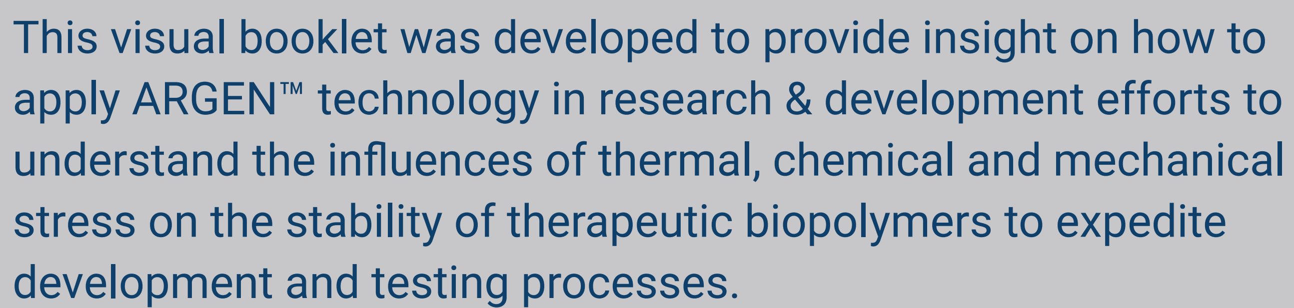
Accelerating Biopharmaceutical Research and Development

Applications of Static Light Scattering

Fluence Analytics



This visual booklet was developed to provide insight on how to apply ARGEN™ technology in research & development efforts to understand the influences of thermal, chemical and mechanical stress on the stability of therapeutic biopolymers to expedite development and testing processes.

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EXPEDITED BIOPHARMACEUTICAL DEVELOPMENT

The increasing demand for shorter development timelines requires a paradigm shift in how we approach formulation development.

Most analytical tools used to monitor biopolymer stability require laborious sample preparation and only provide a periodic "snapshot" of solution behavior at a specific time point. There is a need for analytical tools to rapidly understand biopolymer stability under various stress and solution conditions in real time.

In theory, traditional analytical methods such as size-exclusion chromatography (SEC) should not disrupt the aggregation state and be capable of detecting aggregates that have loose association, but this is not the case.

THE BIOPOLYMER STABILITY CONUNDRUM

Successful development depends upon the proper characterization and stabilization of the target therapeutic

Finding the optimal candidate can be time and resource intensive depending on techniques and methodologies used

Many instruments only allow for a single measurement per experiment which requires laborious sample preparation and experimental set-up

What is the solution to expedite biopolymer stabilization and development?

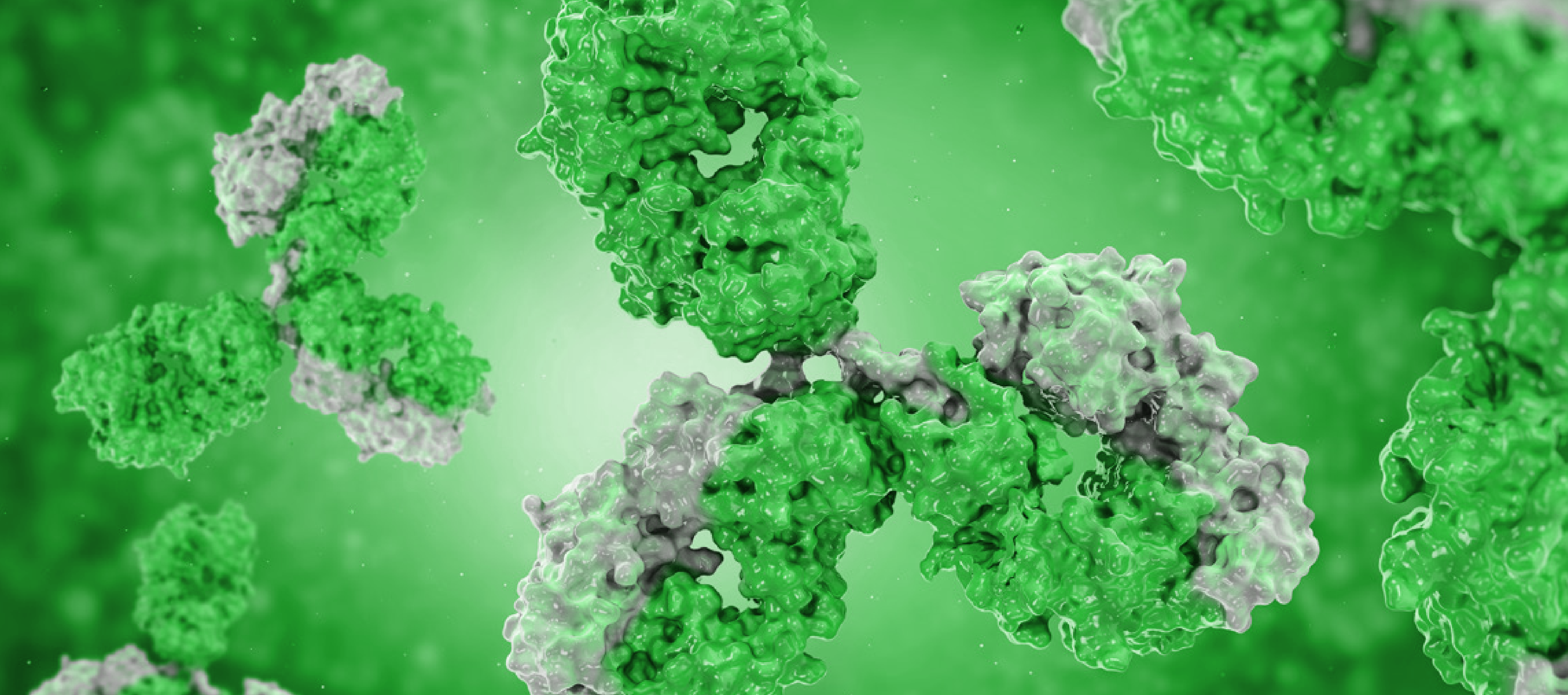
Construct A

Aggregation

STRESS
Chemical
Mechanical
Thermal

Construct B

Protein remains monomeric and viable



INTRODUCTION TO ARGEN™ TECHNOLOGY

ARGEN™ technology provides an 8 to 16 fold reduction in time and resources to identify the optimal target formulation, with rapid early detection of aggregation and stability of target molecules for rapid decision-making in formulation development.

In addition, ARGEN™ technology delivers continuous flexible operation for temporal stability studies and establishing shelf-life, as well as Matrix DOE capability for rapid screening of thermal, chemical and mechanical stress impact.

Smart & Rapid Therapeutic Biopolymer Development

Throughout this eBooklet, all case study data was generated using ARGEN™, a high throughput SLS (static light scattering) instrument from Fluence Analytics for the rapid assessment and real-time monitoring of the stability and viability of therapeutic proteins, peptides, and biopolymers.

ARGEN™ utilizes fixed angle (90°), SMSLS (simultaneous multiple sample light scattering) technology coupled with a multi-stressor platform with a thermal range of ambient to 100ºC and provides rapid, real-time, continuous data collection for characterizing qualitative and quantitative properties of target molecules.

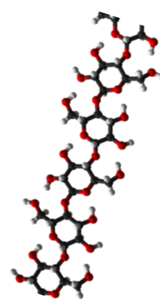
The ARGEN™ instrument is equipped with 8 or 16 independently controlled sample cells, permitting the user to establish thermal and mechanical (stirring) stress parameters on each sample concurrently to fully leverage matrix DoE (design of experiments) capability to accelerate new formulation and development testing.

Key Outputs

- $M_w(t)/M_0$
  Normalized molecular weight with respect to native molecular weight (unaggregated)
- $M_w(abs)$
  Absolute molecular weight
- **AR**
  Aggregation Rate
- **Degradation Rate**
  Rate of polymer breakdown
- **Time to Dimerization**
  Transition time from monomer to dimer
- **Arrhenius Plots**
  Characterize kinetics of aggregation, degradation

Target Biopolymers

ARGEN™ is suitable to characterize the stability of all classes of soluble biopolymers, including (but not limited to) proteins, peptides, nucleic acids (RNA, DNA), polysaccharides, viral vectors, lipid nanoparticles (LNP), and biopolymer complexes.



