High-pressure sample environment and spectroscopic studies of biomolecular solutions

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RET project this summer (A tale of Two Talks)

HP Sample environment for BioSANS: equipment commissioning

Manual System
- cart-ready assembly
- backup for PMI
- For benchtop measurements (DWS, fluorescence,ex-beamline incubations, etc)

PMI automated system
- testing/creating documentation

LIPSS system
- McHugh cells: test orings on old cell; test new cell performance

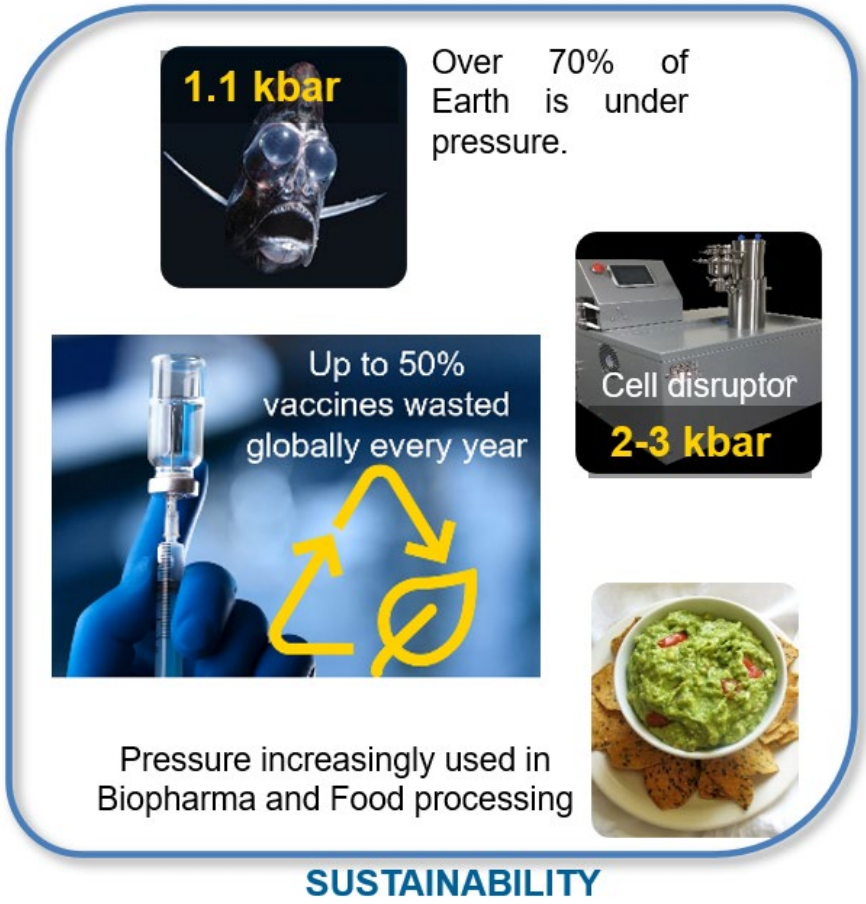
Optimizing HP-BioSANS experiment planning: a case study (BSA binding to polyphenols)

Pre-incubating samples under pressure
- pre-characterization
- testing more pressure steps, holding times, reversibility

Sample characterization using benchtop techniques
- Circular dichroism
- FTIR

Background: Why do we need high-pressure SANS to study biomolecules?

High-pressure is ubiquitous: in-vivo, in laboratories and industrial environments



- SANS accesses sizes and concentrations in environments more relevant to in vivo or in industry
- SANS is non destructive: samples can be equilibrated at different pressures for various holding times (with HP-SAXS we are limited to 5min equilibration), different steps can be explored and pressurization rates can be explored

LIPSS : Liquid Insertion Pressure System for SANS Studies

in-situ studies of P|T effects for Biomolecules **- how it works**

Critical parameters for Biomolecules:

Sample volume: 2 - 5 mL

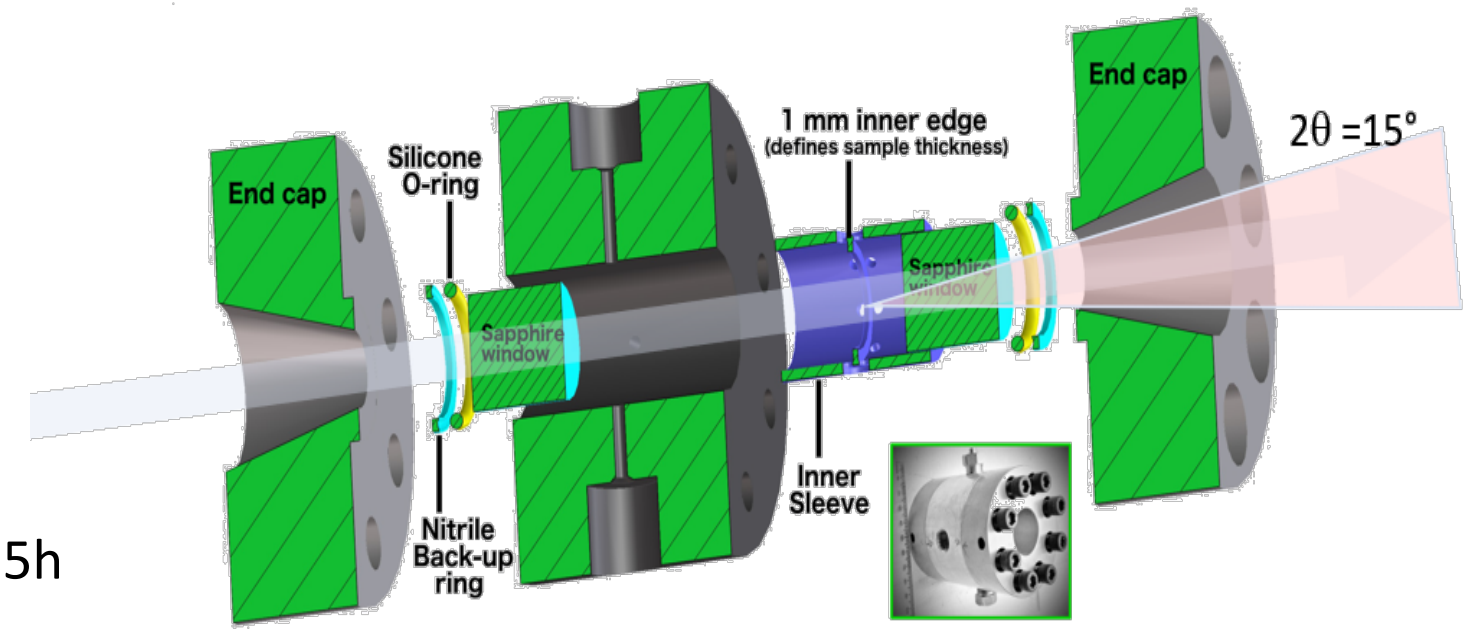
Pressure: ambient to 3.5 kbar

Temperature: -20°C to +65°C

Temperature equilibration: 10 min to 2.5h

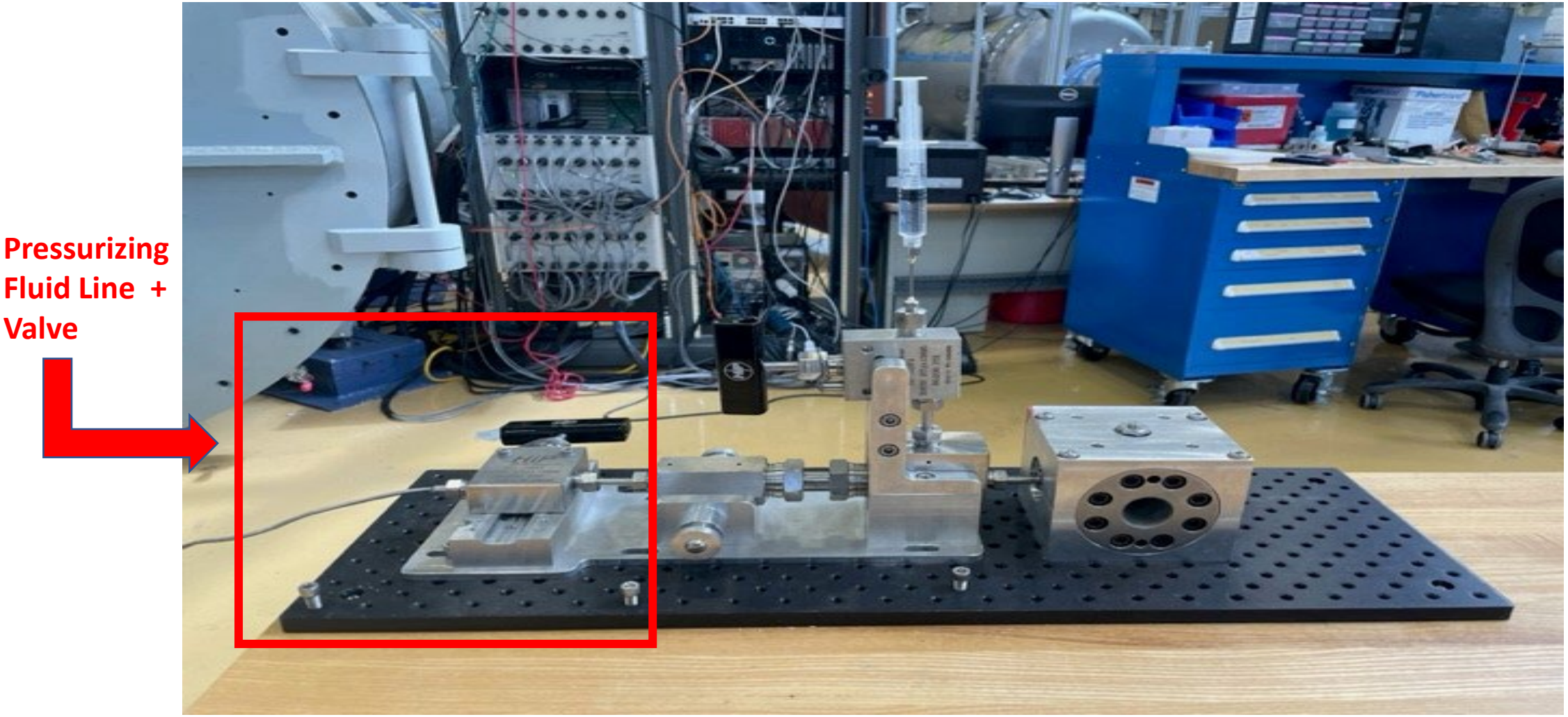
d-spacing: 20 - 6000 Å (0.001 - 0.3 $Å^{-1}$)

Concentration: > 5 mg/mL



Teixeira *et al.* (2018). *J. Neutron Res.* **20**(1), 13.
Teixeira S. (2019). *Cur. Op. Colloid & Interf. Science* **42**, 99.

LIPSS : Liquid Insertion Pressure System for SANS Studies



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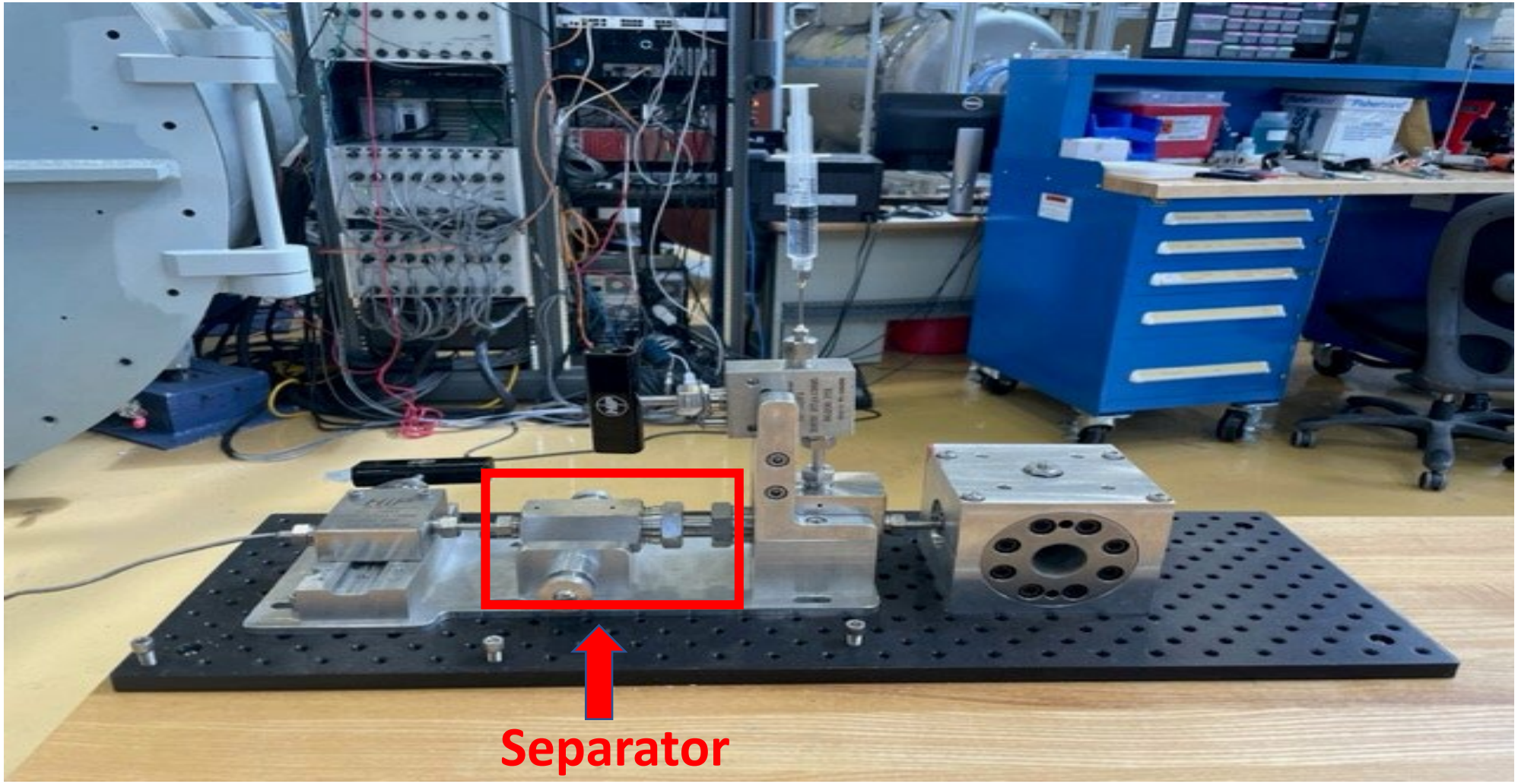

