Planning and Performing SAXS Experiments

Everything BioSAXS 5
Getting Started in Biological Small-Angle X-ray Solution Scattering
Tuesday 11/5/19

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Suggested Reading

- Sample and Buffer Preparation for SAXS. Graewert MA, Jeffries CM. Adv. Exp. Med. Biol 2017; 1009:11-30. doi: 10.1007/978-981-10-6038-0_2 PMID: 29218551

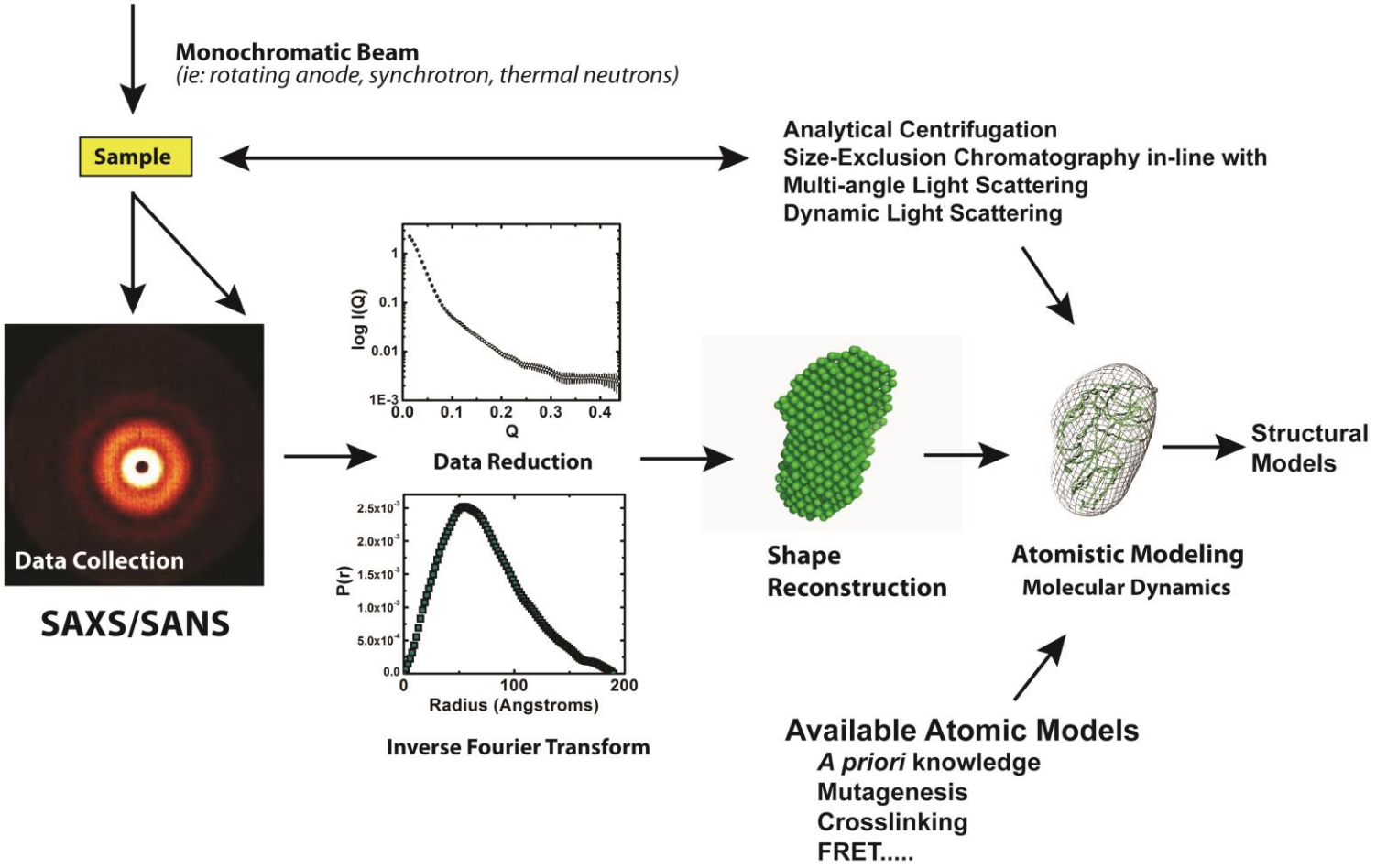
- Considerations for Sample Preparation Using Size-Exclusion Chromatography for Home and Synchrotron Sources. Rambo RP. Adv. Exp. Med. Biol 2017; 1009:31-45. doi: 10.1007/978-981-10-6038-0_3 PMID: 29218552

- Preparing monodisperse macromolecular samples for successful biological small-angle X-ray and neutron-scattering experiments. Jeffries CM, Graewert MA, Blanchet CE, Langley DB, Whitten AE, Svergun DI. Nat Protoc. 2016 Nov;11(11):2122-2153. doi:10.1038/nprot.2016.113. Epub 2016 Oct 6. PMID: 27711050

- Synchrotron-based small-angle X-ray scattering of proteins in solution. Skou S, Gillilan RE, Ando N. Nat Protoc. 2014 Jul;9(7):1727-39. doi: 10.1038/nprot.2014.116. Epub 2014 Jun 26. PMID: 24967622

- Small-angle scattering for structural biology--expanding the frontier while avoiding the pitfalls. Jacques DA, Trewhella J. Protein Sci. 2010 Apr;19(4):642-57. doi:10.1002/pro.351. Review. PMID: 20120026

