) showin repeatability of 99.2-99.8%. (b) Higher Order Structure plot of the same monoclonal antibody dilution series (1 - 150 mg/mL) shows consistent higher order structure between replicates.

Monitoring STABILITY of a Formulation

Understanding the stability of a protein is an important part of developing a quality pharmaceutical. Stability can be monitored by means of quality-by-design approaches in discovery and formulation all the way to forced degradation studies. The result of stress-induced structural changes can include decreased potency, degradation of product, and an increase in particle content, potentially increasing the risk of an immunogenic response in a patient.

Microfluidic Modulation Spectroscopy supports accurate assessment of the stability of a protein throughout the entire formulation, development, and manufacturing processes, derisking the development pipeline and enabling control strategies to be developed for each critical quality attribute. A necessary experiment in formulation design and development is the exposure of a protein to a range of pH conditions in order to determine of the optimal pH range for stability.

In the study presented in Figure 6, insulin was formulated across a pH range from 2.5 to 7.5. The second derivative of the Amide I region of the IR spectrum from 1720 to 1580 cm^{-1} was plotted. With the ability to See Change even on very small magnitudes, the MMS data measured the most change in the region of 1700-1670 cm^{-1} as a downward trend with decreasing pH at 1675 cm^{-1}, and a similar downward trend at 1658 cm^{-1}.

When further analyzed using the AQS^{3}delta software package, Figure 6b shows trends that correlate to a shift in structure from alpha-helix to unordered structure by about 5%. This is consistent with literature which indicates that acidic conditions can change insulin structure, but generally do not degrade helical content enough to prevent formation of higher order dimers and hexamers.[4]

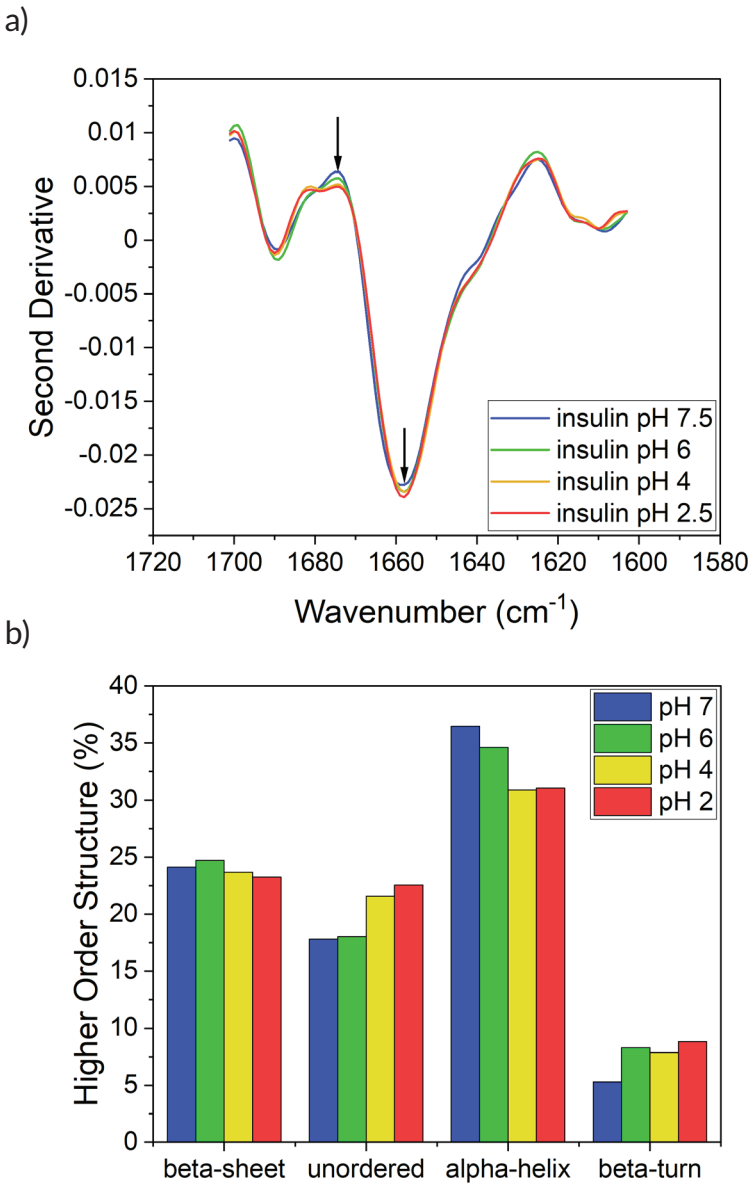


Figure 6: (a) Second Derivative data from Insulin formulated a pH 7.5, 6.0, 4.0 and 2.5 measured at room temperature, (b) HOS insulin exposed to pH 7.5, 6.0, 4.0, and 2.5 showing an increase in unordered structure with decreasing pH.

Quantifying Protein SIMILARITY

Determining similarity for biologic drug products is significantly more challenging than small molecules due in part to their larger size and greater structural complexity. Along with functional comparisons, the measurement and analysis of the structural similarity between proteins are effective methods of demonstrating bioequivalence. Microfluidic modulation spectroscopy measures protein secondary structure and reveals conformational differences between proteins with high sensitivity and resolution. These capabilities make MMS a powerful tool in the analysis and development of biosimilars.