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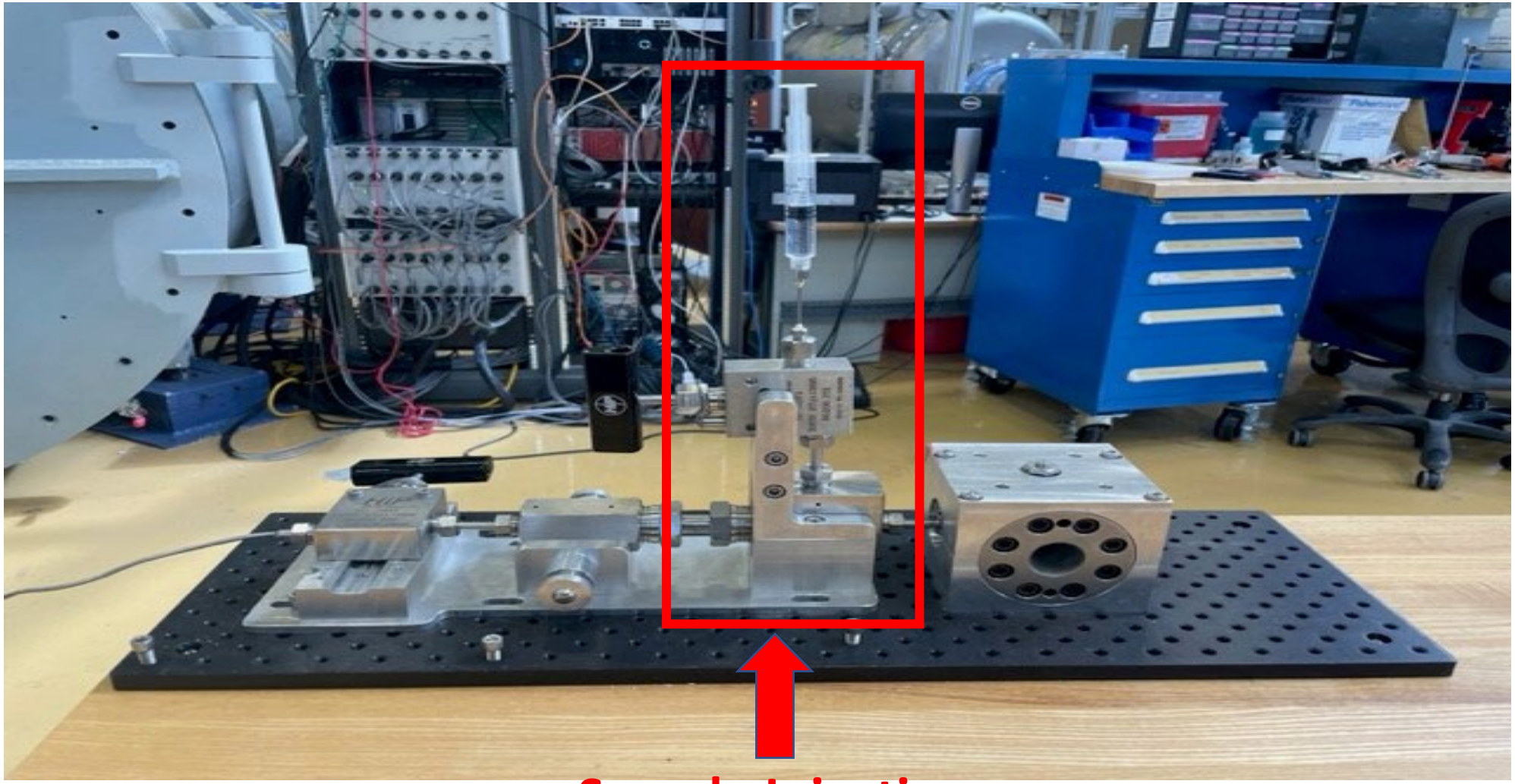
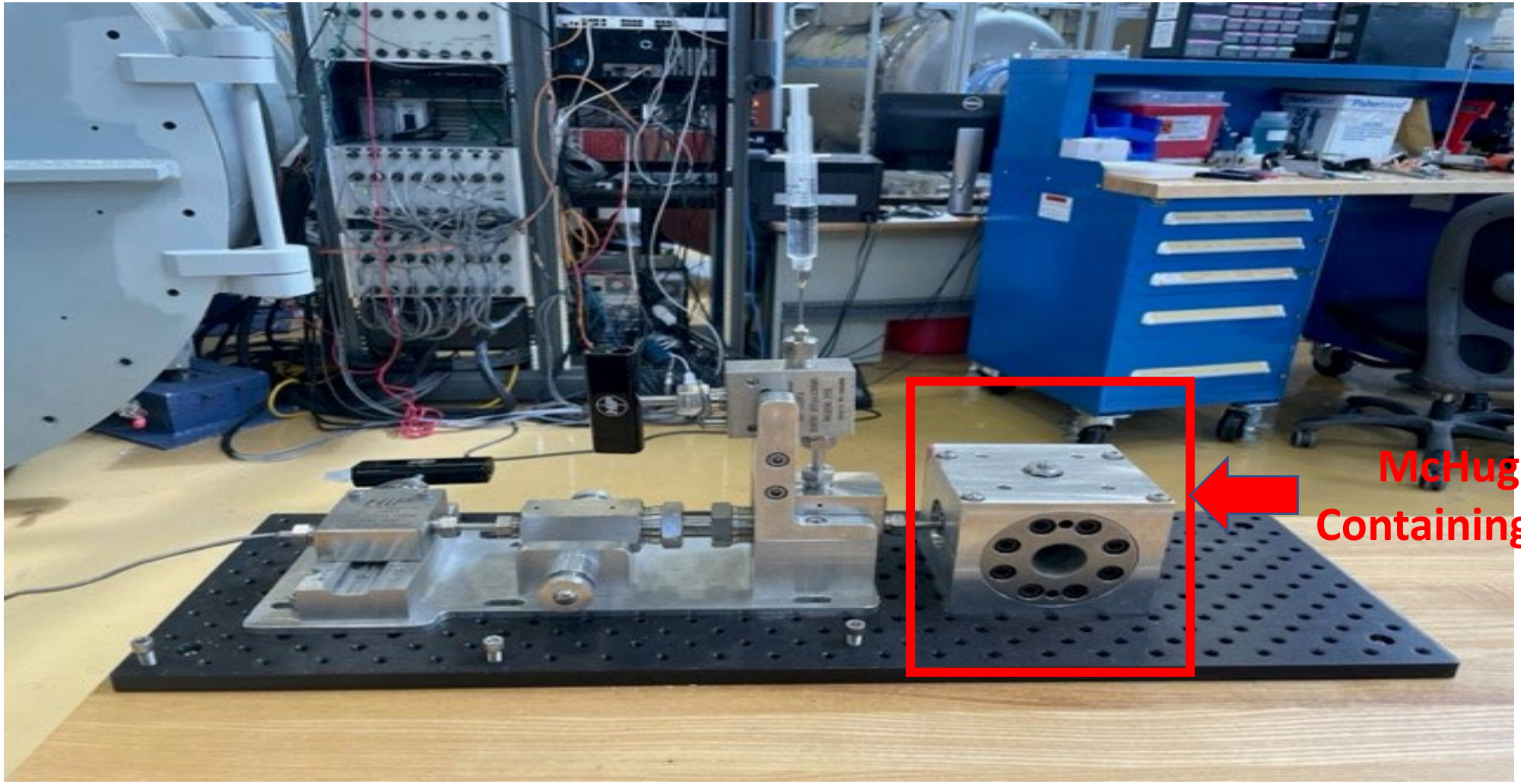