Polysaccharides

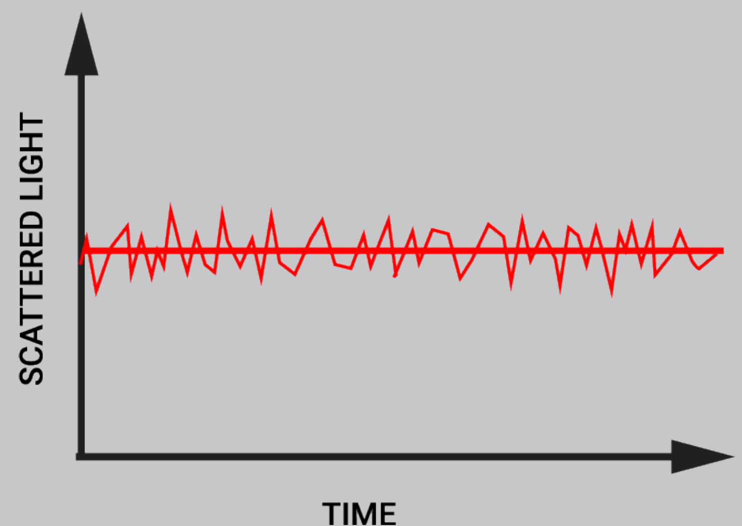
Proteins

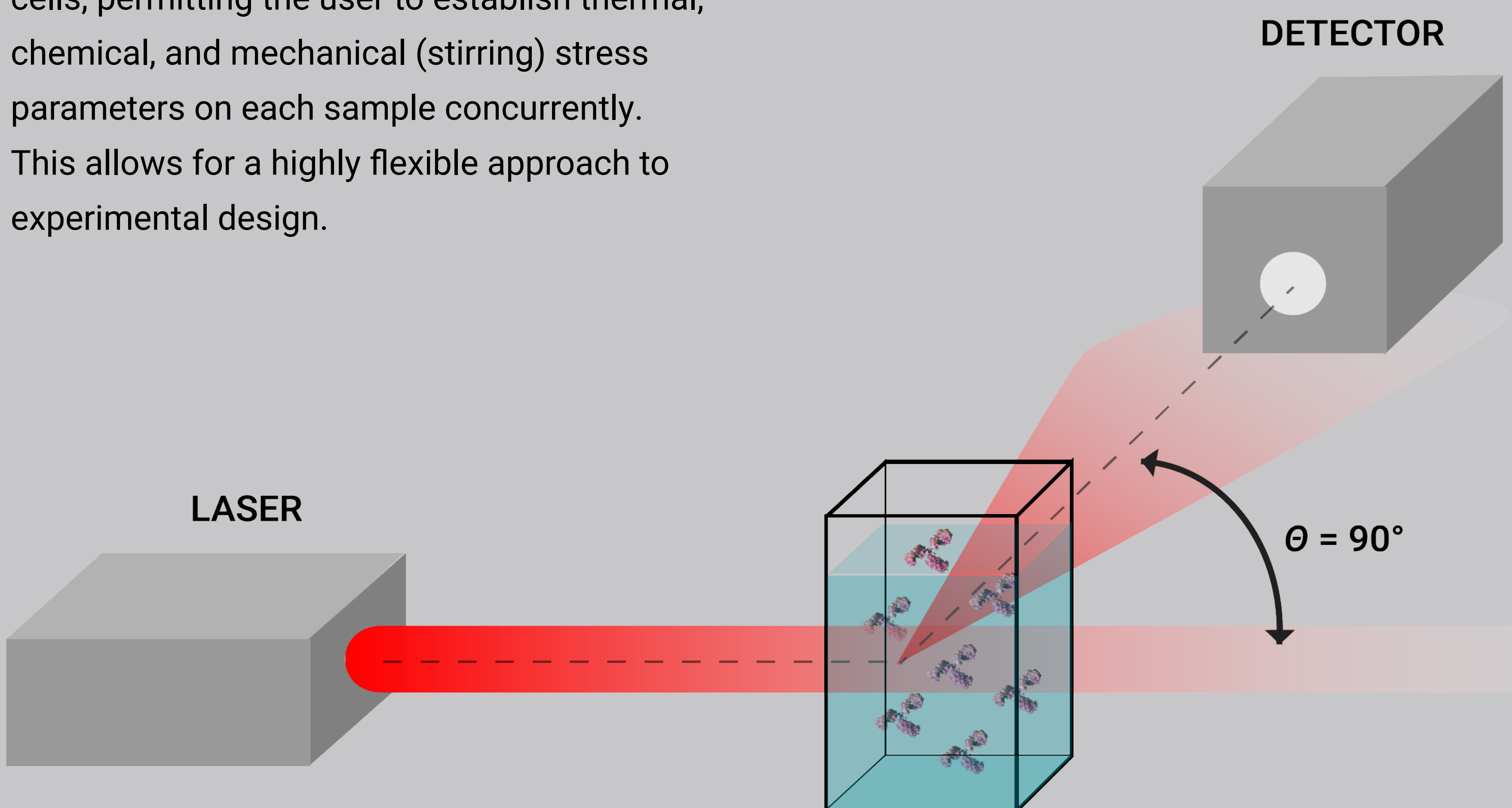
Nucleic Acids (DNA, RNA)

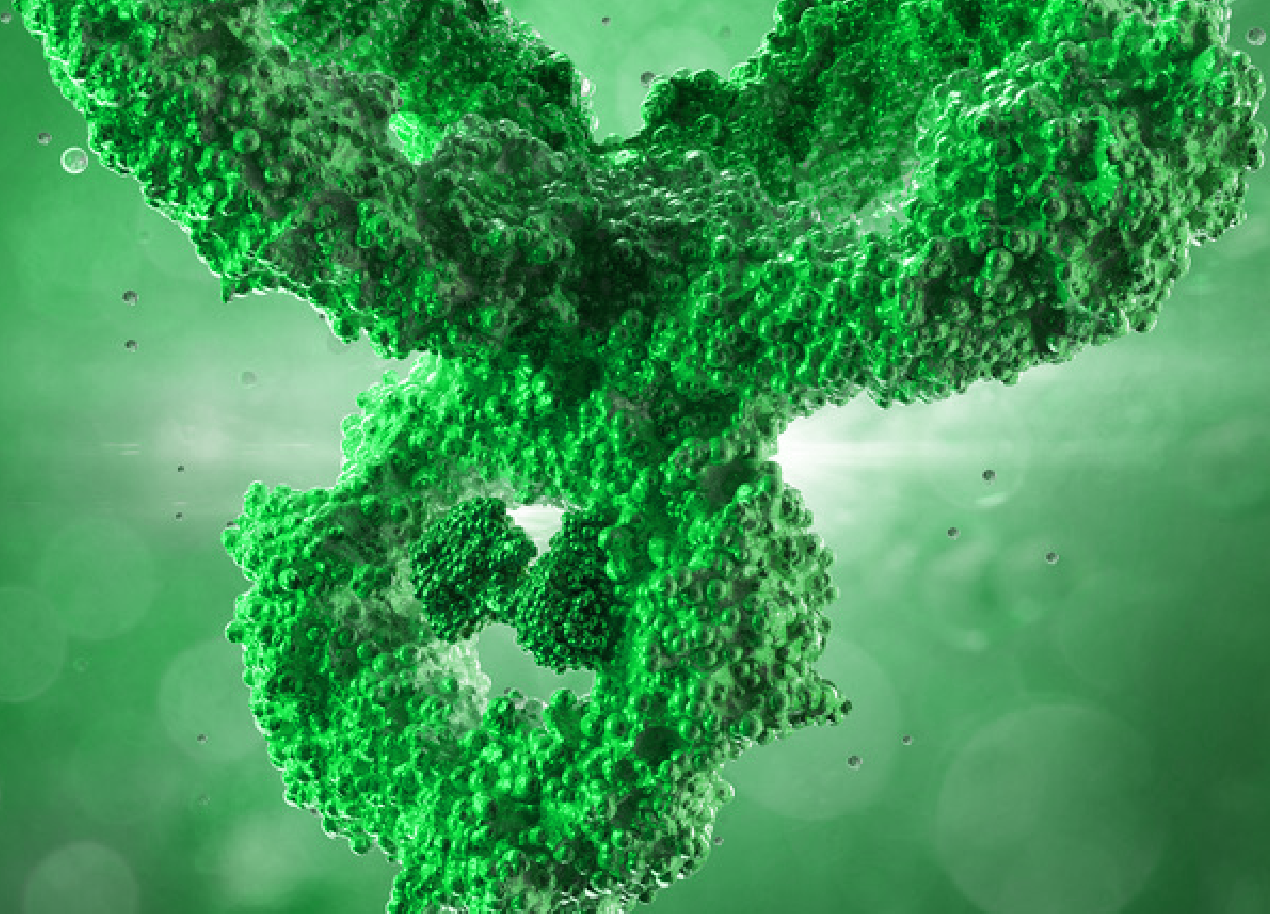
Peptides

How ARGEN™ Technology Works

ARGEN utilizes fixed angle (90°), SMSLS technology to provide rapid, real-time, continuous data collection for characterizing qualitative and quantitative properties of target molecules. The device is equipped with 8 or 16 independently controlled sample cells, permitting the user to establish thermal, chemical, and mechanical (stirring) stress parameters on each sample concurrently. This allows for a highly flexible approach to experimental design.







CASE STUDIES USING ARGEN™ TECHNOLOGY

Developing stable biotherapeutics is challenging, and understanding the factors affecting stability is the key to success. The SLS technology in ARGEN is a very well-established technique used to determine qualitative and quantitative properties of biopolymers, including normalized molecular weight ($M_w(t)/M_0$), absolute molecular weight (M_w), aggregation rates (AR) and degradation rates.

Real-time *in situ* monitoring of molecular weight changes can provide insight into the mechanisms of aggregation and degradation under various thermal, mechanical, and chemical stress conditions. Some of the conditions affecting biopolymer stability include pH, ionic strength, surfactants, divalent metals, excipients, buffer type, temperature, thermal cycling, stirring, air/liquid/material interfaces, and lyophilization.

The following case studies provide insight into the utility of ARGEN™ technology to understand the effects of thermal, mechanical and chemical stress as well as characterize the absolute molecular weight (M_w) of Human IgG and a monoclonal antibody.

Case Study #1

RAPID DESIGN OF A BUFFER SYSTEM TO MAXIMIZE THE STABILITY OF HUMAN IgG USING ARGEN™

Introduction

The successful development of biotherapeutics requires an in-depth understanding of solution conditions, e.g., buffer choice, ionic strength, pH, excipient(s), surfactant(s), as well as sample concentration and their effect on the stability of target molecule(s). Many techniques are not capable of monitoring or resolving subvisible aggregation or degradation in real time and require laborious sample and column preparation after long-term storage under varying conditions to obtain a quasi-accurate assessment of stability. Expediting this process and accurately modeling thermal, chemical, and mechanical stress and subsequent effects on protein stability can save valuable time and resources. The following case study demonstrates how data generated using ARGEN™ technology can expedite the process of optimizing buffer conditions to maximize the stability of Human Immunoglobulin (IgG).

Experiment 1 (Step 1): Determination of Aggregation Rates & Arrhenius Behavior for Human IgG

Aggregation Rate (AR) is the time derivative of the normalized molecular weight during the early, linear phase of aggregation represented by the following equation:

$$AR(s^{-1}) = \frac{d\left(\frac{M_w(t)}{M_0}\right)}{dt}$$

where $M_w(t)$ is the molecular weight of the sample as represented by time resolved light scattering intensity, and M_o is the initial molecular weight of the sample prior to aggregation.

Data generated using ARGEN™ technology (**Figure 1**) permitted the characterization of the AR for Human IgG, pI 7, over a broad thermal range and was utilized to determine Arrhenius behavior to classify thermally induced aggregation regimes at pH 5.

