# Forced Degradation of Proteins Through Mechanical Stress

Fluence Analytics

realtime data. realtime optimization.

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# ARGEN

- Continuously measures light scattering from 16 independently Temperature and Stirring controlled cells

- Early detection of aggregation and degradation

- Key Outputs
  - Aggregation Rate (AR)
  - Normalized Mw
  - Mass AR

- Applications
  - Formulation stability
  - Interfacial studies
  - Material interaction
  - GPC scheduling
  - Dissolution kinetics


Specifications:

- Temp Control: 18 – 100 C
- Stirring Control: 0-2000 RPM
- Power: 90 – 220 VAC
- Dimensions: 30”w x 22”d x 14”h
- Weight: 85 lbs

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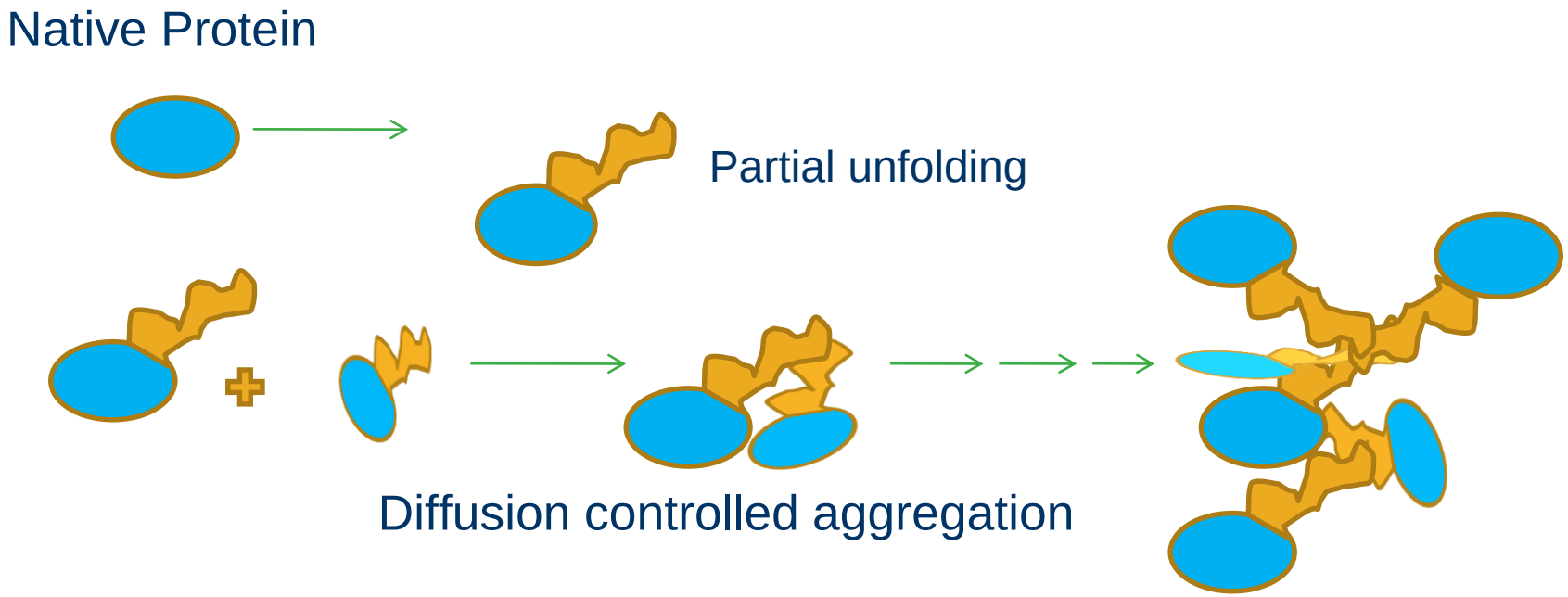
# Protein Aggregation




- Major problem in development of new therapeutic proteins
- Several mechanisms exist, highly protein dependent
- Need for high throughput screening

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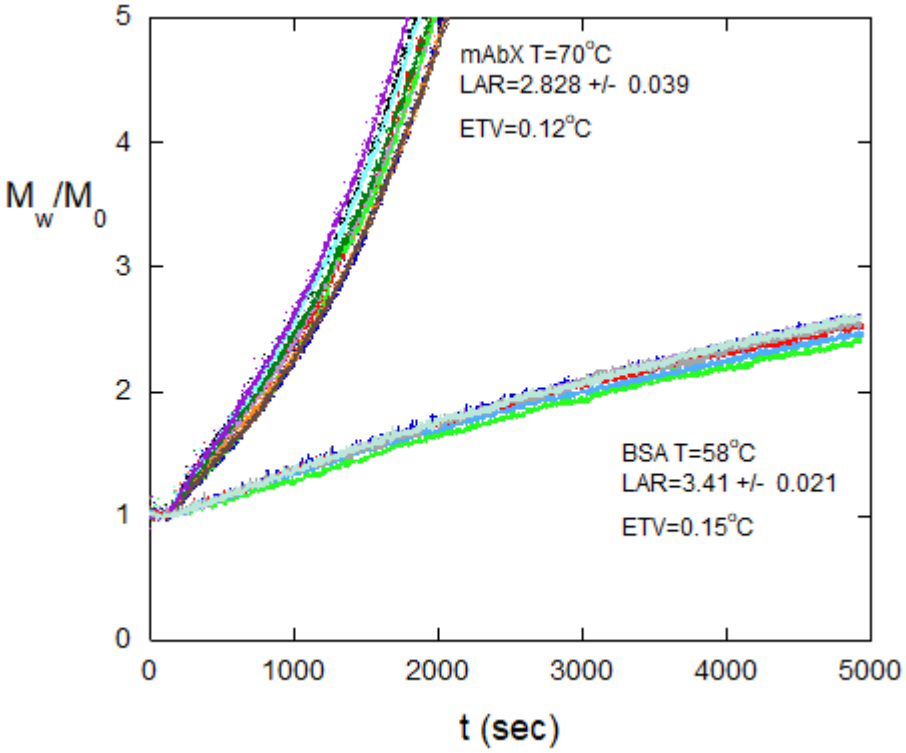
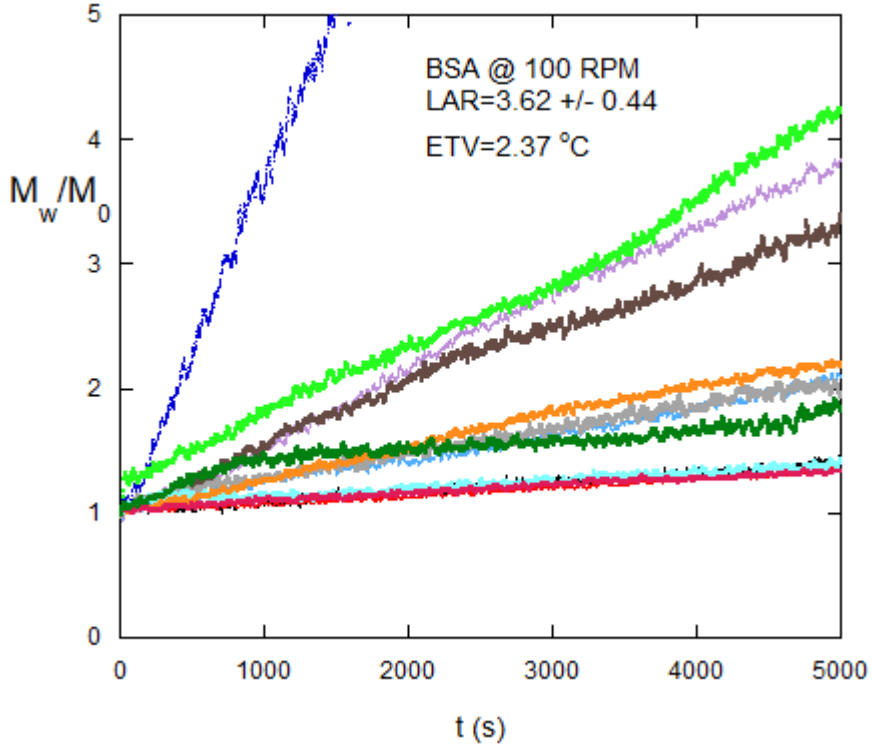
# Routine Aggregation Monitoring via ARGEN