Similarity assessment with MMS is a quantitative approach for detecting small changes in protein secondary structure by analyzing and comparing changes in the Amide I band (1700-1600 cm^{-1}) between proteins. The Amide I band is very sensitive to changes in protein secondary structure as discussed previously, and the ability to measure small differences in the spectra can be a powerful tool in monitoring the biosimilarity of a protein.

The data shown in Figure 7 represents a thermal stress experiment of a biosimilar drug product, versus a control sample. The sample has been exposed to 4 °C and room temperature and shows good similarity and repeatability, indicating good stability of the sample under these conditions. Table 1 shows a comparison of the higher order structural data obtained for both the innovator product (Phenex CRM) and the biosimilar product (EcoCRM®). This data shows a high similarity of structural components with the predominant structure being beta-sheet at approximately 32%, with alpha-helix and turn structure representing approximately 26% and 28% respectively.

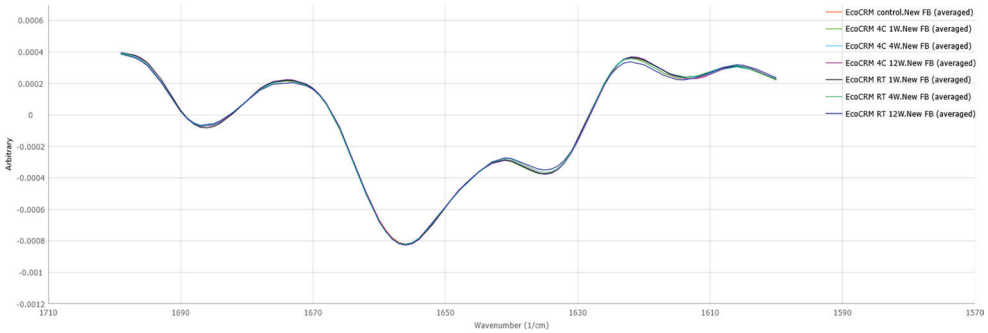


Figure 7: Second Derivative data from a thermal study of ecoCRM exposed to 4 °C and room temperature. Data shows good reproducibility, indicating good stability of the sample.

Structure %	**Phenex CRM** (rep 1 & rep 2)		**EcoCRM®** (rep 1 & rep 2)	
Turn	27.48	29.29	29.08	28.45
Alpha	26.20	28.10	25.81	27.06
Unordered	14.45	10.93	12.60	12.27
Beta	31.86	31.67	32.50	32.22

Table 1: Comparison of higher order structure composition of innovator product (Phenex CRM) as compared to the biosimilar product (EcoCRM®) indicating good structural similarity between the two products.

Chapter 3:
Stability Assessment in Formulation Development

Higher order structure (HOS) assessment of molecules traditionally has not been used as a Critical Quality Attribute (CQA) for drug substances or formulated drug products because of technology limitations.[5] Processes such as filtration, viral inactivation, pH holds, and other typically used to manufacture biologic drugs can affect protein structure and quality, resulting in a compromised drug product.[6]

- Traditional characterization tools such as dynamic light scattering (DLS), size exclusion chromatography (SEC), microcalorimetry, spectrometry and spectroscopy generally represent the bulk of analytical tests performed.
- Analytical gaps exist within the measurable datasets, particularly in relation to formulated drug products in complex formulations and high concentrations.
- There is a need for new tools capable of measuring protein structure across a wider range of measurement conditions.
- The AQS^{3}pro, powered by Microfluidic Modulation Spectroscopy (MMS), significantly eases the possibility of adding this structural assessment tool in manufacturing due to its expanded concentration range, sample compatibility, and improved data quality.

When developing a formulation for a biological drug substance, the use of a quality-by-design rationale at all stages of development ensures a systematic approach to quality criteria and assessment. The list of excipients commonly added to improve stability of a protein, increase solubility or provide pH control includes buffers, salts, sugars, surfactants, and amino acids. Excipients such as antioxidants and preservatives can also be added to improve the shelf life of the product.

In Figure 8, a simplified overview of potential pathways to aggregation in biopharmaceuticals is depicted. Aggregation processes can proceed in two directions: one mediated by particle-particle interactions (upper pathway), and the other enthalpically driven (lower pathway). Colloidal stability directly impacts particle-particle interactions, moderating the ability of proteins to proceed on the lower pathway, and therefore is considered a primary method of engineering product quality.