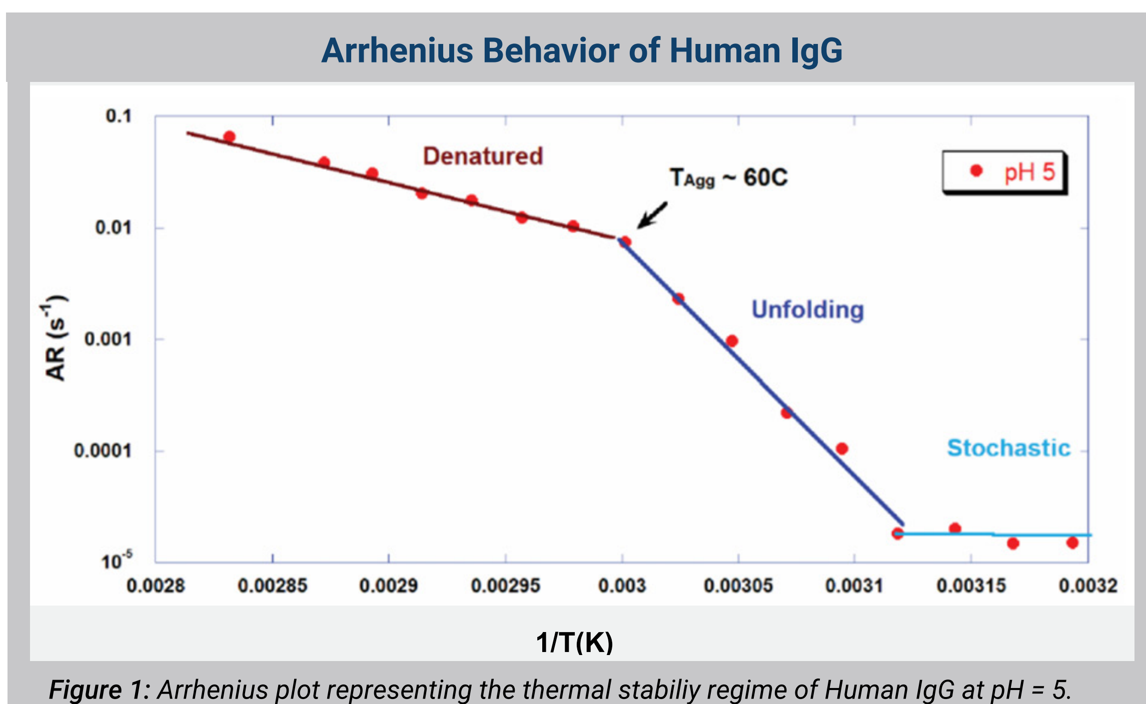


Figure 1: Arrhenius plot representing the thermal stabiliy regime of Human IgG at pH = 5.

Experiment 2 (Step 2): Determination of Optimal Buffer Concentration for Maximum Stability

To further stabilize the sample, data collected with ARGEN™ was utilized to optimize the sodium acetate concentration to minimize intermolecular interactions and mitigate the aggregation of Human IgG. The Arrhenius plot generated from the previous experiment (**Figure 1**) permitted the determination of T_{agg} = ~60°C, and therefore, the experiment was performed at

55ºC to force aggregation over an extended time interval (24 hours). As seen in **Figure 2**, AR decreases as [NaAc] is increased up to 25 mM. A buffer concentration above 25 mM promoted aggregation, confirming the optimal [NaAc] = 25 mM.

Experiment 3 (Step 3): Excipient Screening for Optimal Stability of Human IgG

The addition of excipients is often necessary to suppress or mitigate protein aggregation. For this set of experiments, data collected using ARGEN™ was used to understand the effect of sucrose at varying concentrations on the stability of Human IgG. The experiments were performed at 55ºC at [sucrose] = 0 mM-600 mM, [NaAc] = 25 mM, pH 5. As shown in (Figure 3), the AR decreases up to a [sucrose] = 200 mM with no marked difference in AR at higher [sucrose]. Compiling data from experiments 1, 2 and 3, the optimal buffer condition to stabilize human IgG proved to be, [NaAc] = 25 mM, [sucrose] = 300 mM, pH 5. Arrhenius plots (**Figure 4**) were generated superimposed for the Human IgG sample (red) at pH 5 and in buffer containing [NaAc] = 25 mM, [sucrosande] = 300 mM at pH = 5 (blue) to demonstrate the stabilizing effect (thermal) of the ARGEN-derived buffer system. As depicted, the ARs for the sample in the optimized buffer system were significantly reduced at each temperature set point accompanied by an increase in T_{agg}. Additionally, the rate limited Arrhenius behavior extended to lower temperatures, and no observable aggregation was detected at temperatures <40°C. This data is significant and indicates a reduction in the propensity of the sample to aggregate due to stochastic effects, allowing for accurate predictions of the shelf life and stability under lower temperature storage conditions.