## Routine Methods:




Thermally induced aggregation




Contact-stir induced aggregation

Curtis W. Jarand and Wayne F. Reed, *The Journal of Physical Chemistry B* **2018** *122* (40), 9361-9372

# Non-Routine Aggregation Monitoring via ARGEN

## Mechanical/Shear Stress

- Non-contact stirring with overhead suspended magnetic stir bars controlled by the ARGEN software (3d-printed construction with inexpensive and readily available components)
- Examination of capillary shear through different tubing diameter and composition via reciprocating syringe pump
- Non-contact stirring by overhead impellers controlled by stepper motors (motors and drivers are inexpensive and readily available)
- Recirculation of protein samples from sample cuvette via peristaltic pump
- Recirculation of protein samples through differing filter materials via peristaltic pump
- Continuous extraction of protein samples from ARGEN through a secondary detector (MALS, fluorescence, UV, etc.) via syringe to provide complimentary analytical methods
- Continuous or discrete extraction of protein samples for GPC analysis with an autoinjector

## Non-Mechanical/Shear

- Direct titration in real time with salts, excipients, acids/bases, denaturation agents, etc.
- Generation of Debye plots
- Probing Host:Guest interactions

# Example of Equipment for Non-Routine Experiments

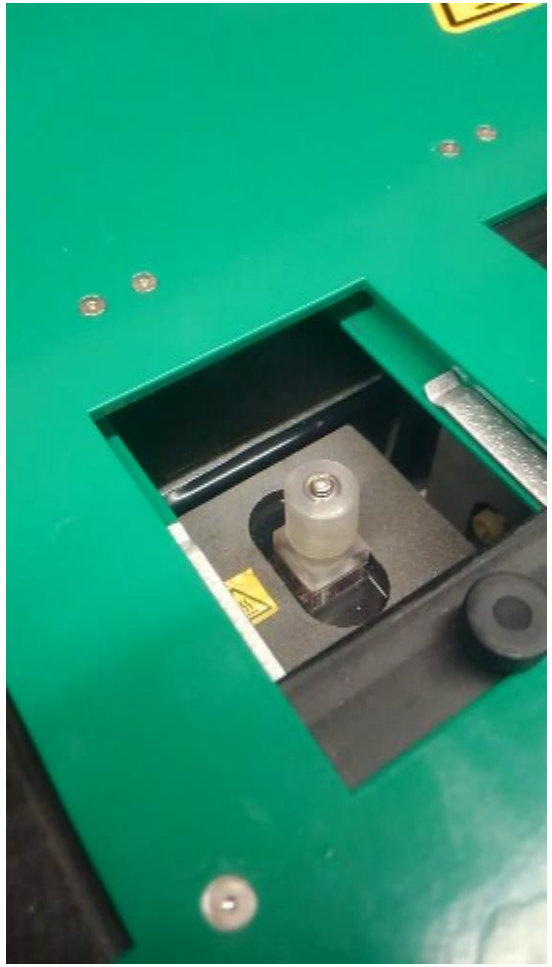
## Mechanical/Shear Stress

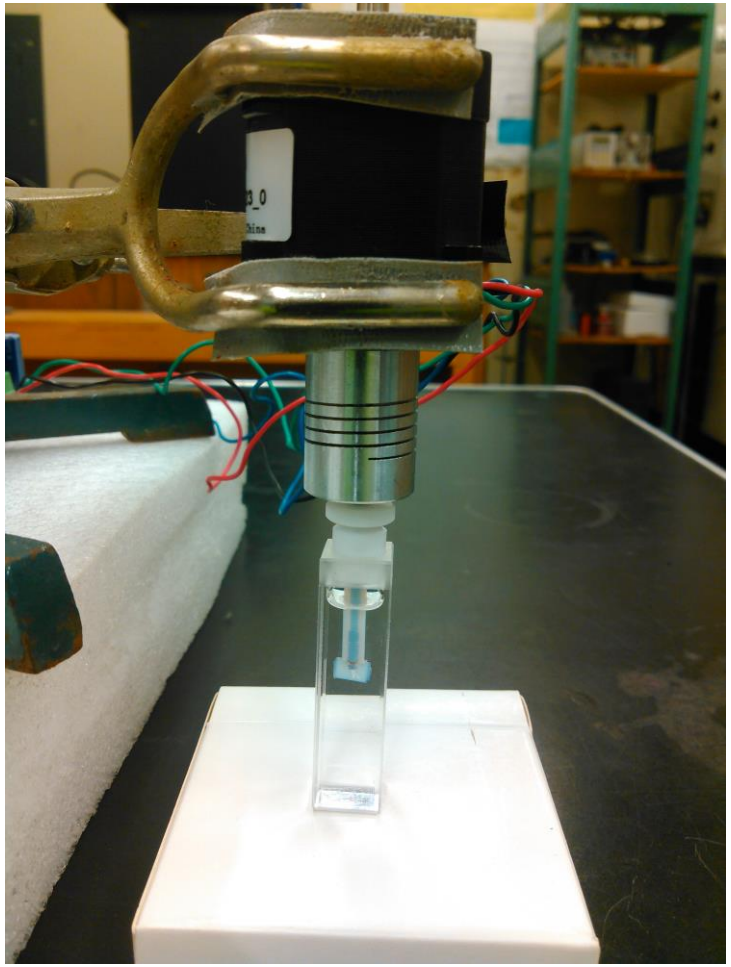
- 3-d printer and/or basic machining equipment (overhead stirring)
- A good source for small parts for stirring is McMaster-Carr, https://mcmaster.com
- Syringe pump (capillary shear stress, withdrawal through secondary detectors)
- Peristaltic pump (recirculation, filtration)
- HPLC tubing and various fittings

## Non-Mechanical/Shear

- Non-routine applications such as titrations and Debye plots are easier to analyze using the data export function, generates .csv file for external analysis

# Non-Contact Stirring





- Can be coupled to instrument control (left) or independently controlled via stepper motor (right)
- Stirrer consists of magnetic stir bar connect to stainless steel or PTFE shaft, miniature bearings pressed into cap (instrument control) or directly inserted through a drilled PTFE cap (stepper motor controlled)
- Shaft/stirrer must not block optical path (~3-4 mm from bottom of cell)

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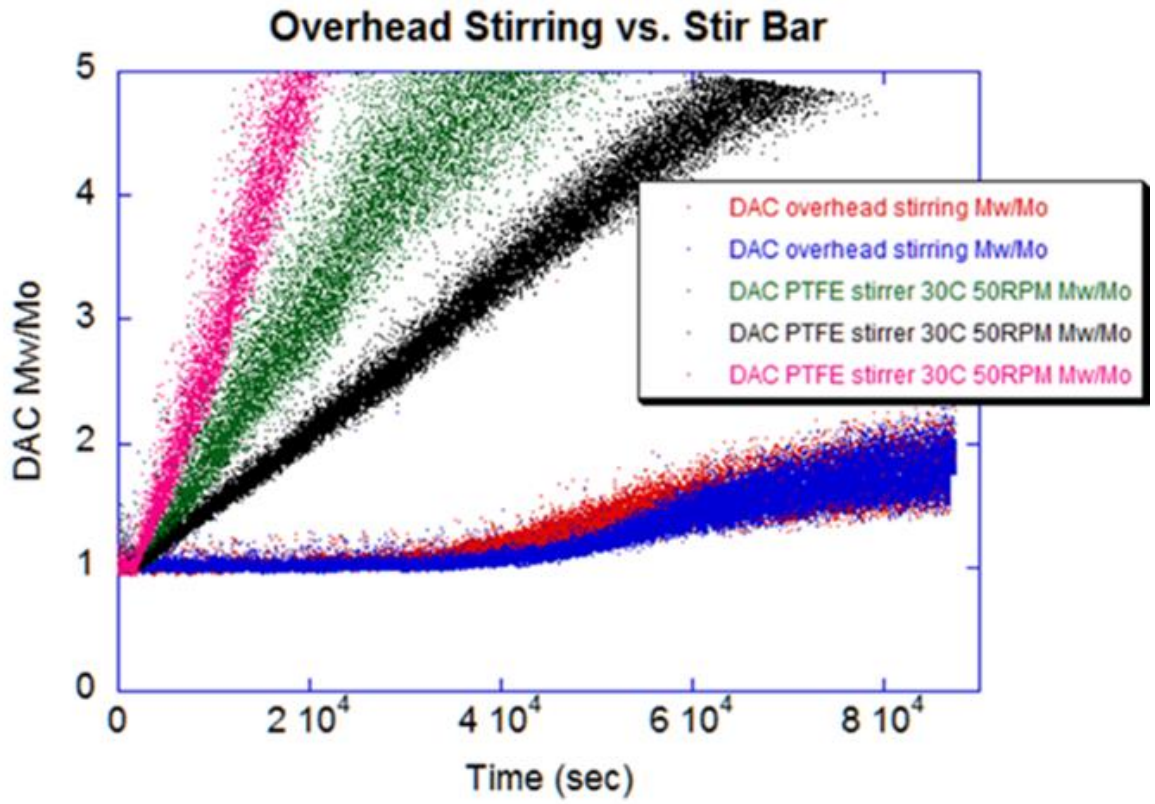
# Overhead vs. Contact Stirring

