# SMALL ANGLE NEUTRON SCATTERING (SANS):
# a complementary technique

Everything BioSAXS 9 – Advanced Topics – March 2, 2023

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# Outline

## What is different about neutron scattering?

- Nature of the radiation used
- Incoherent Scattering
- Contrast

## Notes on experimental planning & SAXS/SAS complemetarity

- Sample volume/concentration
- Measuring match points
- Deuteration & HDX
- Where can I apply for beamtime?

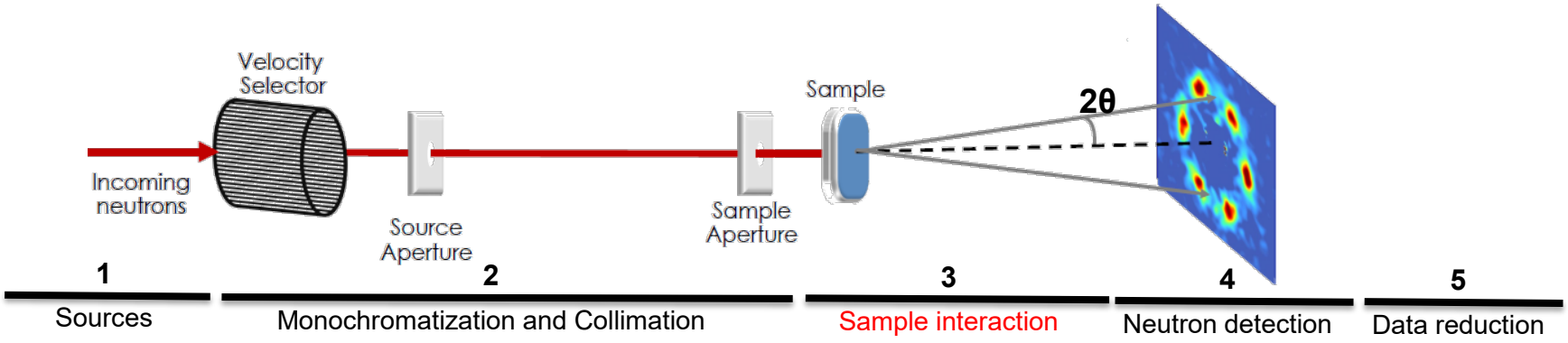
## Examples of applications

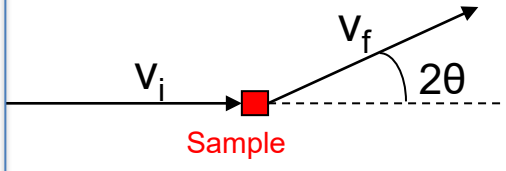
- Contrast variation studies
- Sample environment effects: pressure and temperature

**See Differently With Neutrons at NIST**


https://www.nist.gov/video/see-differently-neutrons-nist

# Nature of the radiation used: neutrons



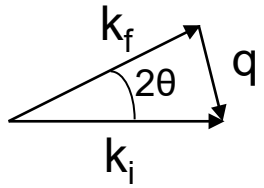

**Real space**

$$\vec{q} \equiv \overrightarrow{k_i} - \overrightarrow{k_f}$$
$$\Delta\omega \equiv E_i - E_f$$

**Elastic scattering**: $|k_i| = |k_f| = k = 2\pi/\lambda$

$$q = 2k\sin(\theta)$$

$$d \approx \frac{2\pi}{q} \qquad q = \frac{4\pi}{\lambda}\sin(\theta)$$




**Reciprocal space**

# Nature of the radiation used: neutrons

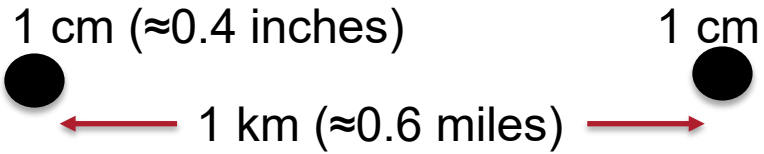
**Neutrons have zero net charge.**

**Relatively low probability of interaction with nuclei:**

- Neutrons are highly penetrating
- thicker materials can be probed
- samples in various containers can be probed (e.g. extreme environments)

**Neutrons causa no radiation damage.**

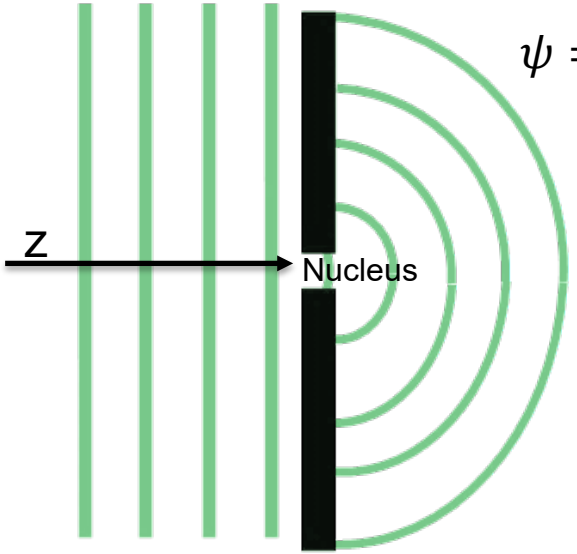
| | |
|---|---|
| Neutron Wavelength: | $10^{-10}$ m (Å) |
| Inter-atomic distances: | $10^{-10}$ m (Å) |
| Atomic nuclei: | $10^{-15}$ m (fm) |
| Strong nuclear forces: | $10^{-15}$ m (fm) |
| Velocity of the neutrons: | $10^{-10}$ m.ps$^{-1}$ |
| Neutron flux at the sample position: | $10^{7}$-$10^{8}$ n.cm$^{-2}$s$^{-1}$ |




**[Neutrons have a magnetic moment and energies appropriate to study molecular vibrations, lattice excitations and dynamics of atomic motion]**

# Nature of the radiation used: neutrons

Incident Plane Wave: $e^{ikz}$




$$\psi = -\frac{\boldsymbol{b}}{r} e^{i\kappa.r}$$

**b: Scattering length** of the nucleus (interaction between the neutron and the nucleus)

Probability of the interaction: $b^2$

**Total Scattering cross section**: $\sigma = \int \frac{d\sigma}{d\Omega} d\Omega = 4\pi|b|^2$

# The concept of Incoherent Scattering

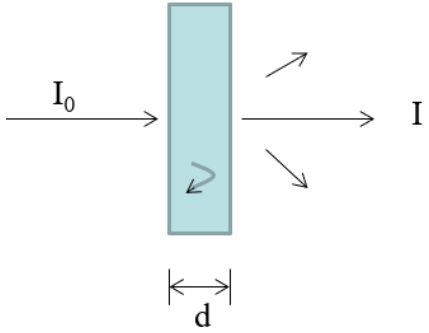
$$\sigma = \sigma_{coh}(q) + \sigma_{inc} + \sigma_{abs}$$

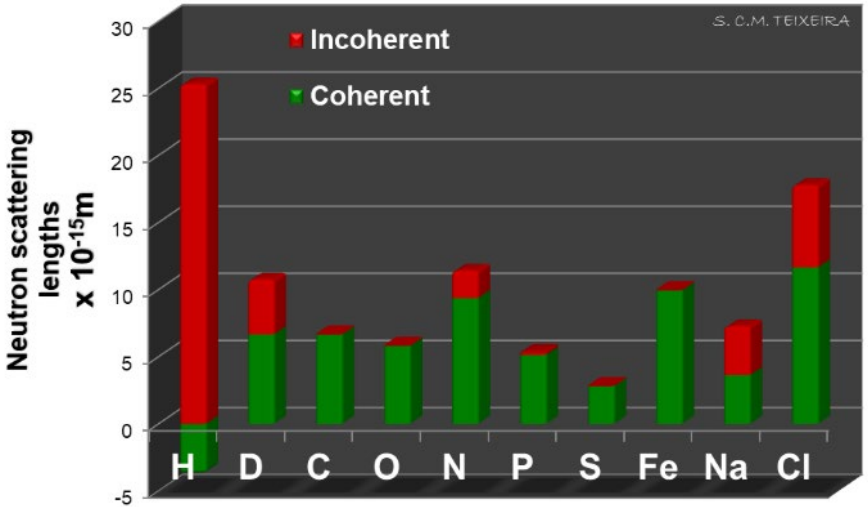
**coherent:**
depends on structure
q-dependent (S)

**incoherent:**
'noise' or 'background'
T-dependent (N)

**absorption:**
Reduces S/N
Wavelength dependent

$$I = I_0 \exp(-\mu d)$$




Data from the Special Feature section in *Neutron News*, Vol. 3, N. 3, 1992, pp. 29-37.