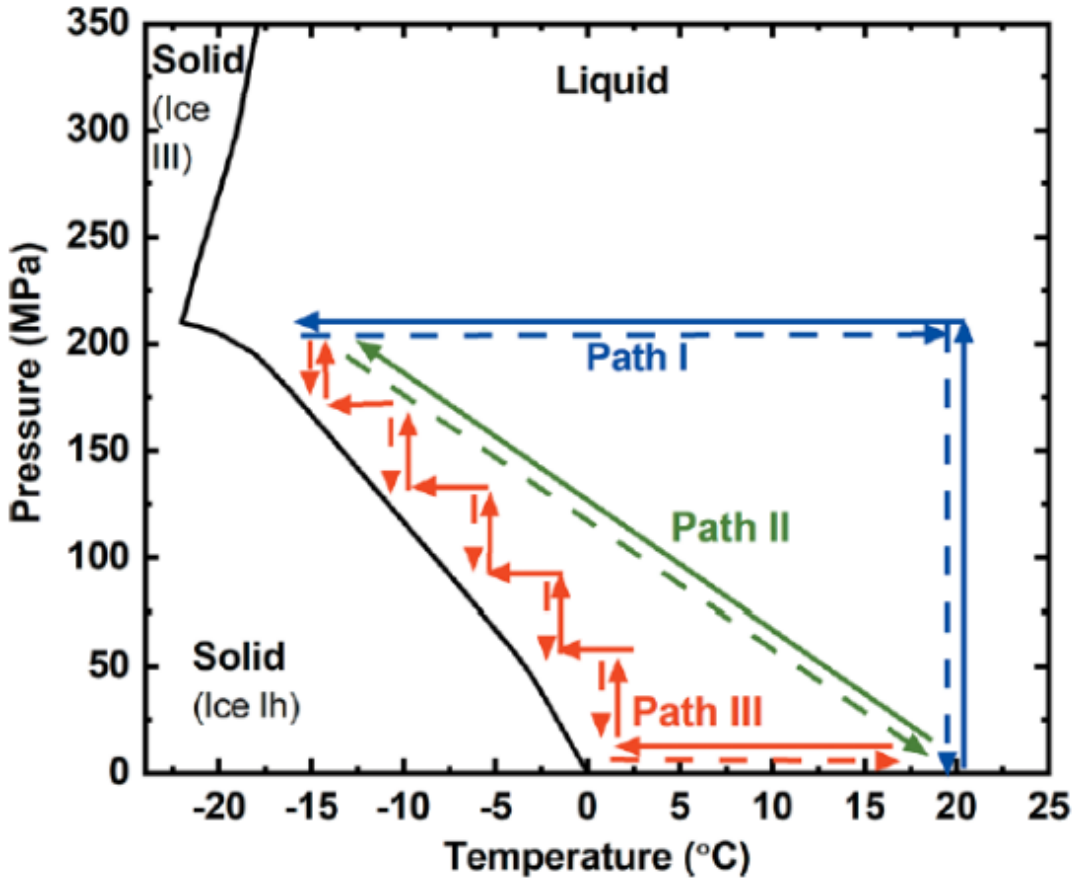
Sample Injection Valve and Block

LIPSS : Liquid Insertion Pressure System for SANS Studies



LIPSS : Liquid Insertion Pressure System for SANS Studies

A typical HP-SANS experiment - how it works



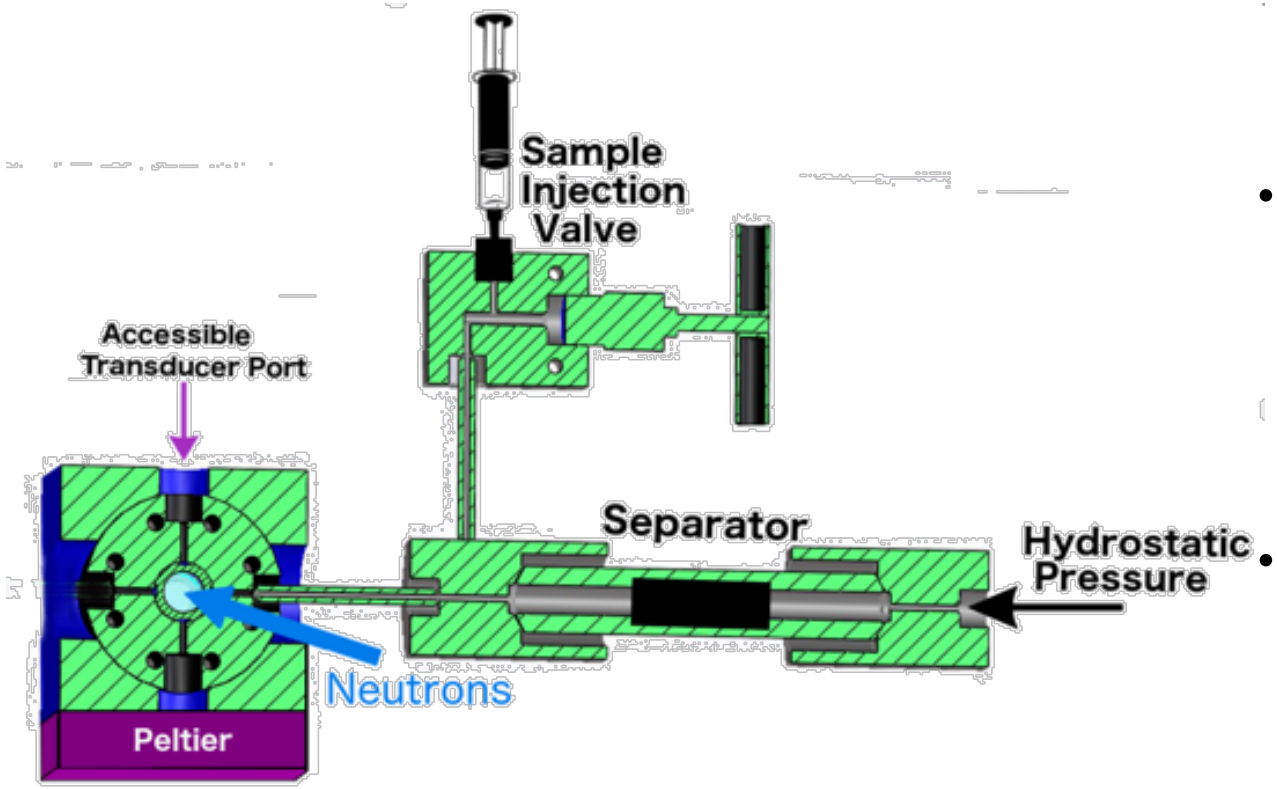
Aggregation of Monoclonal Antibodies: Role of the Fc and Fab Fragments. Berger *et al.* (2022). *J. Phys. Chem. B*, **126** (24), 4431.

Blue path (forced degradation studies):

1. Collect data at ambient pressure and temperature	~2hr
2. Pressurize to target pressure, keep T constant. Equilibrate to avoid heterogenous samples. Collect data.	2min 10-30min ~2hr
3. Lower the temperature to -20C. Equilibrate and collect data.	~2hr 10-30min+2hr
4. Warm up at constant P. Then depressurize. Collect data to monitor reversibility.	10min 10-30min ~2hr

LIPSS : Liquid Insertion Pressure System for SANS Studies

in-situ studies of P|T effects for Biomolecules – **what can be improved**

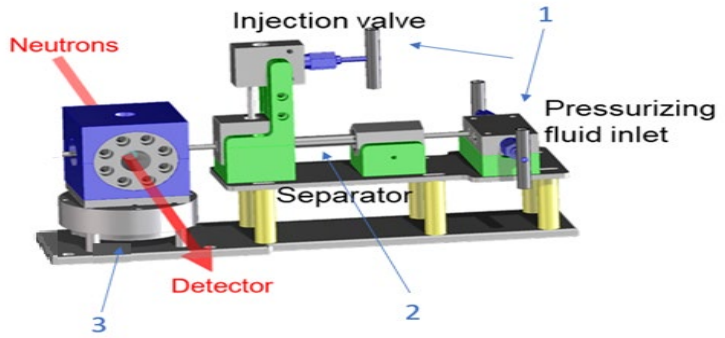


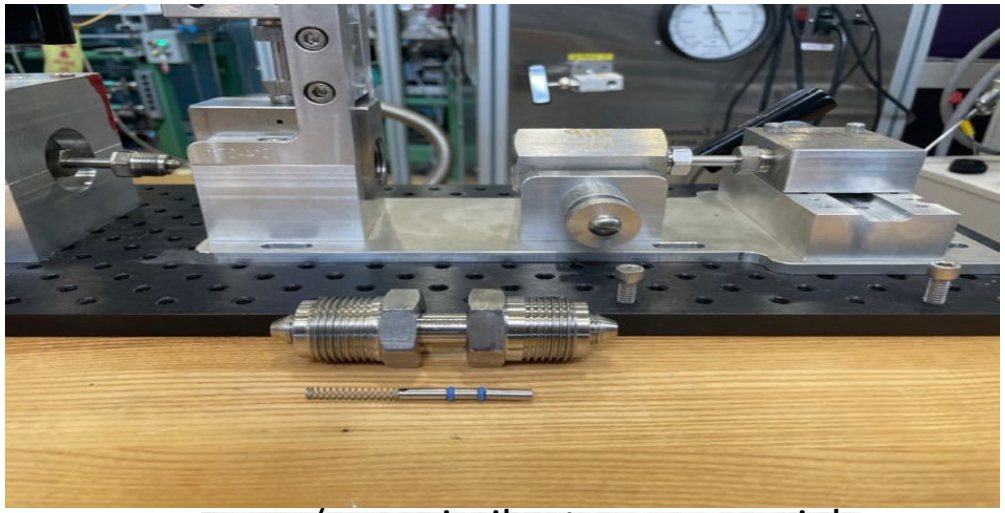
- Reliability of operation: leaks are common, particularly at low temperatures. Continued pressurization while there are leaks can damage the separator.
- Assembly is difficult and time consuming. Sample changes require priming (vacuum to ensure injections fill the entire volume of the sample side of the assembly), this is a slow step (up to 45min)
- Lost time waiting for radioactive decay of one of the two McHugh cells available (3h)
- Temperature equilibration is very slow at subzero temperatures – Not addressed during RET project

What's new with HP-SANS?

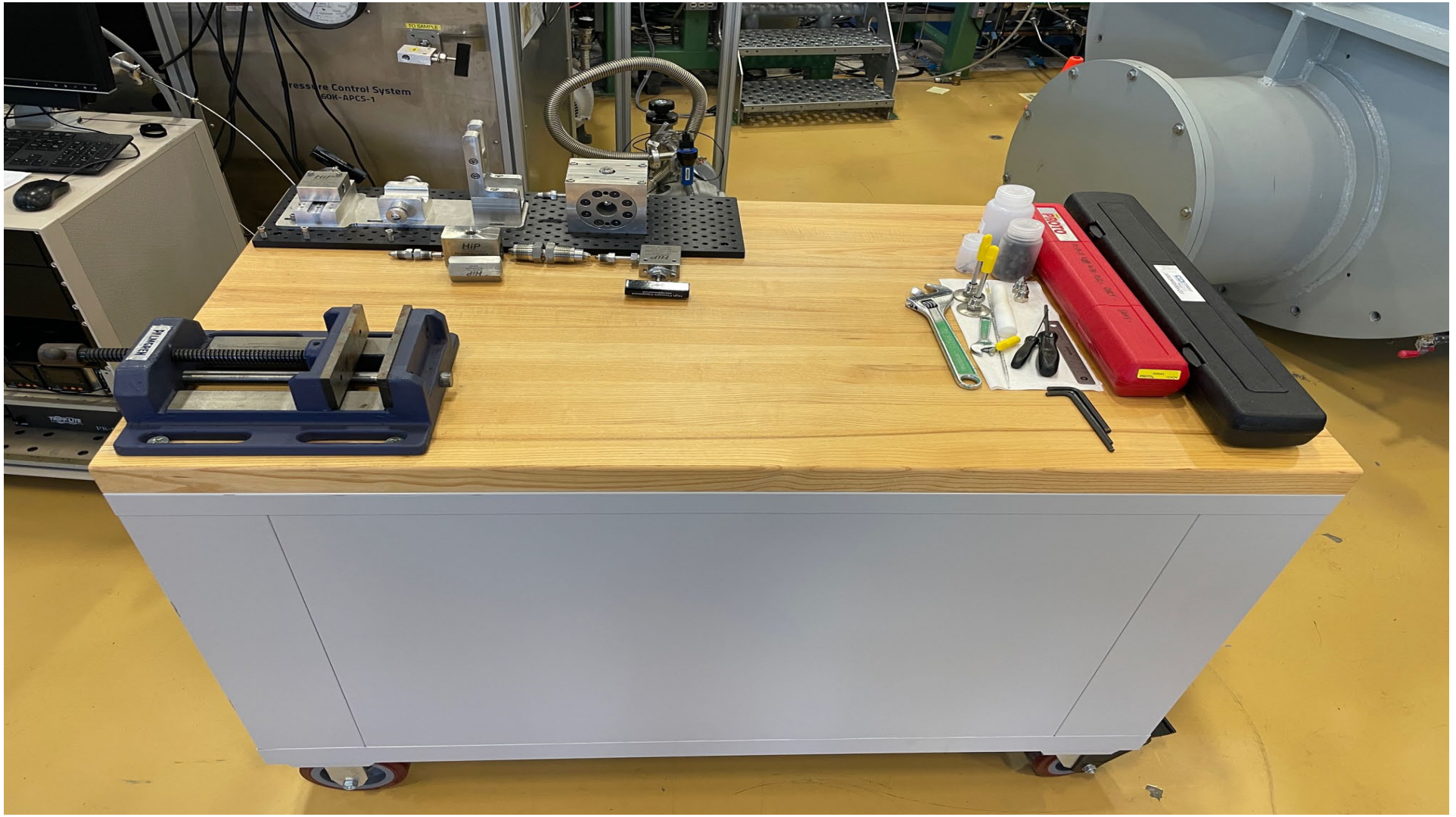
NEW: **"The LIPSS Cart" – New Platform and Assembly Procedure**

1. **Plug and Play Valves**
   - **Loaded** Pressure cell can be placed into sample chamber.
   - Minimizes mistakes and risk of damage to materials.
2. **New separator** (previous version was very difficult to assemble without damaging the o-rings → Note about Manual/Tutorial)
3. **New McHugh cell**
   - 3 assemblies ready
   - Cells activate. ~3hr downtime
4. **New chiller baths** to cool the Peltier system





The LIPSS Cart – “They See Me Rolling…”



What's old with HP-SANS

OLD

1. **McHugh cells** are ~ 30 years old. They are starting to leak at lower pressures and are not meeting the demands of lower temperature operations (materials contract) → Test new O-rings and sapphire window performance.

2. **The automated PMI system** is showing signs of degradation (it is 4 years old). We measure pressure losses even when it is not connected to LIPSS. → Investigate the origin of the problem. Characterize the inherent pressure drop of the PMI to determine:
   - if there is a 'normal' pressure drop that we can assume is not caused by leaks
   - if the system needs servicing

My work with LIPSS

McHugh Cell O-ring Tests:

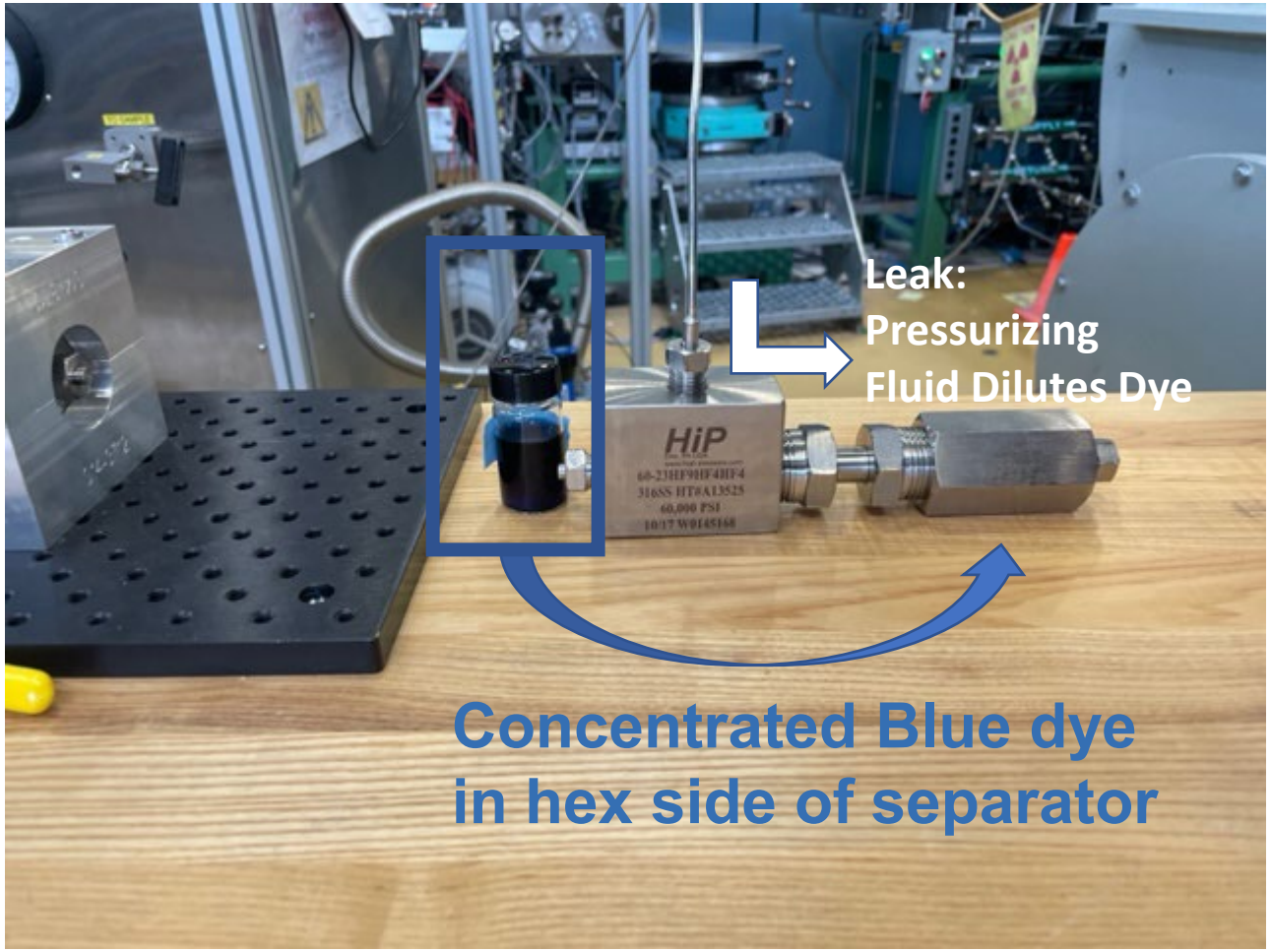
- Tested several different combinations of primary and back-up O-rings.
- Identified a no-leak candidate.
  - Higher Durometer Primary o-ring
- Need more tests. PMI currently down.