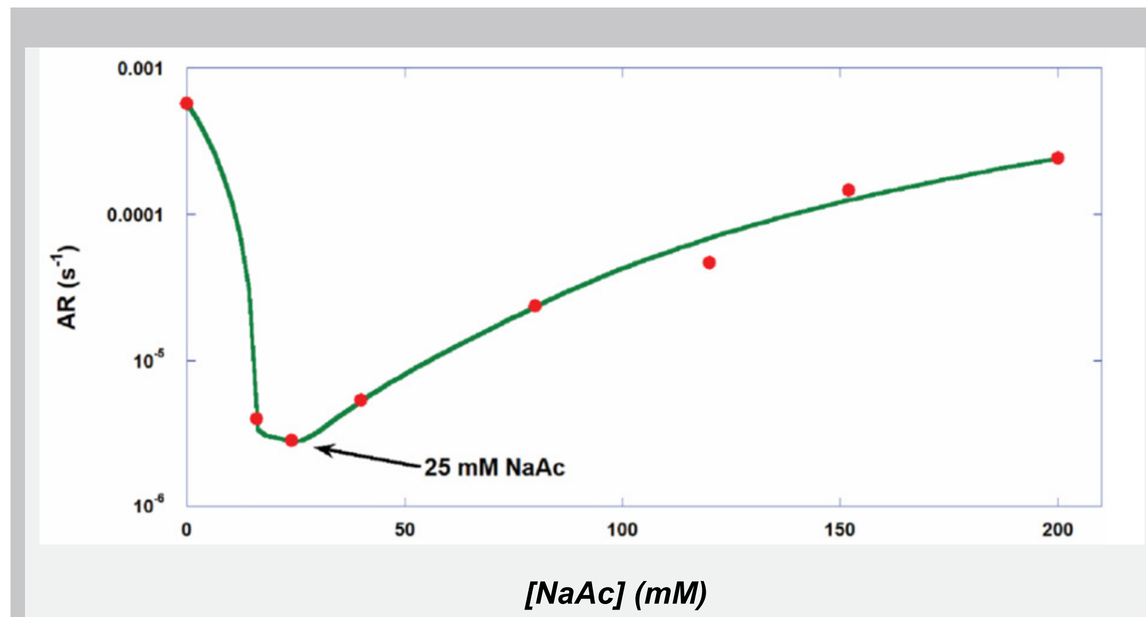


Figure 2: *Aggregation rate of Human IgG vs. [NaAc] (buffer)*

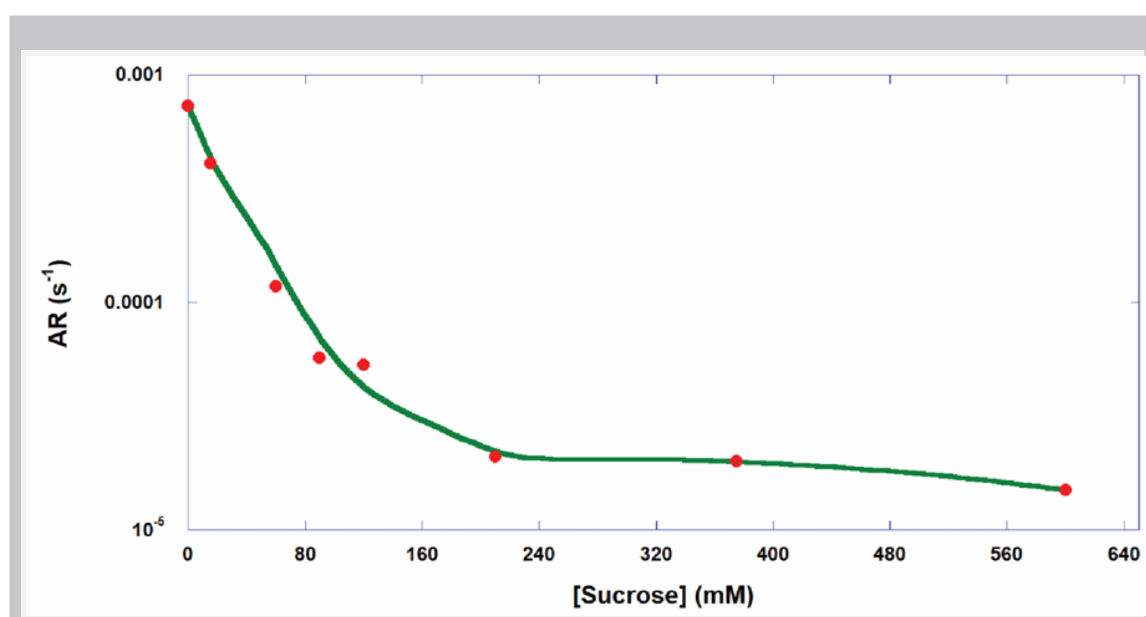


Figure 3: *Aggregation rate (AR) vs. [sucrose] mM*

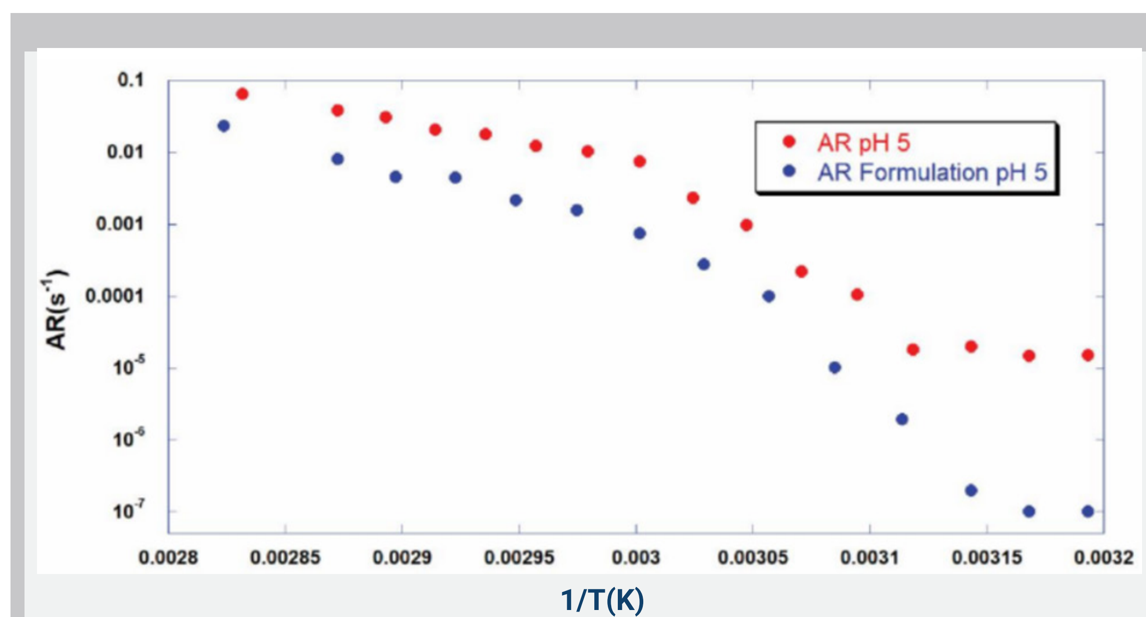


Figure 4: *Arrhenius plots of Human IgG at pH = 5 in the ARGEN-derived, stabilizing buffer system*

Conclusion

The threshold in developing a successful biotherapeutic is understanding the stability of the target molecule under various solution conditions and subsequently designing a buffer system to maximize stability. These experiments demonstrate the utility of ARGEN™ to rapidly determine solution conditions such as excipient(s), ionic strength and pH and allowed for the optimization of the buffer system to augment the stability of Human IgG. The capability of ARGEN™ to screen multiple parameters and samples in parallel vastly reduced the time required to stabilize the molecule. Understanding the kinetics of aggregation (AR) and the dependence on buffer conditions, comparatively, gives required insight into the target biopolymers propensity for aggregation, instability and clinical efficacy.

Case Study #2

MONITORING THE THERMAL AND CHEMICAL STABILITY OF A MONOCLONAL ANTIBODY USING ARGEN™

Introduction

These experiments evaluated real-time molecular weight changes of a monoclonal antibody (mAb) across a temperature spectrum. (**Figure 1**) illustrates the unfolding and subsequent aggregation versus time of a mAb under isothermal regimes with a temperature range of 65°C - 83°C. Each curve (11 total) represents the relative molecular weight ($M_w(t)/M_0$) of the antibody versus time under an isothermal condition. $M_w(t)/M_0$ is the normalized ratio of the molecular weight with respect to the initial, non-aggregated sample mass. M_o is the molecular weight of the native, unaggregated protein, and $M_w(t)$ is the weight average molecular weight of all native or aggregated species at a time point *(t)*. Changes in the $M_w(t)$ represent proportional changes in the aggregate mass and concentration, and this output is used to derive the aggregation rate (AR). These experiments clearly allow for the characterization of the thermal stability of the monoclonal antibody.

Effects of pH on mAb Stability

Figure 2 illustrates the effects of pH on the stability of a monoclonal antibody. Under identical temperature and mechanical stress conditions, changes in pH significantly perturbed stability with varying aggregation rates, as indicated by the change in $M_w(t)/M_0$ versus time. Interestingly, the aggregation rates (inset of **Figure 2**) were different under each condition, providing pertinent insights into ideal storage and purification conditions. Performing experiments with ARGEN™ under various solution conditions can provide valuable data for ab initio development and during downstream production and processing.

Coupling ARGEN™ Data with Size Exclusion Chromatography

Data collected with ARGEN™ allows users to rapidly vet the quality and viability of candidates. M_w/M_0 output quickly permits

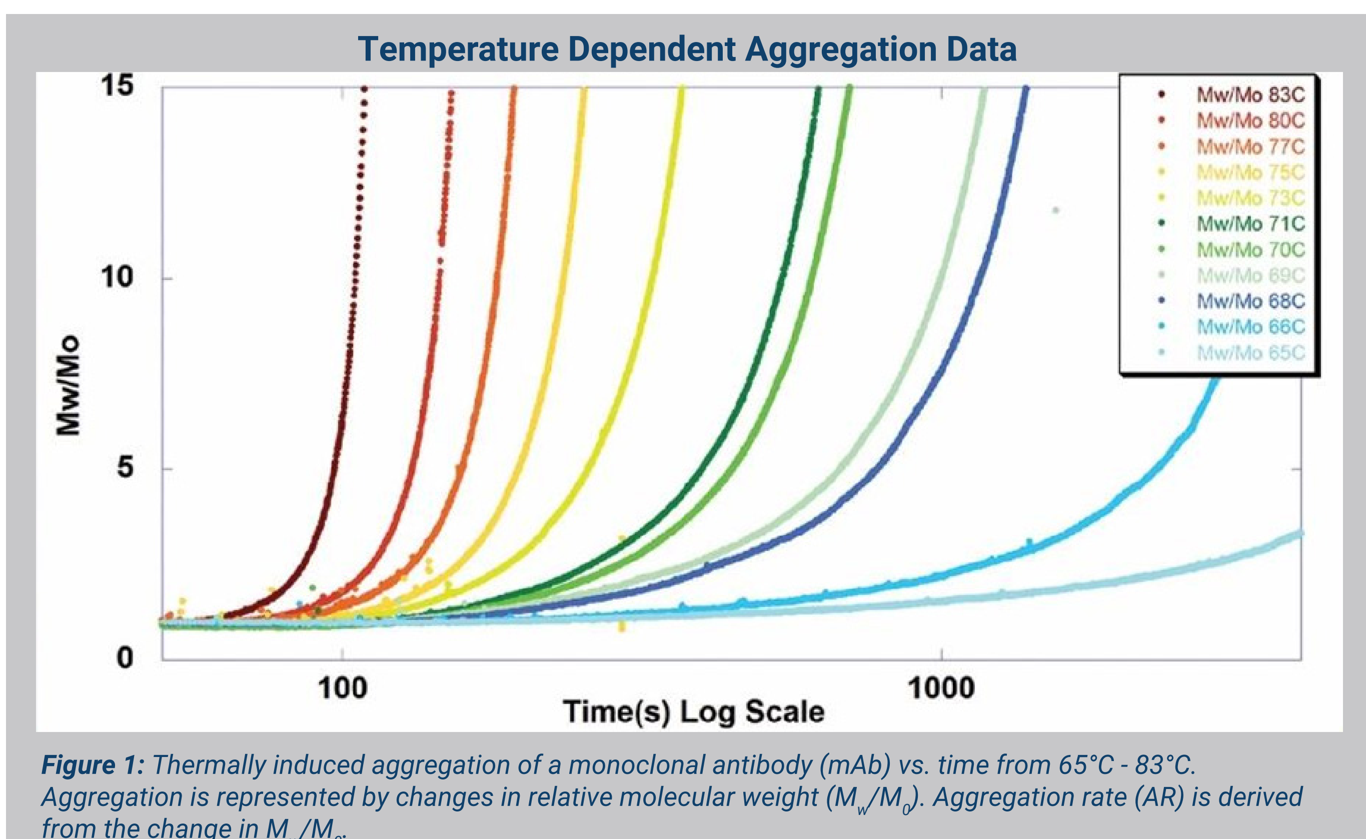
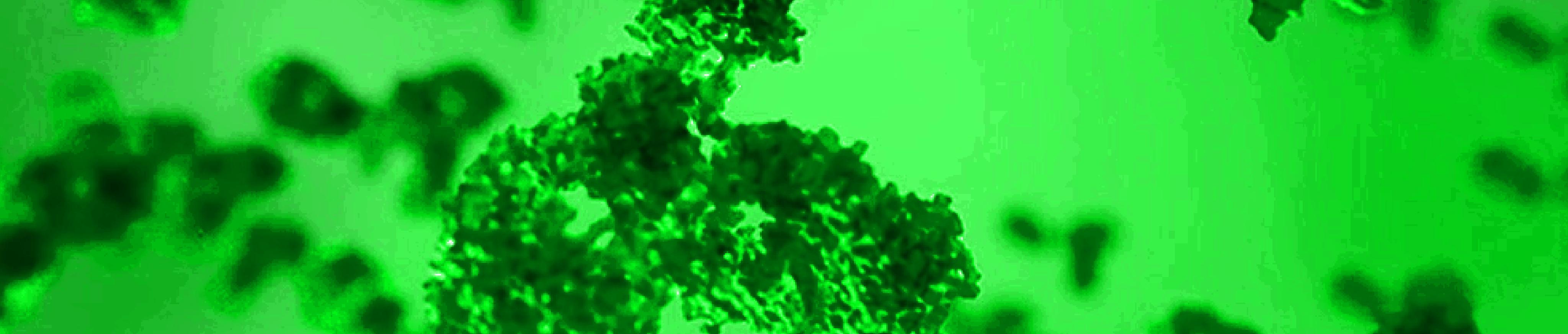


Figure 1: *Thermally induced aggregation of a monoclonal antibody (mAb) vs. time from 65°C - 83°C. Aggregation is represented by changes in relative molecular weight (M_w/M_0). Aggregation rate (AR) is derived from the change in M_w/M_0.*