For elements or relatively simple molecules:

**Scattering lengths ($cm^{-1}$) of elements:**
https://www.ncnr.nist.gov/resources/n-lengths/

**SLD ($cm^{-2}$) calculator:**
https://www.ncnr.nist.gov/resources/activation/

# Neutrons vs X-rays

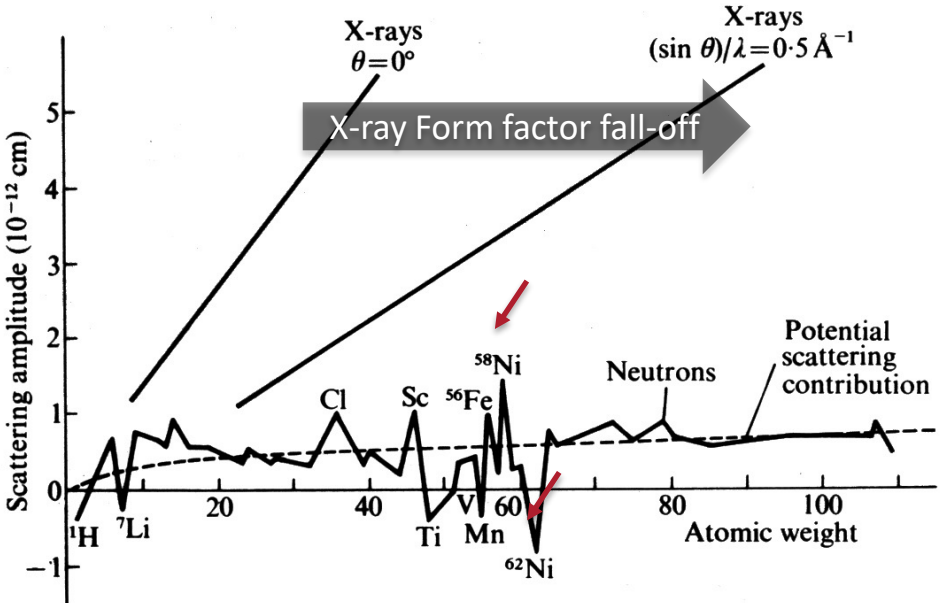
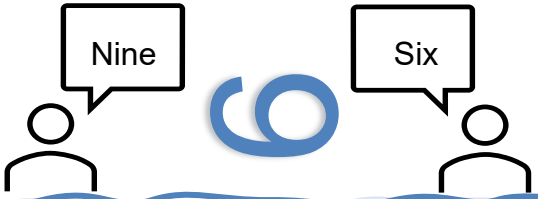



Image adapted from: Fig. 22, Research (London) 7, 257 (1954)




| X-rays | Neutrons |
|---|---|
| - High brilliance beams and larger cross sections | - Need more sample, longer exposures |
| - Scattering length depends on atomic number and varies with q | - Depends on nuclei and no angular dependence |
| - Light or similar Z elements are difficult to resolve | - light elements (H, D) have large cross sections; sensitive to different isotopes |
| - keV energies | - meV energies |

# Contrast

Fourier transform of the SLD distribution

$$I_{molecule}(q) \propto \frac{d\Sigma}{d\Omega}(q) = \frac{N}{V}\frac{d\sigma}{d\Omega}(q) = \frac{1}{V}\left|\int \boldsymbol{\rho}(\boldsymbol{r})e^{iqr}\,dr\right|^2$$

Rayleigh-Gans Eq.

N atoms occupying a volume V

$I_{molecule}(q)$: We measure intensities

$\frac{d\Sigma}{d\Omega}(q)$: Macroscopic cross-section

$\left|\int \boldsymbol{\rho}(\boldsymbol{r})e^{iqr}\,dr\right|^2$: We want to know the structure [shape of the molecule, or F(q) ]

$$\boldsymbol{\rho} = \frac{\sum_i^N \boldsymbol{b_i}}{\boldsymbol{V}} = b_i\delta(r - r_i)$$

Molecule in solution

$$I_{molecule-solvent}(q) \propto \frac{1}{V}\left\langle\left|\int \Delta\boldsymbol{\rho}e^{iqr}\,dr\right|^2\right\rangle$$

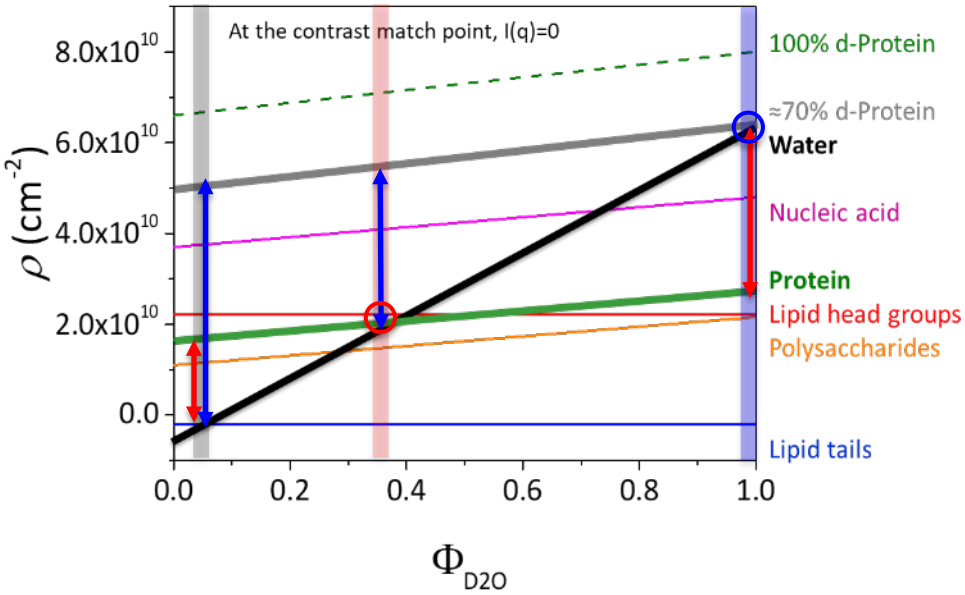
$$\overline{\Delta\rho} = \bar{\rho}_{mol} - \rho_{solv}$$

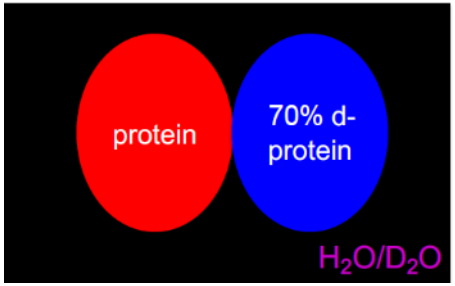
# Generic plot: contrast variation and solvent matching

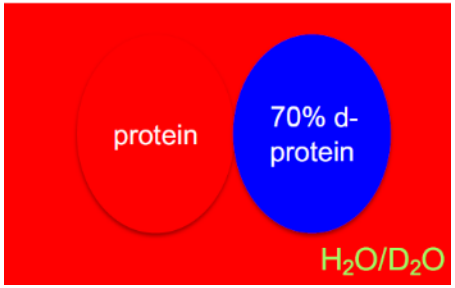
For N molecules with a density n (N/V), averaged over random orientations:

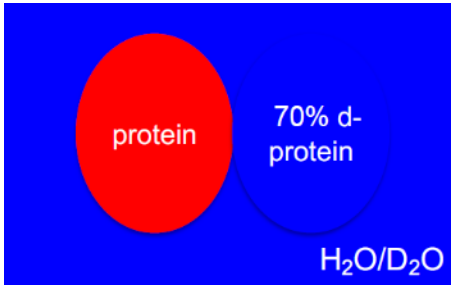
$$I(q) = n\,\Delta\rho^2\,V^2\,<|F(q)|^2>$$

