OAK RIDGE National Laboratory

Sample Preparation and Experiment Planning

Wellington Claiton Leite

Pingali, Sai Venkatesh

Center for Structural Molecular Biology

Neutron Scattering Division

Oak Ridge National Laboratory

ORNL is managed by UT-Battelle, LLC for the US Department of Energy

BER Structural Biology Resources

U.S. Department of Energy

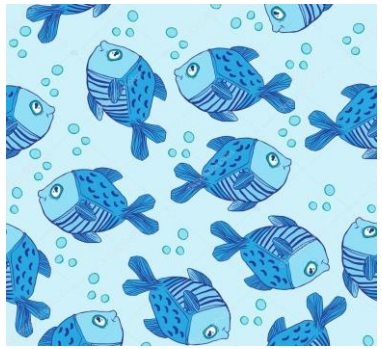
U.S. DEPARTMENT OF ENERGY

Sample preparation

Expression or chemical synthesis	Purification	Preliminary characterization	Sample preparation pre-beamtime	Sample requirements for SANS	Sample ready for SANS
Proteins: E. coli, yeast, Insect cells Nucleic acids: RNA/DNA polymerases Lipids: Commercially available or extracted from cells	Column Chromatography Gel Filtration/Size Exclusion PAGE - (PolyAcrylamide Gel Electrophoresis) Native/SDS	Gel Filtration/Size Exclusion Dynamic light scattering Small-angle X-ray scattering	Ultrafiltration centrifugal devices Dialysis membranes Gel Filtration/Size Exclusion Spectrophoto meter	✓ **Volumes are critical** ✓ **Concentrations already optimized** ✓ **Deuteration levels and scattering length density of sample components must be known a priori** ✓ **The deuteration level in the solvent must be precise** ✓ **Monodispersed species**	
Deuteration strategy 1. Defines contrast matching points 2. Higher the contrast the better – reduces bakgrounds	Characterized monodisperse versus aggregated samples Interparticle interferences Characterize heterogeneities Is your sample happy in D_2O?	Optimized concentrations Separated multiple species Get the correct % D_2O level in sample and solvent Match % D_2O in sample solvent with solvent background			

Monodispersed samples for solution scattering

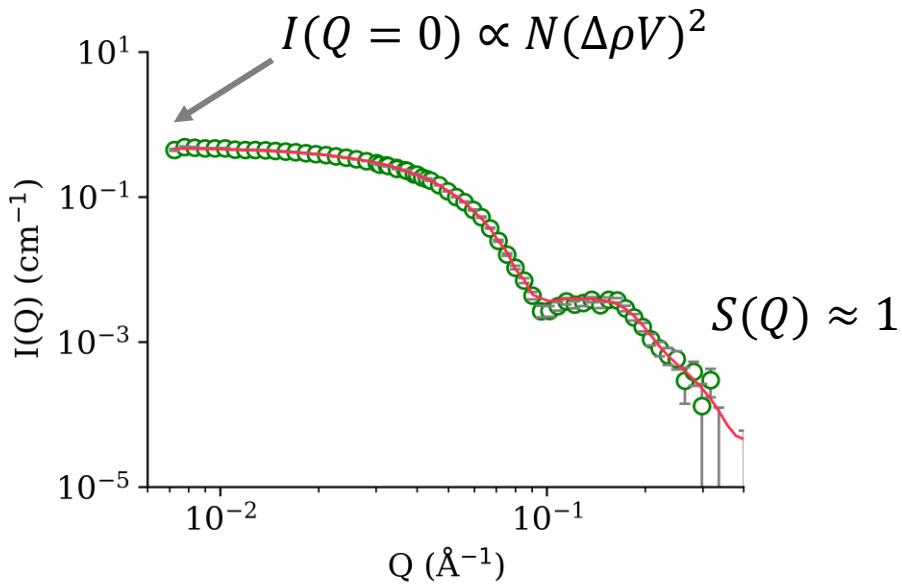
Homogeneous particles



$$I(q) = S(q) \sum_i^n [(\Delta\rho_i V_i)^2 P_i(q)] .$$

1. Pair distance distribution - Dmax
2. Overall size – Rg
3. Conformation – Globular vs extended
4. Oligomerization state
5. Molecular mass – MW
6. Comparison with PDB structures (AlphaFold)
7. Molecular envelope

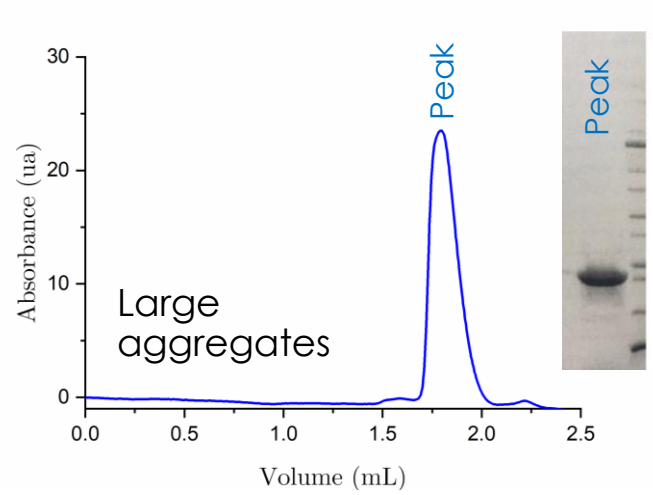
Scattering 1D profile of monodispersed sample



Experiment details:
- Protiated protein in 100% D_2O Buffer
- Buffer exchange to 100% D_2O applying concentrations/dilution cycles
- Concentration used – 2 mg/mL (41.7 KDa)
- Data collected for 2 h (good S/N)
- Volume used 320 uL
- Oligomer: Hexamer

Preparation of monodisperse samples

Size Exclusion Chromatography and SDS-PAGE analysis



- Single peak in the SEC profile
- Single band in the SDS-PAGE analysis

High purity samples

Good changes to be monodispersed (not always ☹)

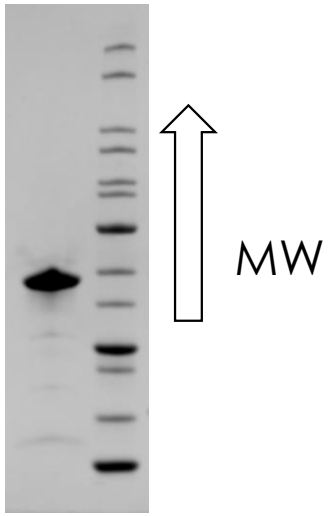
Centrifugation (30 min) prior to measurement can save you 12h of lost beamtime

Monodispersed samples will not present

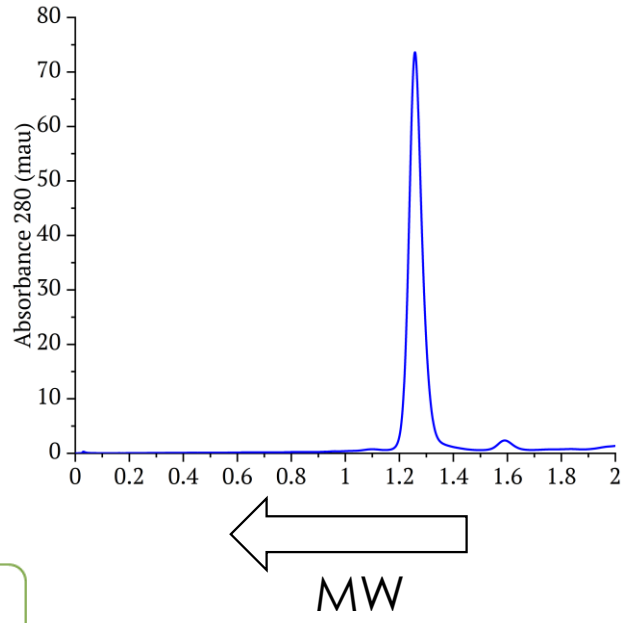
- Intermolecular interactions (Aggregates/Repulsion)
- Precipitates