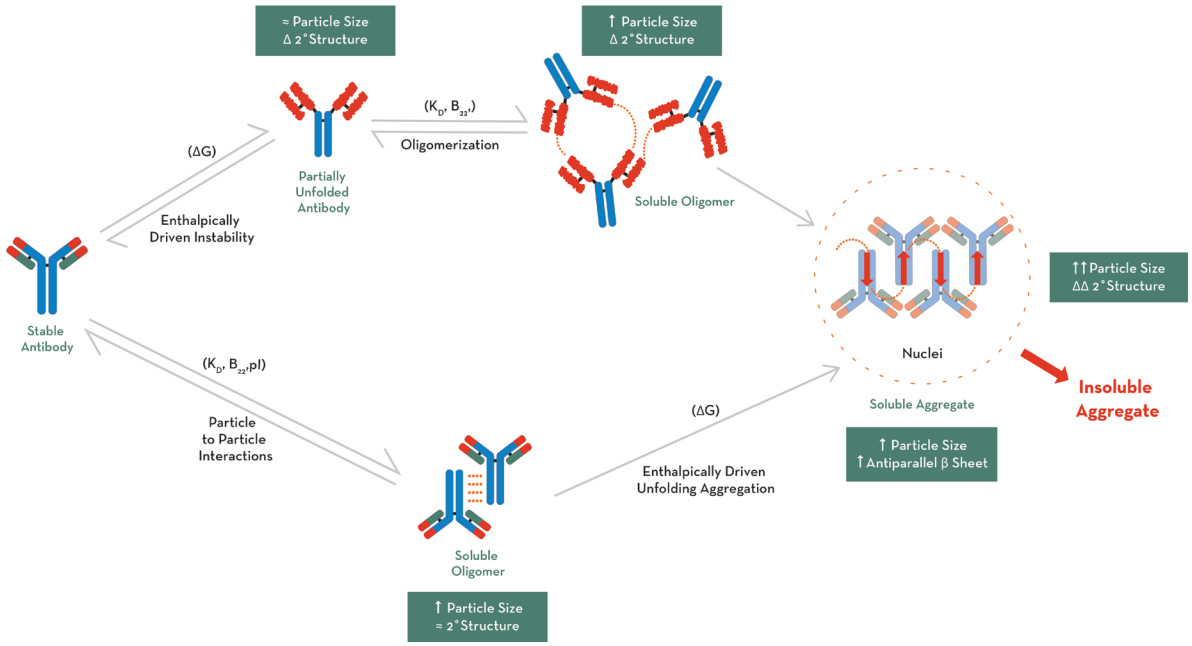


Figure 8: Generalized schematic of protein aggregation pathways in Biopharmaceuticals.

APPLICATION: Thermal Stability of a Formulated Protein

To demonstrate the utility of MMS for the examination of formulation stability, a sample set using 2.5 mg/mL human lysozyme was formulated at pH 2 and pH 4 in 10mM phosphate buffer. Samples were exposed to 60 °C for 2 and 7 days. The sample secondary structure was measured using the AQS^{3}pro MMS system from RedShiftBio. Figure 9 summarizes the results, showing the absolute absorbance spectra (Figures 9a and 9c) of samples measured at time points 0, 2 days and 7 days as blue, green, and red traces respectively. The graphed data for the sample at pH 2 (Figure 9c and 9d) indicated significant change in secondary structure across the time points with a decrease in alpha-helix, and formation of both beta-sheet and beta-turn structure. The graphed data for the samples at pH 4 (Figure 9a and b) in contrast showed little to no change in secondary structure across the same time span, which is consistent with previous reports.[7]

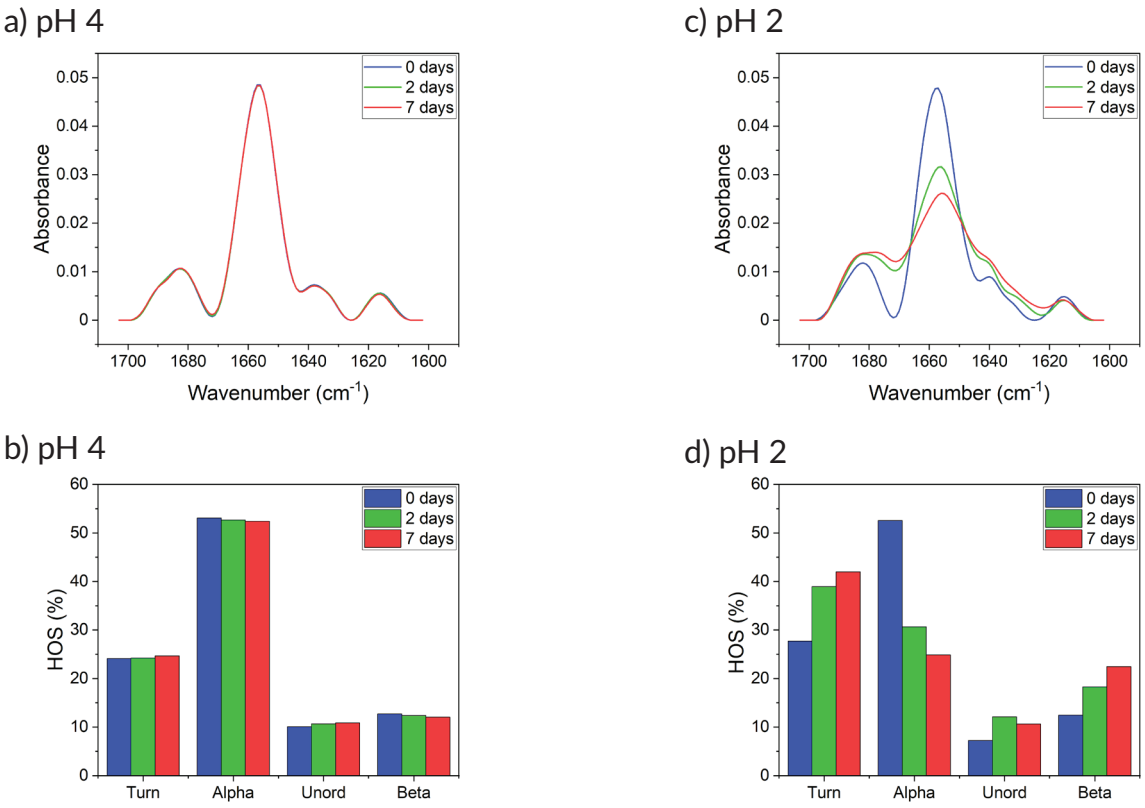


Figure 9: Absorbance spectra and secondary structure distribution of Lysozyme at pH 4 and 2 (left and right) acquired at time 0 (blue) and after heating at 60˚C for 2 days (green) and 7 days (red).