- Overhead stirring results in markedly decreased aggregation
- Proteins examined to date have demonstrated vastly improved reproducibility relative to contact stir
- Lag phases are commonly observed

Note:

- Coupled magnetic stirring suitable for samples which aggregate at <500 rpm, stepper motor control required for higher rpm

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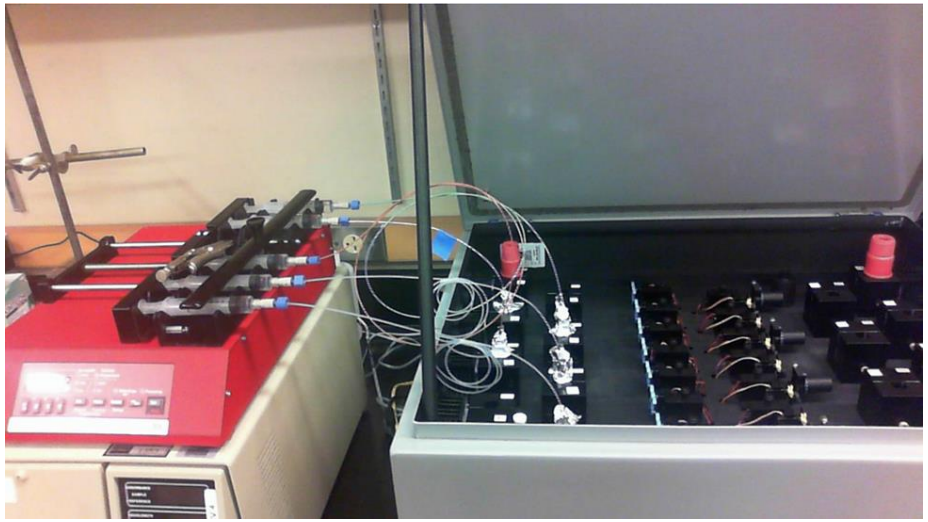
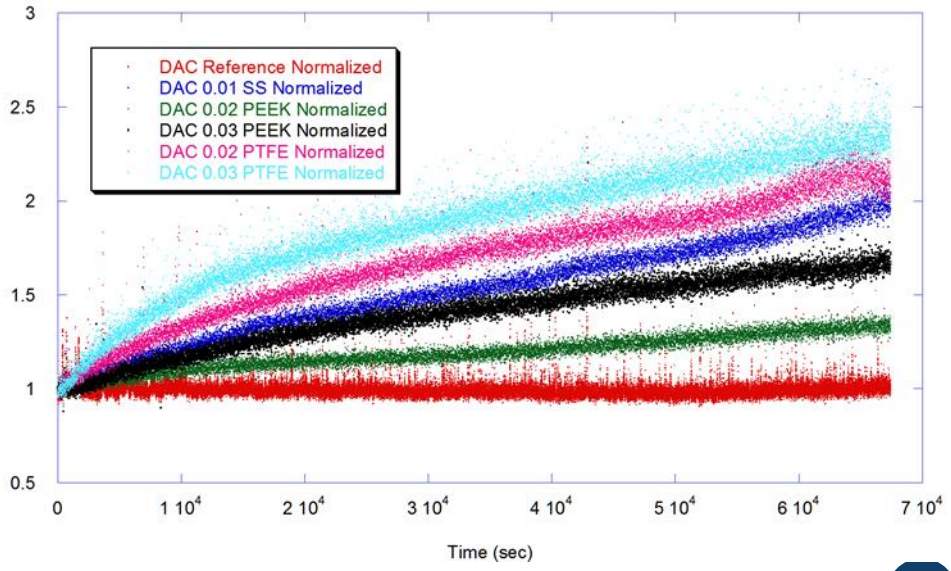
# Capillary Shear Stress

- Utilizes a reciprocating syringe pump to continually extract/inject samples into cuvette under ARGEN monitoring
- Equal lengths of tubing used (1m is sufficient)
- Equal starting volumes of samples required for direct comparison
- Consistent flow rates used
- Tubing material and diameter can have a significant impact on observed aggregation rates, for samples shown in the plot the AR as a function of tubing material is PTFE>SS>PEEK



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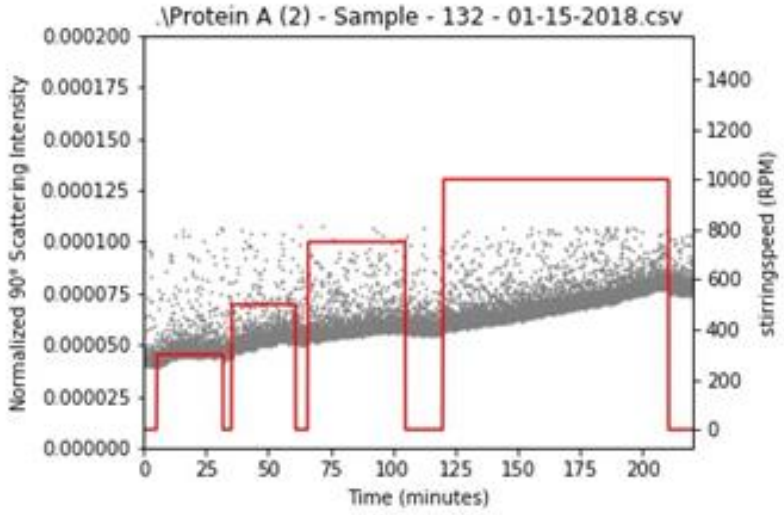
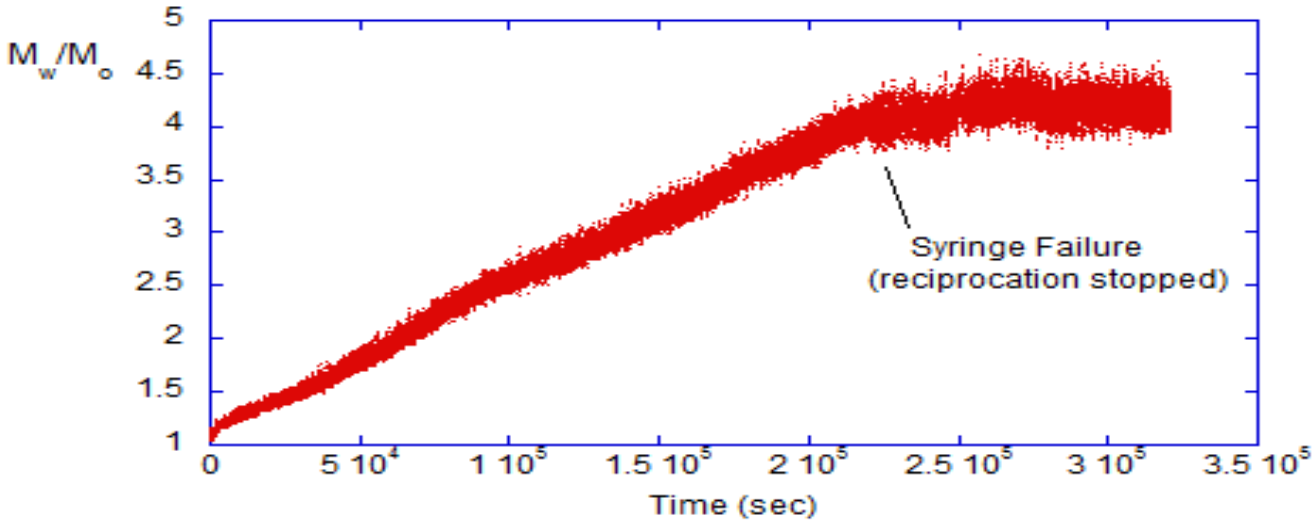
# Stir and Shear Stress Aggregation Halt when Stress Removed