Scattering length density (SLD) contrast



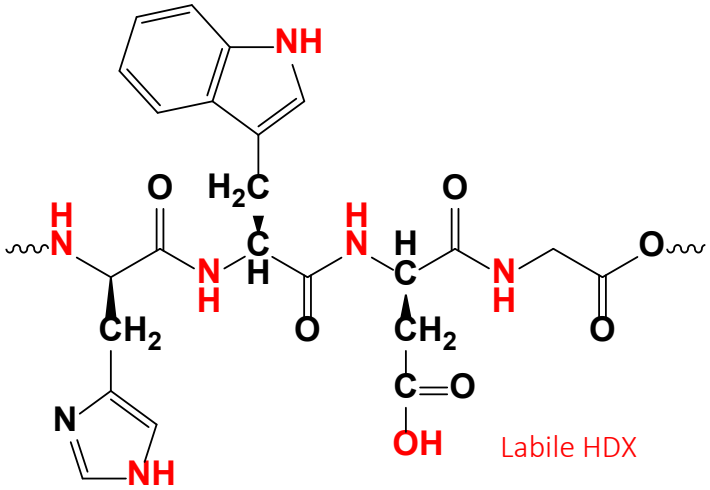



# Deuteration: isotope labeling for higher or selective contrast

The **Biomolecular Labeling Laboratory (BL$^2$)** is a joint NIST/UMD facility. The laboratory provides the infrastructure for labeling proteins, nucleotides and other biomolecules with stable isotopes so they can be used for structural and biophysical methods such as small angle neutron scattering (SANS). The BL$^2$ supports labeling of **proteins expressed in bacteria and yeast** and of **chemically synthesized peptides**.





# Match point : experimental determination

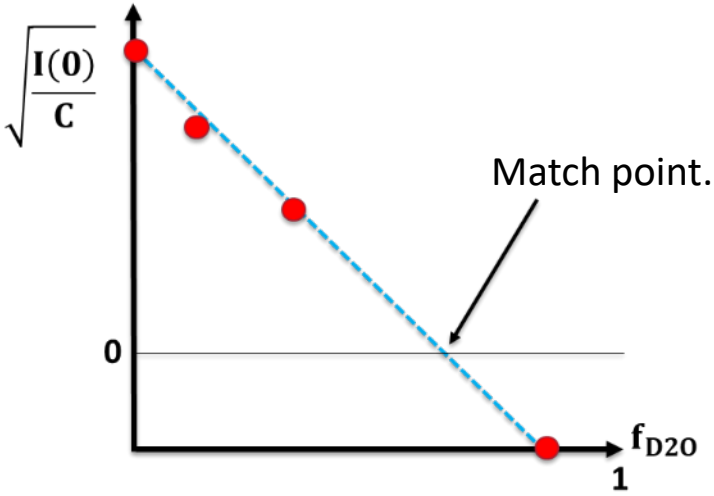
$$I(q=0) = n\,\Delta\boldsymbol{\rho^2}\,V^2 = \frac{C\,M_w}{N_A}\Delta\boldsymbol{\rho^2}\boldsymbol{v^2}$$

C: concentration (g/cm$^3$)
$v$: partial specific volume (cm$^3$/g)
$M_w$: molar mass (g/mol)

Δρ varies with the % $D_2O$ in the solvent

$$\sqrt{\frac{I(0)}{C}} \propto \Delta\rho^2 \propto f_{D2O}$$

Collect data at various % $D_2O$ to find the experimental match point:




See (slide 14) for software to calculate contrast from sequence or PDB structures

# Inhomogeneous SLD particles: beyond the Guinier approximation

Stuhrmann extended the Guinier approximation to account for the contrast dependence of the radius of gyration extracted from a fit to the scattering data. [Ibel, K. and Stuhrmann, H. B. (1975). J. Mol. Biol. 93, 255–265]

## Stuhrman analysis

$$R_g^2 = R_0^2 + \frac{\alpha}{\Delta\rho} + \frac{\beta}{\Delta\rho^2}$$

Overall shape of the particle

**$R_0$:** $R_g$ of an equivalent homogeneous complex

Distribution of inhomogeneities

$$\alpha = \frac{1}{V}\int_r \rho_F(r)|r|^2 d^3r$$

$$\beta = (\frac{1}{V}\int_r \rho_F(r)|r|d^3r)^2$$

**α:** second moment of the internal density fluctuations within the scattering object:

- α = 0 for a homogeneous scattering particle
- α > 0 higher SLD towards the outside of the particle
- α < 0 higher SLD towards the inside of the particle

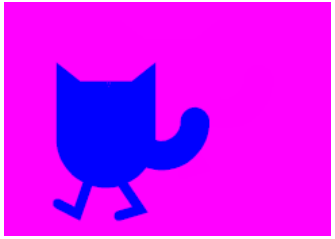
***β***: Distance of the center of mass of the particle to the center of SLD of its heterogeneous components (if ≠0 the centers of mass of the 2 components are not concentric)

# Two component system with different SLD: Parallel-axis theorem

Relating the measured Rg to the distance D between the centers of SLD of each subunit [Moore, P. B. (1982). Methods Exp. Phys. 20, 337–390]:

$R_g$ vs Δρ

$$R_g^2 = \frac{\Delta\rho_1 V_1}{\Delta\rho V} R_1^2 + \frac{\Delta\rho_2 V_2}{\Delta\rho V} R_2^2 + \frac{\Delta\rho_1 V_1 \Delta\rho_2 V_2}{(\Delta\rho V)^2} D^2$$



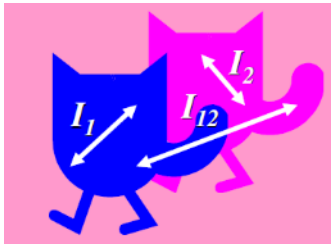
See **MULCh** (http://www.mmb/usyd.edu.au/NCVWeb/); *Rg module* analyses the contrast dependence of the radius of gyration to yield information relating to the size and disposition of the labelled and unlabeled components in a complex. The module *Compost* can also extract composite scattering profiles (essentially deconvoluting the data) for 2 component systems:

$$I(q) = \Delta\rho_1{}^2 I_1(q) + \Delta\rho_1 \Delta\rho_2 I_{12}(q) + \Delta\rho_2{}^2 I_2(q)$$

cross term

$$I_1(q=0) = n\,\Delta\rho_1{}^2\, \boldsymbol{V_1}^2$$

volume of individual components can be determined for component 1 and 2



# A contrast variation experiment

**Calculations prior to the experiment:**

- predict contrast values and match points based on chemical composition (primary structure, type of lipid, etc)
- Predict SANS curves at various contrasts if a low or high res model starting model is available

**Experimental match point determination:**

- determine the contrast match point of the complex and individual components

**Stuhrmann Analysis and Parallel Axis Theorem**

• Determine Rg for the individual components and the spatial relationship between the two components. Typically, 5 contrasts required.

**Structure Modeling**

• Model structures must fit data at all contrasts & SAXS data

Use scattering from individual components as constraints if available.

# Contrast variation software

**MULCh: two component complexes**

**Rg, Contrast and compost** modules

[Whitten et al. (2008), J Appl Crystallogr 41:222–226]

http://smb-research.smb.usyd.edu.au/NCVWeb/

**SASSIE: (unlimited nr of components)**

[Curtis, J.E., Raghunandan, S, Nanda, H. and Krueger, S. (2012), *Computer Physics Comm.* **183**, 382-389; Sarachan, K.L., Curtis, J.E. and Krueger S. (2013), *J. Appl. Crystallogr.* **46**, 1889-1893]

http://www.smallangles.net/sassie/SASSIE/SASSIE_HOME.html

http://sassie-web.chem.utk.edu/sassie2/

**Contrast calculator:** I(0) analysis to plan experiments from sequence information or from coordinate (PDB) files.

**SasCalc:** Calculates theoretical SANS curves from all-atom structures taking contrast and H-D exchange into account.

# A typical SANS experiment – Practical considerations

**Typical sample volumes:** 200-300 µL

**For a labelled complex and a contrast variation experiment:**

Concentrations ≥ 5 mg/mL
Total of 1-1.5 mL solution

**Because of incoherent scattering:**

- samples with more $H_2O$ require higher exposure times for better statistics after solvent subtraction
- samples with less than 50% $D_2O$ may need higher concentrations
- typical sample thickness 1 mm

**Perform consistency checks before modeling:**

- Is there a valid linear Guinier region ($qR_g < 1$)?
- Calculate the $M_w$ from I(0). Is it reasonable?
- Does $R_g$ change with $D_2O$ content of buffer*?