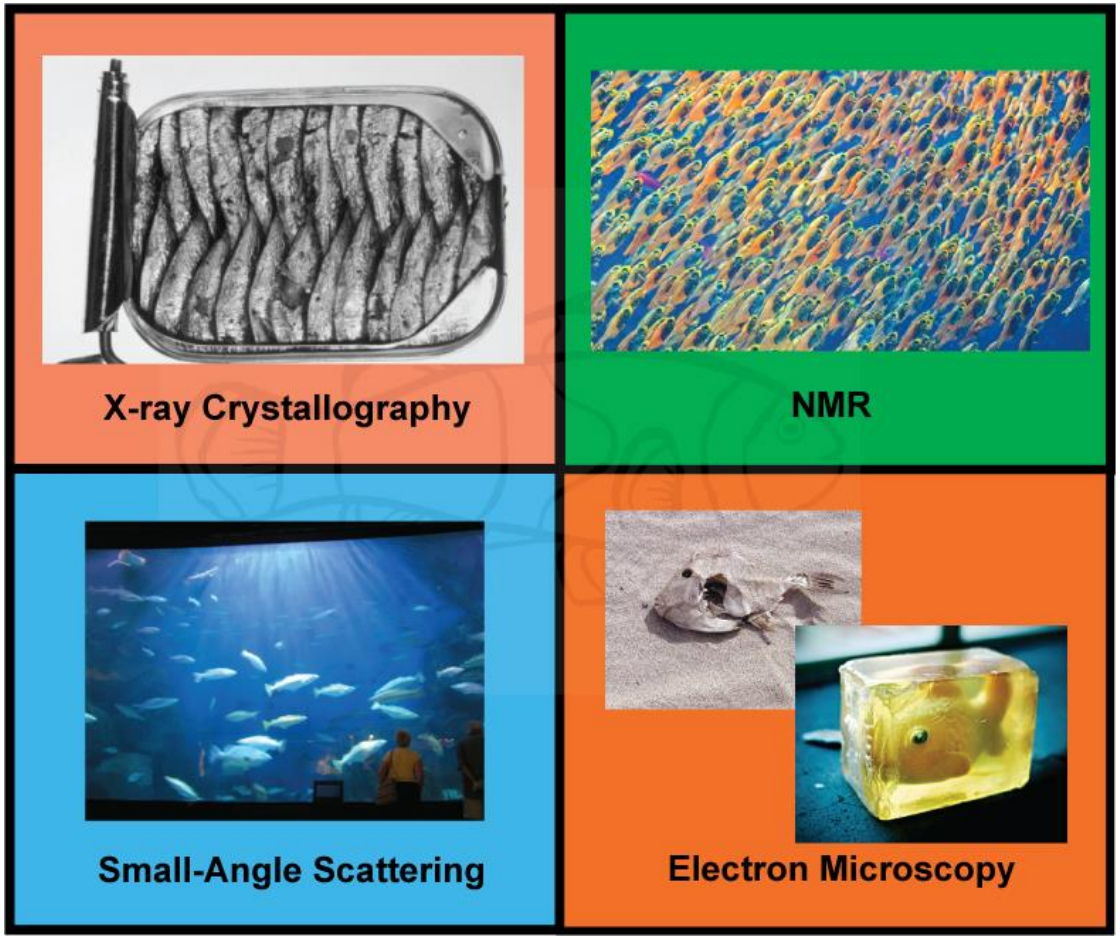
Small-angle X-ray and Neutron Scattering (SAXS/SANS) as part of an integrative approach to structural biology



Planning and Performing Your Experiment

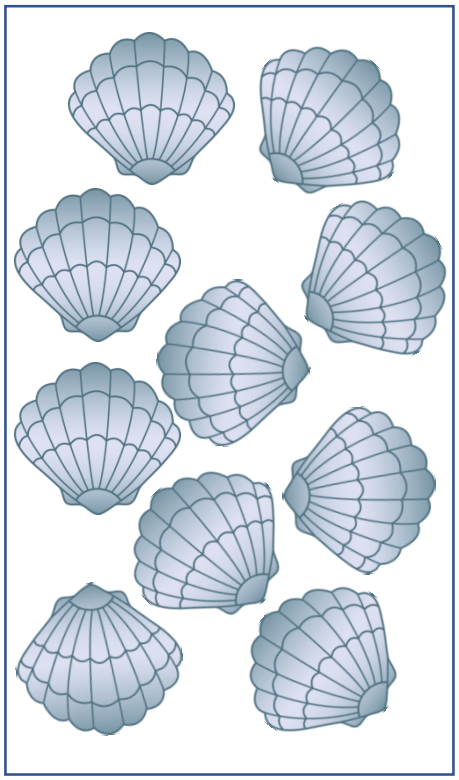
- **Effects of Polydispersity on SAS**
- Design Your Experiment
- Sample Purity
- Sample Quantities and Concentration
- Sample Environment (Buffer)
- Characterization: Methods to Assess Polydispersity
- At the Beamline

Small-angle X-ray and Neutron Scattering (SAXS/SANS) as part of an integrative approach to structural biology



Purity and Monodispersity is important for Small-angle scattering!

Incident Beam



Homogenous, Rigid Species

- With current best practices, the resulting scatter can be readily interpreted.

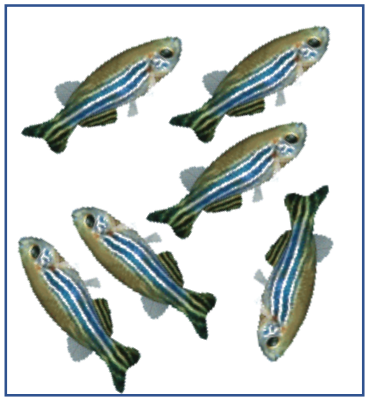
$$I(s) \approx FF(s)$$

e.g. MBP
Lysozyme
Glucose Isomerase
Cytochrome C
Isolated Domains....

Purity and Monodispersity is important for Small-angle scattering!

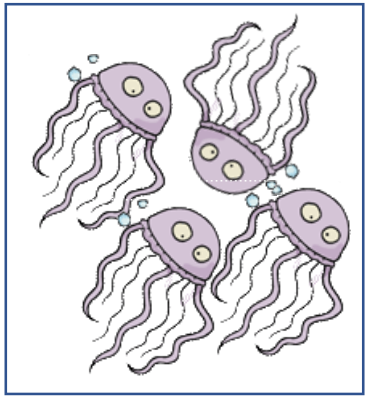
Discrete Conformers

Incident Beam



Intrinsic Disorder

Incident Beam



The resulting scatter is a volume fraction-weighted average of the contributions of the individual components in solution.

$$I(s)=\sum_k v_k I_k(s)$$

Purity and Monodispersity is important for Small-angle scattering!

Heterogeneity

Incident Beam

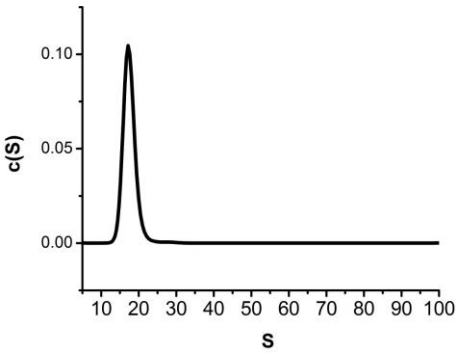
The resulting scatter is a volume fraction-weighted average of the contributions of the individual components in solution.

$$I(s)=\sum_k v_k I_k(s)$$

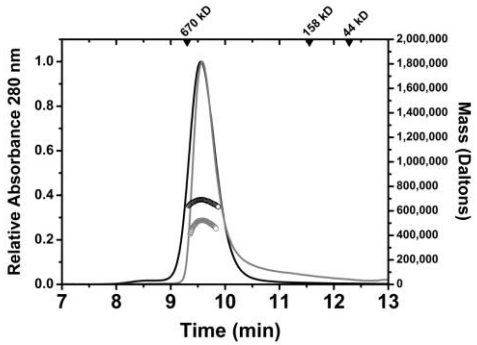
A priori Knowledge and Model-Independent Analysis is key to the reliable interpretation of SAS data