Initial sample characterization

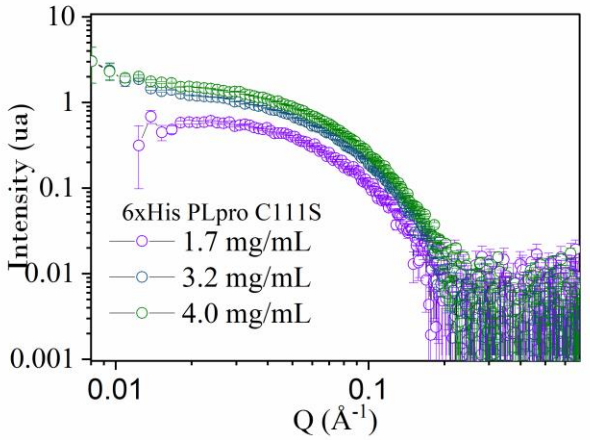
PAGE analysis



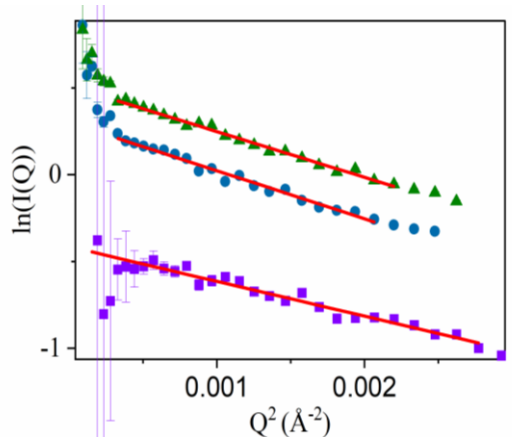
Size-Exclusion Chromatography



SAXS profiles of 6xHis PLpro C111S at different concentrations



Guinier Analysis of 6xHis PLpro C111S



Expressed in *E.coli*

Purified by affinity chromatography

Size-Exclusion Superdex 200

Centrifugation for 30 min at 16000 rcf

SAXS was performed using concentration series

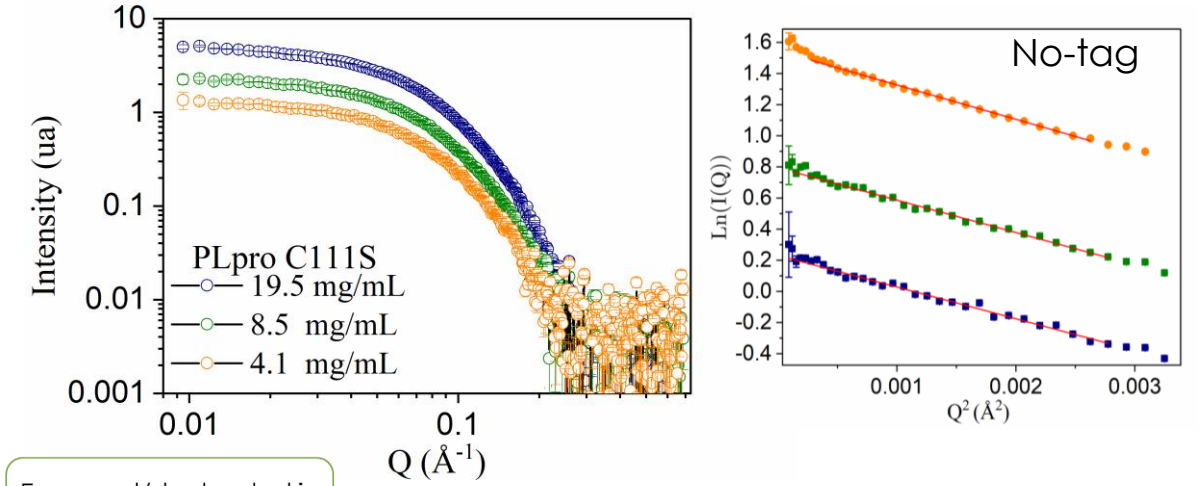
- PAGE analysis shows a sample with high purity
- Size-exclusion chromatography indicates a single population
- SAXS on 6xHis PLpro C111S shows signs of aggregation with increasing concentration

Aggregation can be caused by salt, pH, buffer condition

Initial sample characterization

Example of a well-behaved homogenous sample in solution

SAXS and Guinier analysis of PLpro C111S after TEV cleavage at different concentrations



Expressed/deuterated in *E.coli*

Purified by affinity chromatography

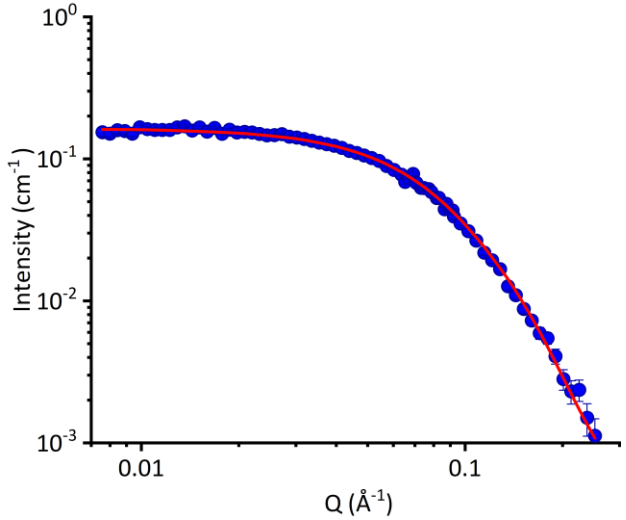
Size-Exclusion Superdex 200

Centrifugation for 30 min at16000 rcf

SAXS was performed using concentration series

SANS experiment

SANS profile of PLpro C111S in 42% D2O



— SAXS analysis of PLpro C111S after removal of the His-tag (4 – 19 mg/mL) shows that after PLpro C111S is monodisperse

— SANS of the deuterated sample is also monodisperse

Experiment details:

- Deuterated protein in 42% D_2O Buffer
- Buffer exchange to 100% D_2O using dialysis
- Concentration used – 5 mg/mL (34.7 KDa)
- Data collected for 2 h (good S/N)
- Volume used 320 uL
- Oligomer: Monomer

SAXS at ORNL and NSLSII for initial characterization

Solutions

— Proteins
— Nucleic acids
— Lipids

Sample requirements:

— 100 uL samples

— MW*Concentration = 100

- 10 kDa – (5 to10 mg/mL)
- 20 KDa – (2.5 to 5 mg/mL)
- 40 - 60 KDa – (2 to 3 mg/mL)

Temperature control at sample position and sample holder

NSLSII joint access program

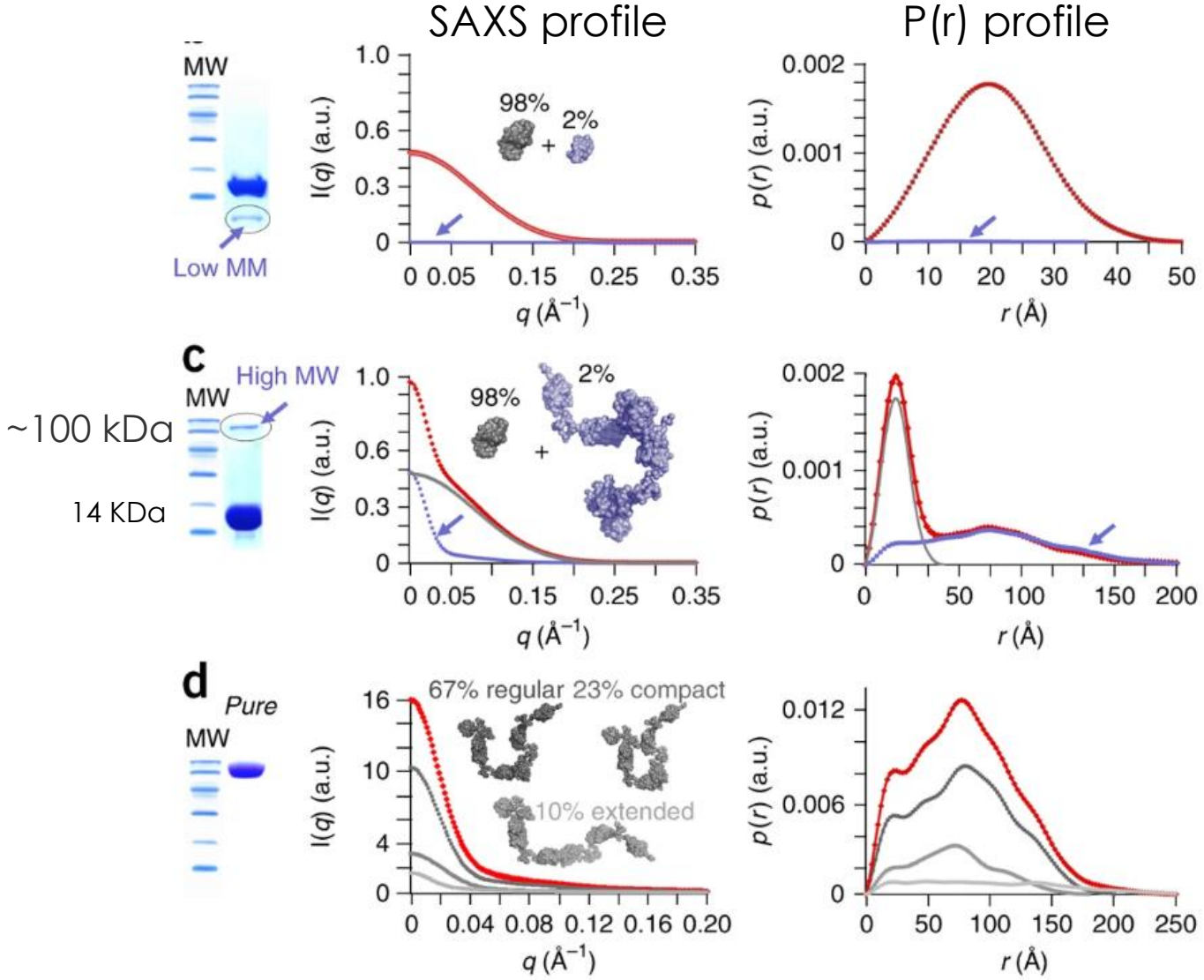
Brookhaven National Laboratory

Mixed populations can be a big problem in SAS studies

Presence of low molecular mass species
- Does not significantly affect the total scattering
- Contribution is proportional to their volume squared and concentration.