- Aggregation stops when stressor removed
- Upon re-application of stress, aggregation will continue along a similar path

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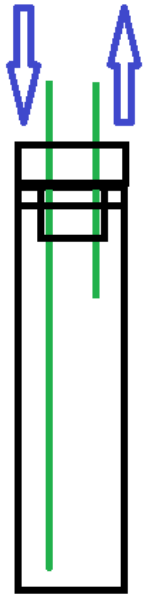
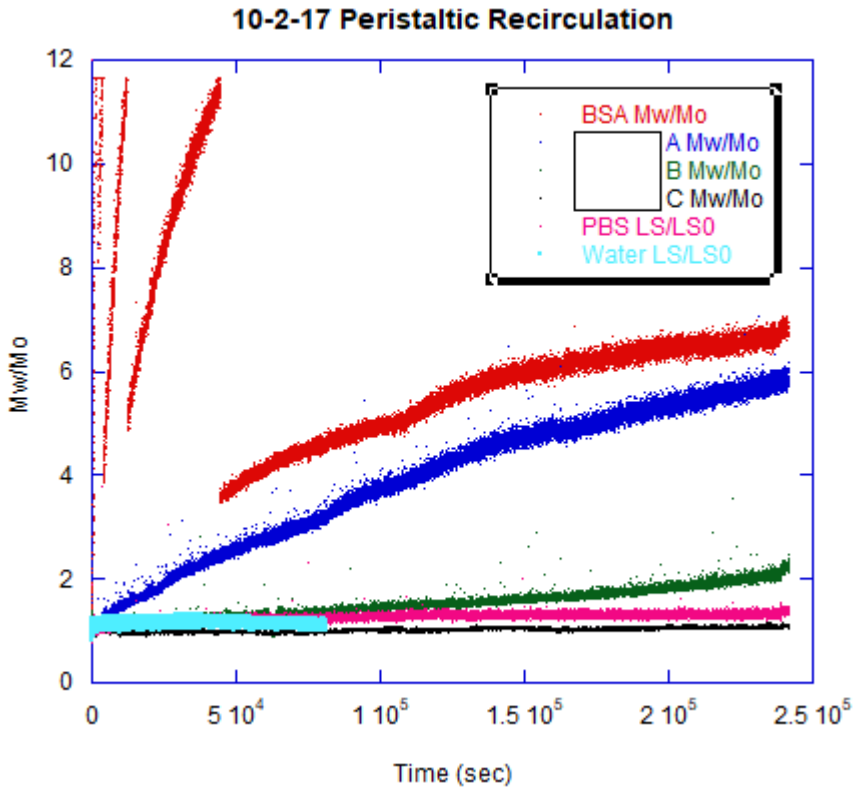
# Peristaltic Recirculation

- Peristaltic pumping generally considered to exert minimal shear stress
- Sample recirculated through cuvette by HPLC tubing
- Water and buffer controls show no increase in light scattering
- The stability of differing proteins varies widely



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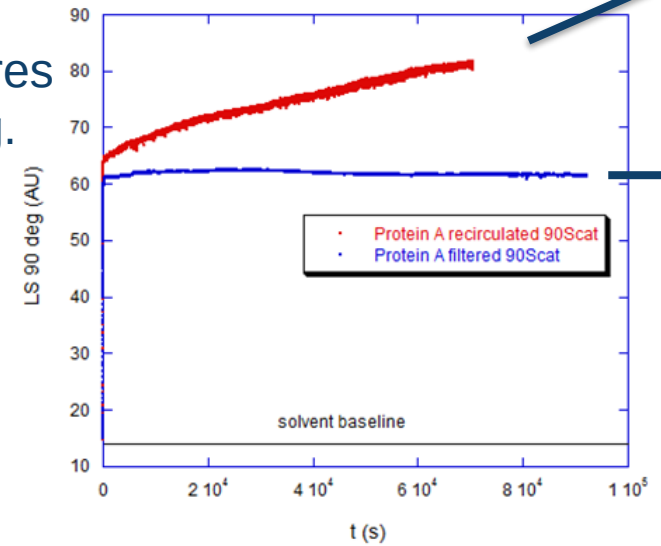
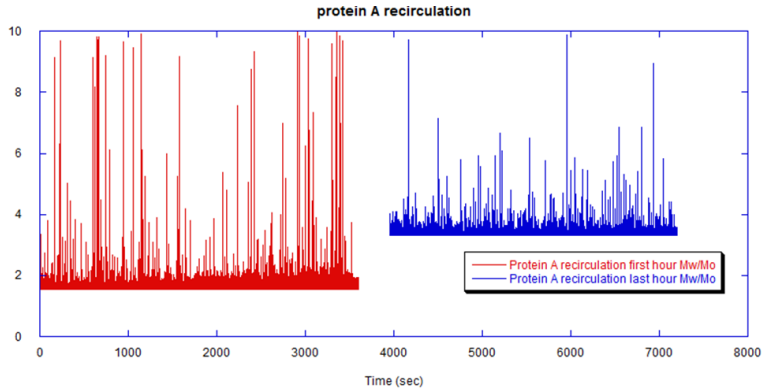
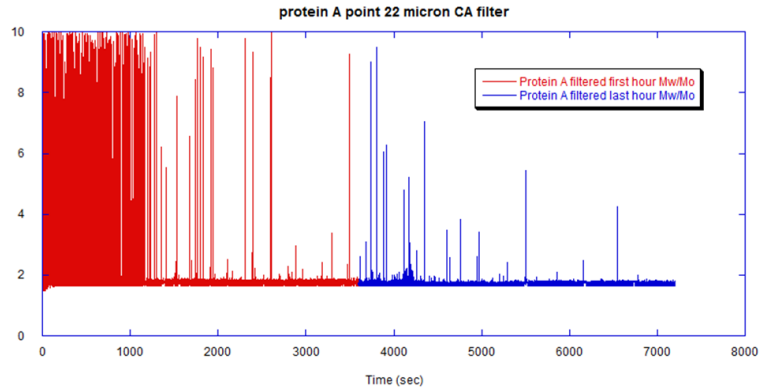
# Peristaltic Recirculation w/ Filtration

- Syringe filter placed in recirculation loop via luer lock fittings
- Filtered sample shows no increase in light scattering and a decrease in scattering spikes caused by large aggregates
- Behavior indicative of a small population of large aggregates leading to increased light scattering signal- SLS intensity is a function of the average population, i.e. a small population of large aggregates can yield an increase similar to a large population of small aggregates
- Confirmation of mechanism requires secondary detection methods, e.g. GPC or MALS