Sweeny EA, Jackrel ME, Go MS, Sochor MA, Razzo BM, DeSantis ME, Gupta K, Shorter J. The Hsp104 N-terminal domain enables disaggregase plasticity and potentiation. Molecular Cell (2014)

Sedimentation Velocity



SEC-MALS



- Purity
- Activity
- Concentration-dependent self-association and aggregation; stoichiometry
- Buffer conditions
- Available structural information

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Design: Construct Design

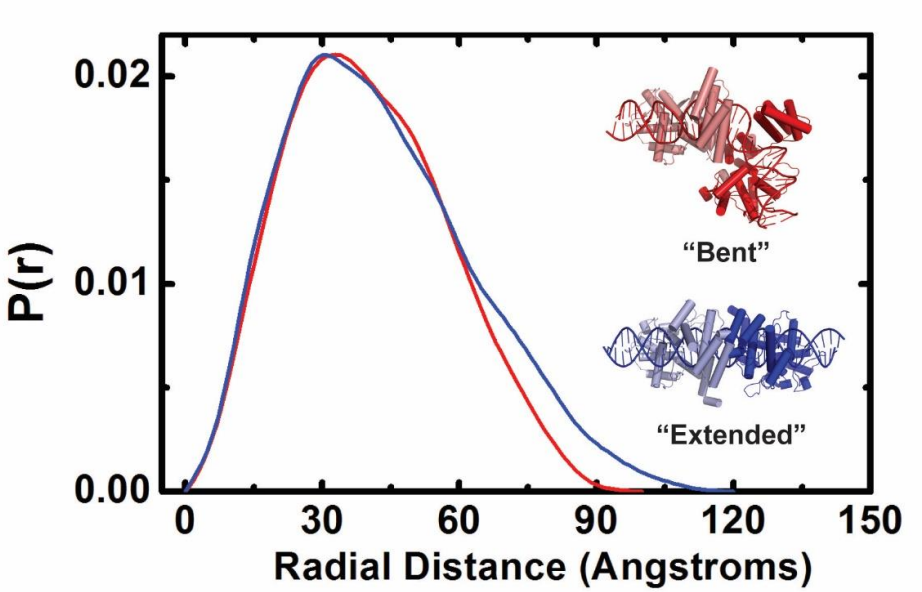
Full-length protein

Available Atomic Structures

- ❑ What is the structure of the missing inventory? Crystal Structures? NMR? Homology Models?
- ❑ Is there Predicted Intrinsic Disorder? Experimental? Disorder-to-Order Transitions? Dynamic Behavior?
- ❑ Are there cofactors?
- ❑ Is the protein soluble? Stable over extended time?
- ❑ Does the protein have post-translational modifications?
- ❑ Are there intramolecular disulphides?
- ❑ How do you remove the purification fusion partner? Cloning artifacts?
- ❑ Can you make milligram quantities in a recombinant expression system or from an endogenous source?

Design: Make Predictions

Example: Cre Recombinase-*loxP*



- ❑ Create Models to Test
- ❑ SAS is generally better at telling what something isn't rather what something is!
- ❑ Great for Beam Time Proposals!

Be Hypothesis-Driven

Design: Make Predictions

Biophysical Properties:

- *Theoretical R_gs, Volumes*
  - *Classical relationships between composition and shape of biopolymers*
- *HYDROPRO*
  - Predict R_g, R_s, S, f/f_o from a crystallographic structure
- SOMO and HYDROPRO
  - bead models
- *SEDFIT, Heteroanalysis, SOMO*
  - Theoretical AUC data, Ellipsoids

Scattering Properties:

- *CRYSOL, CRYSON, Xtal2SAS, FoxS, AXES, WAXIS (and many more)*:
  - Predict Scattering profiles from an atomic structure; create P(r) functions.
- *Molecular Dynamics and Modeling*
  - What type of structures do you expect?
- *MulCH and SASSIE (SCC)*
  - Contrast Calculations

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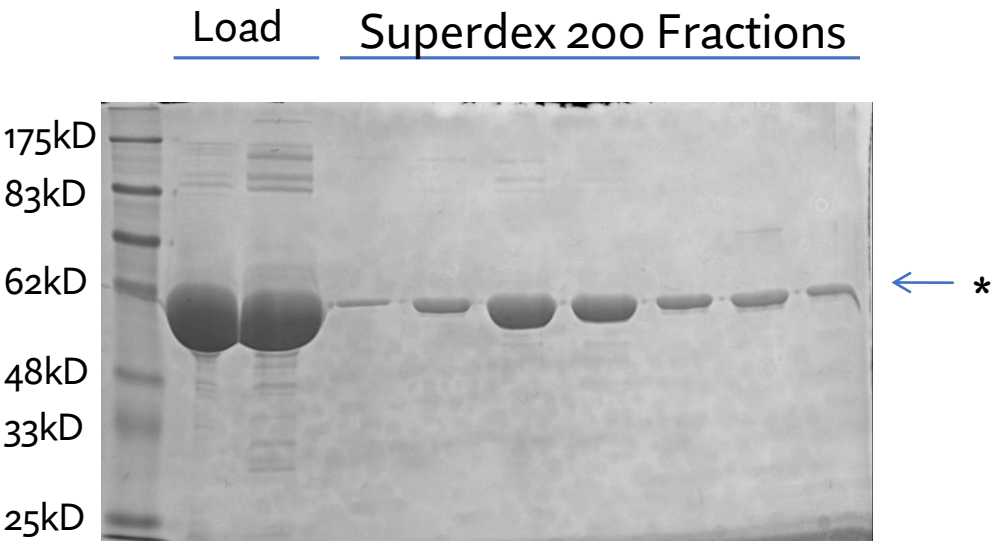
Sample Purity



12.5% SDS-PAGE
Acidic Commassie Stain

- DNA/RNA
  - Gel-purified or HPLC-purified
  - Careful annealing and further purification
  - Ratio 260/280
  - Salts from Purification
- Protein
  - "crystallography grade"
  - >98% purity by SDS-PAGE or Silver Stain
- Ratio 260/280
  - Nucleic Acid Contamination?