Presence of high molecular mass species
- Does significantly affect the total scattering
- Any impurities with the high molecular mass mask the scattering from the low molecular mass protein

Conformational heterogeneity
- The target protein is pure and monomeric. However, the protein is flexible and the total scattering is from different conformations



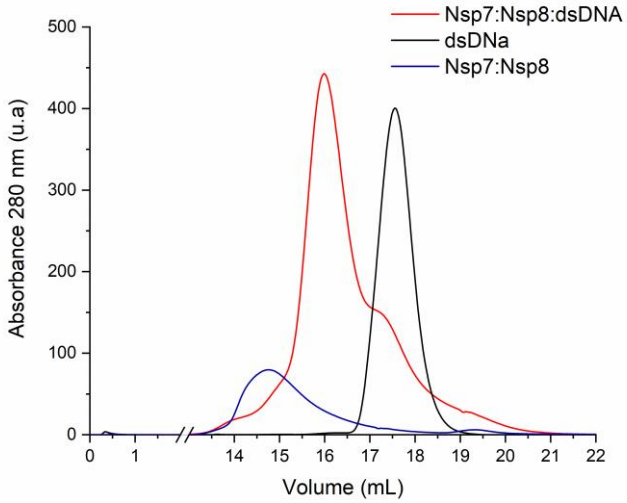
(Adapted from: Jeffries CM, et al . Preparing monodisperse macromolecular samples for successful biological small-angle X-ray and neutron-scattering experiments. Nat Protoc. 2016 Nov;11(11):2122-2153.

Mixed populations can be studied if sample is well-behaved in solution

Example of a well-behaved heterogeneous sample in solution:

PAGE analysis

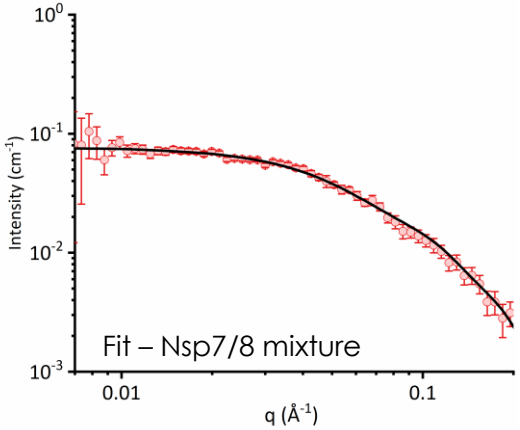
SEC profile of Nsp7/8 complex in the presence of nuclei acids



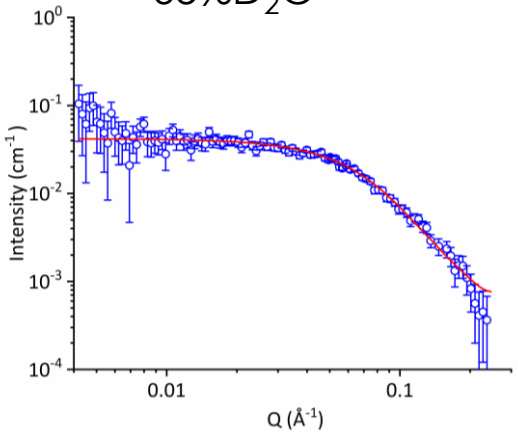
- SEC profiles do not show a bell shape of eluted species, indicating the presence of mix population of proteins or protein complexes

Experiment details:
- Protiated protein in 100% and 65% D_2O Buffer
- Buffer exchange to D_2O using dialysis
- Concentration used – 3.5 mg/mL (~30 KDa)
- Data collected for 2 h (100% D_2O) and 6 h (65% D_2O)
- Volume used 320 uL

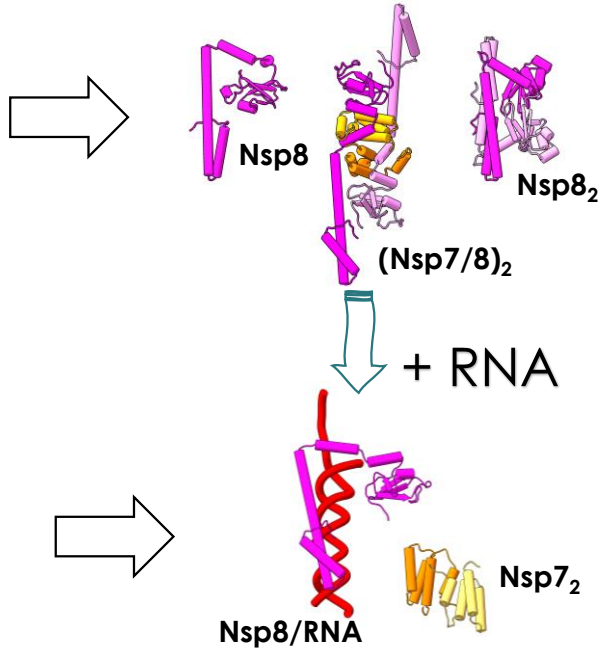
SANS of Nsp7/8 complex in 100% D_2O



SANS of Nsp7/8/RNA in 65%D_2O



Transient model



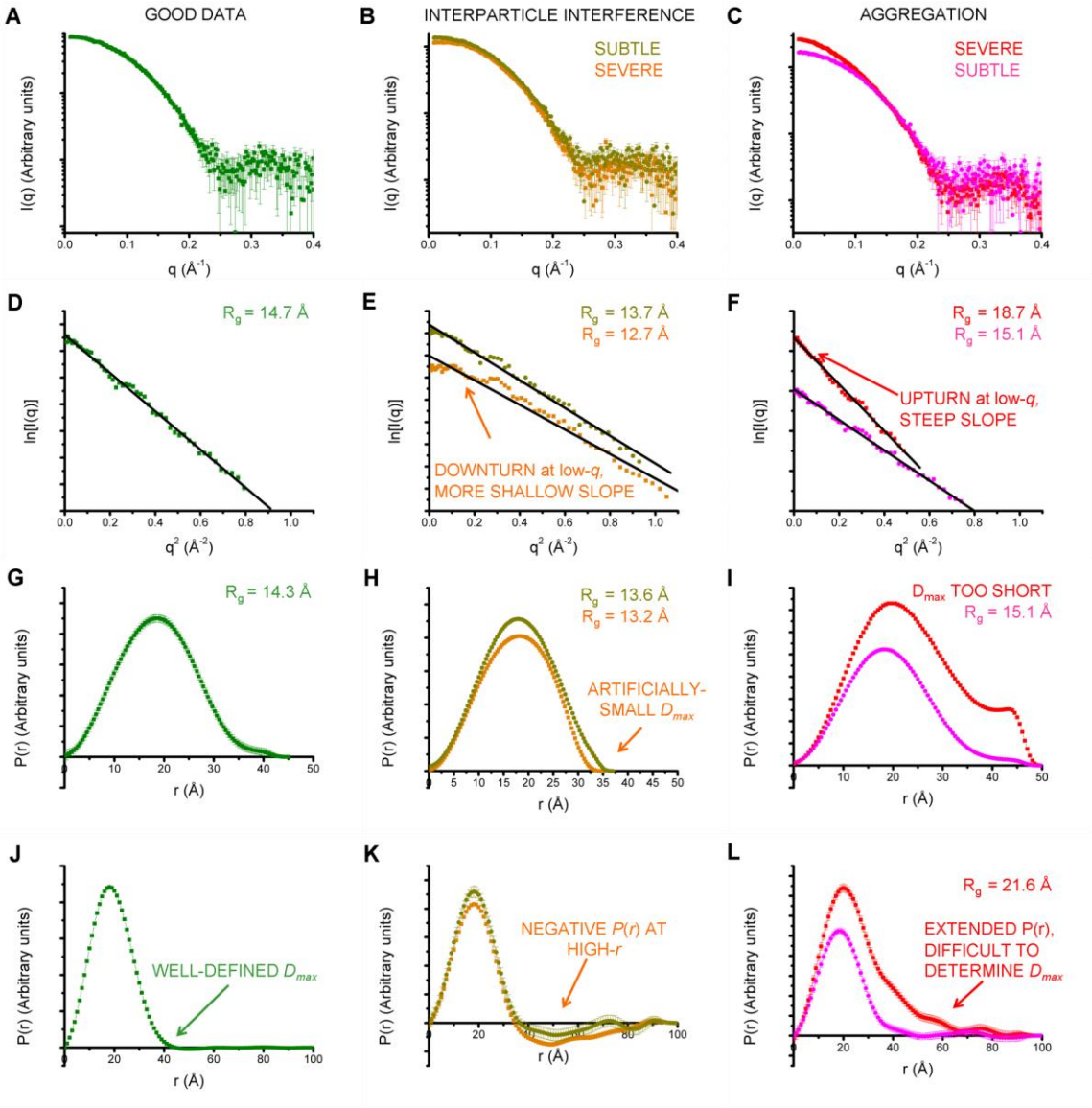
Mixed populations of well-behaved samples can be solved computationally