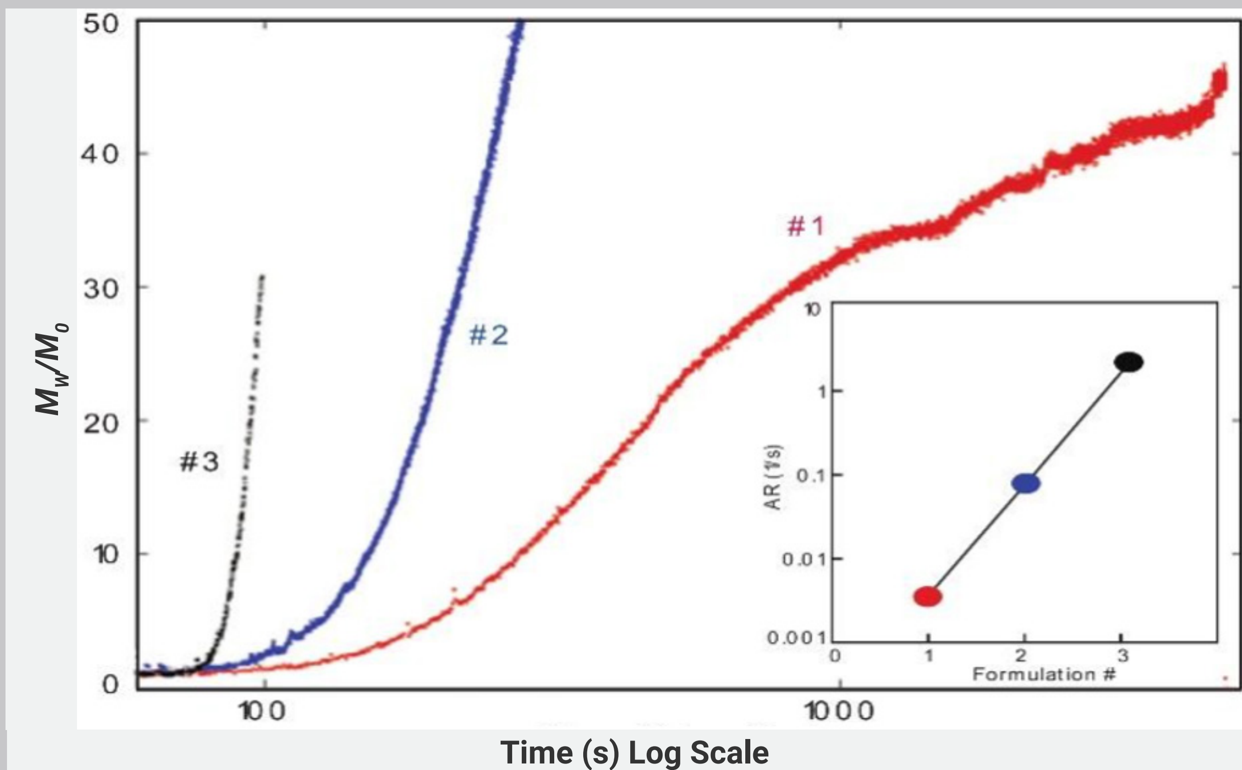


***Figure 2:** Effects of pH on Aggregation Rate (AR) of a monoclonal antibody (inset). Aggregation is represented by relative molecular weight (M_w/M_o)*

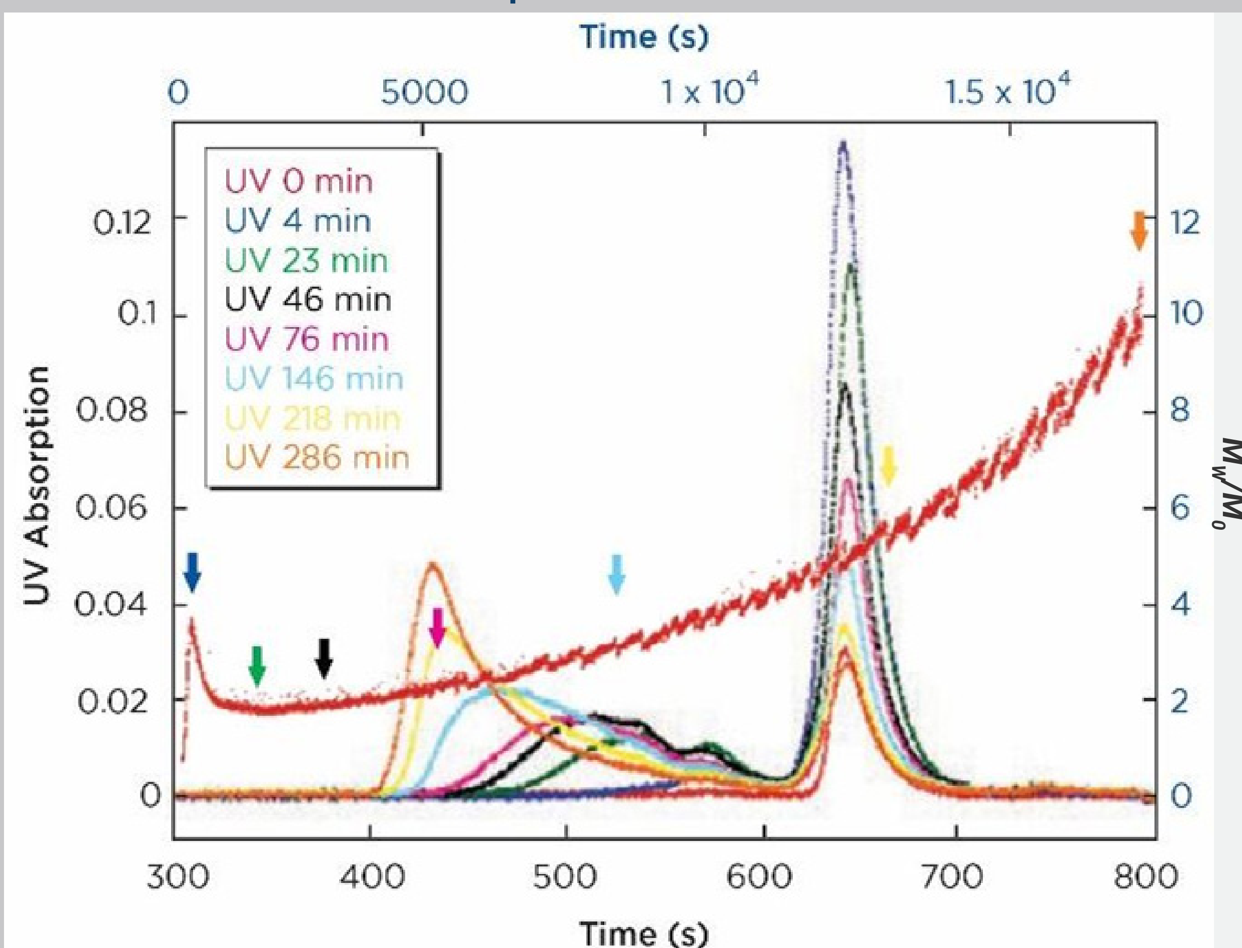


***Figure 3:** SEC elution profiles overlaid with (M_w/M_o) output (ARGEN) vs. time (t) for a monoclonal antibody.*

the identification of abnormal aggregation rates and molecular weight changes, saving time and resources required for orthogonal technique verification. Once a viable candidate is identified using ARGEN™, this data can be reconciled with SEC experiments or similar techniques, leading to an expedited understanding of ideal formulation development conditions. **Figure 3** illustrates the super imposition of the SEC elution profile and variations in M_w/M_0 ratios of a mAb over time. ARGEN™ reveals the aggregation rates and integrated changes in oligomerization states versus time, whereas SEC shows only discrete transitions from monomer to higher order oligomers. Coupling data from these techniques provides robust evidence for the identification of biopolymer stability.

Conclusion

For these experiments, ARGEN™ was used to characterize the chemical and thermal stability of a monoclonal antibody and subsequently establish quantitative and qualitative properties such as pH and temperature dependence on aggregation kinetics. Additionally, SEC elution profiles were superimposed onto M_w/M_o output, providing a robust comparison and identification of the stability landscape for the mAb. These studies demonstrate the power of ARGEN™ and its capability to provide pertinent stability data necessary to expedite the development of therapeutic biopolymers.

Case Study #3

RAPID DETERMINATION OF MOLECULAR WEIGHT AND SECOND VIRIAL COEFFICIENT OF A MONOCLONAL ANTIBODY USING ARGEN™

Introduction

Protein aggregation generally occurs as the result of either conformational or colloidal instability. Conformational stability is the free energy difference (*ΔG)* between folded and unfolded states. Although not direct measurements of free energy, melting temperature value (T_m) and aggregation temperature (T_{Agg}) can be used as qualitative assessments of conformational stability. Colloidal stability is determined by the balance of repulsive and attractive intermolecular interactions between protein molecules to conserve the native folded state. Simply stated, the propensity for aggregation is reduced by less intermolecular interaction. Therefore, the determination of the second virial coefficient (B_{22}) is a valuable screening tool to predict aggregation propensity. ARGEN™ is the ideal tool to assess quantitative and qualitative properties of all classes of biotherapeutics and determine T_{Agg}, B_{22}, molecular weight (M_w) as well as changes in weight average molecular weight (aggregation) under various thermal, chemical, and mechanical stressors. This case study outlines the method in which ARGEN™ is utilized to determine the second virial coefficient (B_{22}) and molecular weight (M_w) of a monoclonal antibody.

Step 1: Calculating the Excess Raleigh Ratio at 90°, *I*(90)

ARGEN™ detects the intensity of scattered light (90°) from a solution subjected to a vertically polarized laser source. The intensity of the scattered light is directly proportional to the size and concentration of the molecules analyzed as defined by the Zimm equation (**Equation 3**) and determined by solving for the excess Rayleigh ratio (**Equation 1**).