My work with LIPSS

Separator O-ring Tests:

- Want to test if the separator is leaking

- Identify leaks using NanoDrop UV-Vis Spectrometer
  - Confirmed nanodrop can detect a 20 µL drop in a volume of 1mL
  - Tests interrupted by torquing issue



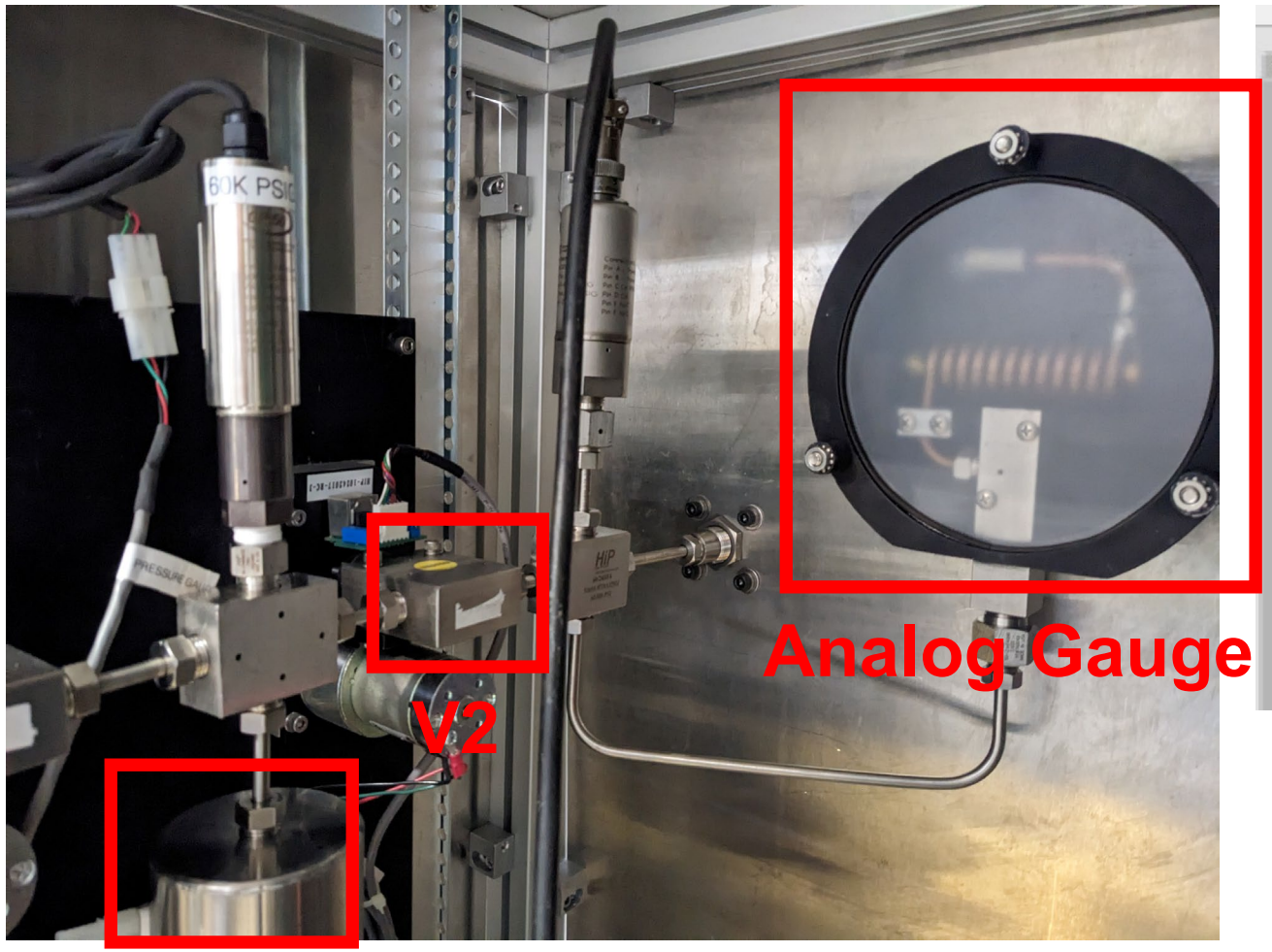
Manual Pressurizing System

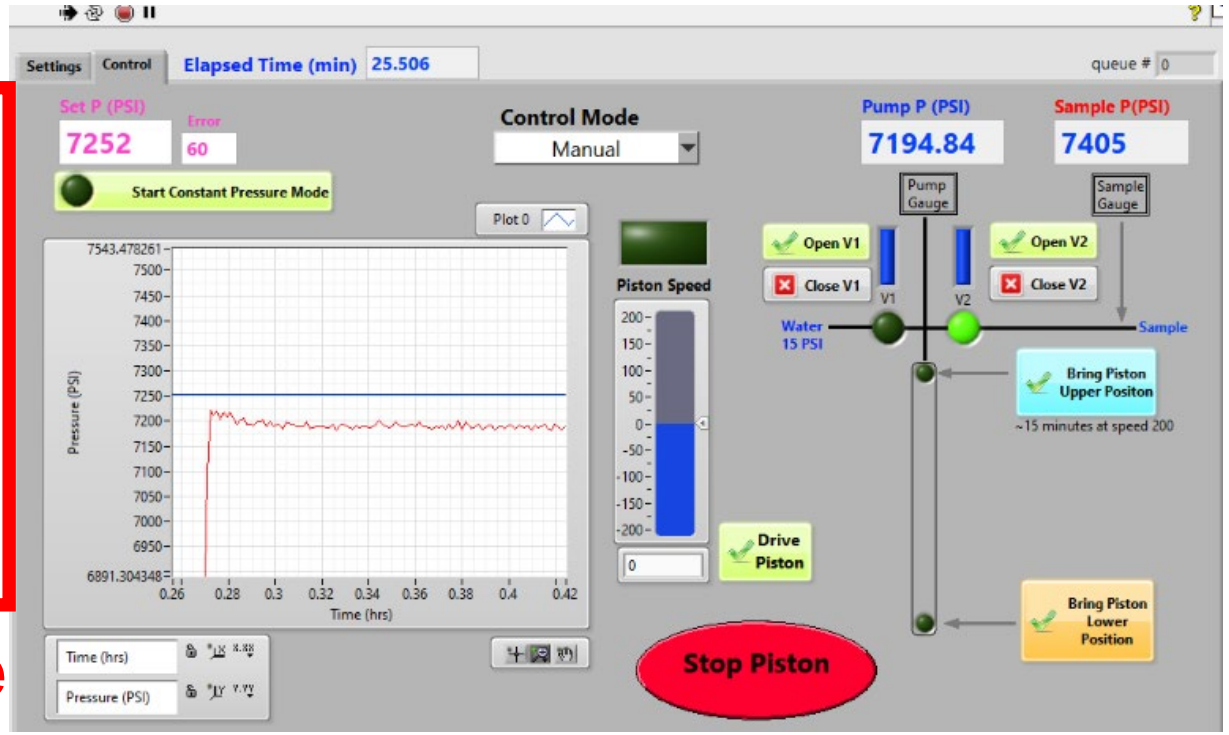
- Previously existed but was to be replaced by automated PMI system.
- Now want as a backup and for benchtop measurements (Diffuse Wave Spectroscopy, Incubations, Fluorometry)
- Ordered necessary parts. When assembled will go on the LIPSS cart.

Automated Pressurizing System – PMI

- For HP SANs need to maintain pressure for several hours.
- Previous behavior was stable. System has aged…
- Pressure reading drifts over time even when isolated (i.e. not connected to LIPSS)