Biophysical Journal, Wilamowski & Leite et al, 2021)

Easy extra step to ensure monodispersity of sample

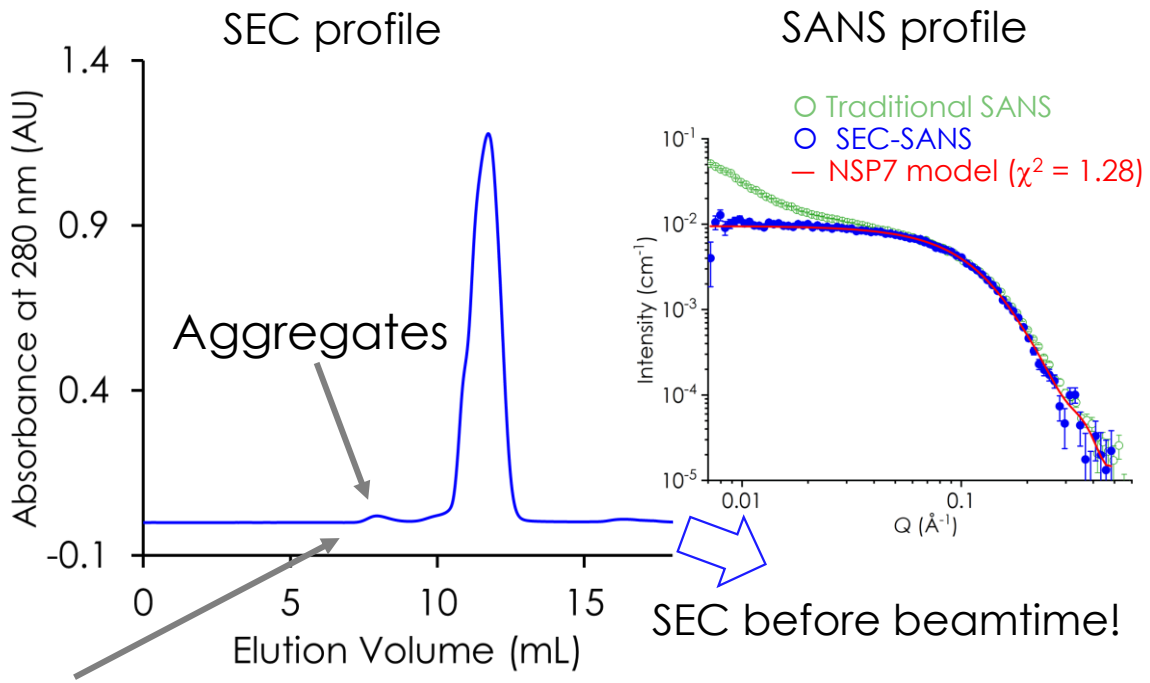
Centrifugation (30 min) prior to measurement can save you 12h of lost beamtime

Effects of interparticle interferences in SAS samples



Interparticle Interferences

Attractive interaction between proteins in solution

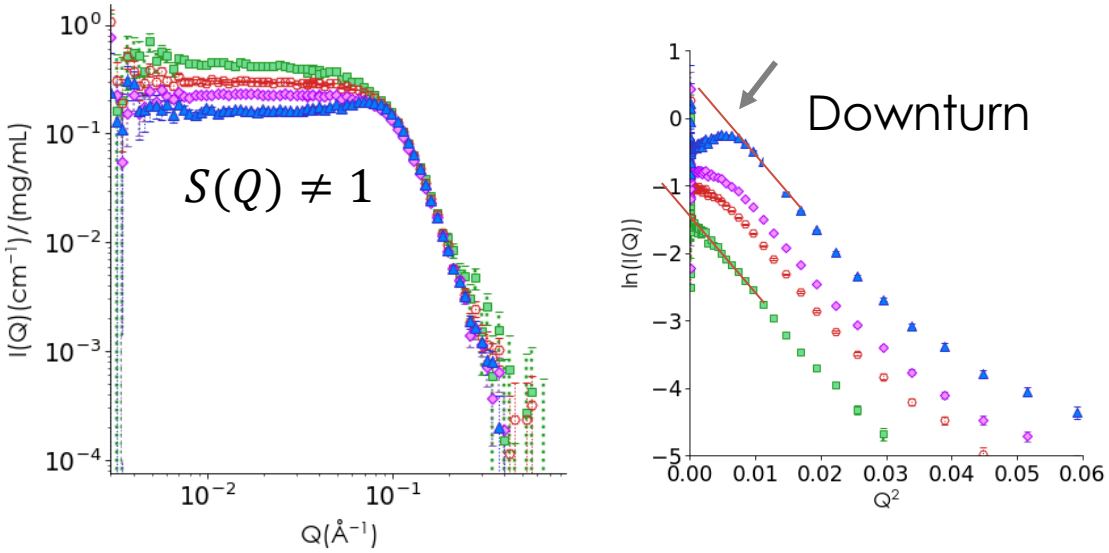


Note: Aggregates have low absorbance at 280 nm, but have high light scattering (MALS)

Experiment details:
- Unlabeled Non-Structural Protein 7 (9.2 KDa)
- Injected ~500µL at ~20mg/mL
- 100% D_2O buffer
- Superdex 75 10/300 GL (24mL CV)
- Data collection triggered manually
- Higher order aggregates removed!
- Nsp7 exists as a dimer (18.4kDa) in solution

Repulsion interaction between proteins in solution

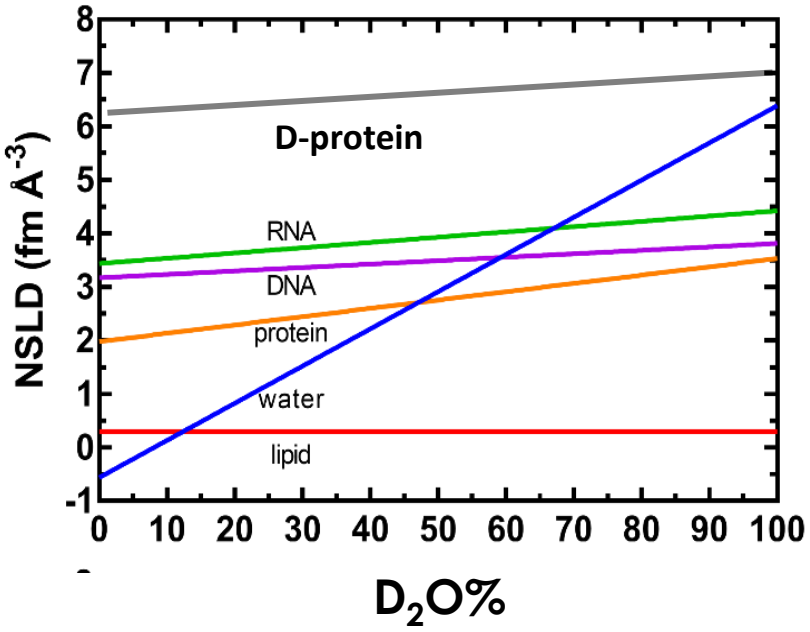
SANS profile and Guinier Analysis



- Membrane protein in micelles
- Sample concentration was reduced 8-fold to reduce attractive interactions