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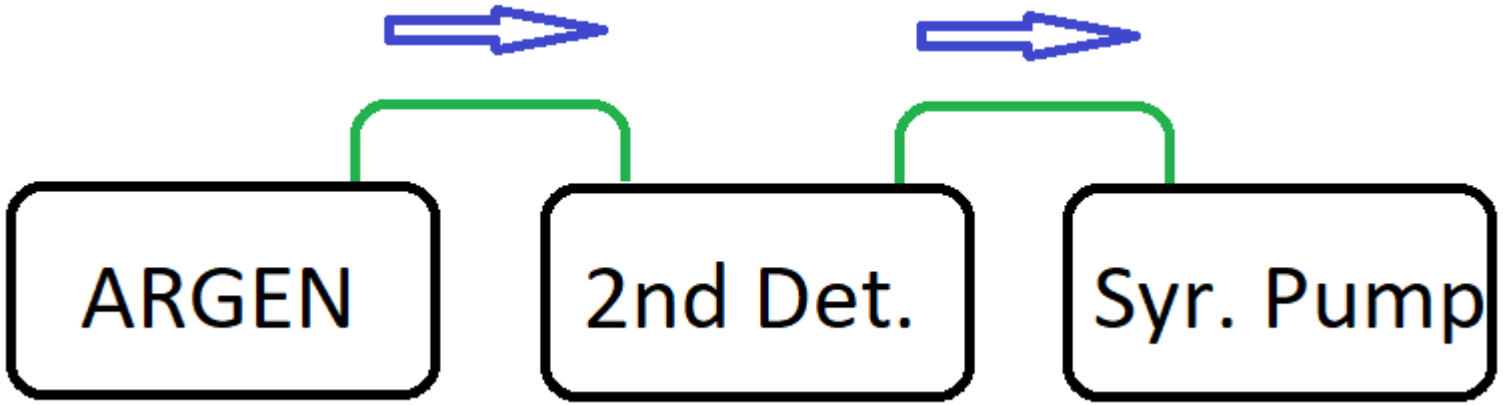
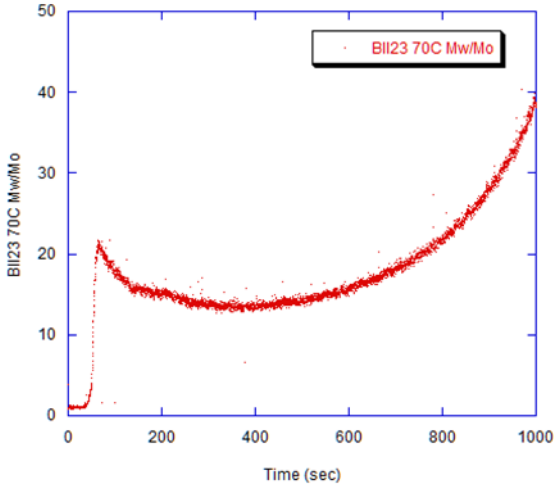
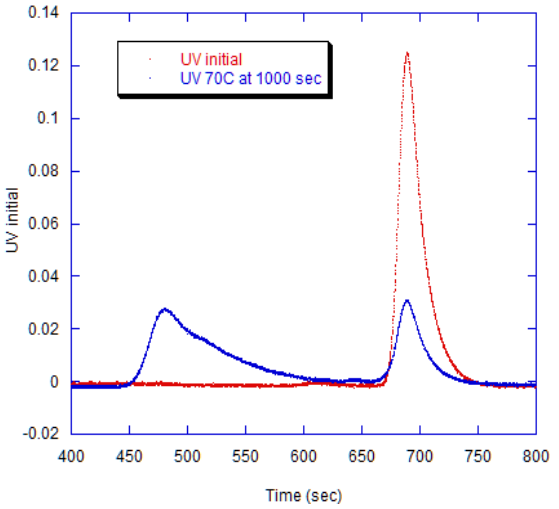
# Coupling to Secondary Detector

- Syringe pump used to withdraw sample via HPLC tubing
- Can be withdrawn directly through detector (MALS, UV, Fluorescence, etc.)
- Can be coupled to autoinjector for GPC (Rheodyne MXP7900)
- Begin pulling sample before stressor applied
- Minimize dead volume from ARGEN to secondary detector

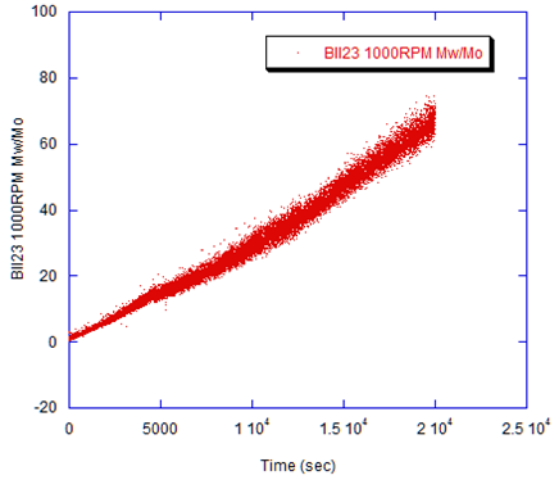


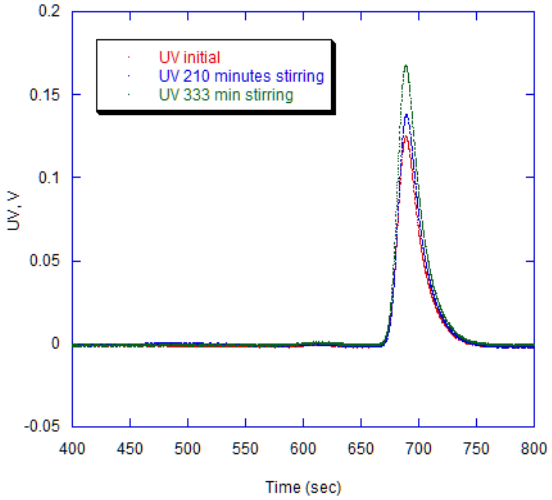
# Analysis of Thermal vs Stir Stress by Coupled GPC




- Sample withdrawn from cuvette through an autoinjector via syringe pump
- Aliquots periodically injected onto GPC column w/UV detection
- Significant monomer loss and aggregate formation observed in thermally stressed sample (left) but not in contact-stir stressed sample (right)

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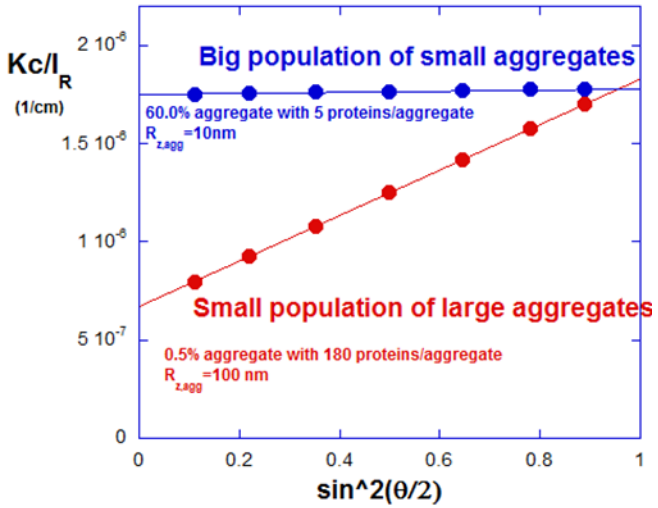
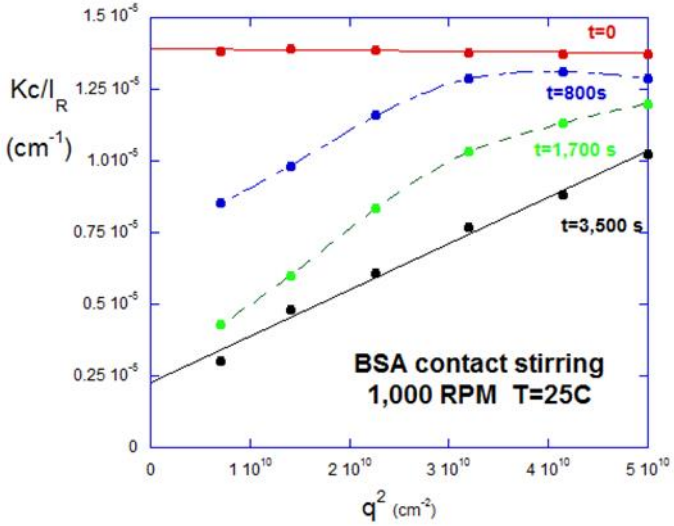
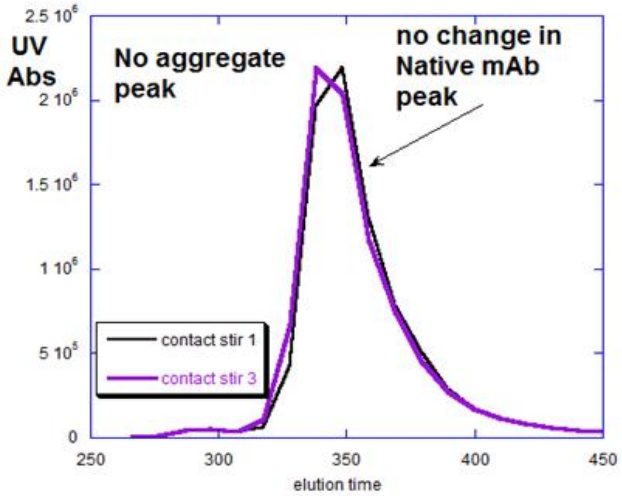
# Analysis of Stir-Stress by Coupled ARGEN-GPC-MALS