The data shown in Figure 9 highlights the capabilities of the AQS^{3}pro platform to quantify secondary structure in biologics with excellent repeatability. Even though the example data set offers a relatively simple set of formulation conditions, the true potential of MMS comes from its dynamic concentration range, compatibility with complex formulations conditions, system automation, and unmatched data quality. This platform in combination with the AQS^{3}delta software offers a solution filling the gap in analytical tools to characterize essential structural attributes of biopharmaceuticals anywhere in the development pipeline. With 21 CFR - Part 11 compliant software, as an available upgrade with the AQS^{3}pro provides a novel tool to strengthen any bioanalytical tool kit, expand measurement capabilities and provide critical insights into protein-based drug development.

Chapter 4:
MMS in the Discovery Phase

The decision to eliminate or progress potential drug substance candidates further along the discovery pipeline relies on gathering relevant and differentiating data typically from very small amounts of sample. After assessing activity through binding studies or similar tests, a barrage of additional properties must be determined including physical, chemical, biological, and pharmacological on the same limited sample.

Uncontrolled conformational changes in a biotherapeutic protein can be directly associated with poor stability. Structural characterization is therefore a powerful tool for the detection of inherent stability especially in early discovery. In addition, data demonstrating structural similarity or comparability is a valuable way of elucidating binding differences, assessing structural integrity, and identifying a lack of stability even before aggregates become detectable.

The AQS^{3}pro is a Microfluidic Modulation Spectroscopy-based technique for the evaluation of protein secondary structure. In late-stage drug discovery and preformulation, MMS offers the opportunity to:

- Compare protein secondary structure with high resolution and precision to quickly detect candidates with poor stability even before aggregates form.
- Efficiently assess structural characterization with automated, easy-to-use technology that is well-matched to preformulation workflows.
- Generate data that has value throughout the lifecycle of the drug, with an instrument that can transition through to manufacturing and QC.

True Sample Flexibility in a Single Instrument

With the flexibility of using both large and small volume well plates, the AQS^{3}pro offers true sample flexibility to meet the testing needs of any lab. The base model AQS^{3}pro system is a 24-well plate system which allows a maximum of 6 replicates per well, where even the most rigorous needs of quality assessment can be met.

Upgrading the AQS^{3}pro to the temperature-controlled 96-well plate configuration offers the ability to screen a higher number of samples with 2 replicates per well to meet the needs of even the most challenging molecular discovery and formulation development applications. Temperature control for the 96-well configuration includes chilling at 10 °C, providing sample stability for long runs.

24-Well Plate Configuration

- Base configuration of all systems.
- Measures up to 7 sample/buffer pairs on a single plate*.
- Room temperature sample storage.
- Up to 6 replicates per well position, great for multi-replicate measurement.

*assumes same protein in same buffer

96-Well Plate Confugiration (Upgrade Kit)

- Measures up to 40 sample/buffer pairs on a single plate*.
- Includes plate chiller set to 10°C.
- Great for small sample volumes (2 replicates).
- Plate loading guide available for easy sample loading.
- Upgrade includes consumable kit.

*assumes same protein in same buffer

Chapter 5:
Quality Assurance and Secondary Structure

Effective quality testing provides a foundation for safeguarding product quality, controlling critical changes in the structure of drug substances, drug products, raw materials, or excipients. Quality assurance (QA) provides a systematic approach that goes beyond the measure of drug product quality attributes and establishes a set of guidelines for all facets of the manufacturing process that could affect product quality. This includes establishing quality guidelines for raw materials, facility and production-line cleanliness, process monitoring, and control all the way to troubleshooting product recalls, product returns, and non-compliance reporting.

Biologic drugs are complex molecules that exhibit microheterogeneity, minor chemical variances such as glycan structural differences, deamidation, oxidation and glycosylation. Casting a wide analytical net helps establish the robust structure-function relationships that define the boundaries of unacceptable risk. The identification of all possible critical quality attributes (CQAs) underpins effective QA.

The AQS^{3}pro facilitates the measurement of secondary structure attributes of biopharmaceuticals in all stages of the manufacturing process. This helps establish quality parameters at stages not possible with traditional techniques. AQS^{3}pro data can be used to establish quality measures relating to protein concentration, purity, stability, as well as aggregate detection and prevention. Implementation of MMS as a comparability tool also provides measures of lot-to-lot comparison to identify potential deviations in manufacturing through the establishment of libraries of fingerprint spectra at all stages of production.

The MMS instrument offers:

- Highly repeatable and reproducible measurements for confidence in monitoring quality characteristics through all phases of development and manufacture.
- Ability to detect differences in secondary structure that cannot be robustly determined with traditional analytical techniques.
- An optional 21 CFR part 11 compliant platform that supports the efficient integration of structural measurements into existing QA workflows.
- Powerful and intuitive software that accelerates data analysis and enables highly informative insights for users at all levels.