Deuteration strategy for SANS with contrast matching experiments

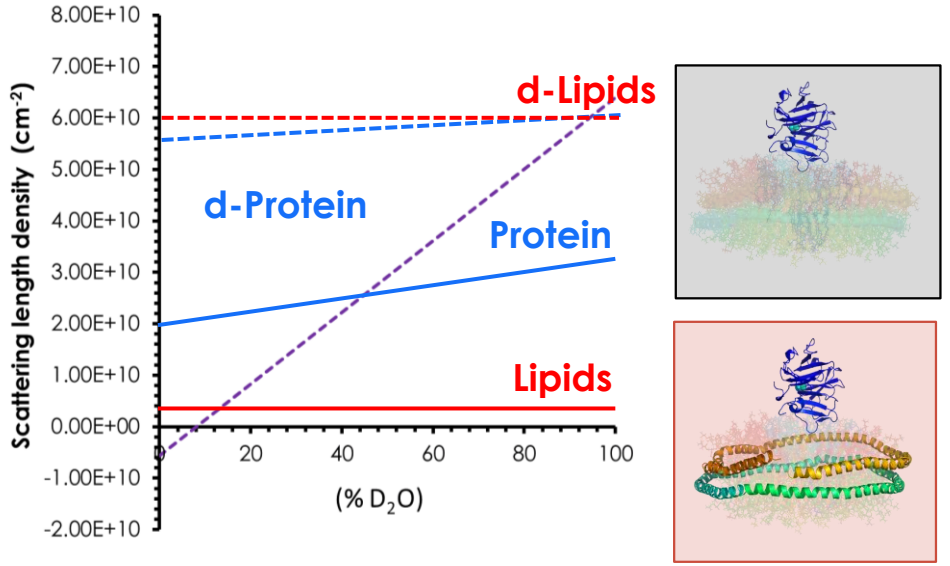
Natural contrast matching points



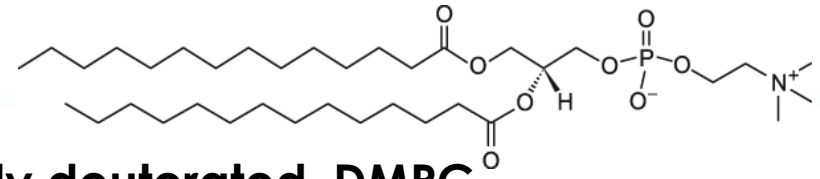
Natural matching points:

Protiated protein – 42%
DNA – 65% D2O
RNA – 70% D2O
Lipid – 10% D2O

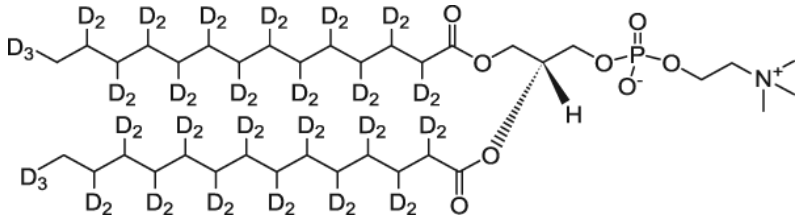
Strategies for lipid deuteration



Protiated DMPC



Partially deuterated DMPC

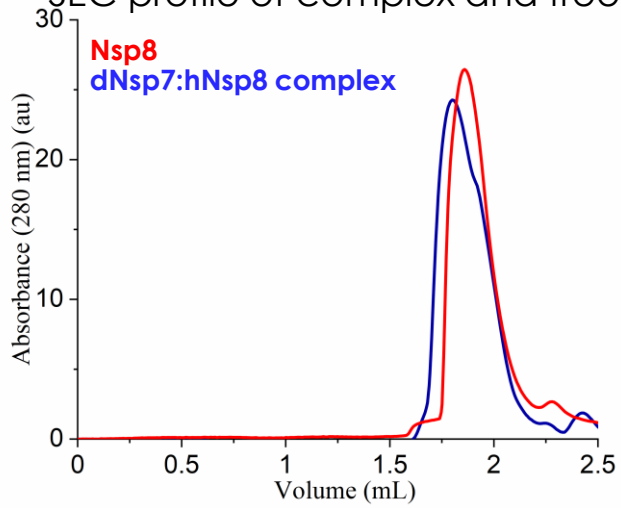


Deuteration strategy for contrast matching SANS

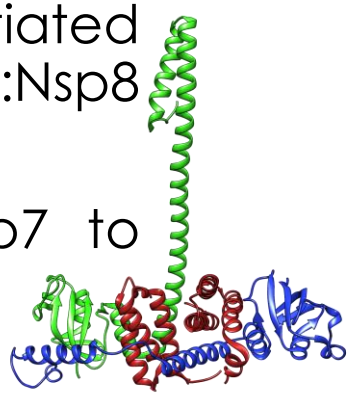
Strategies for protein deuteration

- Expressed deuterium labeled SARS-CoV-2 Nsp7 and Nsp8 in 75% D_2O medium
- Determined the contrast match point of the deuterated subunits (dNsp7 or dNsp8) (~90% D_2O)
- Plan to run a contrast variation series with at last 5 samples
- Co-purification of deuterated Nsp7 and protiated Nsp8 to form a partially deuterated Nsp7:Nsp8 complex (dNsp7:Nsp8)
- SANS at the contrast match point of dNsp7 to highlight the scattering from Nsp8