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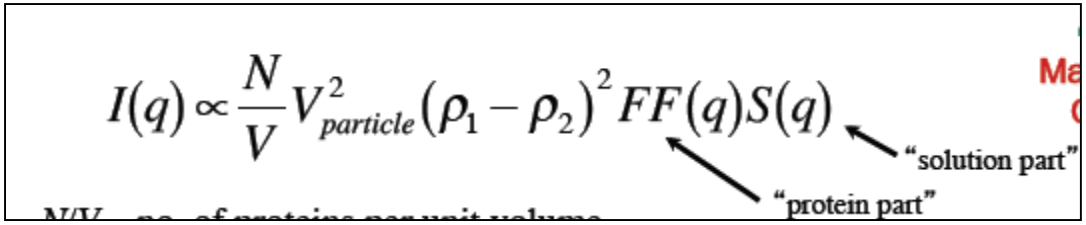
Sample Quantities and Concentration



"The Master Equation"

- What concentration to scatter?
  - Intensity of scattering is proportional to concentration and mass.
  - Series of concentrations are analyzed
  - Interparticle Interference and Aggregation!
  - Generally, 1-10 mg/mL concentrations; much less for big things
  - Rule of Thumb: MM (kD) * conc (mg/mL) ~ 100
- Synchrotron Data Collection
  - Most synchrotrons use capillaries – 30-80 µLs material/exposure
- Small-angle Neutron Scattering
  - "banjo cuvettes" – 350-700 µLs x several samples
  - Multiple contrast points

Perelman School of Medicine University of Pennsylvania

Sample Quantities and Concentration

Table 2.1 Rough estimation of sample requirements and amounts

Experiment	Sample amounts/ volumes[a]	Duration per measurement[b]	Comments
Lab source	20–50 µl; >2 mg/ml	15–90 min; depending on type of lab source	Less sensitive to radiation damage, but check in advance that samples will be stable over the time of the experiment
Synchrotron (proteins)			
No flow	5–20 µl; >0.5 mg/ml	Approx. 1–5 min[c]	Radiation sensitive
Flow	20–50 µl; >0.5 mg/ml	Approx. 1–5 min[c]	Less sensitive to radiation damage at the cost of more sample
SEC-SAXS	50–100 µl; >5 mg/ml	10–90 min; depending on column	Strong dilution of the sample
			Capillary fouling can occur during the elution/X-ray exposure process
Synchrotron (nucleic acids)	5–50 µl; >0.25 mg/ml	Approx. 5 min[c]	Take difference of electron density compared to protein into account for MW calculations
			If measuring RNA, ensure Rnase–free environment

[a]For complete data set concentration series (at least four different concentrations) should be measured
[b]Includes measurement of sample and buffer
[c]Includes automated washing of the measurement cell

Sample Quantities and Concentration

Extinction Coefficients

- Theoretical ones can be unreliable by upwards of 5-10%
- Consider experimentally determined figure using denatured material

> (see *Calculation of protein extinction coefficients from amino acid sequence data Stanley C. Gill, a and Peter H. von Hippel Analytical Biochemistry Volume 182, Issue 2, 1 November 1989, Pages 319-326*)

- Dnase-1 Digestion of Oligonucleotides

Bradford Assays and other colorimetric protein assay kits

- Can vary by as much as 5-10%
- Do multiple concentrations!
- Work within your assay range!

Complementary measures of mass:

- I(o) from SAXS
- SEC-MALS analysis (RI)
- FT-IR

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- **Sample Environment (Buffer)**
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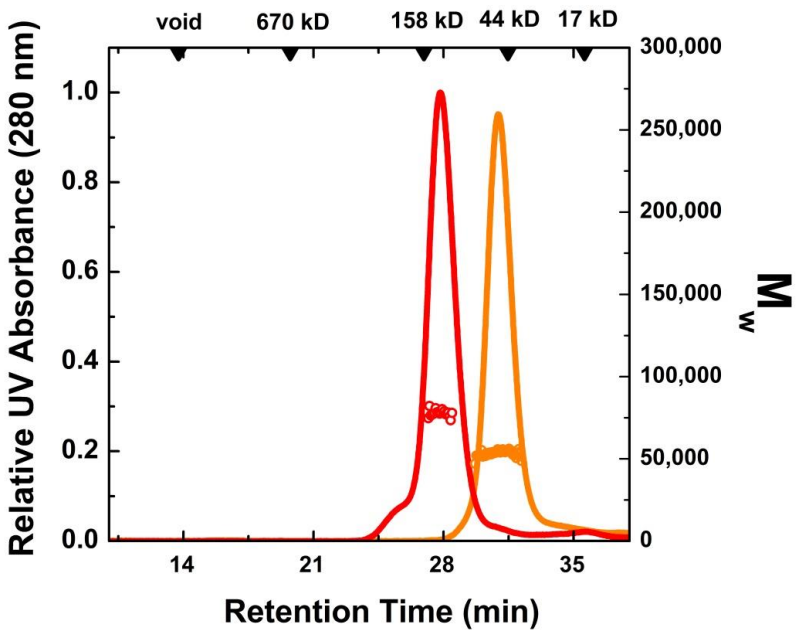
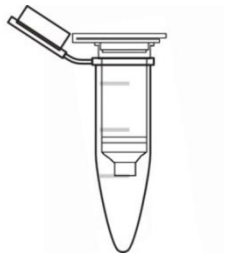
Sample Environment (Buffer)





- ❑ Fresh Samples
- ❑ Final Gel Filtration Step
  - ❑ Isolate Half-peak
  - ❑ Use sizing buffer as match
  - ❑ If needed, concentrate at 4°C and use flow-thru as match buffer.
- ❑ Use concentrator for extensive buffer wash
  - ❑ use flow-thru as match buffer.
- ❑ Additional Dialysis – use Fresh DTT
- ❑ Don't make matches by hand!
- ❑ *SEC-SAXS*

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Characterization: Many Methods Available

- Bioinformatics – Intrinsic Disorder, Normal Mode Analysis, Charge-Hydropathy
- Specific Activity – *in vitro* activity assays
- SDS-PAGE and Native PAGE
- Mass Spectrometry (MALDI, LC/MS/MS, etc.)
- Circular Dichroism (CD) , Infrared Spectroscopy (FTIR)
  - Secondary Structure and Folding
- FRET, Crosslinking, etc.
  - intra and intermolecular restraints
- Microscale Thermophoresis (MST)
  - Binding affinities and cooperativity
- Isothermal Titration Calorimetry (ITC)
  - Binding affinities and cooperativity
- Surface Plasmon Resonance (SPR)
  - Binding affinities and cooperativity
- Differential Scanning Calorimetry (DSC)
  - Binding affinities and cooperativity

- Dynamic Light Scattering (DLS)
  - D_t, R_h, Derived Mass
- Sedimentation Velocity (SV-AUC)
  - S value, f/f_o
- Sedimentation Equilibrium (SE-AUC)
  - Buoyant Mass, K_d of association
- Size-Exclusion Chromatography (SEC)
  - R_h, Apparent Mass
- Size-Exclusion Chromatography in-line with Multi-angle Light Scattering (SEC-MALS)
  - R_h, Absolute Mass profiles
- Composition Gradient Multi-angle Light Scattering (CG-MALS)
  - Mass, K_d of association