The result is a valuable analytical tool for assessing the consistency of materials and processes, from early stage development through the final product. Comparability and similarity testing with the AQS^{3}pro is simple, fast and highly differentiating.

APPLICATION: Benchmarking AQS^{3}pro Sensitivity Using Spiked IgG

The performance of the AQS^{3}pro in quantifying, comparing, and contrasting critical attributes of a biologic formulation, sets it apart from other technologies. Elion Labs used the AQS^{3}pro in a model experiment to determine the theoretical limit of quantitation (LOQ) of MMS in comparison to both FTIR and CD. In this experiment, a 20 mg/mL IgG formulation was measured. To determine the LOQ of contaminants, BSA was spiked into the sample at a range of 2 to 10% (w/w) of protein content.

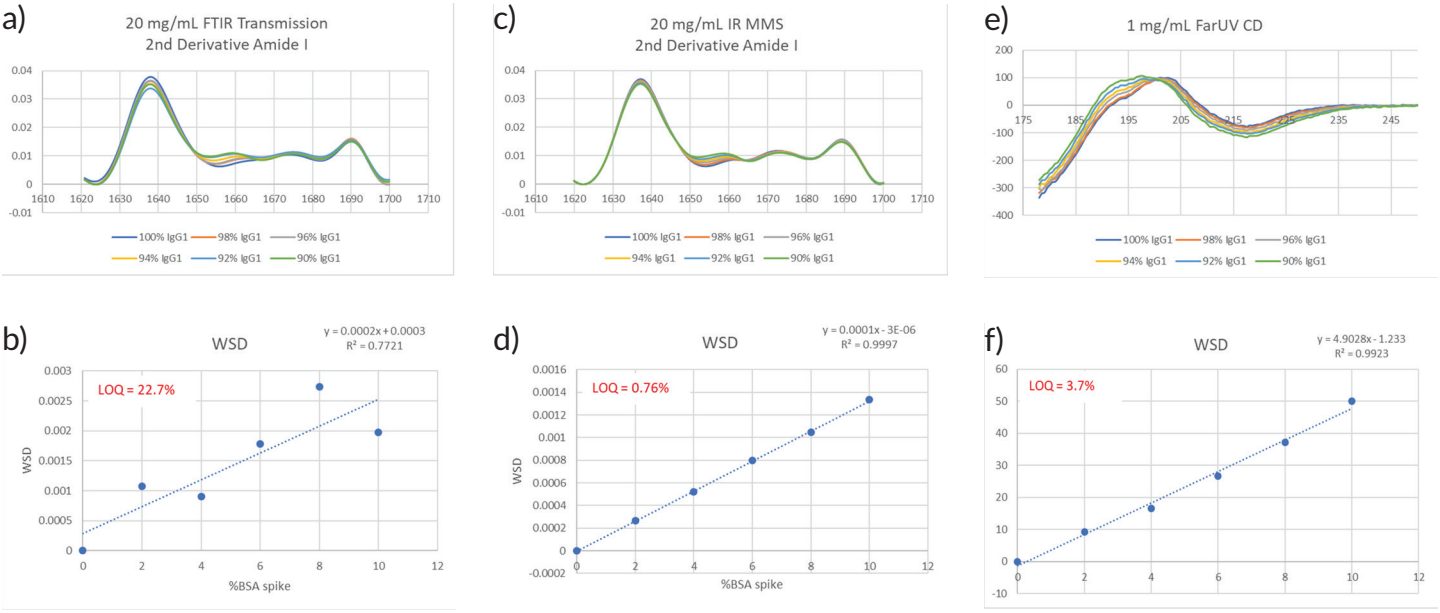


Figure 10: Spectral comparison of an IgG sample spiked with 0 to 10% BSA to identify the limit of quantitation of each technique. Data acquired from FTIR (a & b), MMS (c & d) and CD (e & f) showing spectral quality, reproducibility and the weighted spectral difference is used to determine the LOQ of 22.7% for FTIR, 0.75% for MMS, and 3.7% for CD.

The data shown in Figure 10 above highlight the true performance capabilities of MMS relative to the incumbent technologies of FTIR and CD particularly highlighting the improved resolution, and repeatability. Two sets of processed data have been compared, one to compare the LOQ of each system and the other to show repeatability and determine the total user time required to complete each set of data acquisition.

Microfluidic Modulation Spectroscopy indicated the presence of alpha-helix at a theoretical LOQ around 0.76%, compared to 22.7% and 3.7% using FTIR and CD respectively. This contrast is also exemplified by the respective linearity results associated with each data set. MMS shows linearity of the data with an R^2 of 0.9997, as compared to 0.9923 for CD and 0.7721 for FTIR. This superior performance by MMS highlights the limitations of the traditional two techniques in terms of repeatability, reproducibility and data quality.

Finally, a comparison of the time required for total data acquisition and processing showed that MMS required approximately 15 minutes for sample preparation, loading, initializing data acquisition and data processing, where, in comparison, the hands-on time required for FTIR was estimated to be greater than 5 hours.