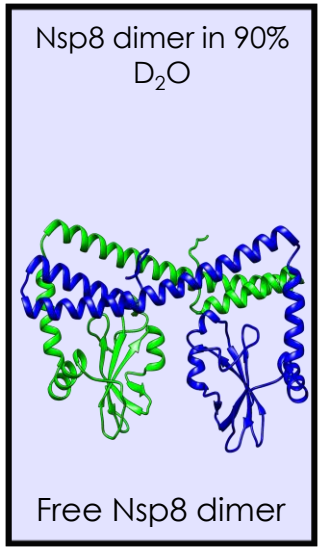
SEC profile of complex and free Nsp8

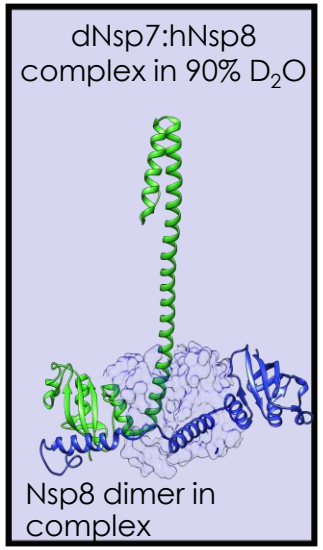


SANS contrast matching experimental design



Crystal structure of Nsp7/8 complex





Buffer exchange to D_2O is an important step

Buffer exchange using dialysis

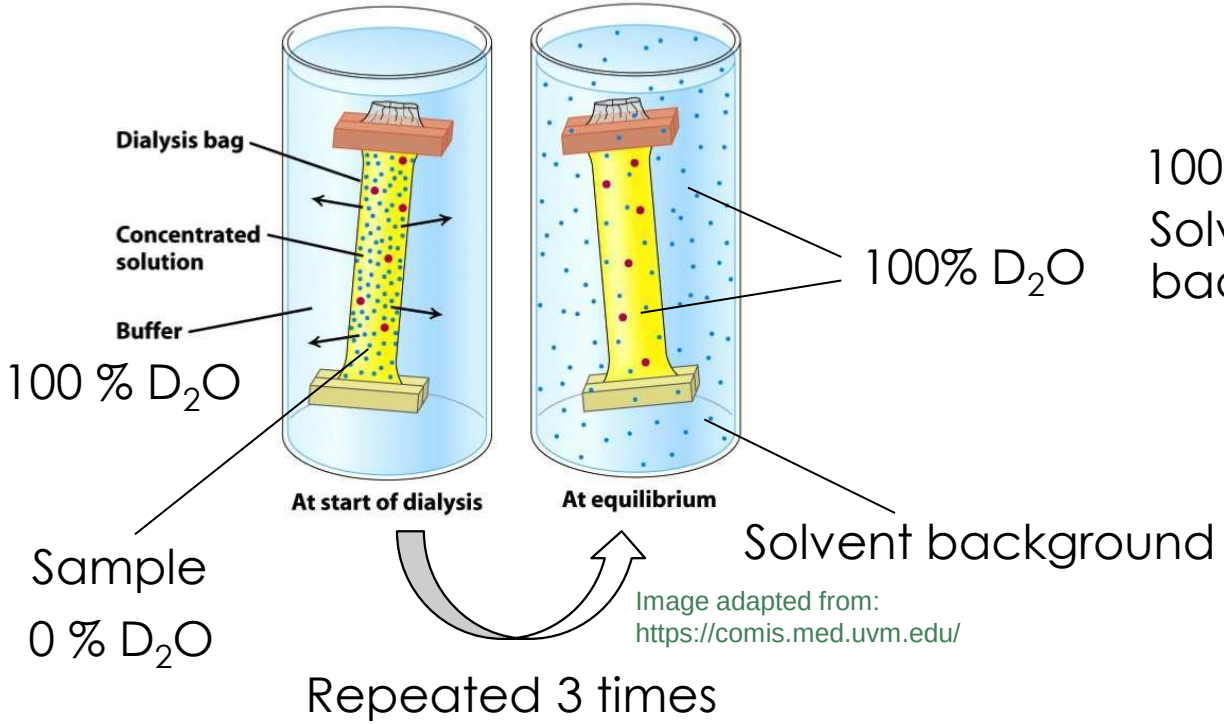
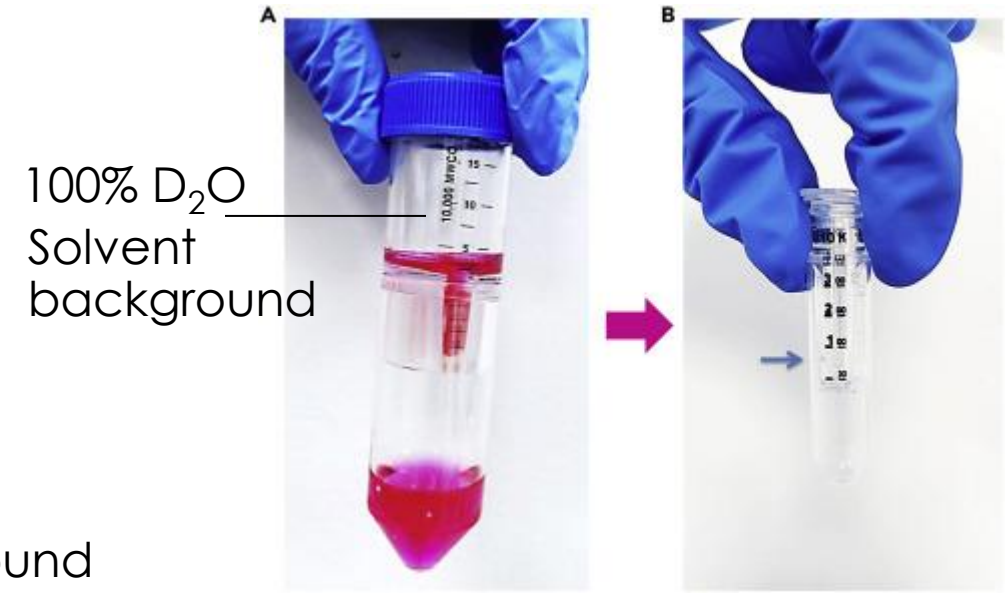


Image adapted from: https://comis.med.uvm.edu/

Buffer exchange using concentration/dilution cycles



(Image adapted from: Vittoria Matafora et al, STAR protocols, 2020)

— The % D_2O defines in solution defines the experimental SLDs of the solvent

— Contrast matching experiments requires your target and buffer have the same SLD

Sample preparation: pre-beamtime

Determine your protein concentration

1) Extinction coefficient (usually A280) - https://web.expasy.org/protparam/
2) Bradford assay or similar if the protein does not absorb in 280nm because of lack of aromatic residues

Does your protein aggregate?

1. Time-dependent aggregation
2. Temperature-dependent aggregation
3. pH-dependent aggregation
4. Salt concentration
5. Wrong pH
6. Behavior of protein through freeze-thaw cycles

How your sample behaves in D_2O/H_2O mixtures?

1. Buffer exchange using dialysis
2. Concentration/dilution cycles
3. Size-exclusion chromatography

— SANS using solutions requires 320 ul for one measurement (Banjo cell)

— Protiated samples must target high $\%D_2O$ solvents (e.g., $100\%D_2O$ buffer)

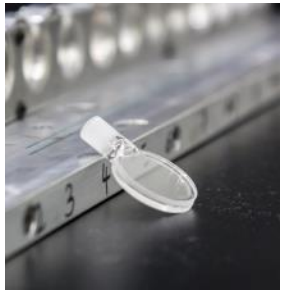
— High protein concentration the better (2 to 5 mg/mL)

Sample requirements:

— **MW*Concentration = 100**

- **10 kDa – (5 to10 mg/mL)**
- **20 KDa – (2.5 to 5 mg/mL)**
- **40 - 60 KDa – (2 to 3 mg/mL)**

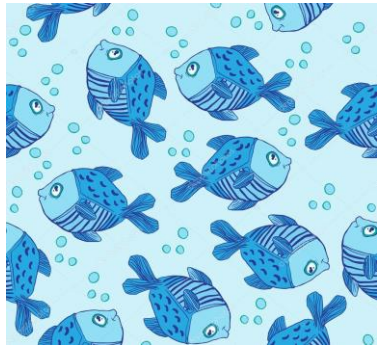
Banjo cell



Bio-SANS Experiments for Hierarchical Systems

Types of Biological Samples

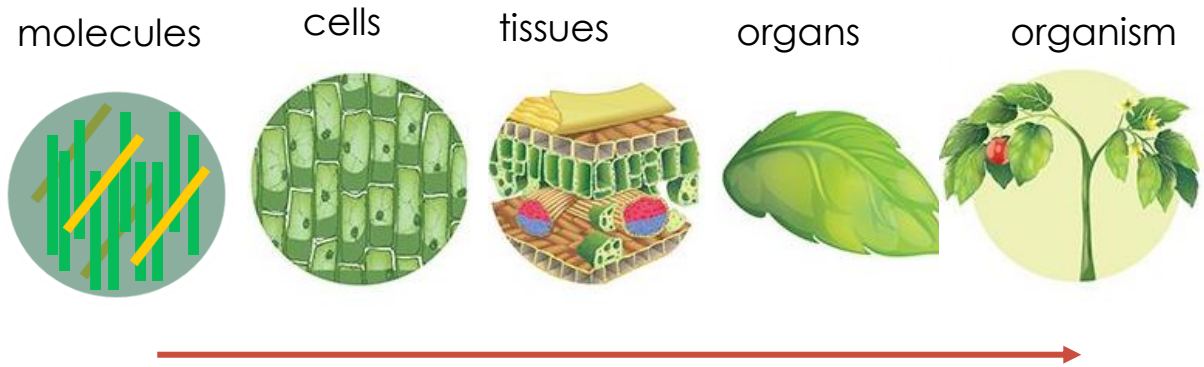
Homogeneous particles



Eg: Proteins in buffer
DNA/RNA in solutions
Virus particles

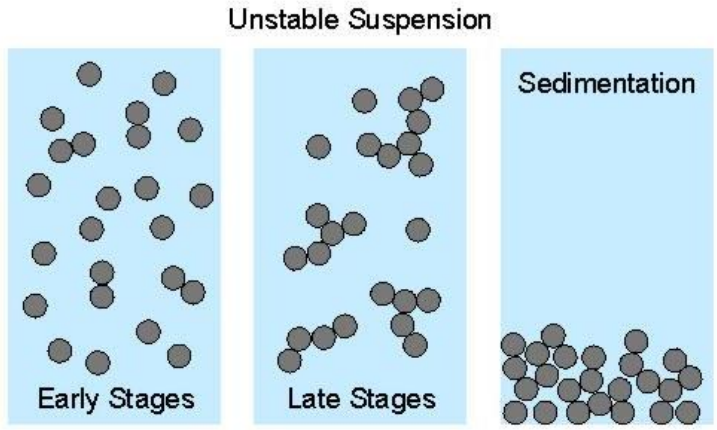
Wellington Covered !!