Characterization: Light Scattering Methods

Technique	Acronym	Radiation	Information
Rayleigh Light Scattering	LS	Visible Light	Molecular Weight (Mw), Molecular Interactions
Angular-Dependent Light Scattering	MALS	Visible Light	Radius of Gyration (R_g)
Dynamic Light Scattering	DLS (or QELS)	Visible Light	Diffusion coefficient Hydrodynamic Radius (R_h)
Small-angle X-ray Scattering	SAXS	X-rays	Molecular Weight (M_w), R_g, Volume, Shape, Flexibility and Compactness, Mixture Analysis
Small-angle Neutron Scattering	SANS	Cold Neutrons	Molecular Weight (M_w), Rg, Volume, Shape, Flexibility and Compactness, Mixture Analysis, internal structure and compositional distribution

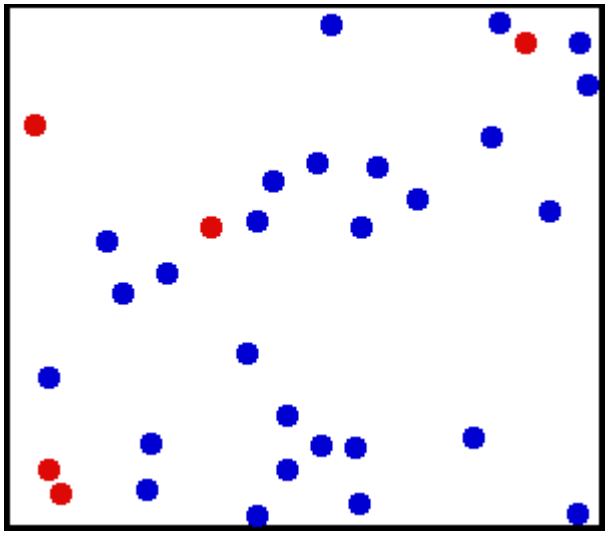
Advantages:

- ❑ No crystals or high sample concentrations needed – small sample quantities
- ❑ More rapid than crystallography and NMR
- ❑ Broadly applicable to all macromolecules of all shapes and sizes
- ❑ Few buffer restrictions – Physiological Buffers

Disadvantages:

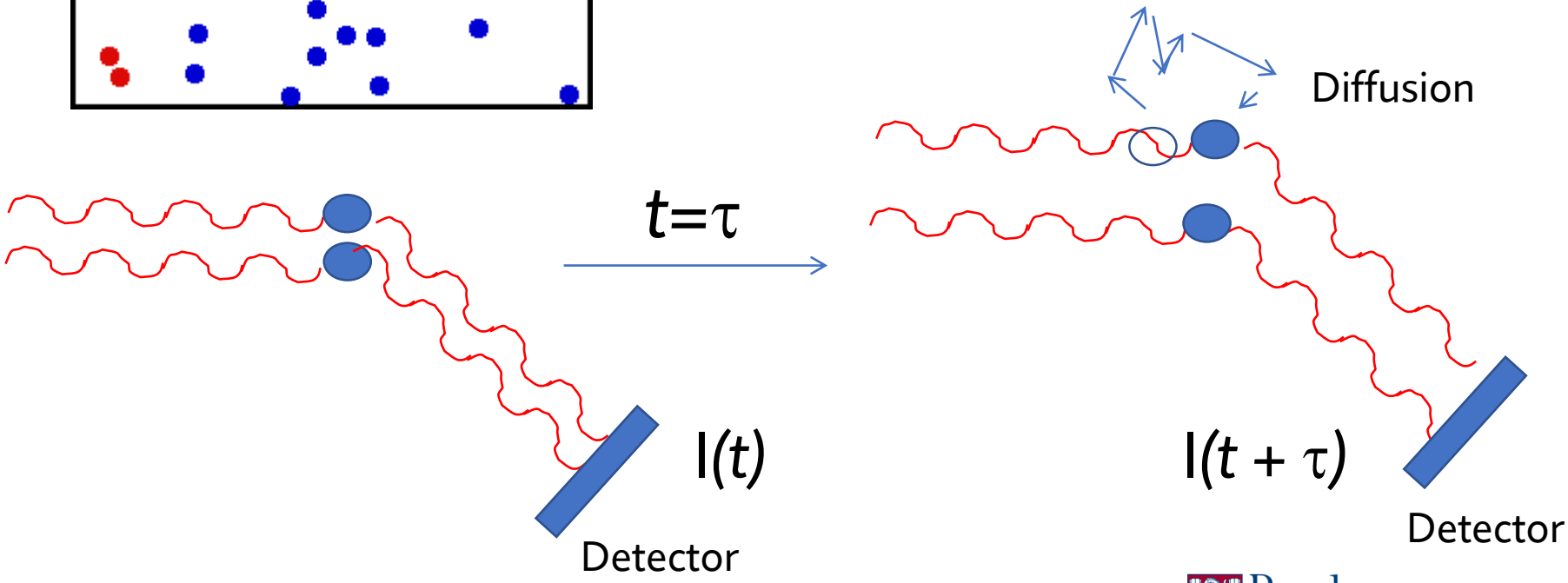
<u>Low Resolution</u>

Characterization: Dynamic Light Scattering



Molecules in solution undergo Brownian Motion, which constantly changes the distance between scattering centers.

The scattered light thus undergoes constructive/destructive interference, resulting in time-dependent fluctuations of the scattered intensity.



Characterization: Dynamic Light Scattering

The intensity of the light scattering recorded from a small volume at short time intervals shows fluctuations. The rate of such fluctuations is proportional to the diffusion coefficient (*D*) of the scattering molecules and, therefore, depends on the size and shape of the molecules and the viscosity of the solvent