Chapter 6:
Assessing Biosimilarity using MMS

The development of effective orthogonal biophysical characterization tools is essential for biosimilar development and helps reduce reliance on clinical and animal studies. This thereby decreases time to market and development costs. For example, measurements of primary and higher order structure (secondary, tertiary, quaternary) of drug products define the active biotherapeutic molecule and are vital elements of reverse engineering as well as supporting bioequivalence claims. Comparative testing of a proposed product and reference product reveals the extent of structural similarity and helps to elucidate differences in stability and aggregation behavior along the development pathway.

The AQS^{3}pro is a powerful and flexible system that uses Microfluidic Modulation Spectroscopy to characterize the secondary structure of proteins. MMS, compared to conventional FTIR spectroscopy, offers much greater reproducibility and utility for biosimilar development. Comparability testing with the AQS^{3}pro is simple, efficient, and precise. Figure 11 gives a typical workflow that could be used to demonstrate bioequivalence of a biosimilar drug product, as reported by Amgen.[8] It is worth noting that higher order structure is an essential part of this process, and the noted publication from Amgen uses a spectral comparison of the Amide I region as evidence of structural similarity. Additional techniques in the workflow include methods to examine primary structure and composition, as well as biophysical and biochemical properties.

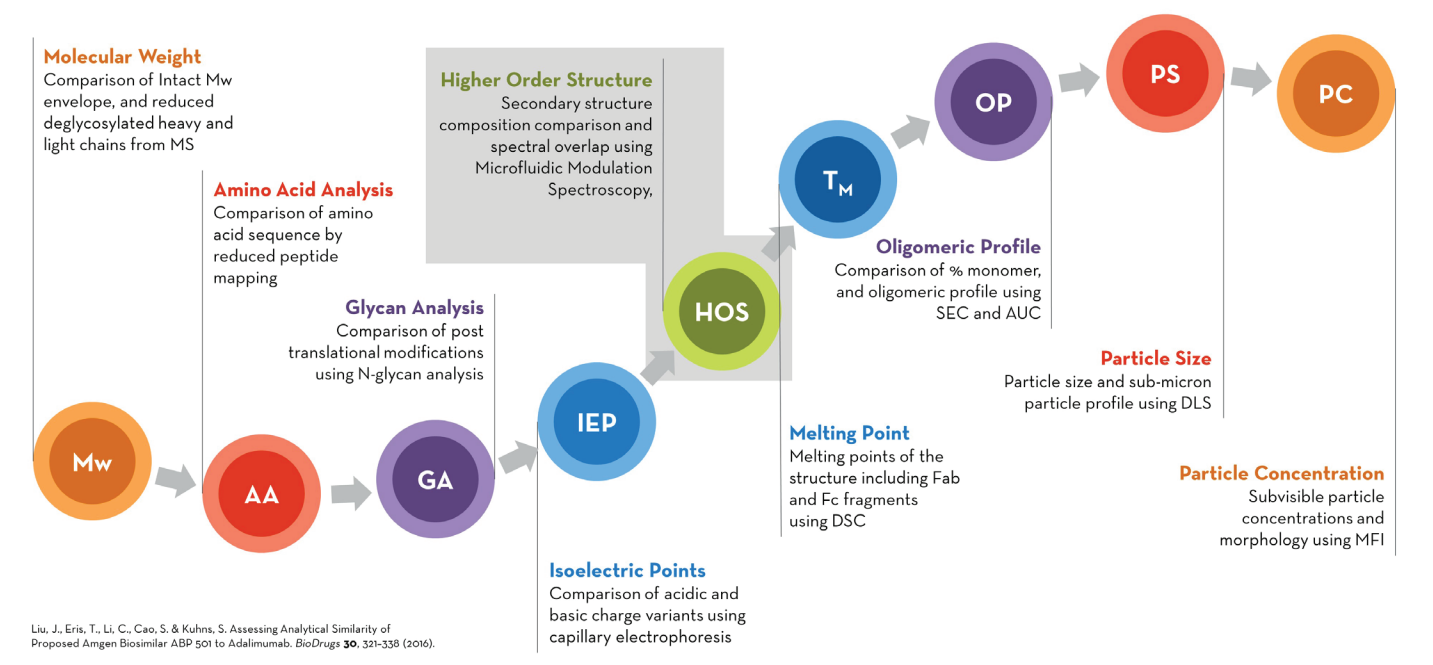


Figure 11: Example workflow for bioequivalence determination of a biosimilar drug product as proposed by Amgen.[8]

AQS³delta for 21 CFR - Part 11

User Role and Permission-based Access

- One Required Administrator role for security oversight and user permissions.
- Ability to configure roles and designate security access per role.
- Activate and inactivate users.
- Set Password login and expiration, Idle time-out, and password reset criteria.

Data Integrity and System Security

- One-time migration option (for upgrade from non-21 CFR software version).
- Export of data but no import permissions.
- Encryption of all database items.
- Lock system, Idle-time lock, and ability to switch user.

E-Signatures

- Assignment of E-signature permission by User Role.
- Ability to require “Reason” field with signature.
- Ability to E-sign protocols, Data Analysis Settings, and processed data.

Audit Trail

- Tracking of all actions within Database.
- Ability to view and print all revisions for specified content or whole database.
- Ability to see and print “differences” in content between revisions.