Hierarchical systems



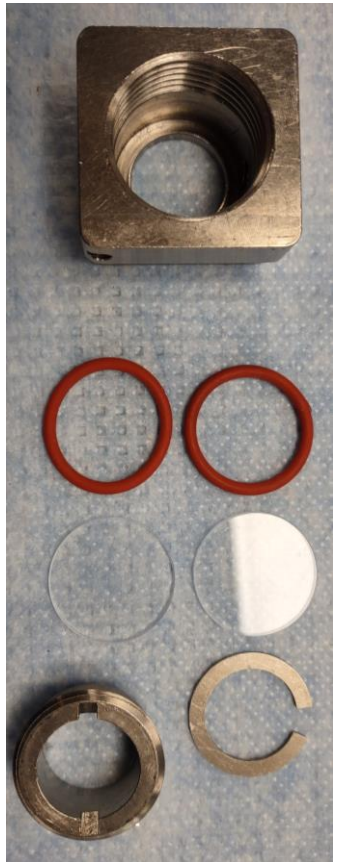
Eg: Plant cell wall
Biopolymeric hydrogels
Cellulose composites
Sedimentary rocks

Colloidal particles

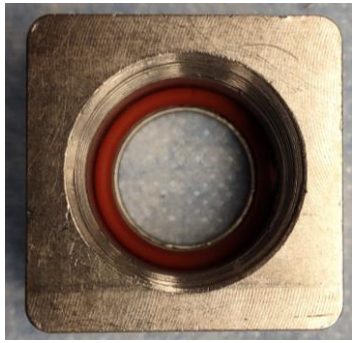


Eg: Cellulose nano crystals
Soil nano particles
Nano fertilizers
Processed biopolymers

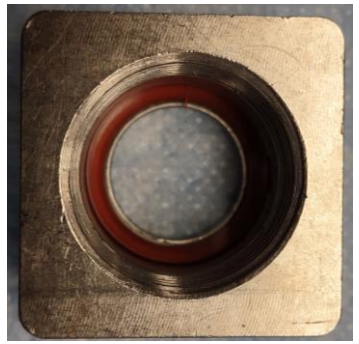
Assembling of Aligned Sample in Titanium Cells



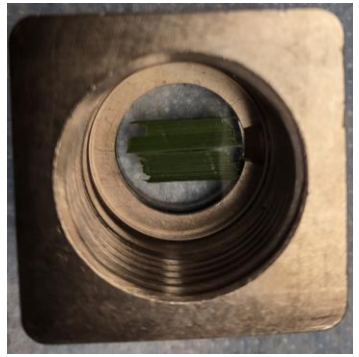
Parts of Titanium cell



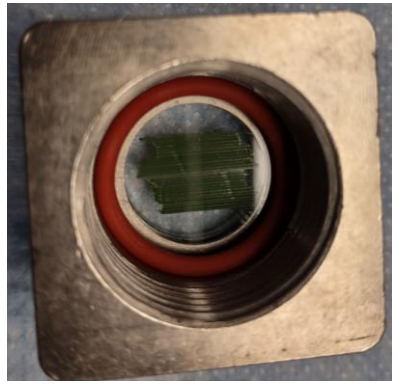
Place o-ring in main cell body



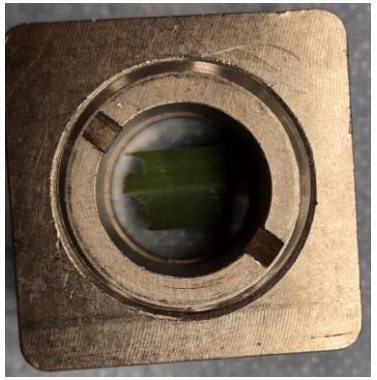
Place bottom quartz window



Place spacer and carefully place sample aligned to the preferred direction on top



Place top quartz window and o-ring



Screw the sample, windows, o-rings tight

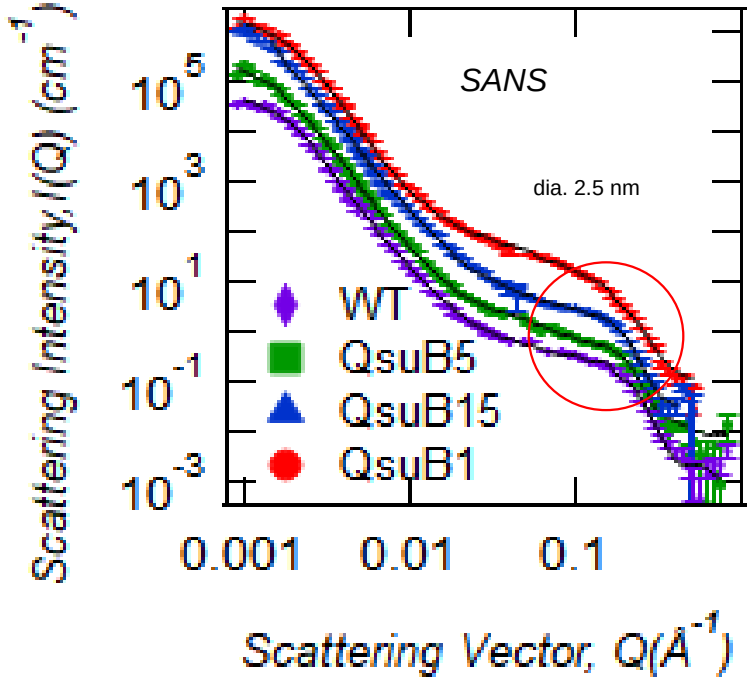
Inject solvent from top and seal cell from top

Plant Cell Wall Study

Titanium cells

- Path lengths: 1mm, 1.5 mm, 2mm
- Good for
  - Large solid pieces in solutions
  - Viscous solvents like slurries
  - Plant stems in solutions
  - Biopolymeric hydrogels
- Easier to assemble and clean

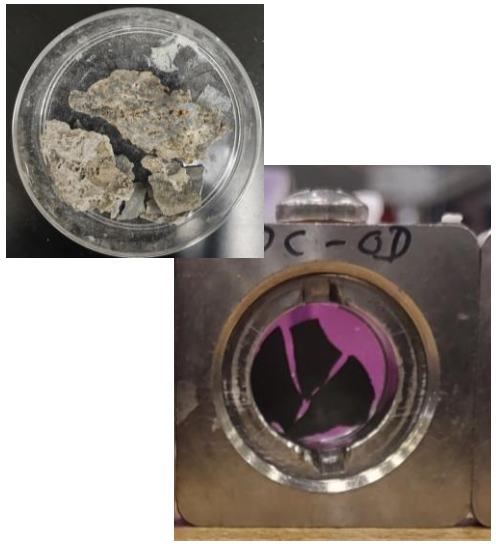
Genetically modified Poplar for Biofuels

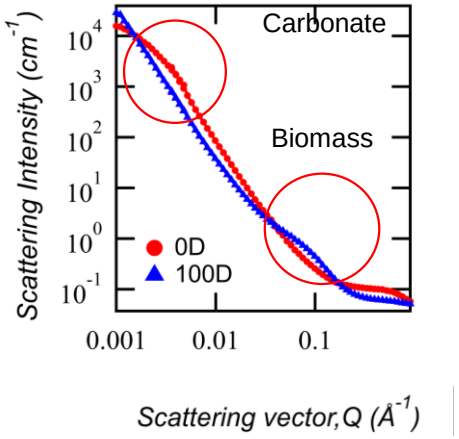


SANS show that lignin plays an important role in cellulose structure and organization that effect biomass recalcitrance.

Other Examples of Hierarchical Systems – sedimentary rocks and hardwood pulp

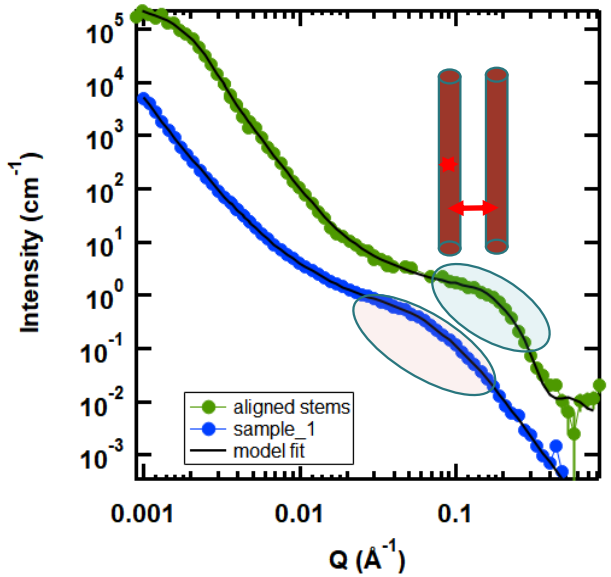
Carbon Capture into Carbonate Sediments





- Correct thickness is important to avoid the multiple scattering!!!
- The ability of contrast variation SANS to distinguish structural components in carbonate sediments to used in anaerobic oxidation of methane at the nanoscale.