Below is the equation used to calculate the excess Rayleigh light scattering intensity "*I*" at a 90° scattering angle:

$$I(90) = \frac{[90Scat_{sample} - 90Scat_{solvent}]}{90Scat_{reference}} \times I_{Abs,Tol}$$

Equation 1

- ***90Scat***$_{solvent}$ is the scattering intensity of the solvent or buffer only (minus protein). This value is automatically stored for each cell when a "Solvent Baseline" experiment is performed.
- ***90Scat***$_{reference}$ is the scattering intensity of the reference (toluene). This value is automatically stored for each cell when a "Reference Baseline" experiment is performed.
- $I_{Abs,Tol}$ is the absolute Rayleigh scattering ratio for toluene at the laser wavelength. ARGEN™ uses a Laser with λ = 660nm, therefore $I_{Abs,Tol}$ = 1.19E-5 cm^{-1}.
- F is determined by the optical correction factor of the instrument. For ARGEN™ this value is F = 0.95.

Step 2: M_w & B_{22} Determination using the Zimm Approximation

When determining the molecular weight of a synthetic or natural polymer, the Zimm approximation is made at a low concentration when $q^2 <S^2>_z$ << 1. The following equation can be used for a polydisperse polymer population:

$$\frac{K \times Conc.}{I(\theta)} = \frac{1}{M_W}\left(1 + \frac{q^2 \times <S^2>_z}{3}\right) + 2 \times B_{22} \times Conc.$$

Equation 2

- ***Conc.*** is the concentration of the sample (mg/ml or mg/cm^3).
- $q^2 <S^2>_z$ is the term for the z-average mean square radius of gyration.
- B_{22} is the 2nd virial coefficient for the sample dissolved in solvent.
- ***K*** is the optical component for vertically polarized light and is determined from the following equation:

$$K = \frac{(2\pi)^2 \times n^2 \times \left(\frac{dn}{dc}\right)^2_{sample}}{(\lambda \text{cm})^4 \times N_{\text{Avogadro}}}$$

Equation 3

» n is the index of refraction of the pure solvent

» $\left(\frac{dn}{dc}\right)_{sample}$ is the differential index of refraction increments of a solvent with respect to the concentration of sample dissolved in that solvent.

ARGEN automatically determines the value of K upon entry of n and $\left(\frac{dn}{dc}\right)_{sample}$ sample on the experiments page.

Within the limitation of measuring scattering at 90°, the Zimm Equation reduces to:

$$\left.\frac{K \times Conc.}{I(90)}\right|_{q=90} = \frac{1}{M_W} + [(2 \times B_{22}) \times Conc.]$$

Equation 4

Or simply:

$$y = b + mx$$

Equation 5

With these values, a Debye plot can be generated using Equation 6:

$$\frac{K \times Conc.}{I(90)} vs. Conc.$$

Equation 6

The Y-intercept is equal to $1/M_w$ of the molecule in solution, and the slope of this plot is equal to 2x the virial coefficient (B_{22}) as displayed in (**Figure 3**).

Sample preparation: Solvent & Standard (toluene) scattering baseline determination

A 2 ml stock solution of [mAb] = 0.05 mg/ml was prepared and subsequently syringe-filtered using a 0.22 µm cellulose acetate filter. After establishing solvent baseline and standard scattering intensities, a filtered buffer solution was used to dilute the stock mAb sample to [mAb] = 0.05 mg/ml. Since there is a significant increase in scattering intensity between the solvent and mAb solution, the neutral density (ND) filter was adjusted to mitigate scattering signal saturation. Next, the sample was

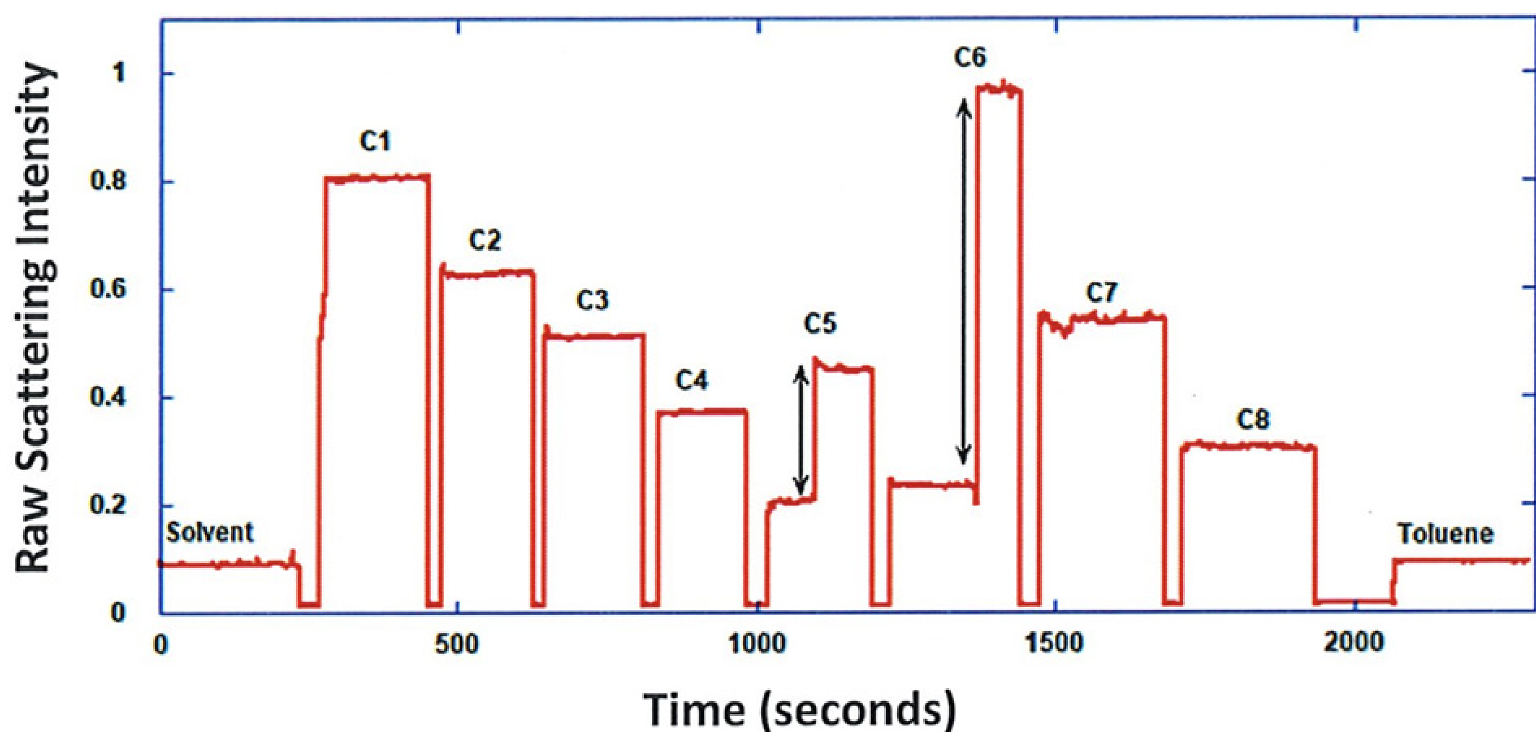


Figure 1: *Raw scattering intensities for solvent, mAb dilution series and toluene*

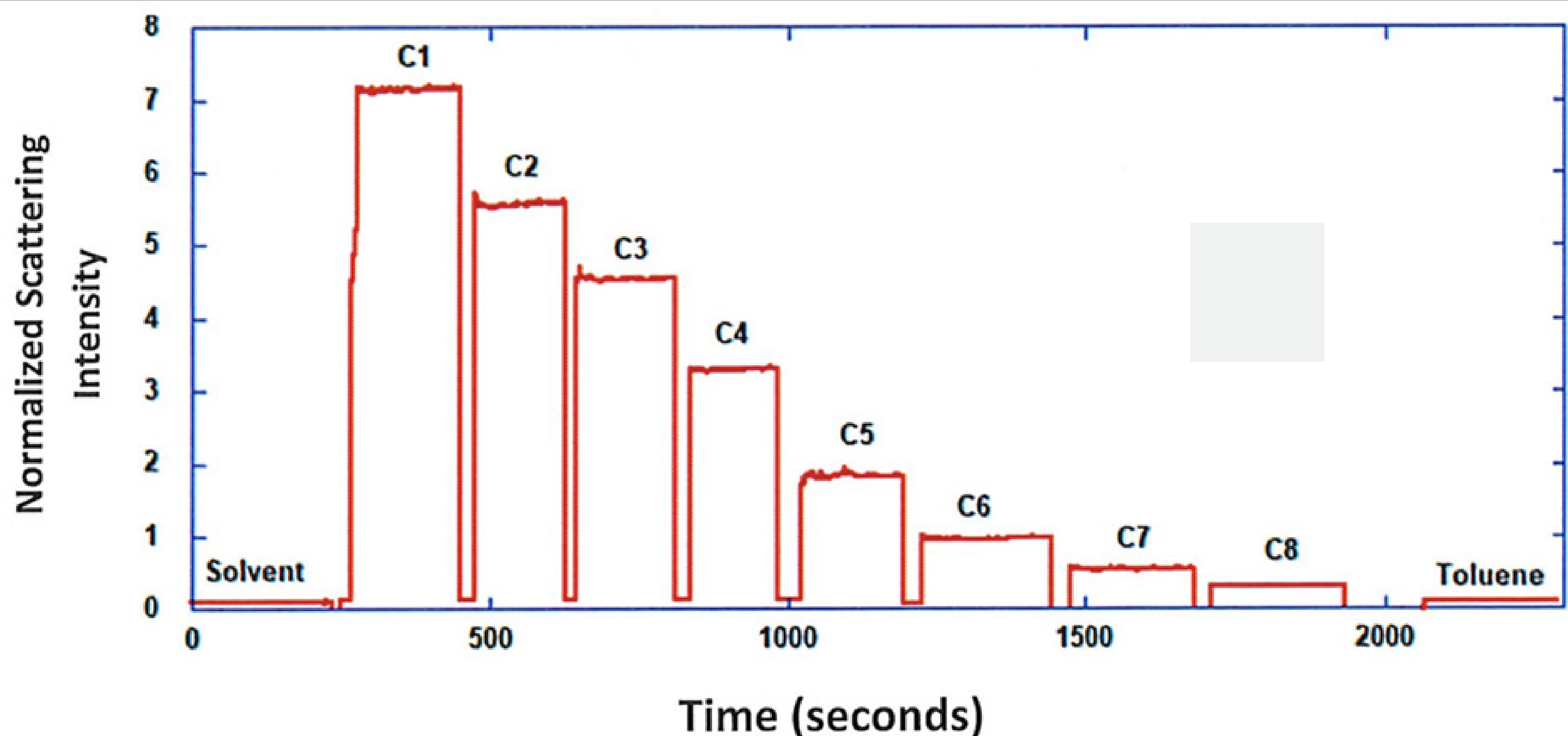


Figure 2: Normalized scattering intensities for solvent, mAb dilution series and toluene

serially diluted, and scattering intensities were collected for each dilution in series. When the scattering signal was <20% of the maximum, the ND filter was adjusted to increase signal intensity as depicted in **Figure 1** (steps C5 and C6). Normalized scattering intensities for each dilution are shown in **Figure 2**. Raw scattering intensities were scaled and normalized to zero neutral density. Scaled scattering intensities (Scaled SLS) for each dilution, solvent, toluene as well as Rayleigh scattering intensities ($I(90)(cm^{-1})$)) and values of $K*Conc./I(90)$(mol/g) are shown in (**Table 1**).