- MALS expands ARGEN capability to discern the nature of aggregate populations
- Angular dependence increases with aggregate size
- Can discern between a big population of small aggregates (thermal stress) and a small population of large aggregates (stir stress)

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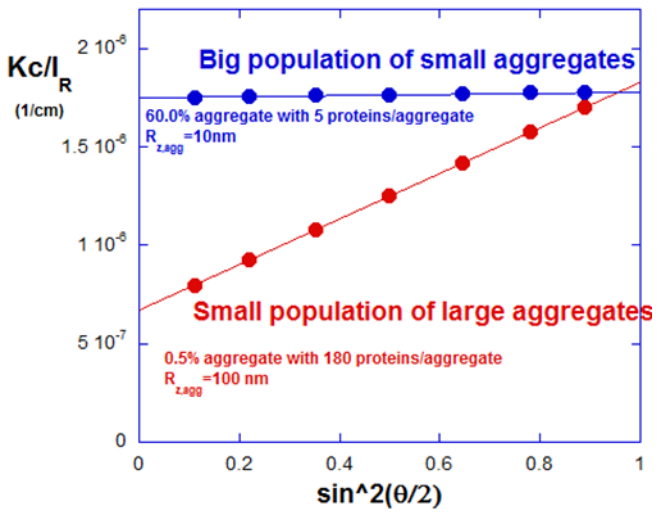
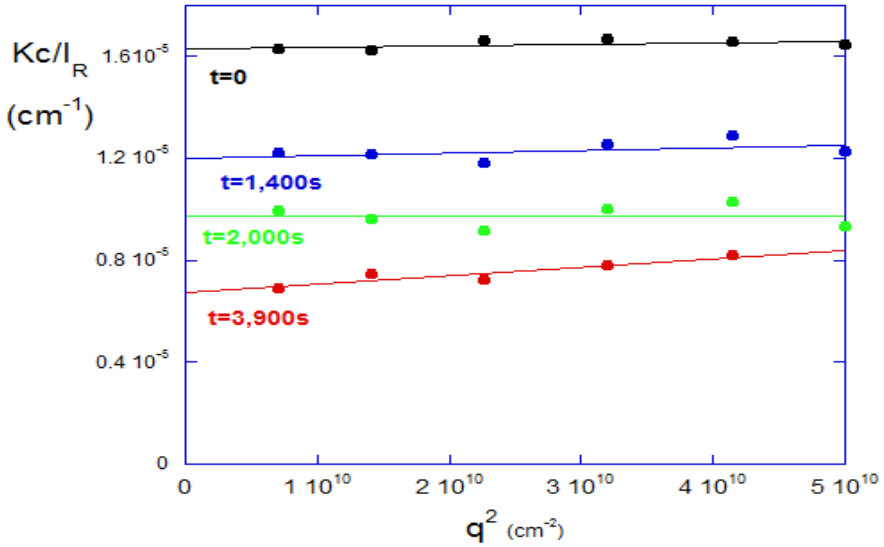
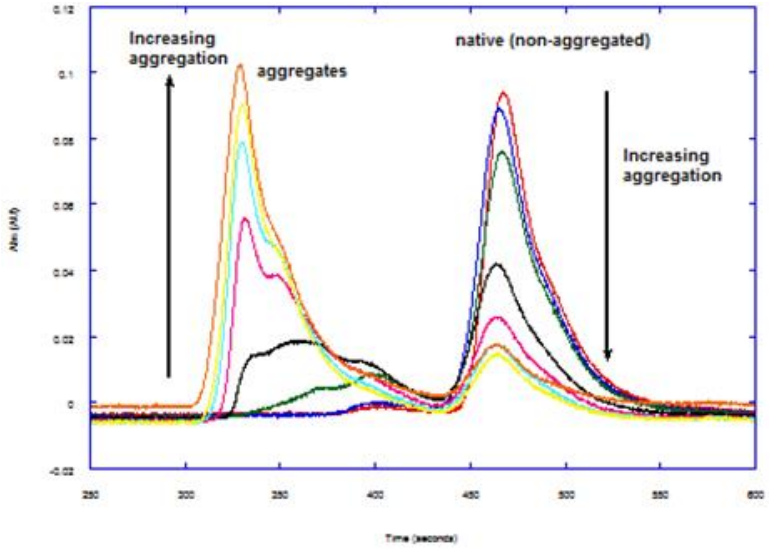
# Analysis of Thermal Stress by Coupled ARGEN-GPC-MALS




- In contrast to stir stress, thermal stress yields a very small change in angular dependence
- MALS data in agreement with GPC data showing small aggregate formation during thermal stress

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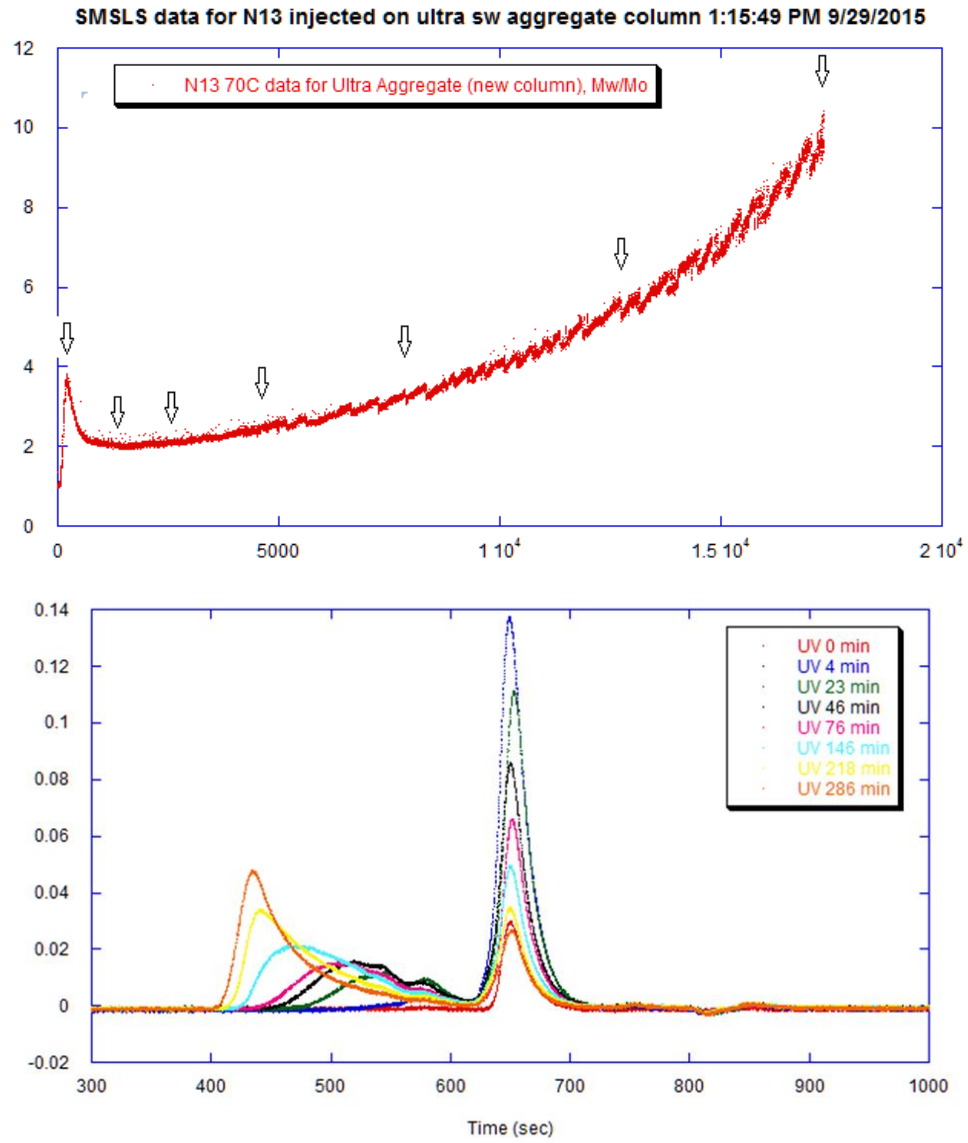
# Observing Aggregate Formation by Coupled GPC