*Complementary SAXS measurements

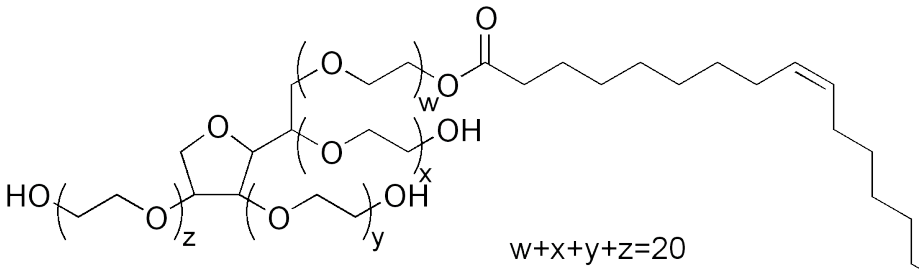
Krueger, S. (2017). Designing and Performing Biological Solution Small-Angle Neutron Scattering Contrast Variation Experiments on Multi-component Assemblies. In: Chaudhuri, B., Muñoz, I., Qian, S., Urban, V. (eds)

***Biological Small Angle Scattering: Techniques, Strategies and Tips.*** Advances in Experimental Medicine and Biology, vol 1009. Springer, Singapore. https://doi.org/10.1007/978-981-10-6038-0_5

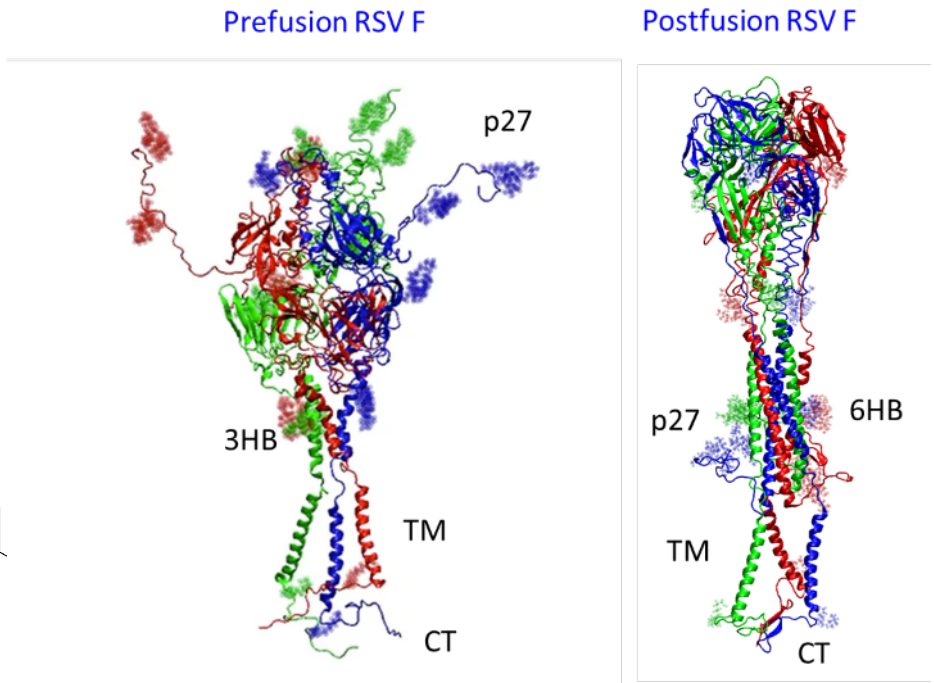
# Example 1 – a contrast variation study | RSV Nanoparticle Vaccine: Protein-PS80 Complex

[Krueger S, Curtis J, Scott D, Grishaev A, Glenn G, Smith G, Ellingsworth L, Borisoz O, Maynard E (2021). *Mol. Pharm.* 18, 359-376]

Nanoparticles represent a powerful and versatile platform for development of vaccines to combat deadly pathogens such as respiratory syncytial virus (RSV), influenza, and recently, SARS-CoV-2.




Polysorbate 80 (PS80): $C_{64}H_{124}O_{26}$
Mw ~ 1310 Da




Prefusion and Postfusion RSV F trimer structures Mw ~ 180 kDa

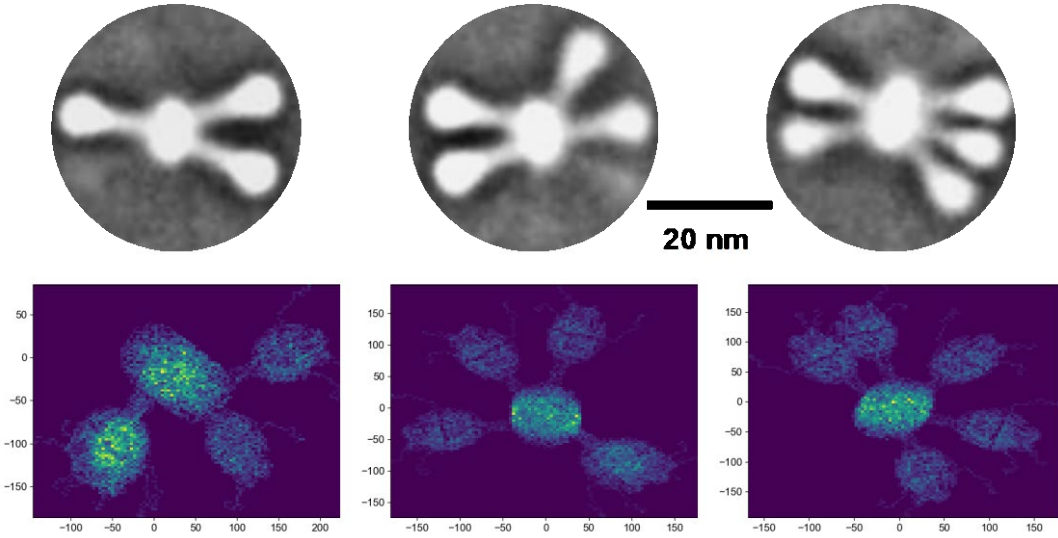
# Example 1 – a contrast variation study: why SANS?

[Krueger, S. *et al*., *Molecular Pharmaceutics* 18, 359-376 (2021)]

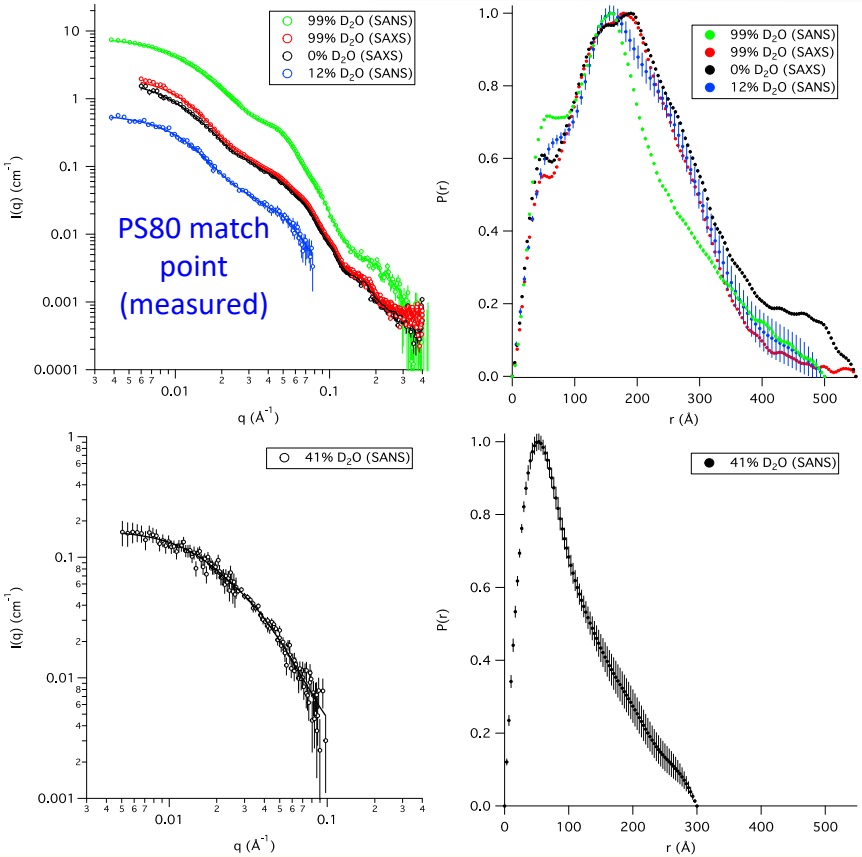
- Nanoparticle vaccine structures are challenging due to the heterogeneity of the membrane protein−detergent complex where the detergent mass may equal or exceed that of the protein.