Debye Plot Analysis

To determine K (optical component), the index of refraction for the buffer solution was assumed equal to that of pure water, therefore, $n = 1.33$. Additionally, the published differential index of refraction increment for monoclonal antibodies is $dn/dc = 0.185$ cm^3/g with $K = 2.089E\text{-}7$ mol*cm^{2}g^{-2}. The Y-intercept of the linear fit (regression) indicated that the molecular weight of monoclonal antibody is 151,600 g/mol, and the second virial coefficient is 3.3E-05 mol*cm^3/g^2.

Conclusion

These experiments demonstrate the utility of ARGEN™ to determine molecular weight (M_w) and the second virial coefficient (B_{22}) of a monoclonal antibody. The quantitative and qualitative measurements permitted classification and an understanding of the propensity for aggregation in a variety of solution conditions. Furthermore, the high throughput capacity of ARGEN™ allows users to analyze up to 16 samples or conditions simultaneously, vastly reducing time and resources required for development.

Conc. (g/cm³)	Scaled SLS	*I (90) (cm⁻¹)*	*K*Conc / I (90) (mol/g)*
Solvent	0.091	NA	NA
C1 = 0.0500	7.275	1.05E-03	9.91E-06
C2 = 0.0333	5.589	8.07E-04	8.63E-06
C3 = 0.0250	4.525	6.51E-04	8.02E-06
C4 = 0.0167	3.283	4.69E-04	7.43E-06
C5 = 0.0083	1.819	2.54E-04	6.86E-06
C6 = 0.0042	0.970	1.29E-04	6.74E-06
C7 = 0.0021	0.535	6.52E-05	6.67E-06
C8 = 0.0010	0.310	3.22E-05	6.76E-06
Toluene	0.092	NA	NA

*Table 1: Sample concentrations (g/ml), scaled scattering intensities Scaled SLS), Rayleigh scattering intensities ($I(90)$ (cm^{-1}))), and K*Conc. / I(90) (mol/g)*

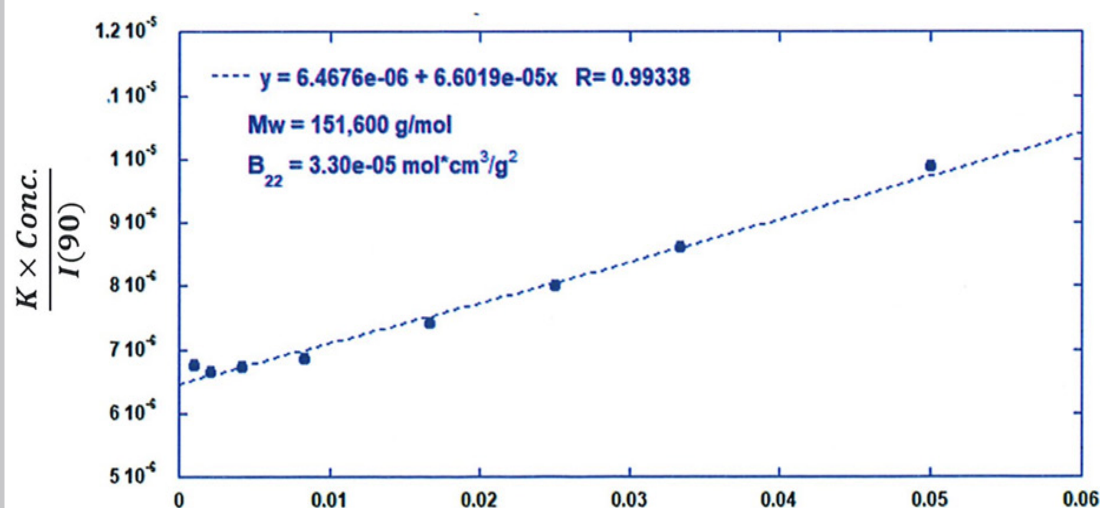


***Figure 3:** Debye plot for molecular weight and second virial coefficient determination for a mAb*

Case Study #4

APPLYING ARGEN™ FOR THE RAPID CHARACTERIZATION OF A MONOCLONAL ANTIBODY & ASSESSMENT OF MECHANICAL IMPACTS OF BIOPROCESSING

Introduction

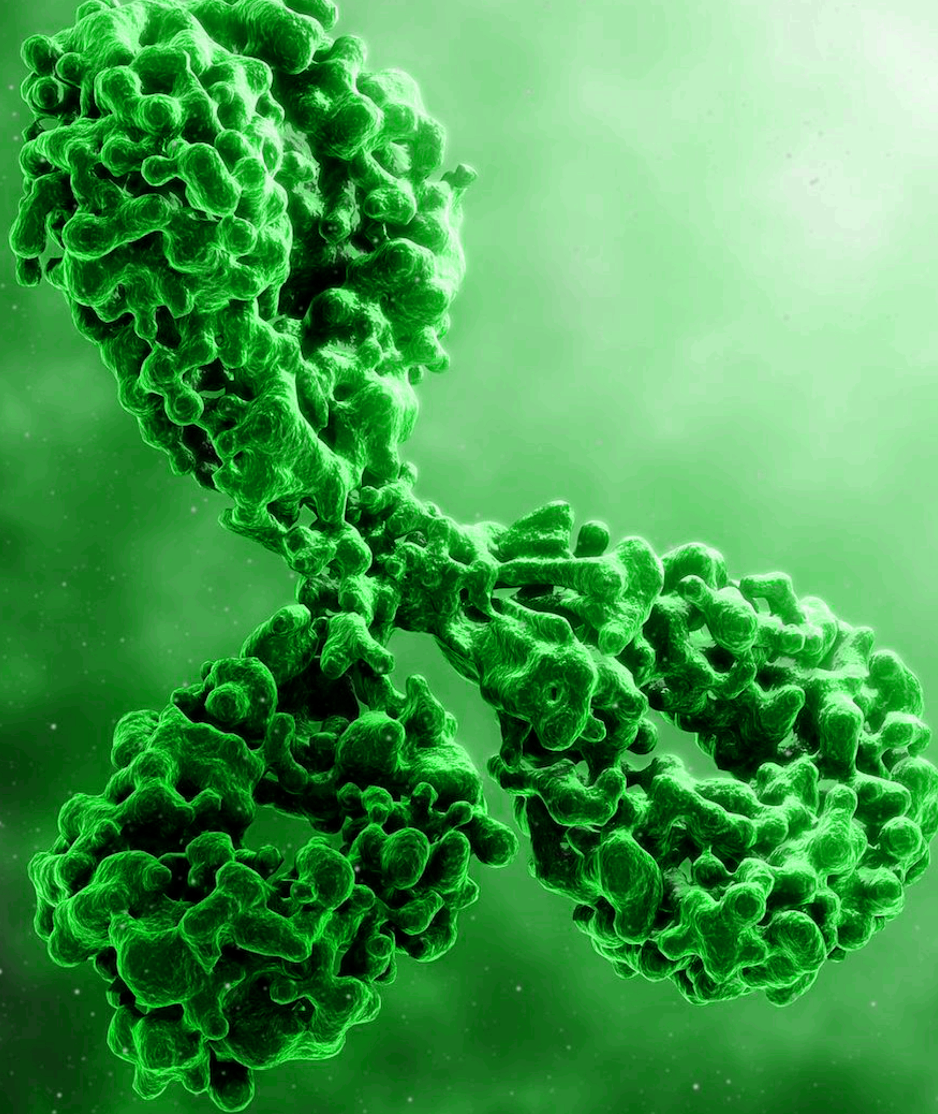
The successful development of biotherapeutics requires an in-depth understanding of the impacts associated with mechanical and physical stress. Instability and subsequent aggregation can render them biologically inactive or even induce an immunological response in patients. Stirring is a notable factor which induces aggregation. Contact stirring represents only one type of stress that can occur during the purification, manufacturing and packaging of therapeutic proteins. Overhead stirring, capillary shear stress, filtration, and peristaltic recirculation can all impact protein stability and induce aggregation. Since many or all of the listed mechanical and physical stressors can be present during therapeutic protein production, it is critically important to qualitatively and quantitatively assess the impact of each stressor. ARGEN™ is the ideal instrument to quantify the impacts associated with stirring stress. With 8 (ARGEN-LT) or 16 (ARGEN™) individual sample cells with fixed optical pathways that can be operated independently, parallel experiments can be performed by modeling stirring stress conditions associated with production and manufacturing, expediting the development process.