- Thermally stressed sample monitored simultaneously by ARGEN and coupled GPC with a Tosoh Ultra SW aggregate column
- Stepwise aggregation is observed which in which the sample can appears to undergo an evolution through dimeric, trimeric, etc aggregate formation
- Mechanically (contact-stir) stressed samples do not exhibit this same evolution

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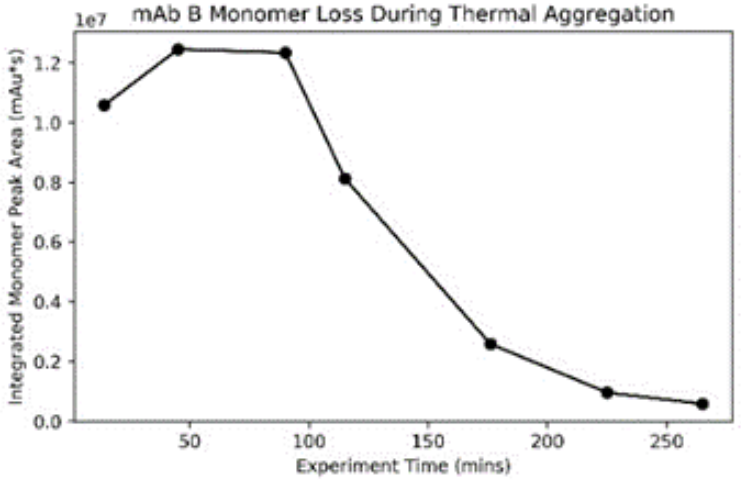
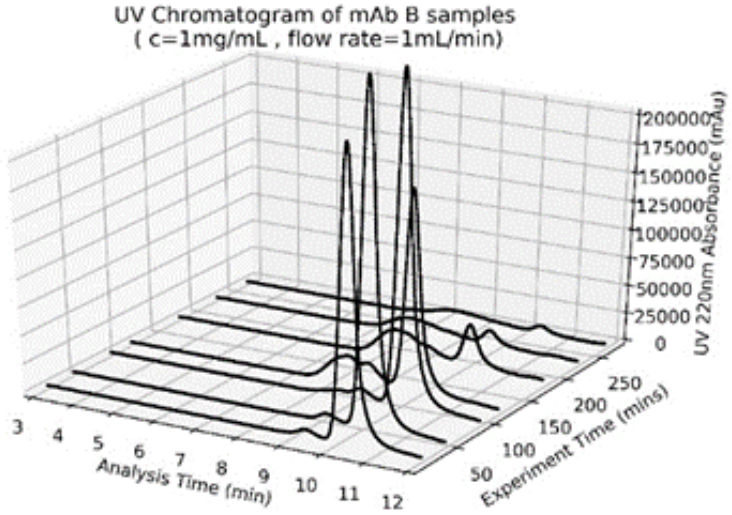
# Protein Monomer Loss by Coupled GPC

- Protein monomer loss can be calculated with a standard GPC column if an aggregate-specific column not available
- Monomer loss quantified relative to initial area of peak prior to application of stress by ARGEN

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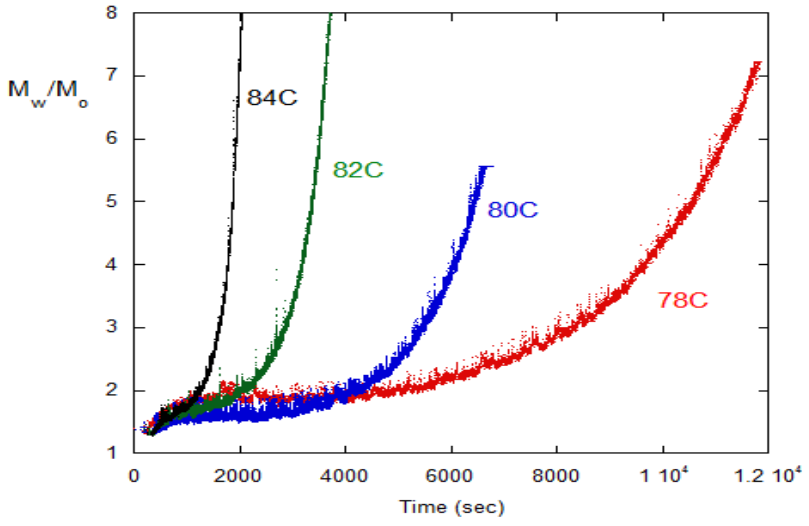
# Superposability of LS Signatures Implies the Same Mechanism

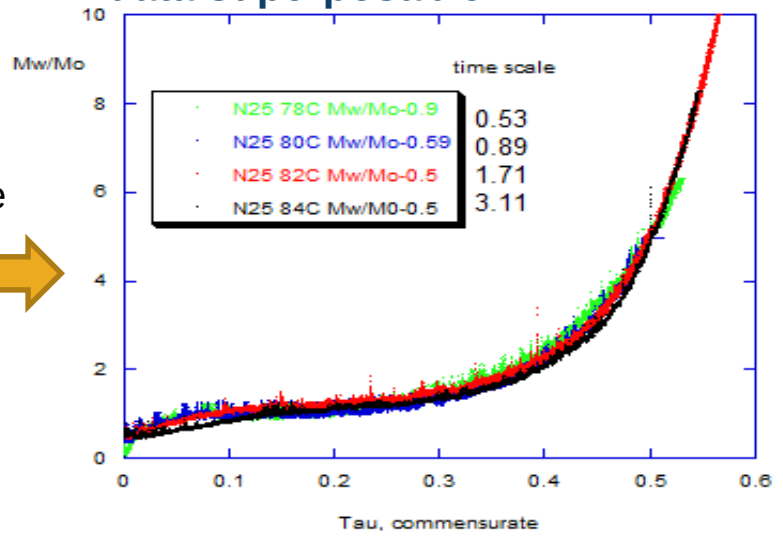
**Hypothesis:** If there exists a proportionality constant between the time bases of non-linear $I_s(t)$ from two aggregation processes which yields an exact superposition of the non-linear $I_s(t)$ then the kinetic pathways for the two processes are of the same kind.