- Atomistic structures are only partially available from X-ray crystallography, where the structure was manipulated to stabilize crystals

- TEM studies require extensive manipulation, including dilution prior to staining, drying and fixing on a grid: is the general morphology meaningful? **What is the stoichiometry and arrangement of the RSV trimers and PS80 in solution?**



# Example 1 – RSV Nanoparticle Vaccine | SAXS and SANS data




[Krueger, S. *et al.*, *Molecular Pharmaceutics* 18, 359-376 (2021)]

## Monodispersity

(1) DLS/AUC

Particle size as a function of RSV F:PS80 ratio could be optimized for SAS studies.

## Knowing your sample

(3) Phosphate Binding Assay

Indicated all PS80 was bound to RSV F with no free PS80 in solution.

# Example 1 – SV Nanoparticle Vaccine: Protein-PS80 Complex | stoichiometry determination

[Krueger, S. *et al.*, *Molecular Pharmaceutics* 18, 359-376 (2021)]

$$n = \frac{CMw_{total}}{N_A} \quad \text{where} \quad Mw_{total} = Mw_1 + Mw_2 \quad \text{(molar masses aka molecular weights)}$$

At each contrast: $$\frac{I(0)}{C} = \frac{Mw_{total}}{N_A}(f_1\Delta\rho_1\bar{v}_1 + f_2\Delta\rho_2\bar{v}_2)^2$$

where $$f_1 = \frac{Mw_1}{Mw_1 + Mw_2}$$ (mass fraction)

Knowns:

$\Delta\rho_1, \Delta\rho_2$: contrast for components 1 and 2

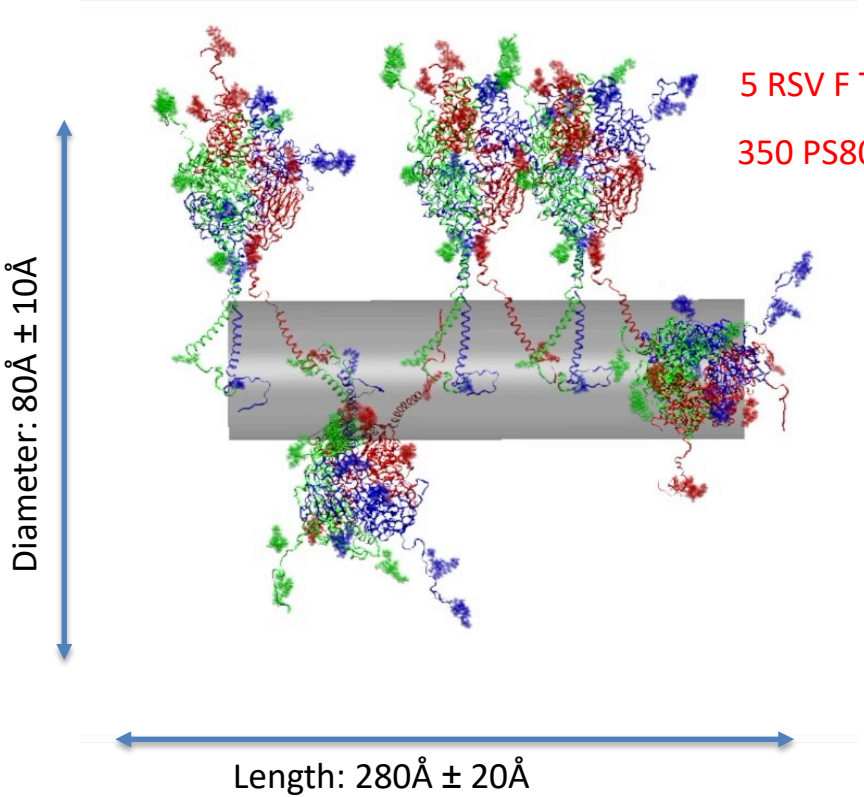
$\bar{v}_1, \bar{v}_2$ : partial specific volume for components 1 and 2

I(0): measured I(0) value

C: measured total concentration of the complex (protein + PS80)

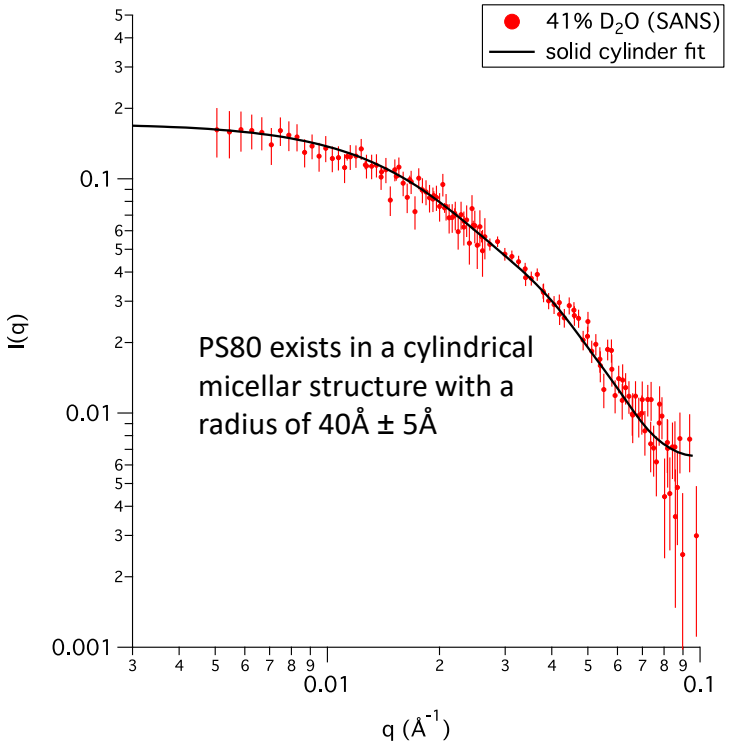
# Example 1 – SV Nanoparticle Vaccine| ensemble structure modeling

[Krueger, S. *et al.*, *Molecular Pharmaceutics* 18, 359-376 (2021)]