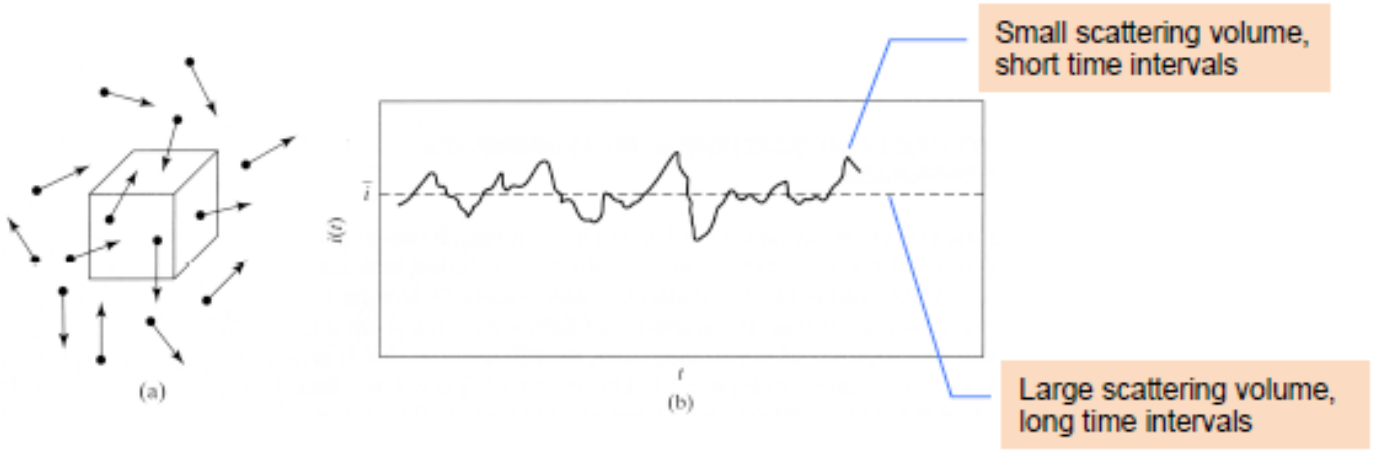


Figure 7.6 Fluctuations in light scattering are used to measure diffusion. (a) A small element of volume from which scattering is being recorded. The number of macromolecules within this volume fluctuates with time, due to their diffusional motion. (b) A recording of intensity fluctuations about the mean value, $\bar{i}$ (dashed line).

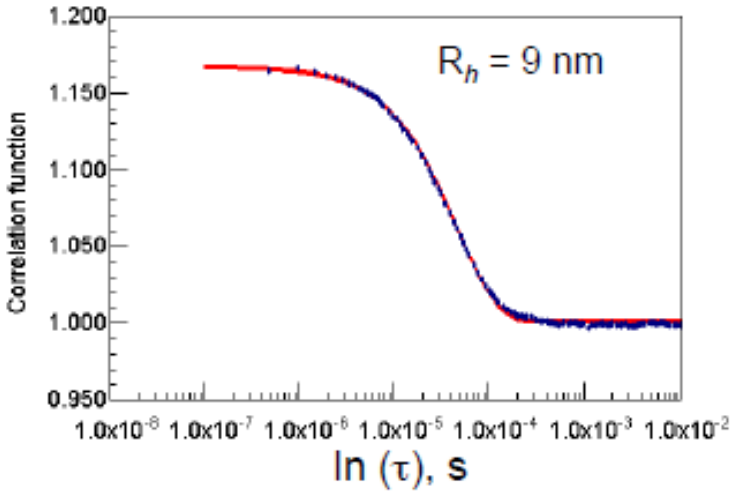
Characterization: Dynamic Light Scattering

Dynamic light scattering fluctuations are quantified via a second order correlation function, i.e. by comparing the light scattering intensities at times t and $t + \tau$

Second order correlation function $g^{(2)}(\tau) = \dfrac{\langle I(t) I(t+\tau) \rangle}{\langle I(t) \rangle^2}$

$\langle I(t) \rangle$ average scattered intensity over time t

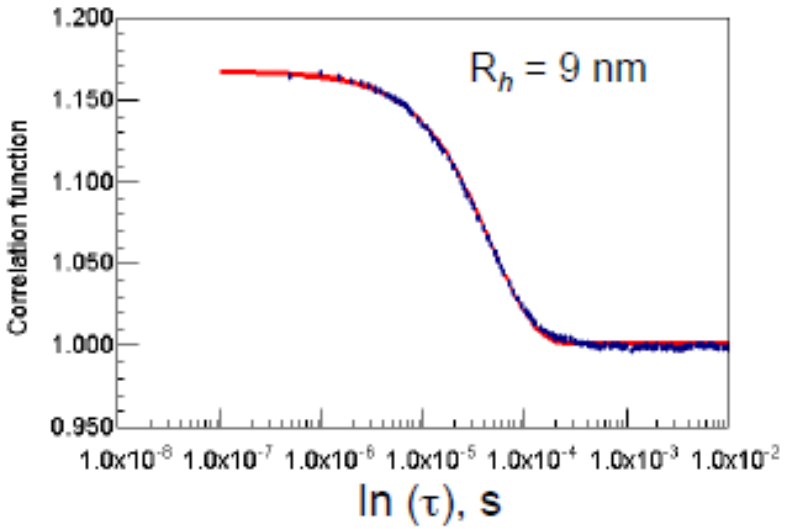
τ delay time (typically 0-1 ms)



Example – Second order correlation function of a monodisperse 9 nm particle. The correlation is maximum at short time delays, and decays exponentially with time.

Characterization: Dynamic Light Scattering

For a monodisperse sample, the second order correlation function can be expressed by the equation:



$$g^{(2)}(\tau) = B + \beta e^{-2\Gamma\tau}$$

Where:

- B baseline of the correlation function at infinite delay (or mean intensity value)
- β correlation function amplitude at zero delay
- Γ correlation function decay rate
- τ time delay

The Diffusion Coefficient is then calculated from the relation:

$$D = \frac{\Gamma}{h^2} \quad ; \text{ where } \quad h = \frac{4\pi n_0}{\lambda_0}\sin\left(\frac{\theta}{2}\right) \text{ is the scattering vector}$$

Characterization: Dynamic Light Scattering

Hydrodynamic Radius (R_H)

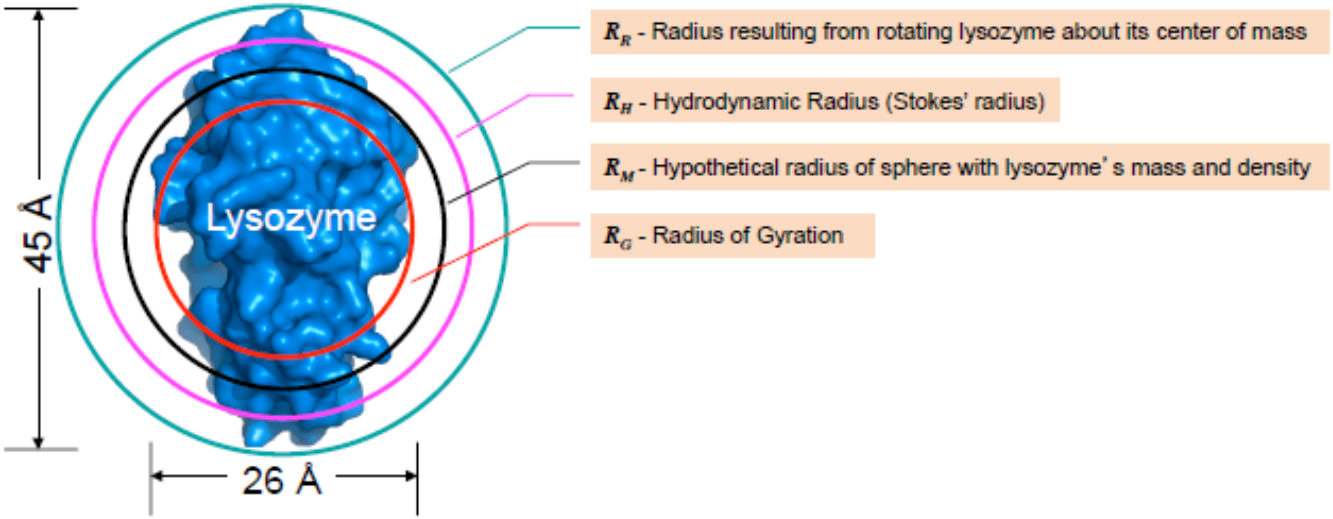
The most interesting measurable is the **hydrodynamic** (or **Stokes'**) **radius** (R_H) of a molecule, determined from the diffusion coefficient using **Stokes-Einstein's equation.** The R_H is the radius of a sphere with the same diffusion coefficient or viscosity as our sample.