**Thermal stress: Real time data**




Superposable

**Thermal stress: Time scaled; data superposable**

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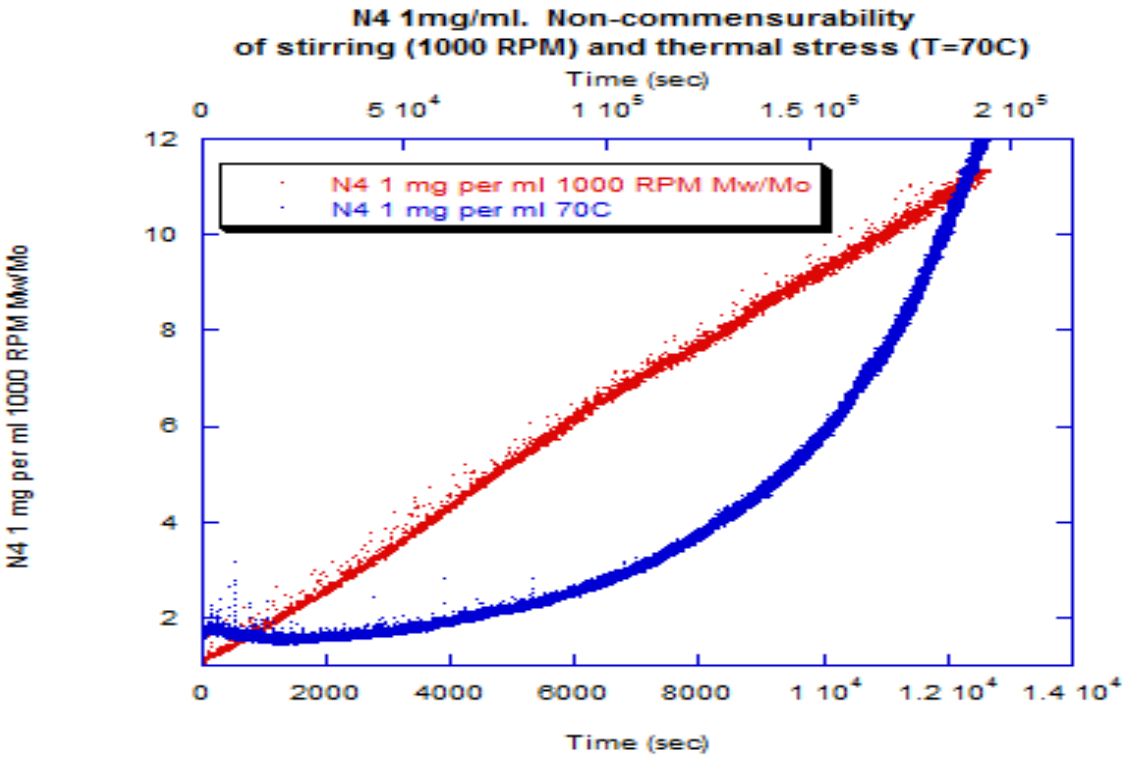
# Distinguishing Kinetic Mechanisms via Superposability- Stir Stress




- Stirring and thermal stress with scaled timebases are not superposable
- Indicative of different kinetic mechanisms

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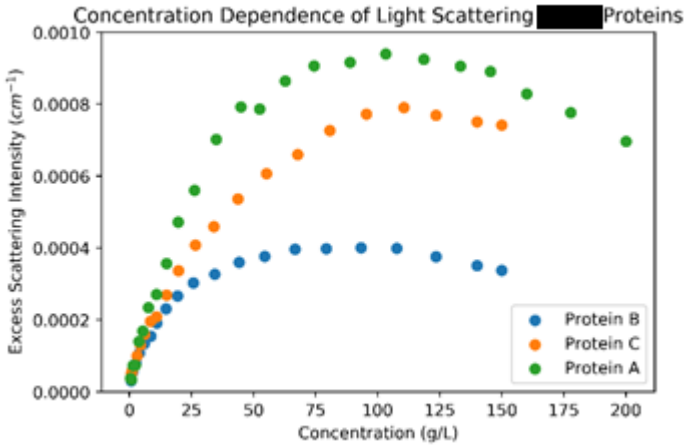
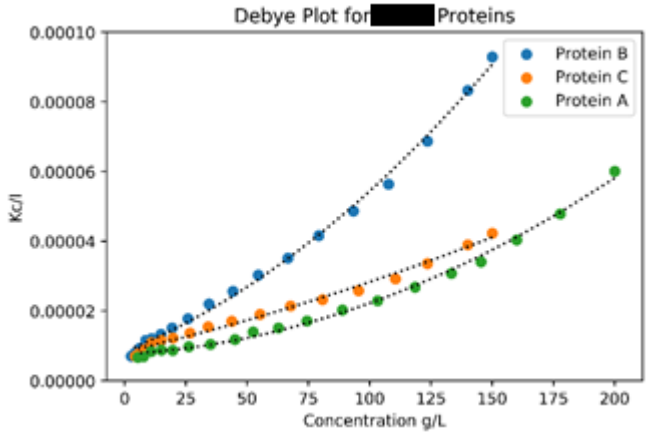
# Generation of Debye Plots

- Samples directly titrated in cell w/ gentle hand mixing
- LS v. concentration used to generate Debye plot for calculation of viral coefficients
- May require several different starting sample concentrations to cover concentration range
- Data can be collected in a single file




| Debye Statistics for Proteins | | | |
|---|---|---|---|
| *Label* | *$A_3$ ($cm^2$ mol $g^{-2}$)* | *$A_2$ ($cm^2$ mol $g^{-2}$)* | *$M_w$ (kDa)* |
| Protein A | 5.35e-4 | 1.9e-5 | 131 |
| Protein B | 8.95e-4 | 1.40e-4 | 119 |
| Protein C | 1.86e-4 | 8.2e-5 | 121 |

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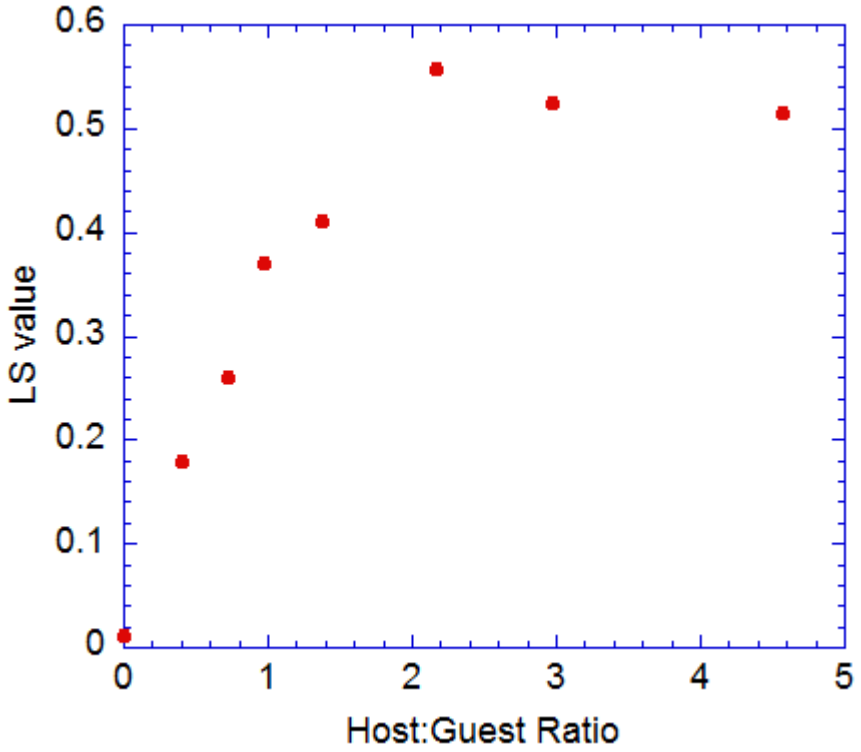
# Probing Supramolecular Host:Guest Interactions

- Host:Guest ratios for large molecule interactions can be determined by ARGEN
- Methodology is similar to the generation of Debye plots, i.e. direct titration of host or guest
- LS signal maxima yields host:guest stoichiometry




Payne, M; Jarand, C.; Reed, W.; Grayson, S., *Abstracts of Papers, 255th ACS National Meeting & Exposition*

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# Summary

- ARGEN provides a simple and robust platform for monitoring protein aggregation by thermal and contact-stir stress
- ARGEN capabilities can be easily expanded to monitor a variety of other phenomena beyond thermal and contact-stir stress
- Examples of expanded methodology include:
  - -non-contact stirring
  - -peristaltic recirculation
  - -monitoring filtration effects
  - -examining capillary shear
  - -secondary detection for monitoring aggregate formation/monomer loss
  - -generation of Debye plots
  - -probing host:guest interactions