Characterizing the Impacts of Different Modes of Stirring Stress

Understanding the impacts of different modes of stirring provides insight into the stability of therapeutic biopolymers and their ability to remain stable during purification and manufacturing processes. Each cell of ARGEN™ is equipped with a stepper motor attached to a rotating magnet which couples to a micro stir bar either at the bottom of the cuvette (contact stirring) or overhead (non-contact stirring) to simulate perturbations during bioprocessing. Stirring/agitation rate (0 - 2000 RPM) is manually controlled for precision via the ARGEN™ intuitive control software. Overhead stirring is performed with a custom engineered cuvette. Simply, the cap of the cuvette is fitted with a spindle which suspends a micro stir bar. Sealed bearings housed in the cap allow the spindle and stir bar assembly to freely rotate in sync with the magnetic stepper motor. **Figure 1** demonstrates the difference in aggregation rates (slope of M_w/M_o) between contact stirring and non-contact stirring. Changes in M_w/M_o and aggregation were observed at the outset of all (triplicate) contact stirring experiments. This data clearly provides evidence that changing the mode of stirring stress applied to the monoclonal antibody results in very disparate aggregation rates and profiles, which can be vital during the vetting process and identifying the most viable construct.

Conclusion

Understanding the impacts of stirring and shear stress on biopolymers during processing and manufacturing are key to expediting development. The ability of ARGEN™ to simulate stirring stress while simultaneously monitoring oligomerization or degradation states is unique and provides the final link in getting a treatment to market. This case study provided evidence of the effects of different modes of stirring stress experienced during bioprocessing on a monoclonal antibody. Monitoring changes in normalized molecular weight in real time demonstrated that contact stirring would not be a preferred method as the protein aggregated immediately. This data is critical in vetting constructs to discover the optimal candidate, as well as designing processing equipment to minimize the impacts and perturbations experienced during bioprocessing.



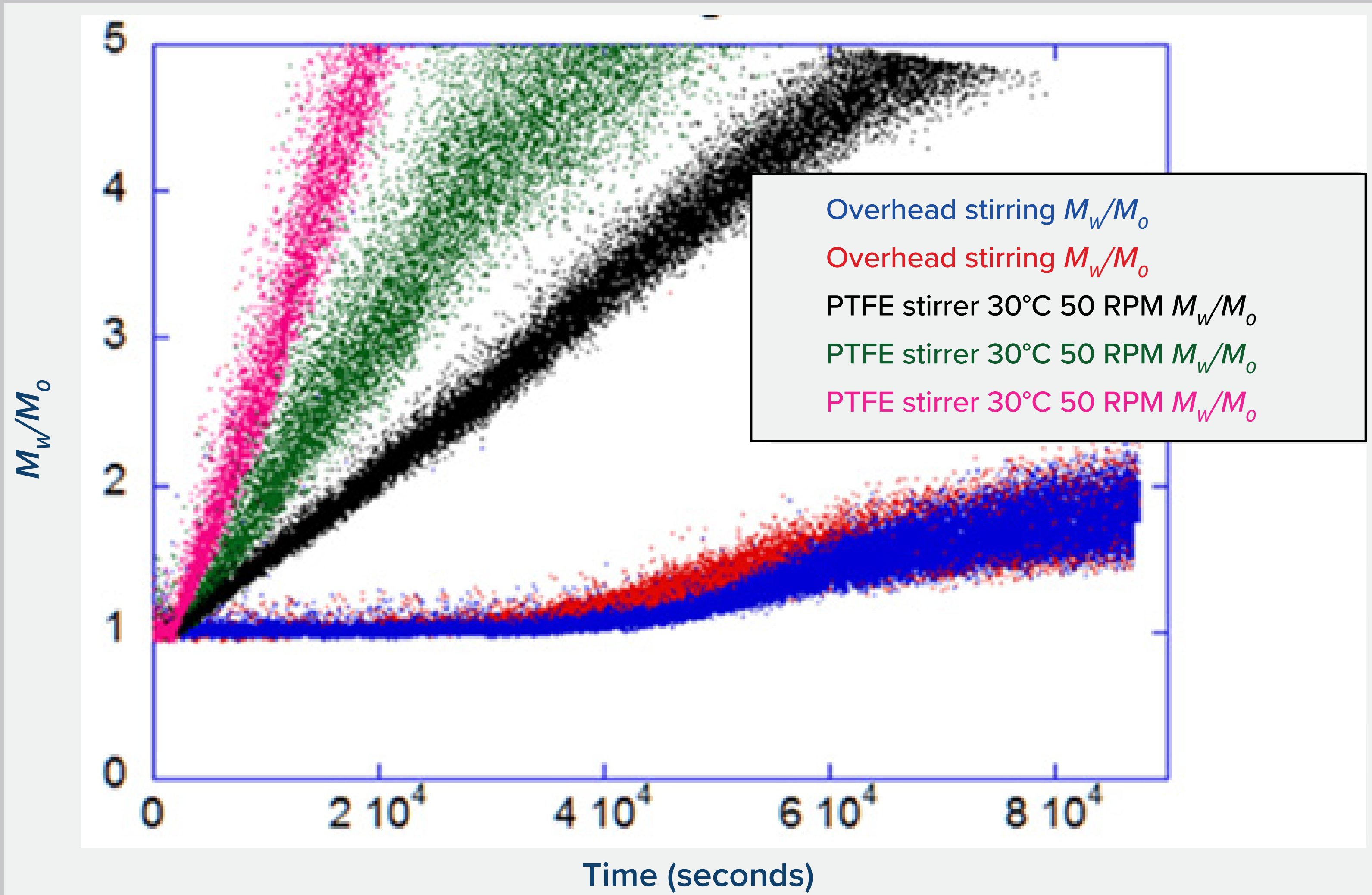


Figure 1: *Change in normalized molecular weight (M_w/M_0) vs. time (sec) of a monoclonal antibody when subjected to non-contact (overhead) vs. contact stirring*

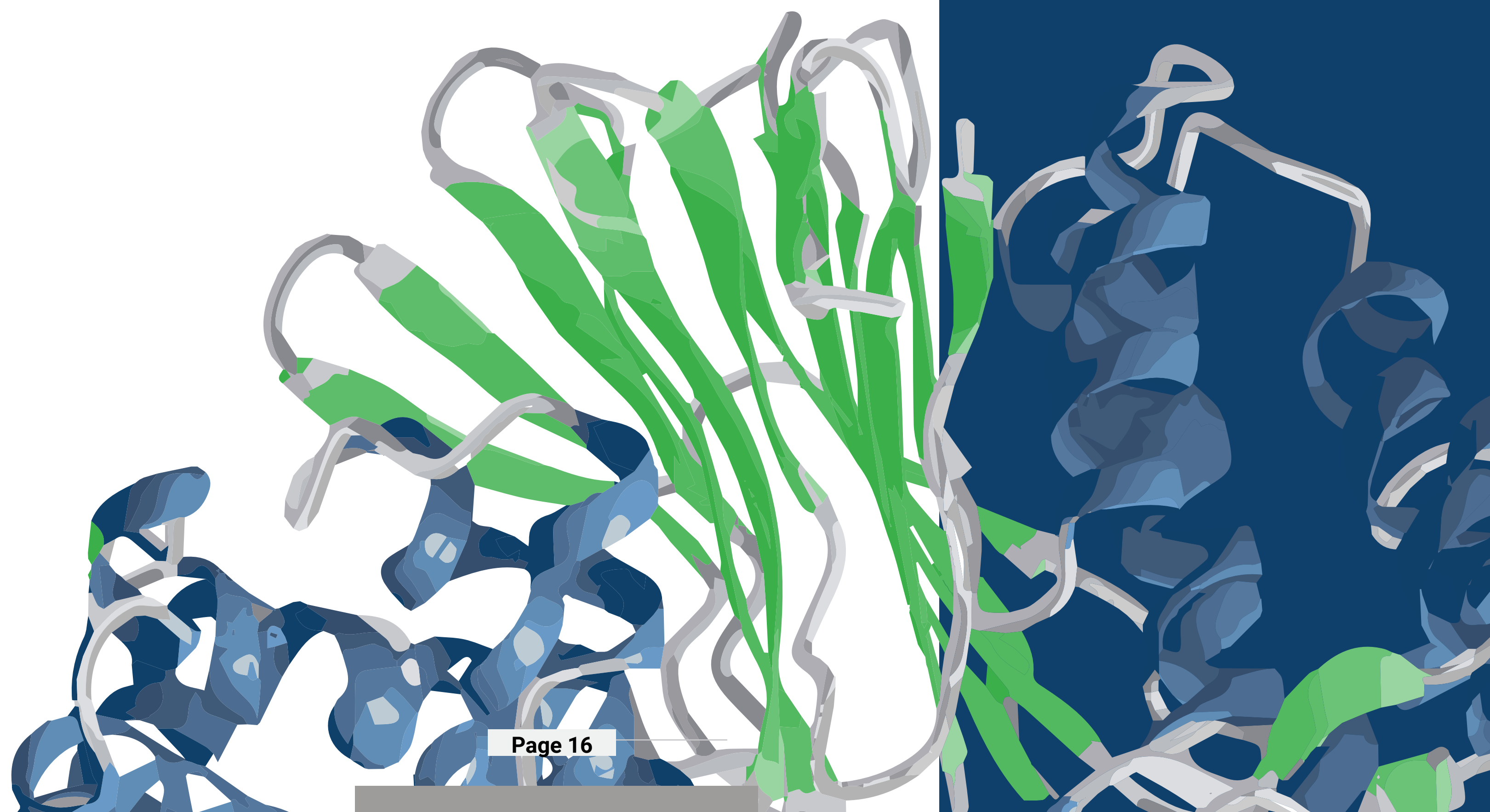
DIGITAL BOOKLET SUMMARY

We hope this digital booklet provided you with an in-depth understanding of the ARGEN™ technology and related applications to deliver a comprehensive stability landscape for all classes of therapeutic biopolymers. Patented simultaneous multiple sample light scattering (SMSLS) technology permits the user to model and monitor the effects of thermal, chemical and mechanical stress in 8 (ARGEN-LT) or 16 (ARGEN™) cells in parallel, vastly increasing efficiency and expediting development.

Additionally, the ARGEN™ team is committed to your success and always available to answer questions or assist with any challenges that may arise. Please feel free to reach out with any questions or concerns.

Very Respectfully,

Fluence Analytics



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