5 RSV F Trimers

350 PS80 molecules

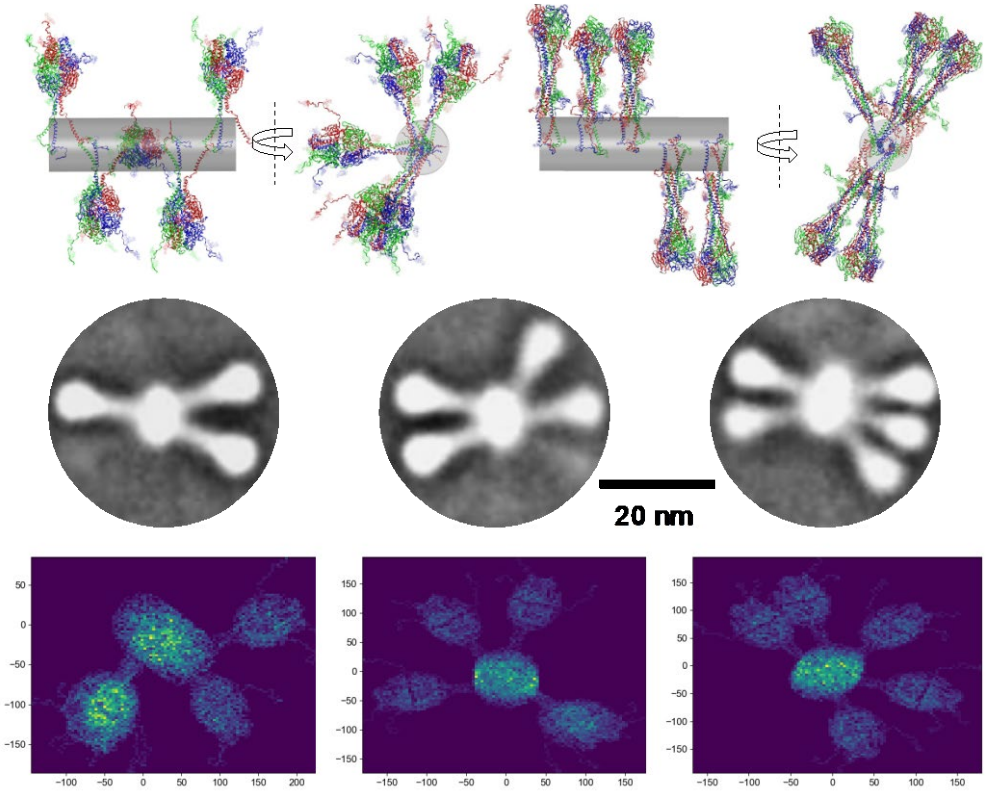


# Example 1 – a contrast variation study – ensemble structure modeling

[Krueger, S. *et al*., *Molecular Pharmaceutics* 18, 359-376 (2021)]

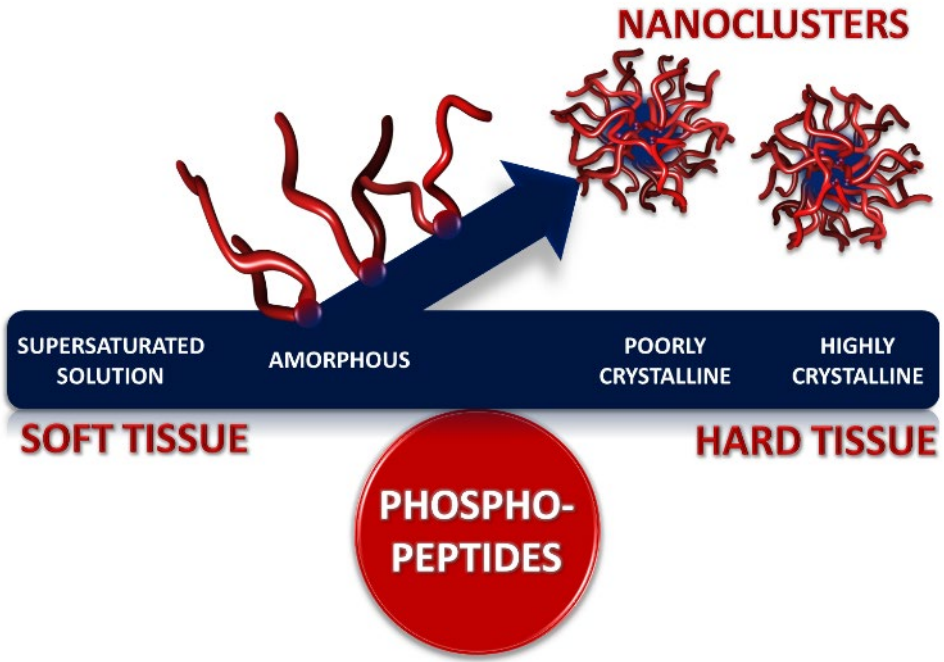
- The RSV F trimers exist as a distribution of prefusion and postfusion conformations and/or intermediate conformation(s), consistent with the broad immunogenic response elicited by the vaccine.

- The results suggest a mechanism of 1-D growth along the axis of the PS80 core with the ultimate size controlled by the RSV F:PS80 ratio.



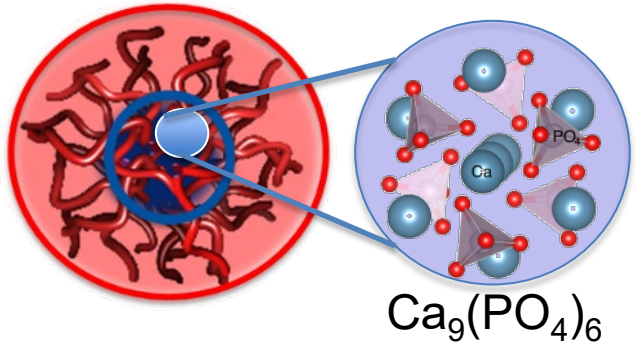
# Example 2 – Solvent matching: Biomineralization requires a delicate balance

See review: Lenton, S.; Wang, Q.; Nylander, T.; Teixeira, S.; Holt, C. *Crystals* **2020**,*10*, 755.




Secreted Calcium Phosphate-binding Phosphoproteins (SCPPs) are involved in every aspect of Biomineralization. Examples:

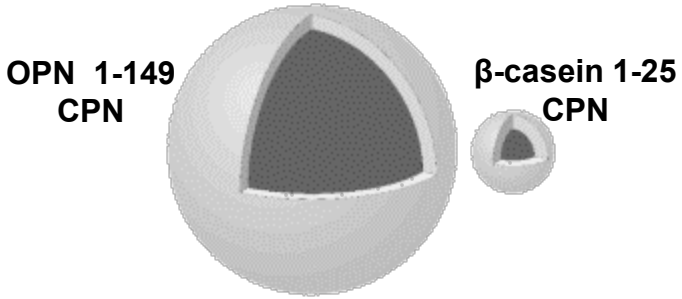
- **Ostepontin (OPN)** ubiquitous in species, tissues and biofluids
- **Caseins** known to stabilize colloidal calcium phosphate in milk



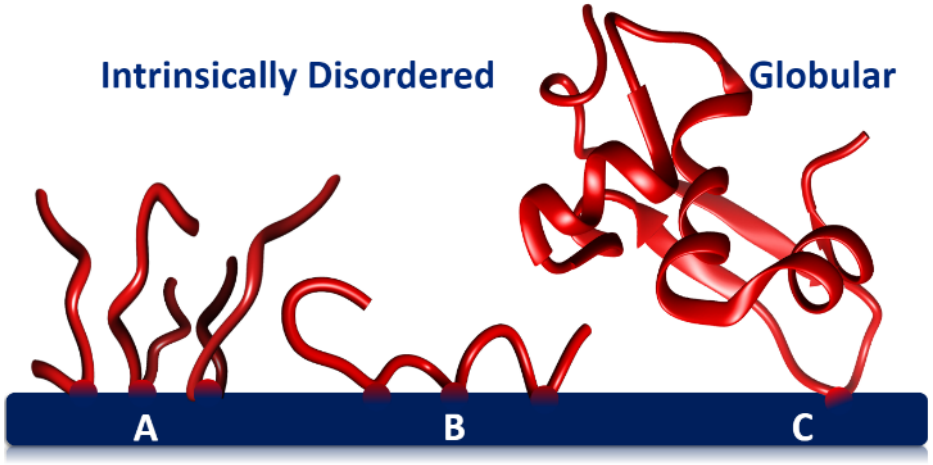

$Ca_9(PO_4)_6$

# Example 2 – Solvent matching: Biomineralization and IDPs

Lenton S., Nylander T., Holt C., Sawyer L., Haertlein M., Mueller H., Teixeira S. (2016). *Eur Biophys J.* ***45***:*405.*




| | <Core Ø> | <Shell> |
|---|---|---|
| OPN 1-149 | ≈ 35 nm | 1.5 nm |
| β-casein 1-25 | ≈ 4.8 nm | 1.6 nm |
| Biological apatite crystals: | few to hundreds of nm | |
| Posner Clusters: | 0.9 nm | |




**There is a preponderance of IDPs in biocalcification due to several key properties:**

**A:** packing advantages

**B:** higher density of PCs to modulate crystal nuclei growth

# Example 2 – Solvent matching: Biomineralization and IDPs

**Before the experiment:**

- predict contrast values and match points based on chemical composition (primary structure, calcium phosphate composition)
- test protein solubility and stability in $D_2O$ buffers
- deuterate one of the components if necessary (use MS to determine final % deuteration)

**Preliminary data:**

- measure the match point of the protein
- ensure no effects from the use of $D_2O$ (SAXS)

**Why SANS was chosen:**

- Non-destructive nature of neutrons allowed for repeat measurements of the same sample (with the same or complementary techniques)
- Natural contrast between complex components due to SLD difference
- No sample preparation artifacts: SANS is a non-invasive tool to investigate the structural properties and stability of multi-component complexes such as CPN

# Example 3. mAb stability: what happens to proteins when we freeze them?

SUSTAINABILITY


Over 70% of Earth is under pressure.