$$R_H = \frac{k_B T}{6\pi\eta D}$$

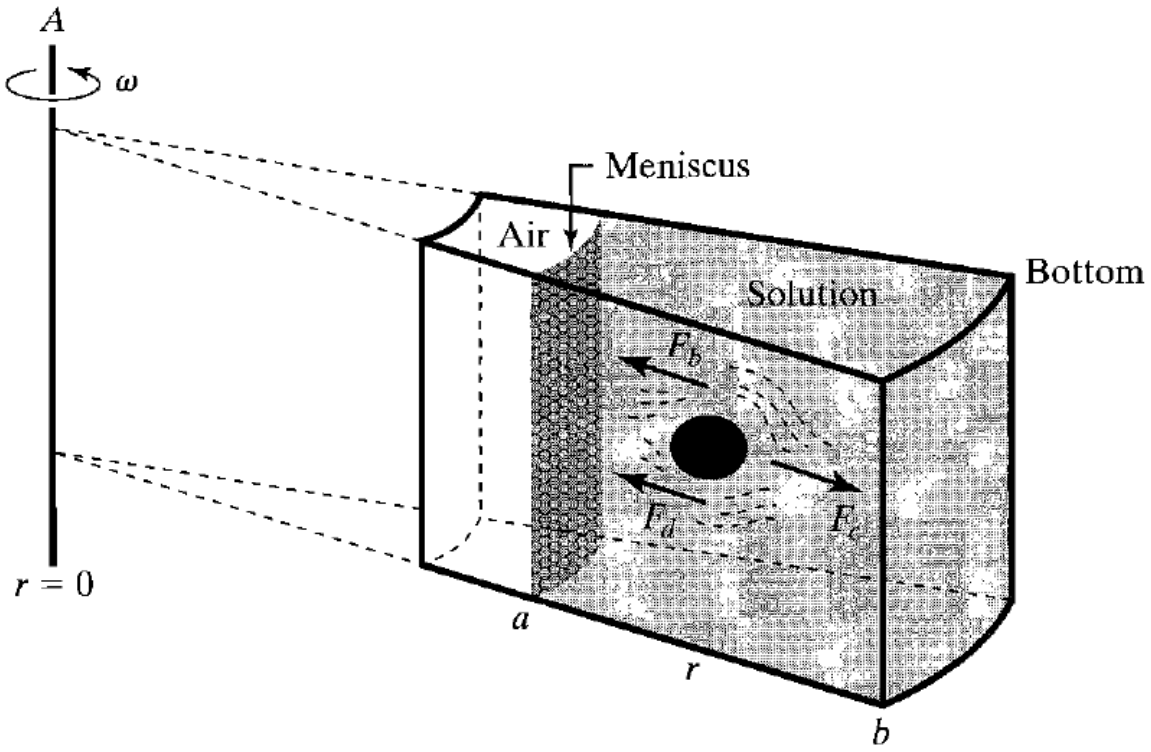
k_B Boltzmann's constant

T Temperature in Kelvin

η Medium viscosity



Characterization: Analytical Ultracentrifugation

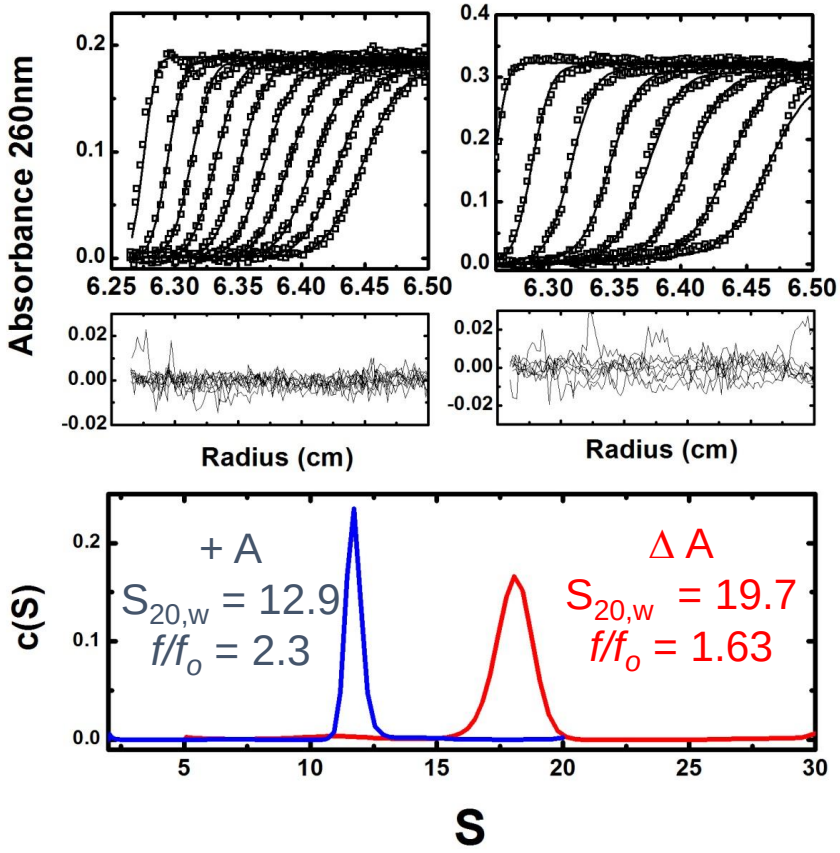


Van Holde

Characterization: Analytical Ultracentrifugation

Sedimentation Velocity

Beckman XL-A



Determine $S_{t,b}$, f/f_o, and from these parameters calculate Mass (M_f)