PMI – Under the Hood

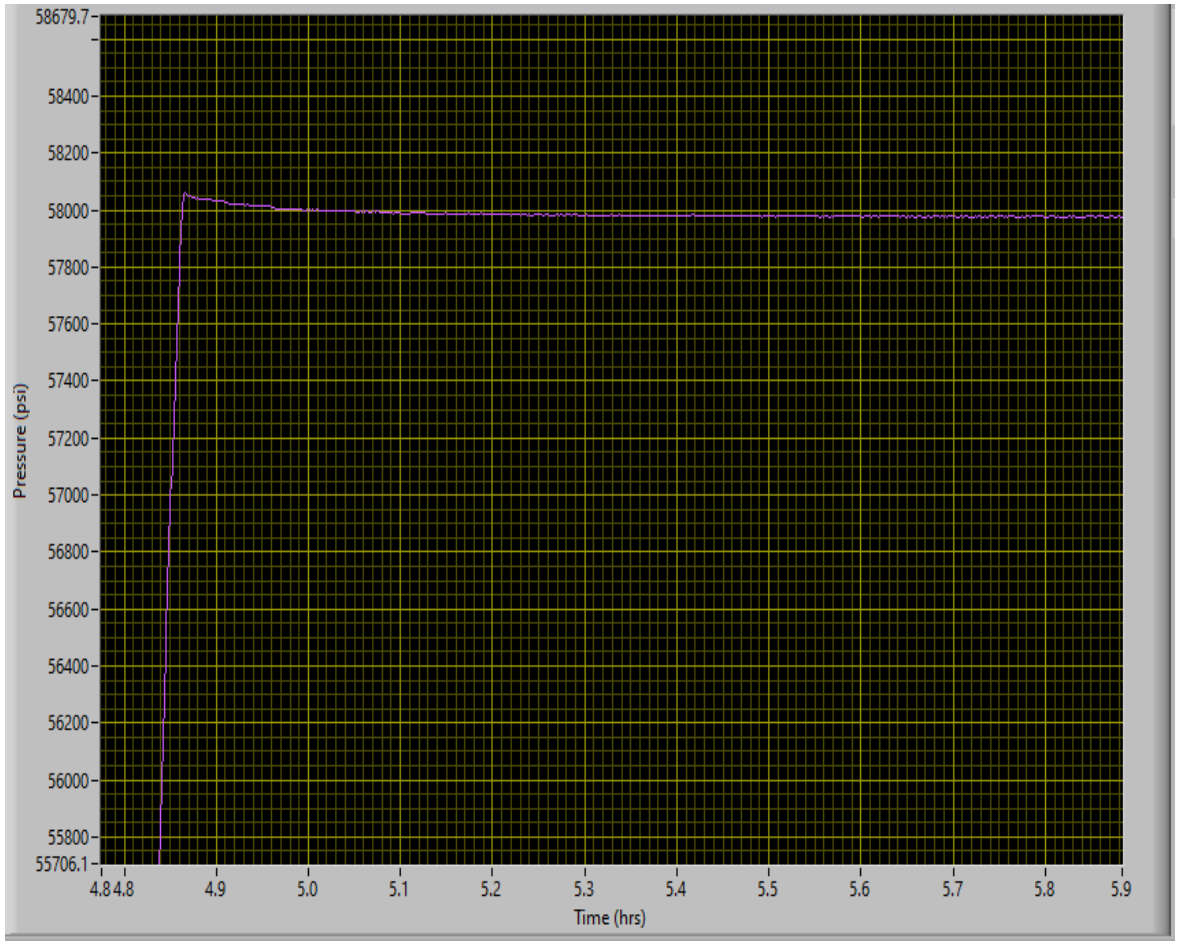




Labview GUI

Automated Pressurizing System – PMI

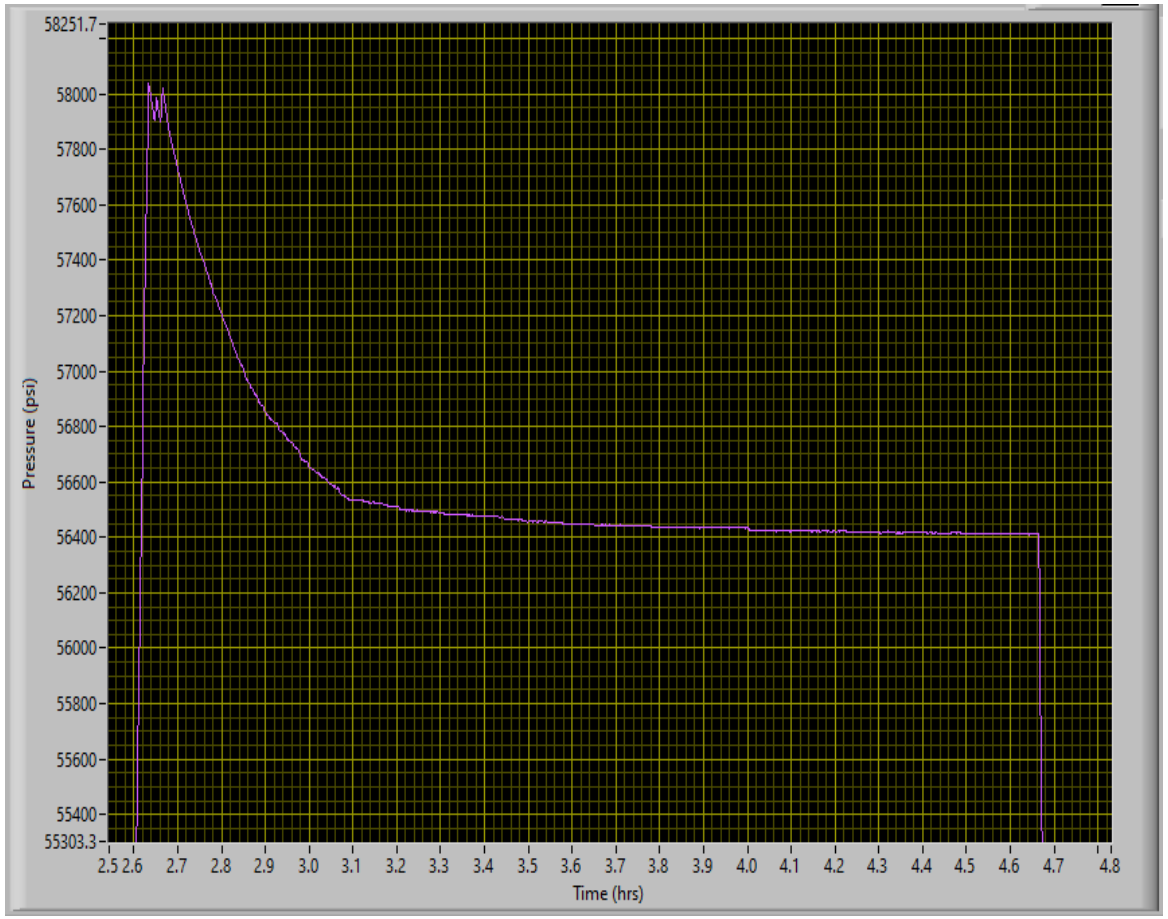
What we want:



- Minimal drift at pressures up to 4kbar (58000 psi)
- This was achievable in the past

Characterizing PMI Drift

What we see now:



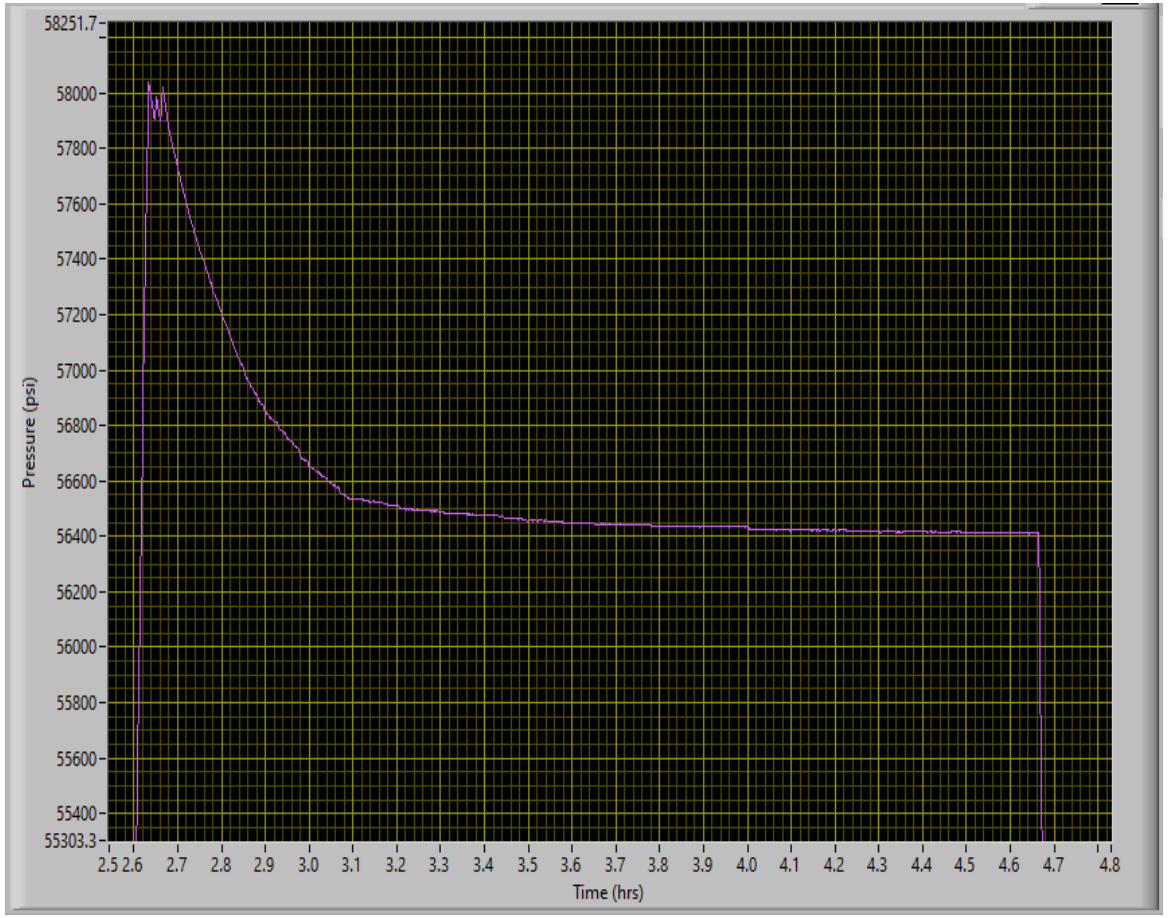
- Pronounced drift followed up by equilibration

- Put yourself in shoes of a user:
  - Starting to pressurize… Is there a leak or is the system just drifting?

- Goal: Develop procedure for user to determine if system is <u>leaking</u>
  - ~30min ideal. These experiments are long, need to optimize for time.

Characterizing PMI Drift

What we see now:

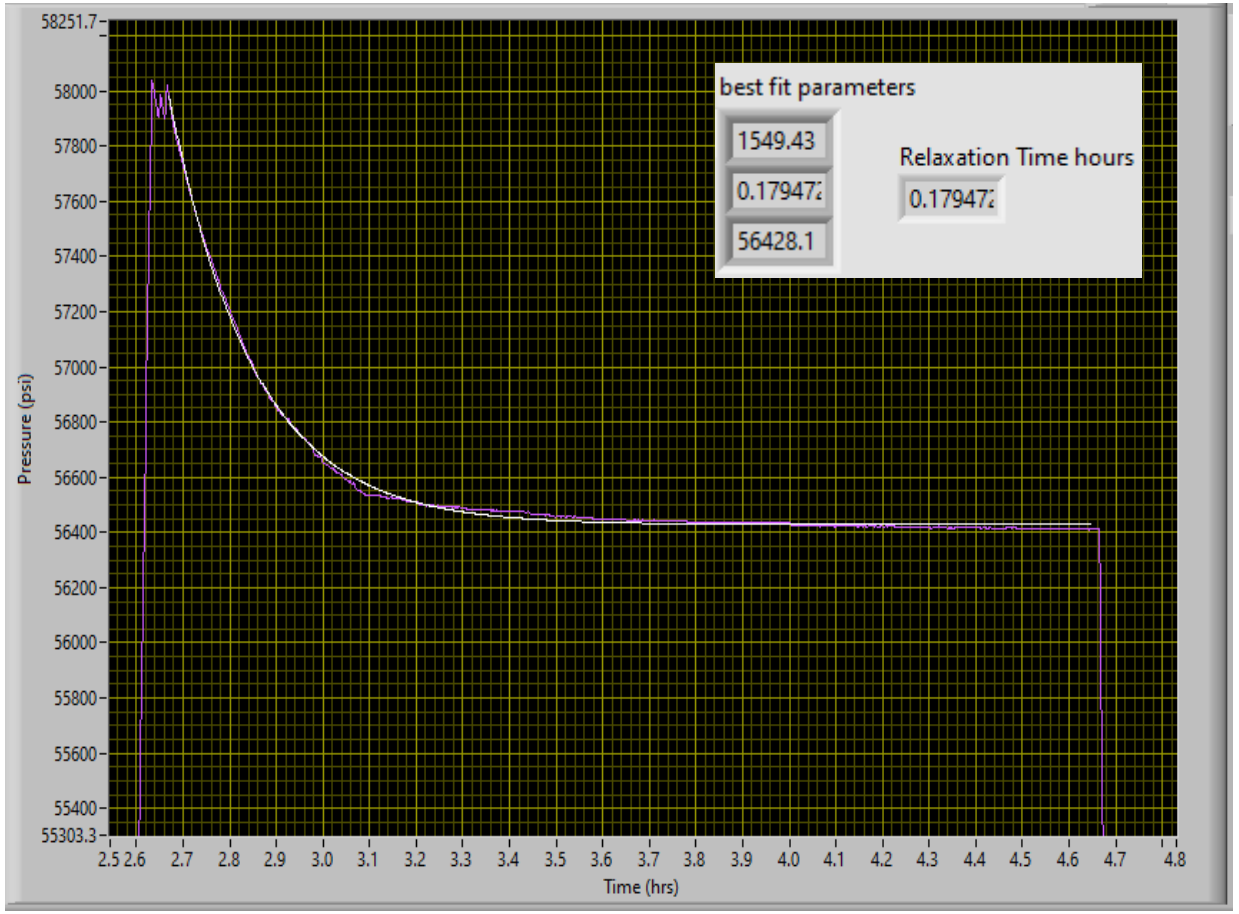


Considered the effects of 3 different pressurizing fluids

1. Regular DI Water
2. In-House Degassed Water
3. Commercially Degassed Water

Characterizing PMI Drift

What we see now:



Collected data at 2 different pressure thresholds and held to examine drift

Used fitting program developed by Alan Ye to fit decaying exponential curves to the drift.

Obtain a time constant τ for the relaxation time.

Characterizing PMI Drift Results

Table 1: PMI Drift Characterization: Commerical Degassed Water

36 kpsi		58 kpsi	
Linear Slope (psi/hr)	Time Constant (hr)	Linear Slope (psi/hr)	Time Constant (hr)
603.2	N/A	N/A	N/A
N/A	N/A	21958*	0.412
3758	0.152	836	0.66
528	0.147	2994	0.179
3894	0.248	658	0.209
4864	0.142	N/A	N/A
8310	0.199	3171	0.227
6354	0.294	1596	0.364
11634	0.242	946	1.56
7182	0.272	160	0.127

Each run takes about 3-4 hours

These data are all with commercially degassed water.

There may be a reuptake of air as time goes on.

Characterizing PMI Drift Results

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More data needed for in-house degassed water and regular DI.

Most importantly need to introduce a known leak, collect data, and compare to natural drift of system.

Hope to be able to quickly distinguish between leak vs drift using time constant.

Testing can continue when V1 is repaired/replaced.

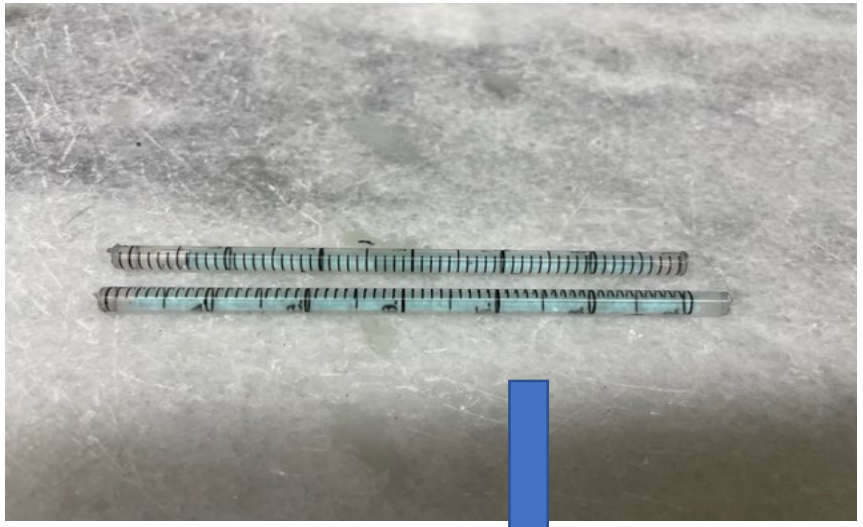
Development of a Novel Pre-Incubation Vessel

Would be ideal to be able to pre-pressurize a sample to cut down on necessary beam time and use complementary techniques (e.g. SAXS)

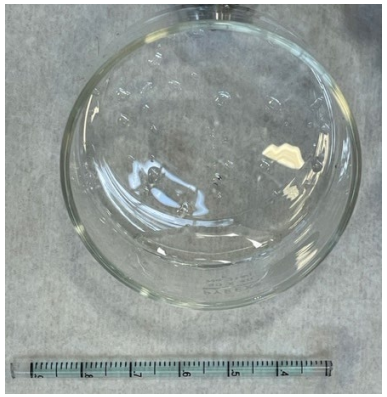
Preliminary data (shown later) indicates this may be feasible.