Pressure increasingly used in Biopharma and Food processing

Monitoring vaccine wastage at country level. Immunization, Vaccines and Biologicals. WHO/V&B/03.18. Rev.1.

Maximally freeze concentrated solution
($T_g' \leq -35°C$)

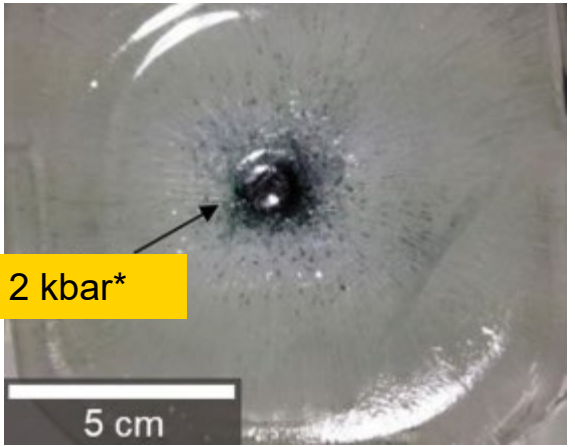
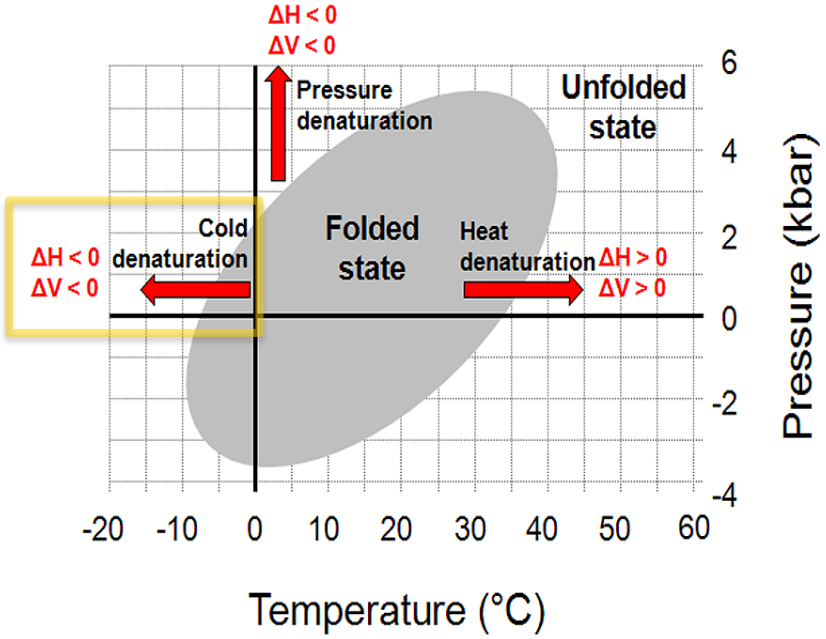



Image adapted from Hauptmann et al. (2019). *AAPS PharmSciTech* **20**: 72.

*Authelin *et al.* (2020). *J. Pharm. Sci*. **109**(1), 44.

Cryo-concentration, pH shifts, pressure, crystallization of excipients, ice/liquid interface

# Example 3. mAb stability: pressure, low T and high T can denature a protein




$$\Delta G = \underline{\Delta H} - T\Delta S$$
$$= \underline{\Delta U + P \cdot \Delta V} - T\Delta S$$

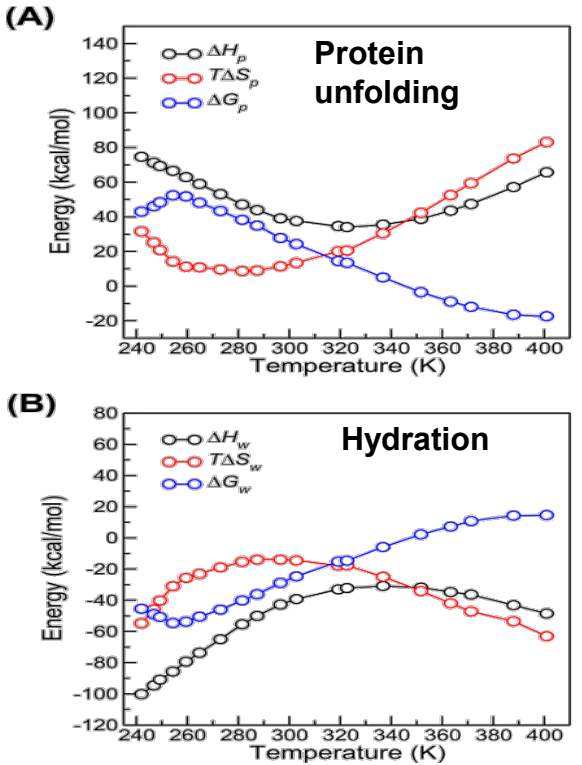
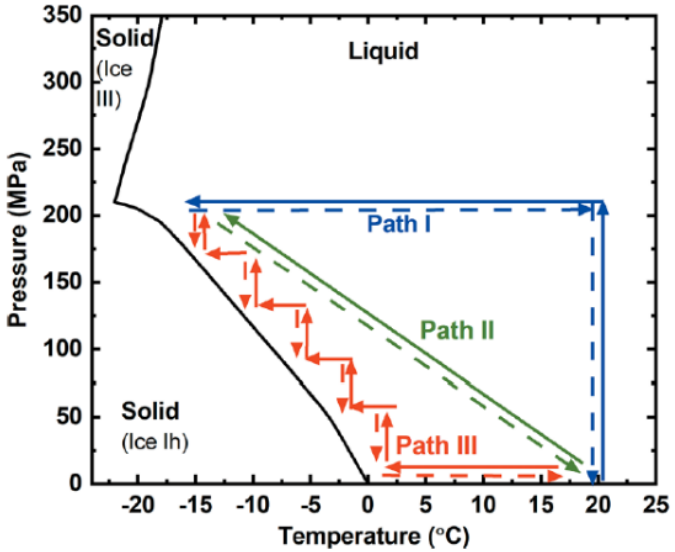
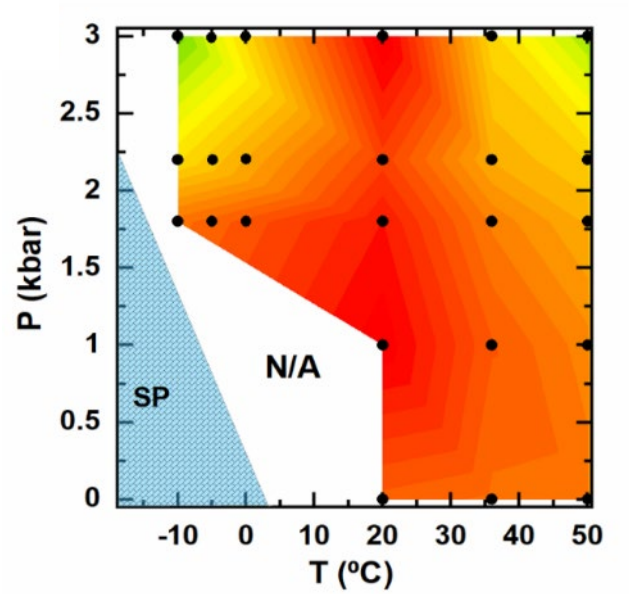


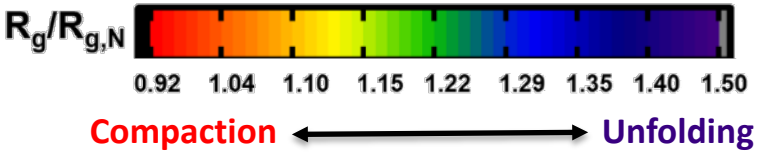

Image adapted from: Yang *et al* (2021). *J. Chem. Theory Comput*. **17**, 515.