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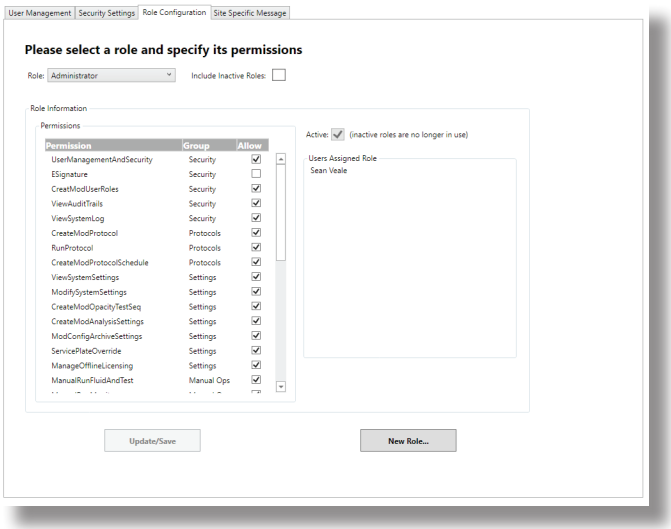

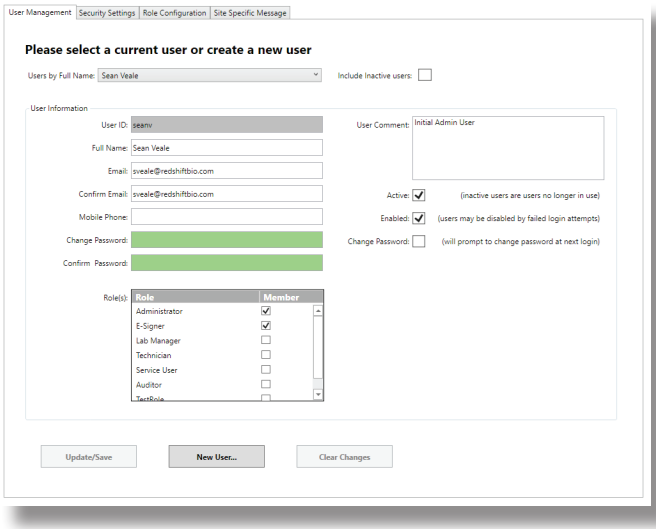

APPLICATION: Differentiating Secondary Structure Changes to Compare Batch to Batch Comparability of a Biosimilar

Microfluidic Modulation Spectroscopy enables very small changes in structure to be confidently differentiated, allowing the technology to be used to measure structural differences that may have an impact on function. Here differences in secondary structure were used to correctly predict which of 13 BSA samples from different suppliers and batches versus would be viable for reagent manufacturing of all samples tested as compared to a control sample. Five samples were found to be similar enough to the control to retain activity (<2% difference). These changes in structure would be extremely challenging to measure using traditional technologies such as FTIR or CD.

Biosimilar Comparison by MMS

Figure 12 shows higher order structure (HOS) plots of 13 BSA samples from different manufacturers and batches compared to a known control sample (BSA_{ref}). The maximum observed difference between samples was ~2%, which would be very challenging to distinguish using traditional technologies. Replicates were acquired for each sample, which gave a maximum internal variance of 0.5% indicating differences observed between these samples was significant.

Concentration, Protein Displacement Factor and Similarity

Sample concentrations were measured automatically and found to vary between batchs. Protein displacement factors (a measure of the volume occupied by the protein in solution) shows all samples are of very similar volume, but similarity assessment of all samples indicates several are slightly closer to the control than the others.

Sample Name	Given Conc (mg/mL)	Calc. Conc (mg/mL)	Displac. Factor	Similarity
BSA_1	10	8.4	0.65	98.88%
BSA_2	10	9.3	0.64	98.92%
BSA_3	10	8.9	0.62	97.84%
BSA_4	10	8.7	0.63	97.77%
BSA_5	10	9.0	0.63	97.89%
BSA_6	10	9.5	0.63	97.68%
BSA_7	10	9.0	0.62	97.51%
BSA_8	10	8.8	0.62	97.64%
BSA_9	10	8.6	0.62	97.66%
BSA_{10}	10	8.7	0.65	98.67%
BSA_{11}	10	9.5	0.63	97.76%
BSA_{ref}	**10**	**8.5**	**0.62**	**100.00%**
BSA_{12}	10	10.4	0.64	99.50%
BSA_{13}	10	9.7	0.64	99.57%

Table 2: Data comparison showing percent similarity of 13 BSA samples compared to a reference material (BSA_{ref}) with the 5 most similar highlighted.

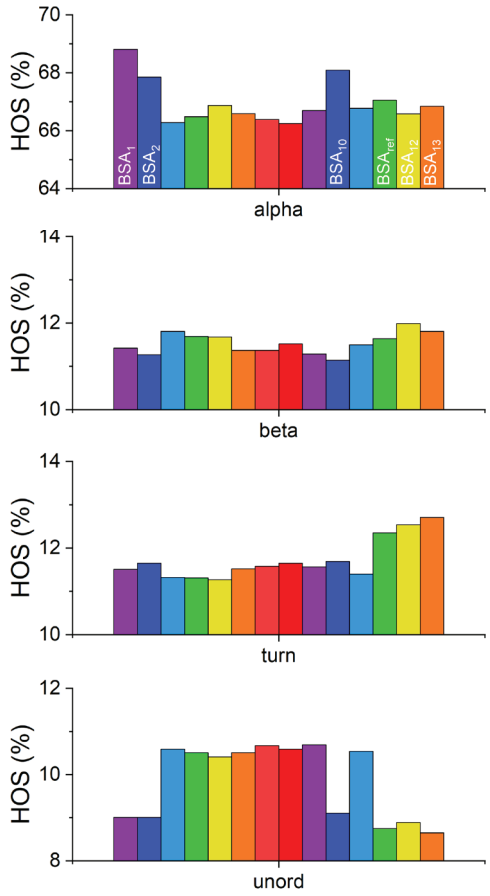

Figure 12: HOS Comparisons of 13 BSA samples compared to reference (BSA_{ref}) showing 5 samples with good similarity based on HOS.