Used pipettes and vacuum grease with an empty separator tube to incubate protein sample.

We incubated Bovine Serum Albumin (BSA).

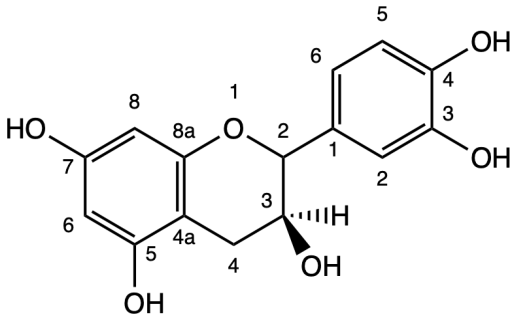


Vessel does not leak.

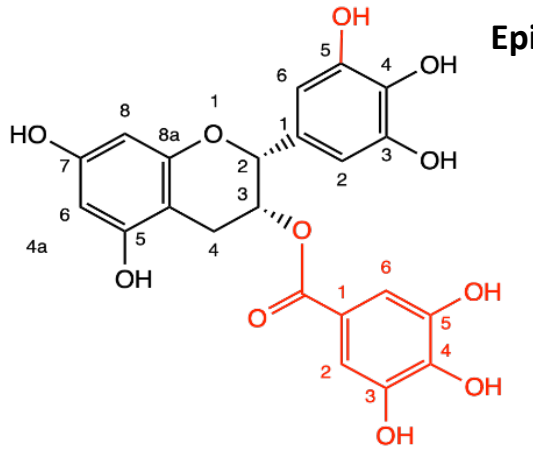


Small Angle Scattering studies of BSA/Polyphenols

- EGCG: principal green tea polyphenol (≈270 mg/L)
- Potential food preservatives
- Therapeutic effect limited by poor stability under physiologic conditions

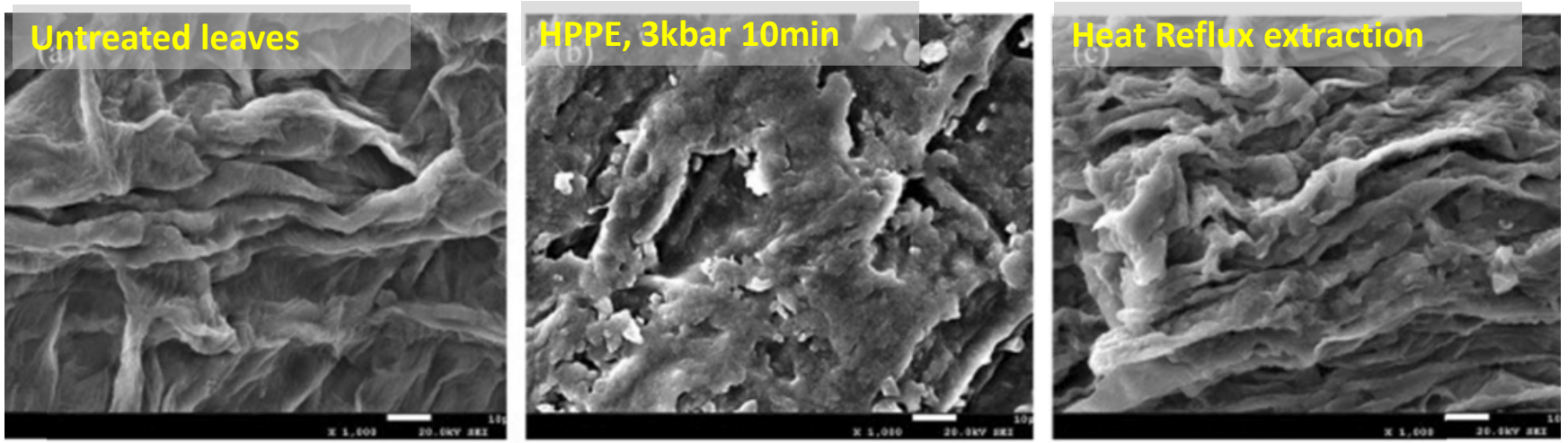


Catechin



Epigallocatechin Galate (EGCG)

SEM images of green tea leaves

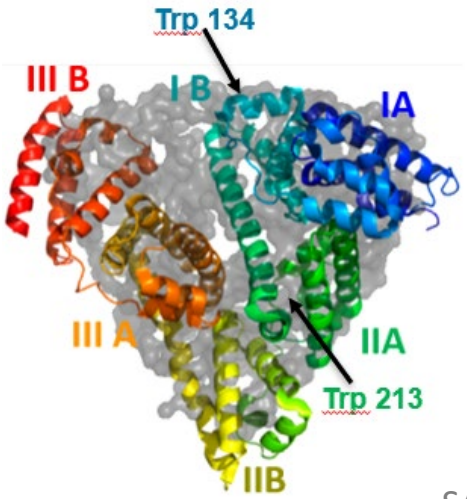


Adapted from Scepankova *et al.* (2018). *Plant Foods for Human Nutrition* **73**: 253.

Health benefits: Ohishi *et al.* (2017). *Molecules* ***26****(2): 453,* 656.

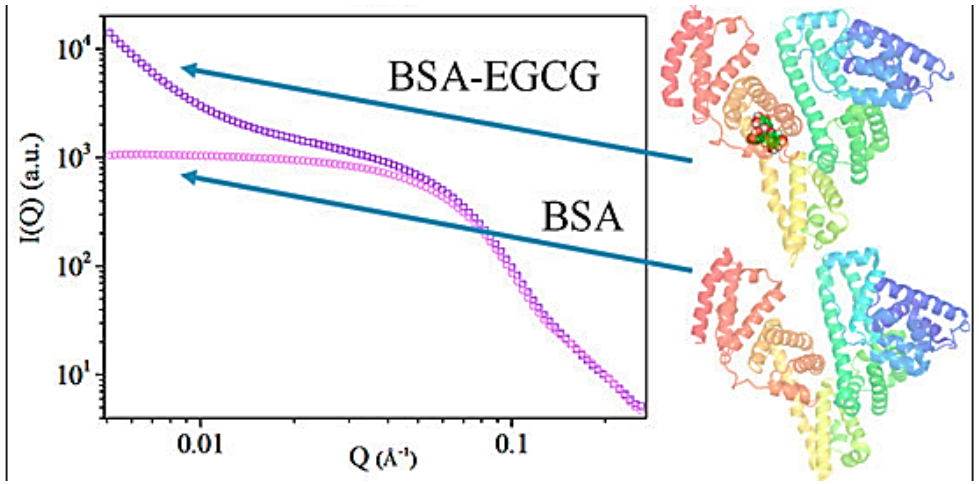
BSA: a model protein in food

- Main carrier protein in blood plasma
- Dietary protein found in beef and cow milk. Used as a food additive (emulsifying properties, enhanced after HPP processing)
- P > 100 MPa: secondary structure affected
- P > 300 MPa: protein unfolds under certain conditions (pH, salt, temperature)

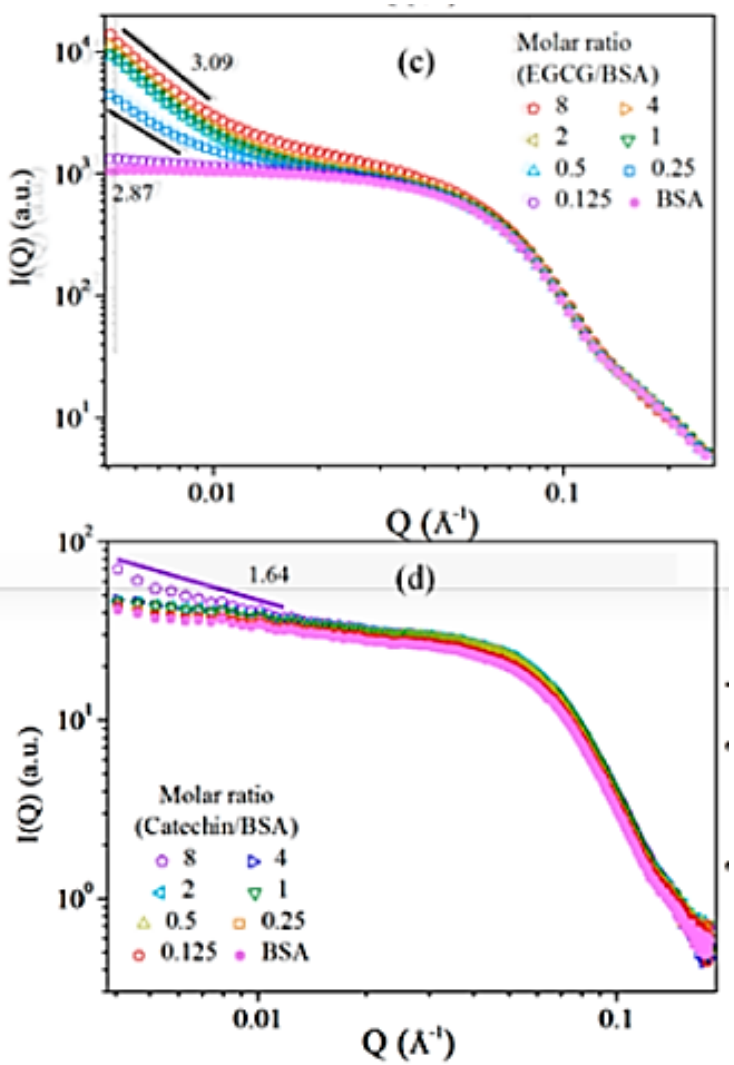


Physiological blood concentration: 40 mg/mL

SAXS

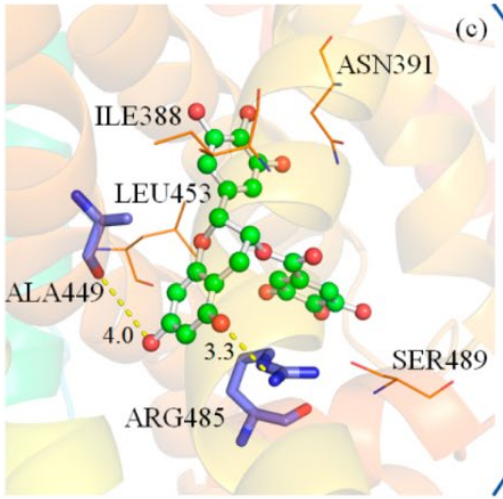


BSA image adapted from:
Tang *et al.* (2017). J. *Agric. Food Chem*. **65,** 656.