# Example 3. Using High-pressure SANS to study mAb stability

Gomes D, Teixeira S, Leao J, Razinkov V, Qi W, Rodrigues M, Roberts C (2021). *Mol. Pharm.***18**(12), 4415.




**5 mg/mL AsIgG1, ≈ 150 kDa**;
5 mM citrate buffer pD 4.0. $T_{CD}$ < -20 °C; pI ≈ 8.6

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D. Gomes
(Lisbon/Delaware)

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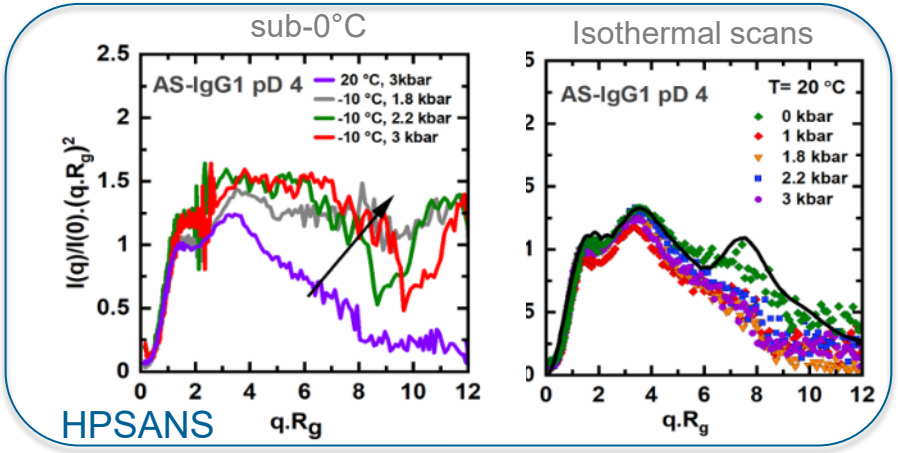
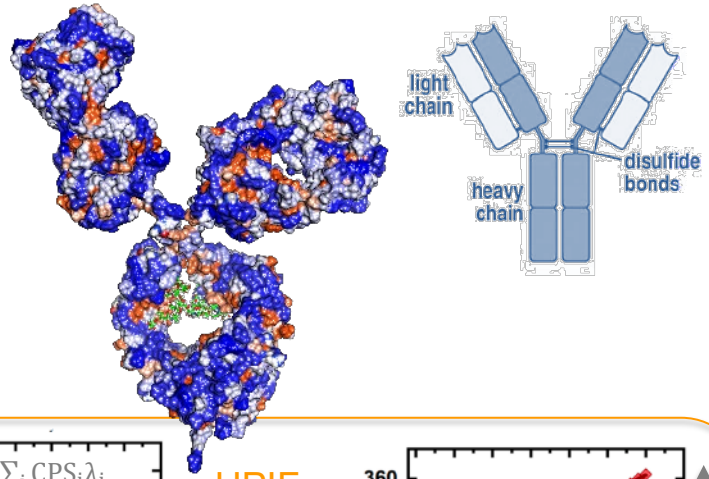
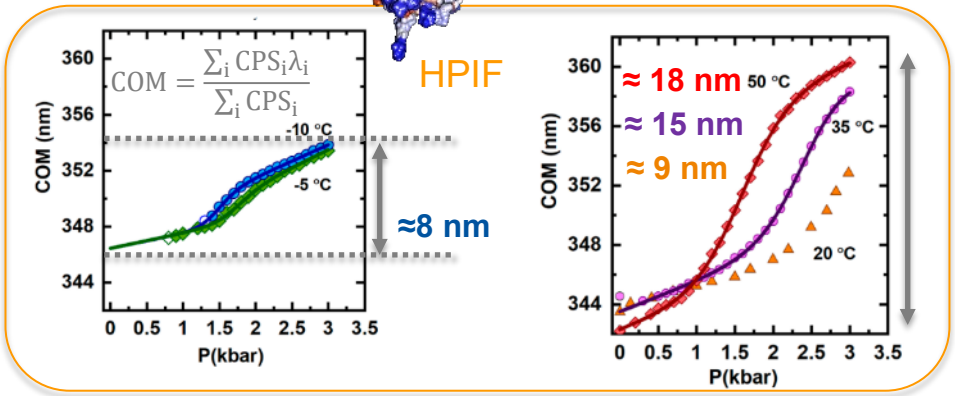



| $T$ (°C) | $\Delta G_{un}$ (kJ/mol) | $\Delta V_{un}$ (mL/mol) | $P_{1/2}$ (kbar) |
|---|---|---|---|
| 50 | 14 (±2) | − 86 (±13) | 1.59 (±0.05) |
| 35 | 22 (±3) | − 89 (±13) | 2.50 (±0.1) |
| −5 | 23 (±5) | − 135 (±30) | 1.70 (±0.06) |
| −10 | 21 (±5) | − 144 (±37) | 1.47 (±0.03) |



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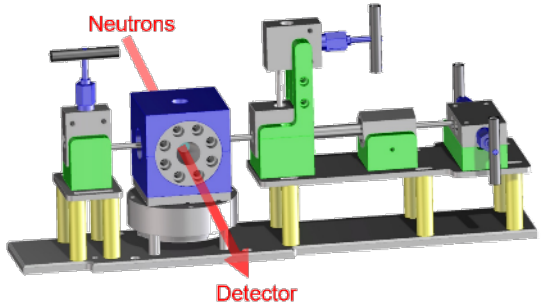
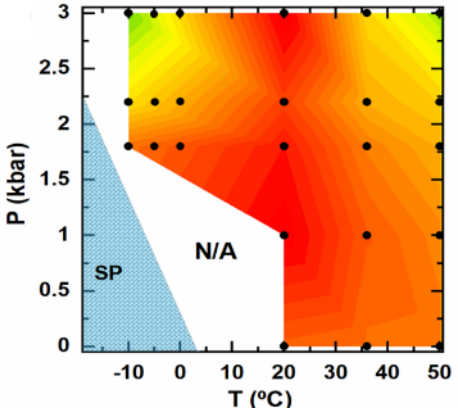



| | |
|---|---|
| Temperature: | -20° to +65°C |
| q-range: | 0.001-0.3 Å$^{-1}$ |
| Concentration | >5 mg/ml |

**https://www.nist.gov/laboratories/tools-instruments/hp-biosans-lipss**

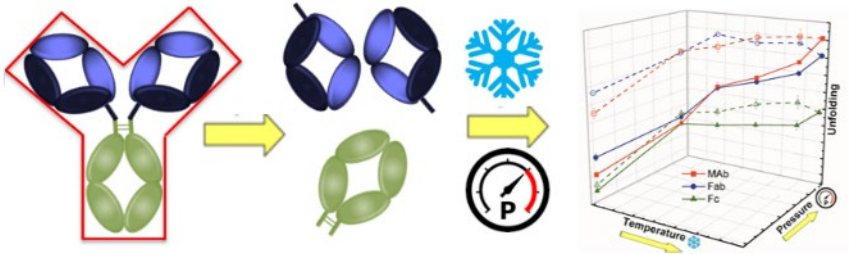



**HP-SANS advantages:**

- No radiation damage
- No protein size limitation
- Access to subzero T in the absence of ice
- Contrast variation

**Disadvantages:**

- larger sample volumes (2.5-5ml)
- slow throughput (use spectroscopies and HP-SAXS first when possible)




*Berger et al. (2022). J. Phys. Chem. B* 126, 24, 4431–4441

# Conclusions & Where can I apply for beamtime

SANS experiments are low throughput (compared to SAXS) an may require isotope labeling (deuteration): SANS should only be used to provide information no other technique can access.

Typical applications of Bio-SANS:

- Rheology
- Studies at various T and P (e.g. freeze-thaw, lyophilization, accelerated degradation studies)
- Studies of Halophiles (high-salt)
- Contrast variation studies
- Studies of radiation-sensitive samples

SAXS and SANS are complementary techniques ☺

https://www.ncnr.nist.gov/nsources.html

**North America**

- NIST Center for Neutron Research
- Oak Ridge Neutron Facilities (SNS/HFIR)
- Los Alamos Neutron Science Center (LANSCE)
- University of Missouri Research Reactor Center
- Indiana University Cyclotron Facility

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https://www.nist.gov/ncnr/chrns/education-and-outreach

SURF, CORE & Summer School

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