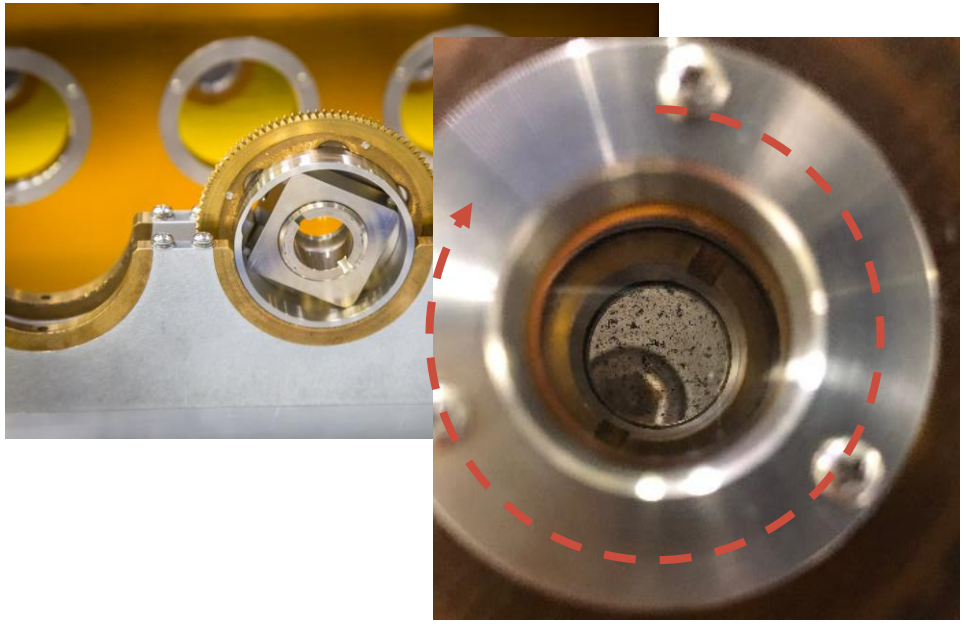
Effects of expansin Proteins on hardwood pulp



- Hardwood pulp has larger cellulose structure compared to native plants and expansin proteins has slight impact on structure.

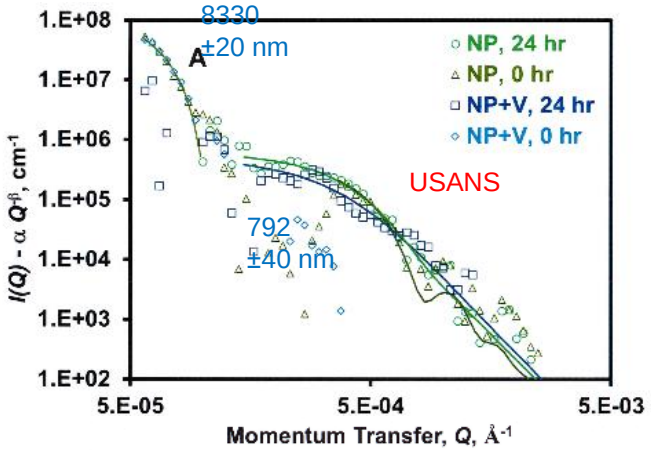
Colloidal Systems settle and need constant tumbling

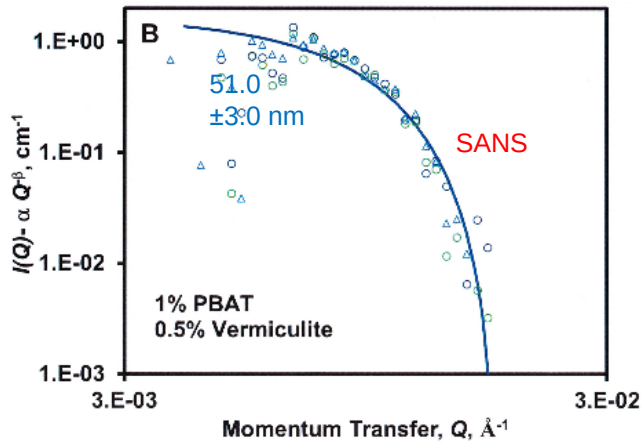
Tumbling setup



- Required for sedimented particles
- Can be used in both banjo or titanium cell setup

NanoPlastics (NPs) in Soil Environment

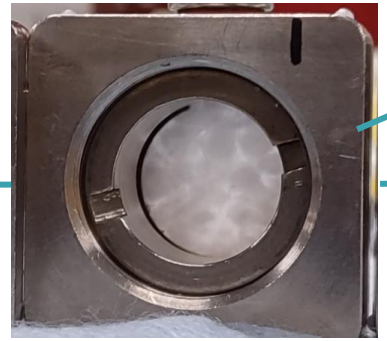




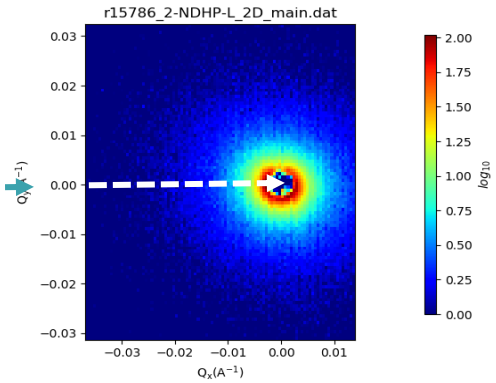
- Contrast matching SANS and USANS showed how different size agricultural NPs interact with soil and also changes in NP-NP and NP-soil interactions under different environmental conditions.

Isotropic vs. Anisotropic scattering

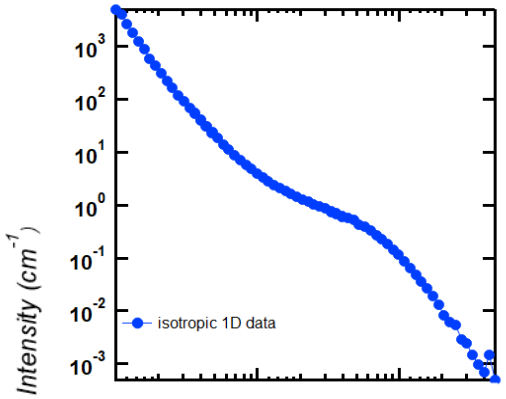
Incident wave vector, k_i

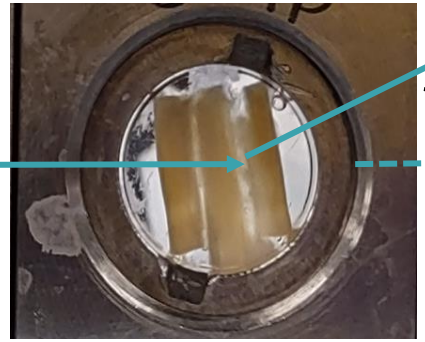


θ

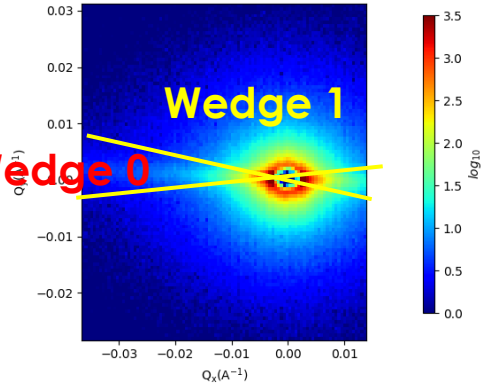


data reduction
(Azimuthal averaging)

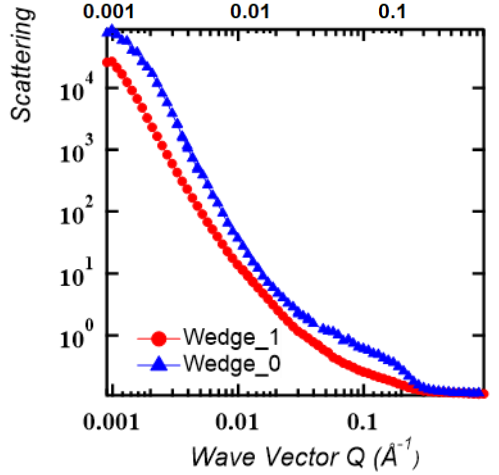




θ



data reduction
(Averaging within
each Wedge)



Sample

Scattering pattern (2D)

Scattering pattern (1D)

Practical Considerations at SANS User Facilities

- Plan your experiment well!
- What Q-range would I like, and what must I have?
- For how long should I measure my samples? – counting statistics, sample size, concentration, experimental contrast
- What are my backgrounds and how to correct for them?
- How can I optimize my sample quality?
- Ask your local contact / instrument scientist for advice well ahead of time

Facility Acknowledgment Statement

- A portion of neutron scattering research presented as examples in this introduction used resources at the High Flux Isotope Reactor or Spallation Neutron Source, DOE Office of Science User Facilities, operated by the Oak Ridge National Laboratory.
- The Bio-SANS of the Center for Structural Molecular Biology at the High Flux Isotope Reactor is supported by the Office of Biological and Environmental Research of the U.S. DOE.