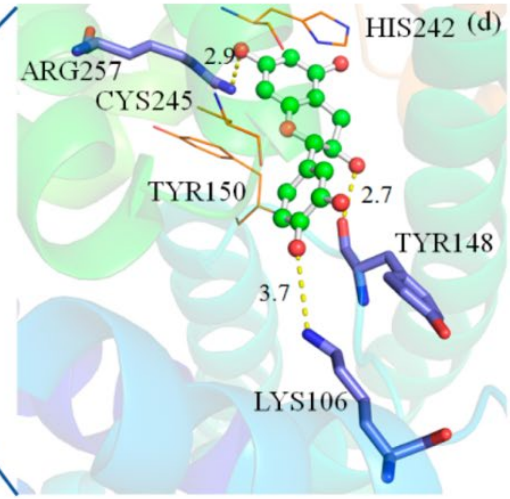


Stronger binding

EGCG



CATECHIN



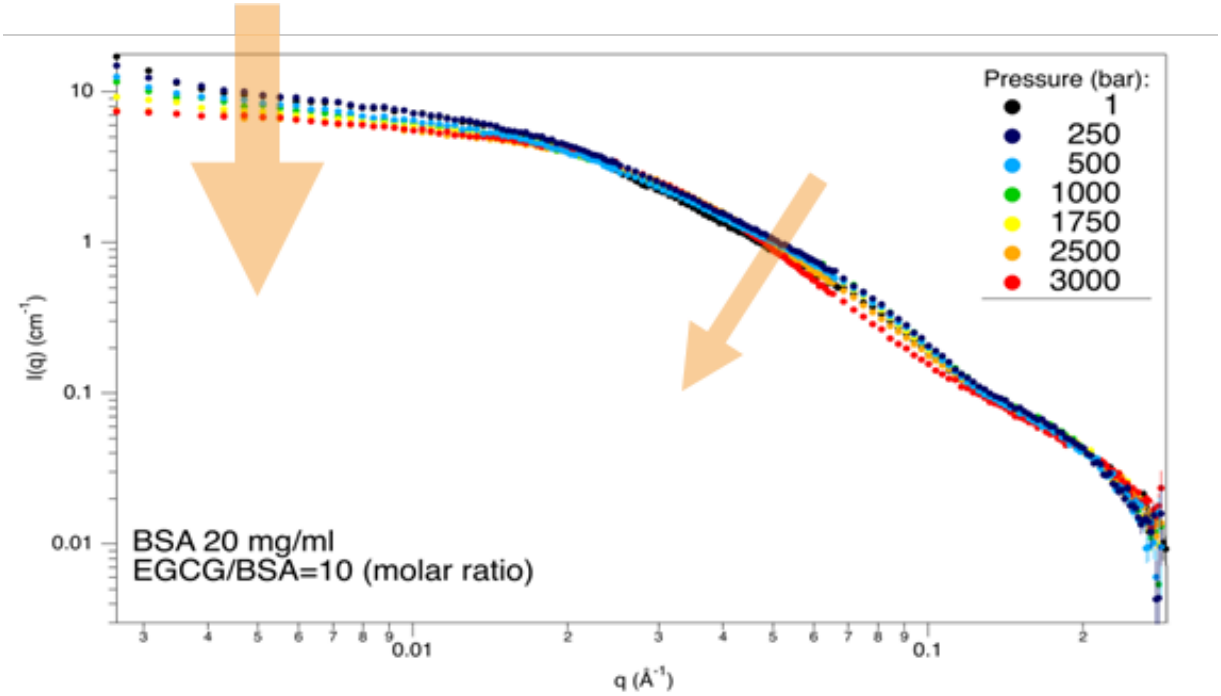
Hydrophobic (VDW + Nonpolar)	-25.11 kCal/mol (EGCG) -18.63 kCal/mol (Catechin)
Hydrogen bonding	7.49 (EGCG) 1.68 (Catechin)

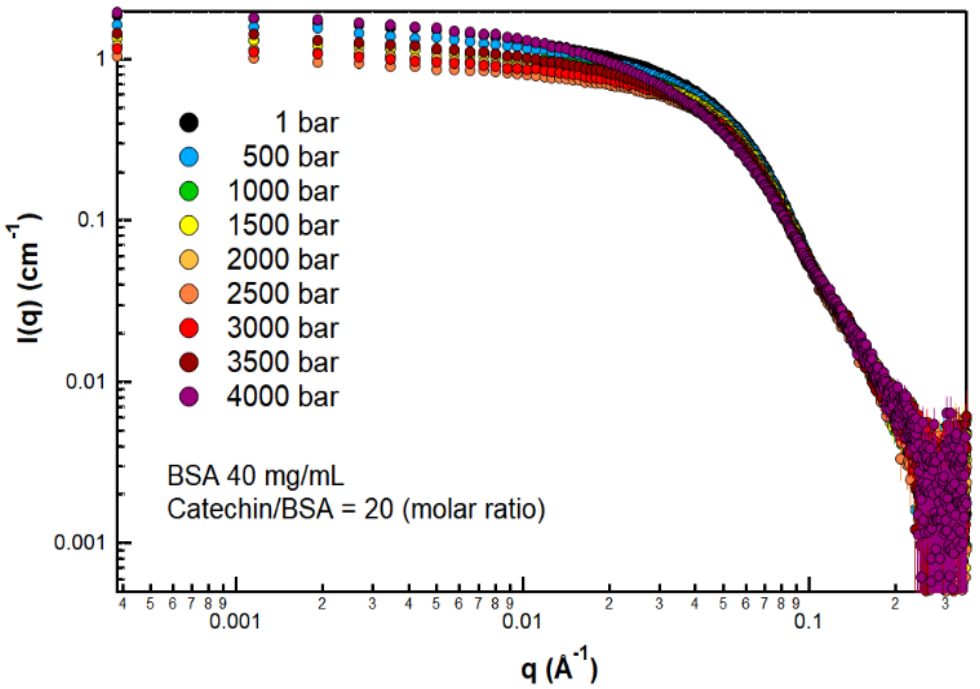
Adapted from:
Tang *et al.* (2017). J. *Agric. Food Chem*. **65,** 656.

DOI: 10.1021/acs.jafc.6b04630
J. Agric. Food Chem. 2017, 65, 656−665

Prior HP-SANS and HP-SAXS studies of complexes

HP-SANS scattering profile





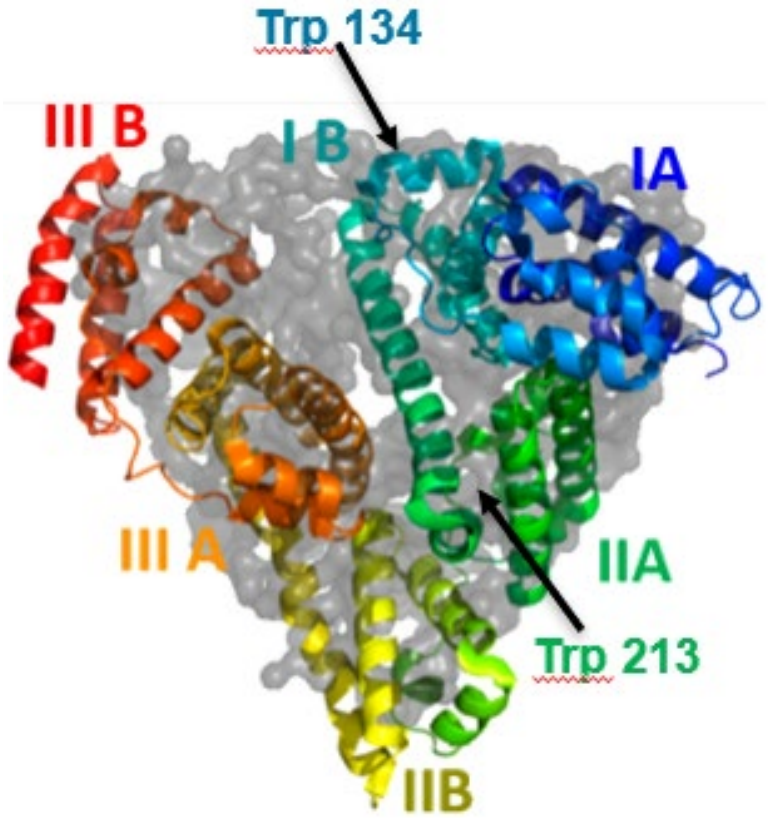
100 mM MES pH 6.5, 50 mM KCl

20 MPa/min ramp
20 min equilibration

Prior Spectroscopic data

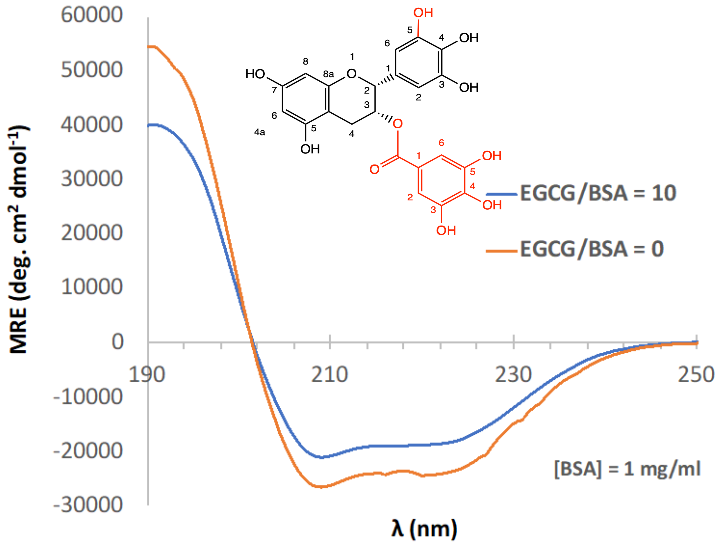
Intrinsic HP- Fluorescence

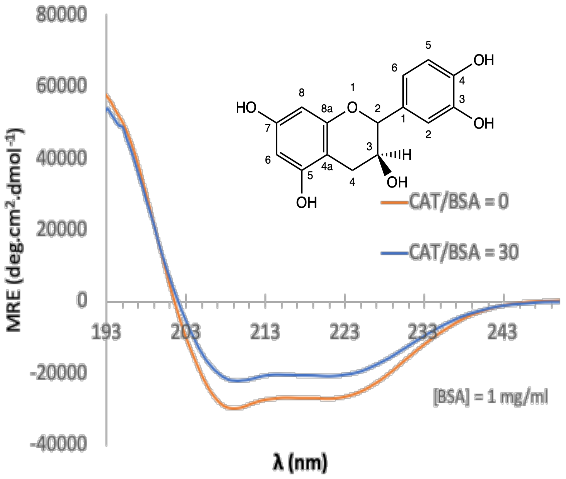
- Cathecin/BSA complex is not very sensitive to pressure. Minor shifts in fluorescence, reversible (local chemical environment of the Tryptophans)
- EGCG/BSA complex partially unfolds under pressure. Effect is irreversible.

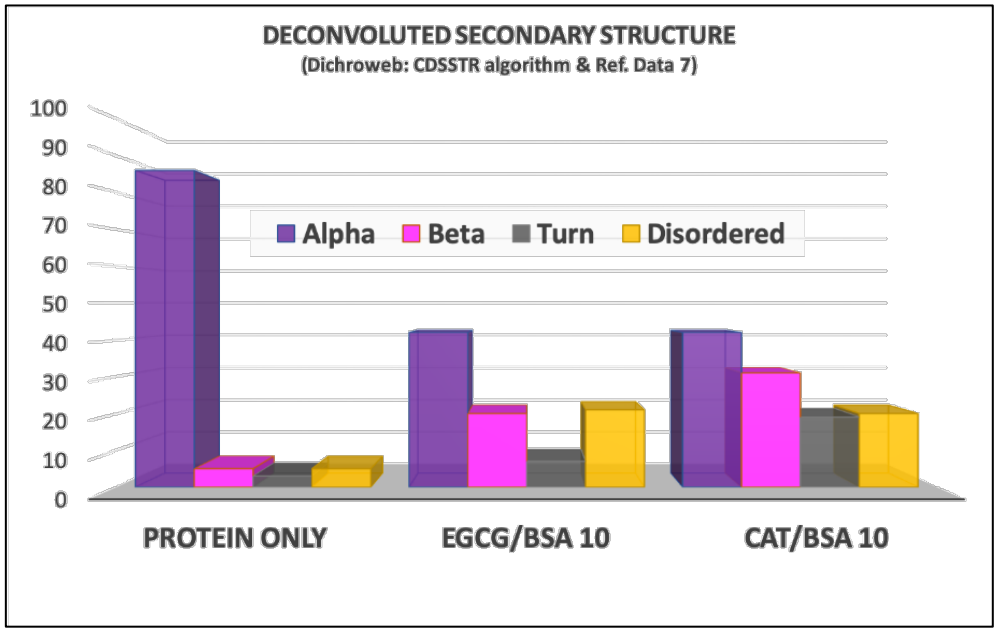


HP-Fluorescence data collected at the University of Delaware

Prior Spectroscopic data – Far UV Circular dichroism



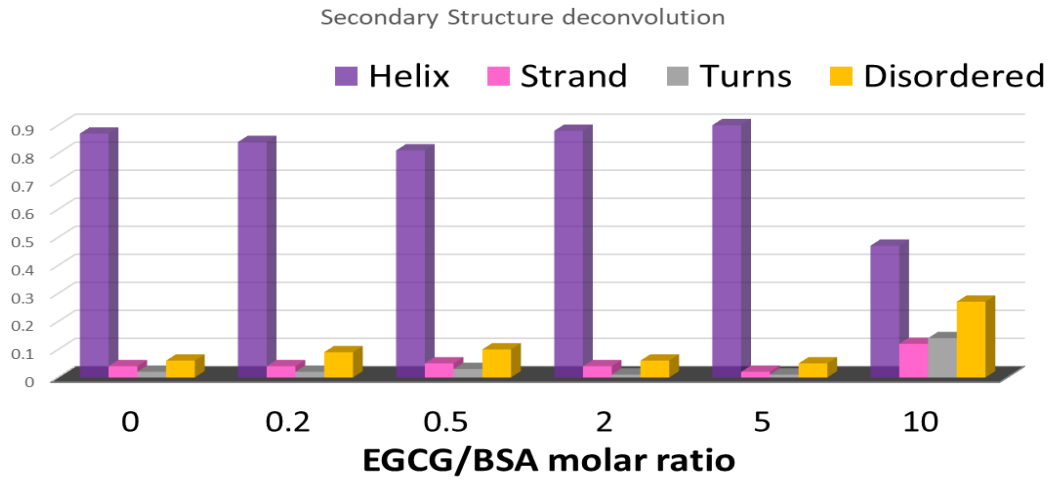


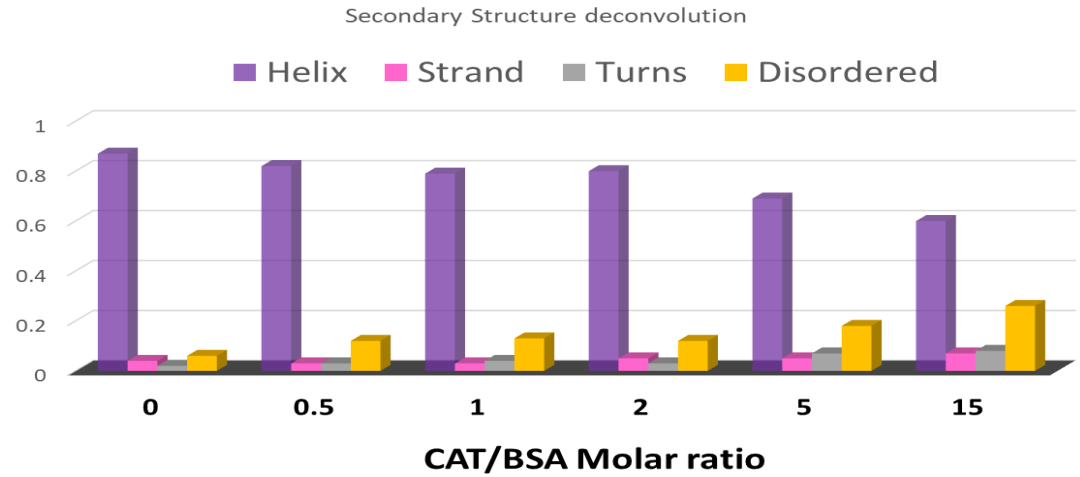


S. Teixeira

CD Deconvolution using Dichroweb
[Miles et al. (2021). *Protein Science*: https://doi.org/10.1002/pro.4153] and
Reference Data #7 [Sreerama et al. (2000). Analytical Biochem.287,252-260]