Conclusion

MMS enables more sensitive and reproducible secondary structure information to be captured for proteins compared to traditional technologies. Here 13 BSA samples were compared to a control sample, as a biosimilar analysis to determine which suppliers and batches had produced samples structurally similar enough to be used for reagent manufacturing. MMS analysis showed that with less than 2% detectable change in secondary structure, only 5 samples were deemed biosimilar to the reference, providing a method by which new suppliers' samples could be validated quickly before use in production.

Chapter 7:
Publications and Journal Articles featuring MMS

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2. Ahl, P. L. et al. (2020) 'Applying Microfluidic Modulation Spectroscopy in Vaccine Formulation to Identify Intermolecular Beta-sheet Aggregation of Antigens Case Study : Applying Orthogonal', American Pharmaceutical Review, (July/August), pp. 1–5.
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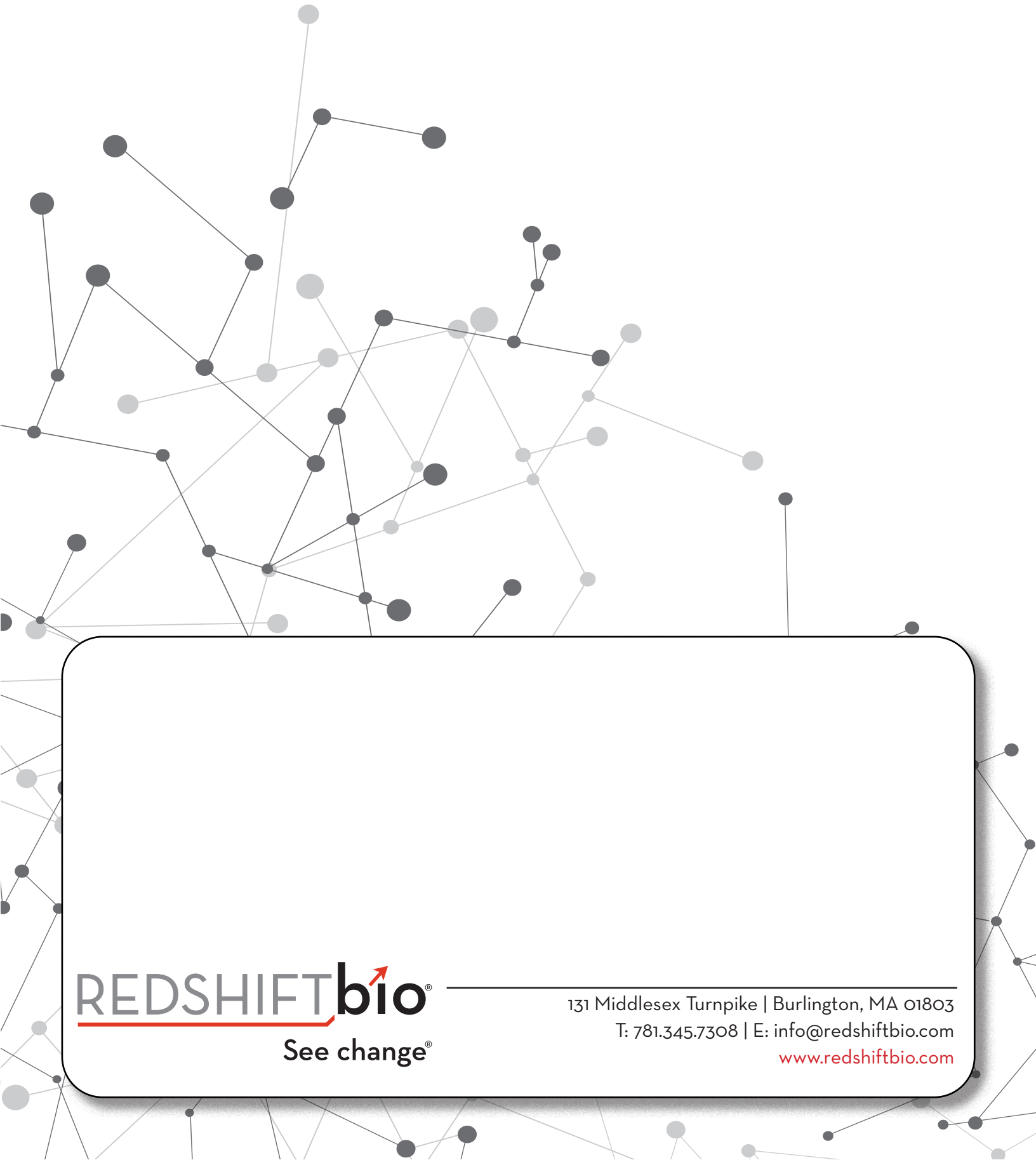

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