Circular Dichroism Far UV Data (D2O solutions, [Protein]=2.5 mg/mL)





100 mM MES pD 6.5, 50 mM KCl

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[Miles et al. (2021). *Protein Science*: https://doi.org/10.1002/pro.4153] and
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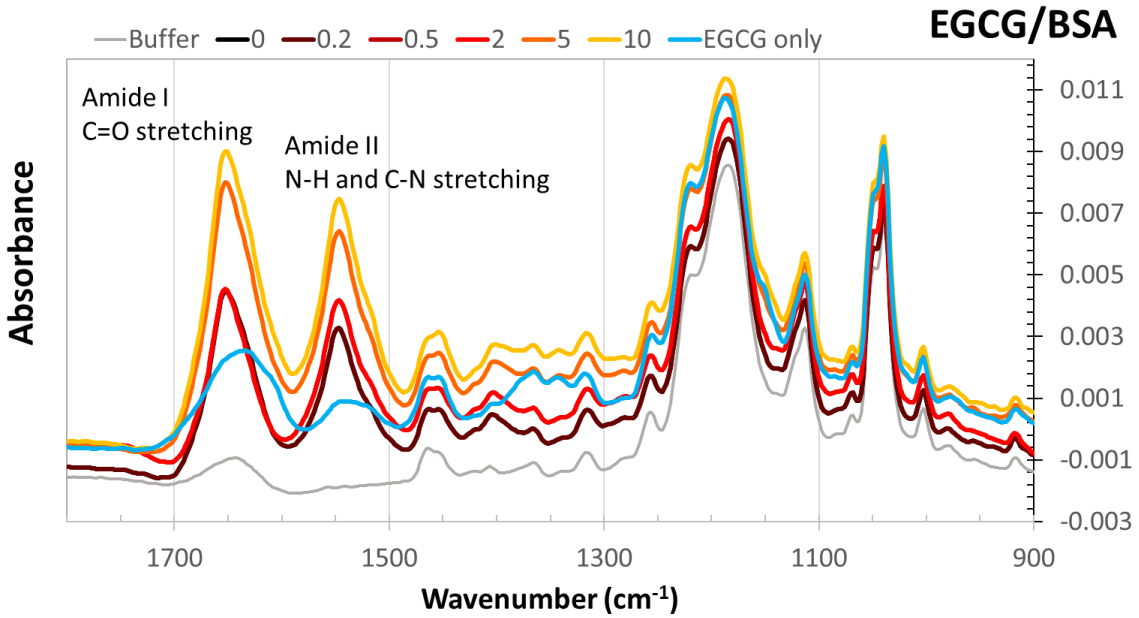
FTIR Data (D2O solutions, [Protein]=10 mg/mL) – Collected at 18 angles (1 angle shown here)

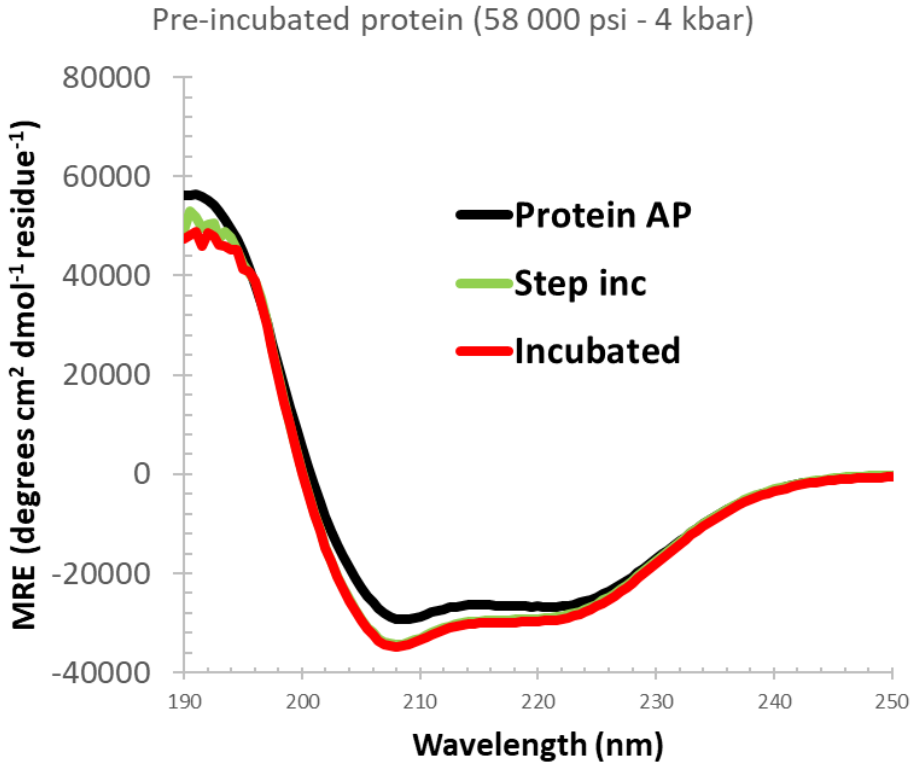


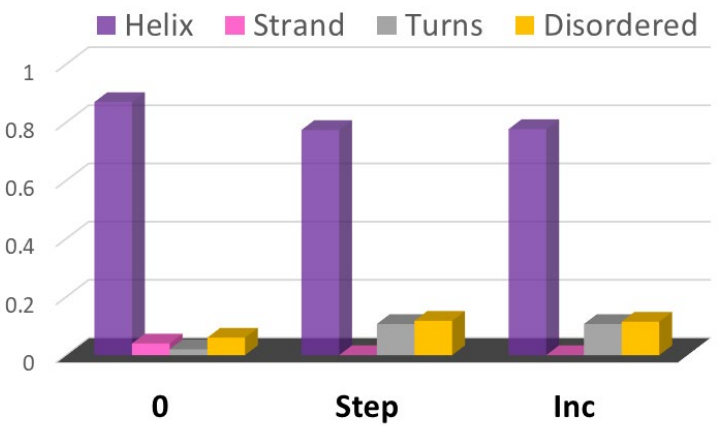
10 mg/mL solutions
100 mM MES pH 6.5
50 mM KCl

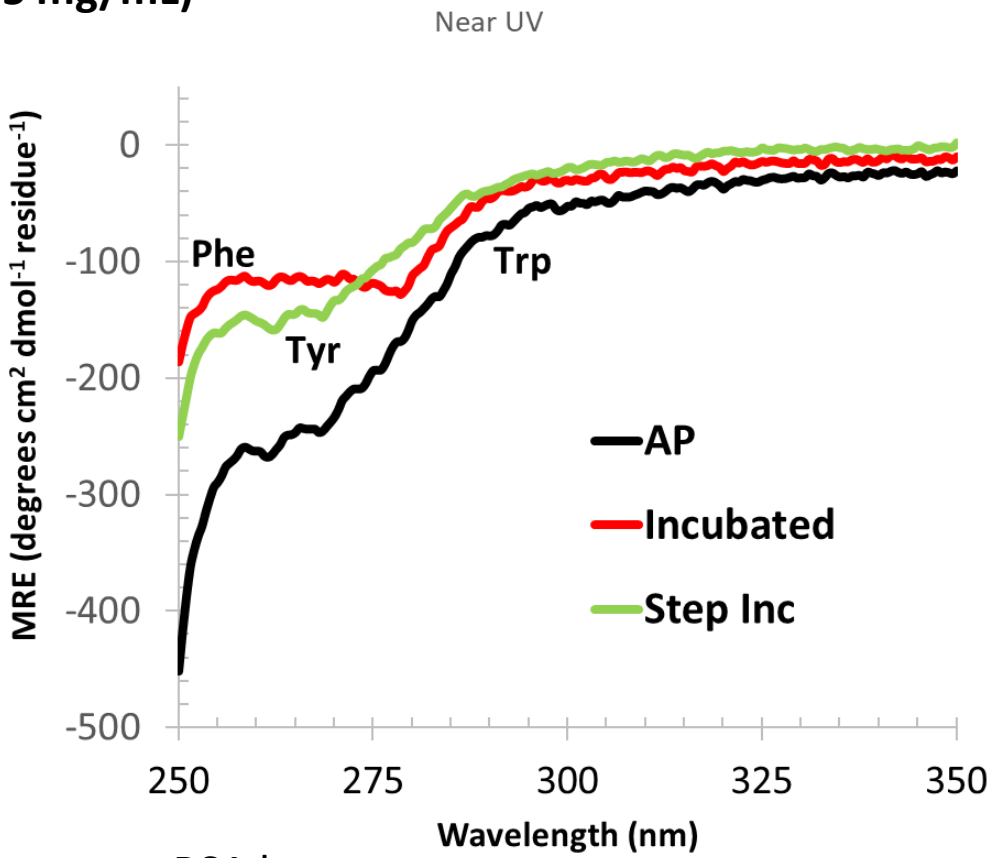
Ongoing analysis

Collaboration with Curtis Meuse (IBBR)

Circular Dichroism Far UV Data (D2O solutions, [Protein]=2.5 mg/mL)







BSA has

- 2 Trp residues
- 20 Tyr, 27 Phe
- 7 intrachain disulfide bonds with one free thiol group at residue 34

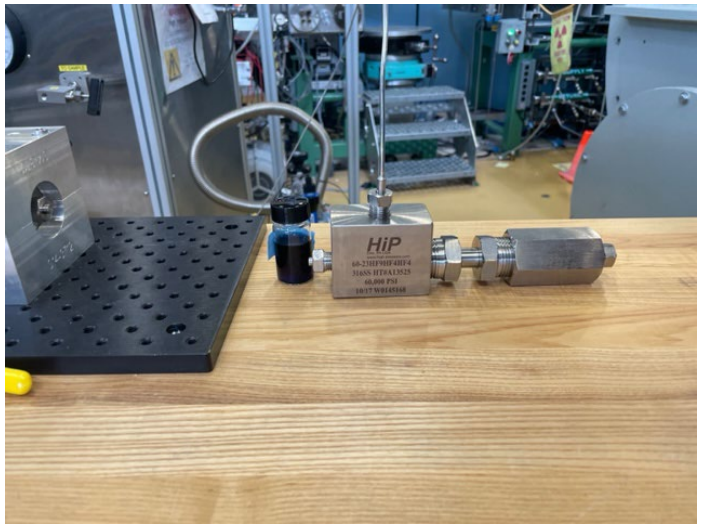
- Can see from Near UV difference in tertiary structure for different incubation procedures

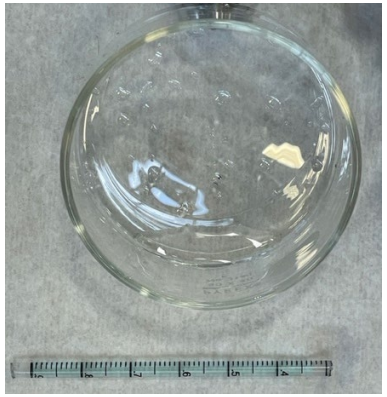
What Will I take Back to the Classroom?

1. The techniques you are introduced to in science courses are used *routinely* in contemporary work.
   - e.g. UV-Vis. Used an elementary technique to tackle a problem and develop a solution.

2. Recognition that scientific progress is not linear.
   - There is a great deal of trial and error, creative exploration, and dead-ends.

3. Newfound patience for students who are learning things for the first time.
   - Interactions with everyone at the NCNR are helpful, positive, and extremely encouraging.





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  - Paul Butler, Juscelino Leao, Alan Ye, Cedric Gagnon for their unwavering support and collaboration

- The one, The only, THE INCOMPARABLE…
  My Mentor: Susana Teixeira