- Lamm Equation
- Van-Holde Weischet
- g(s*) distributions

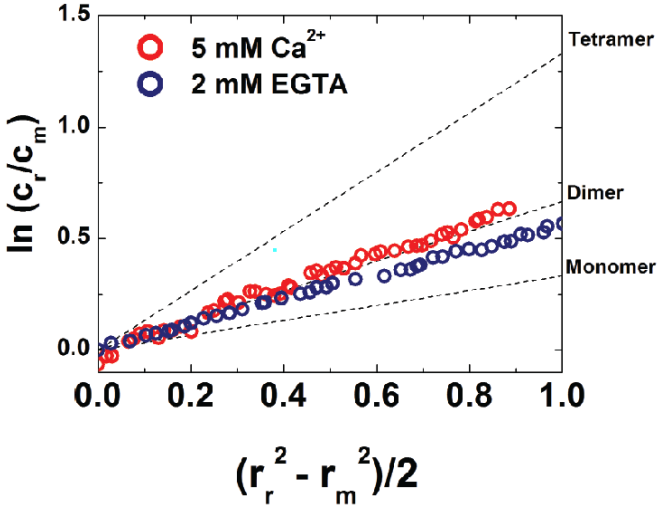
Gupta K, Contreras LM, Smith D, Qu G, Huang T, Spruce L, Seeholzer S, Belfort M, Van Duyne GD. Quaternary Arrangement of an Active, Native Group II Intron Ribonucleoprotein Complex revealed by Small-angle X-ray Scattering. Nucleic Acids Research 42(8):5347-60 (2014).

Huang T., Shaikh T., Gupta K., Contreras-Martinez L., Grassucci, R., Van Duyne G., Frank J., Belfort M. The group II intron ribonucleoprotein precursor is a large, loosely packed structure. Nucleic Acids Research (2010)

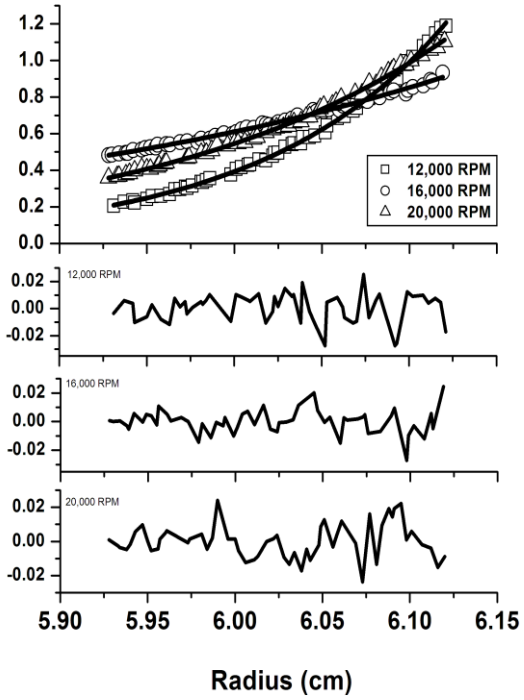
Characterization: Analytical Ultracentrifugation

Sedimentation Equilibrium

Beckman XL-A



Linear Plots



Global Fitting

- Multiple Speeds and Multiple Concentrations
- Global Fitting
- Models of Association (K_d)
- Buoyant Mass → Mass Determination
- With interference optics, higher concentrations than with absorbance

Characterization: Analytical Ultracentrifugation

Sedimentation velocity (SV)

- short experiment (hours)
- get S for each species
- known M: calculate f and D
- unknown M: fit M, f, D
- identify oligomers
- demonstrate complex formation
- need ~500 µL, abs~0.5

(250 µg of a typical protein)

Sedimentation equilibrium (SE)

- long experiment (days)
- fit M, K_a
- no assumptions made
- independent of shape
- need ~120 µL, abs~0.5 for

each concentration/stoichiometry

Instruments

Beckman XLA (absorbance optics)
Beckman XLI (interference optics)

References

Lebowitz, Lewis, Schuck (2002) Modern analytical ultracentrifugation in protein science: A tutorial review. *Prot. Science*, **11**, 2067-79.

Ralston (1993) Introduction to analytical ultracentrifugation. Beckman handbook.

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At the Beamline

bio.aps.anl.gov/pages/how-to-design-saxs-exp.html

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How to design an equilibrium SAXS experiment

SAXS has emerged as a standard biophysical tool deployed routinely for characterizing macromolecules of biomedical interest. The relative logistical simplicity embodied in a technique that provides easy access to the size and low-resolution shape of macromolecules makes it an essential part of the biophysicists' tool kit. With the introduction of Size-Exclusion Chromatography (SEC)-SAXS at BioCAT, which ensures monodispersity of even the most biochemically challenging molecules, structural parameters such as the Radius of Gyration (Rg), Maximum Dimension (Dmax), Volume and Molecular weight estimates can be determined with a high degree of success for a large variety of samples.

There are three equilibrium data acquisition strategies available at BioCAT: SEC-SAXS, SEC-MALS-SAXS, and batch mode SAXS.

Below we give some general guidelines for designing your SAXS experiment. If you have questions, or are a new user, please contact a beamline scientist.

Topics:

- What technique should I use?
  - SEC-SAXS is the right choice if …
  - SEC-MALS-SAXS is the right choice if …
  - Batch mode SAXS is the right choice if …
- What sample concentration and volume do I need?
  - Concentration
  - Volume
- How many samples should I bring?
  - Experiment time
  - Equilibration
- What column should I use?
- How much buffer should I bring?
  - SEC-SAXS
  - SEC-MALS-SAXS
  - Batch Mode

What technique should I use?

BioCAT strongly encourages all users to use either SEC-SAXS or SEC-MALS-SAXS. There are some rare cases where sample concentration and volume are inadequate for SEC-SAXS, in which case you will use batch mode.

SEC-MALS-SAXS allows highly accurate quantification of molecular weight, making it generally superior to SEC-SAXS. However, the equilibration times for the SEC-MALS system are quite long (at least 6 hours), which limits the number of buffer changes. Additionally, the SEC-MALS columns have a limited pH range (3-8). Finally, because of the sensitivity of the system, the requirements on the sample quality are much higher than for SEC-SAXS.

SEC-SAXS is the right choice if …

- Your system has a single well defined peak or several well resolved peaks (not including large aggregates that show up in the void)
- You will need to make several buffer changes during your experiment
- You need to use a wide range of pH in your buffers (3-12)

SEC-MALS-SAXS is the right choice if …

- You have a complicated elution with several overlapping or poorly resolved peaks
- You need at most one buffer change
- You can use a narrower range of pH in your buffer (3-8)
- There is a small amount of elution in the void

Batch mode SAXS is the right choice if …

- You can't meet the concentration and volume requirements for SEC-SAXS (see below)

What sample concentration and volume do I need?

Concentration

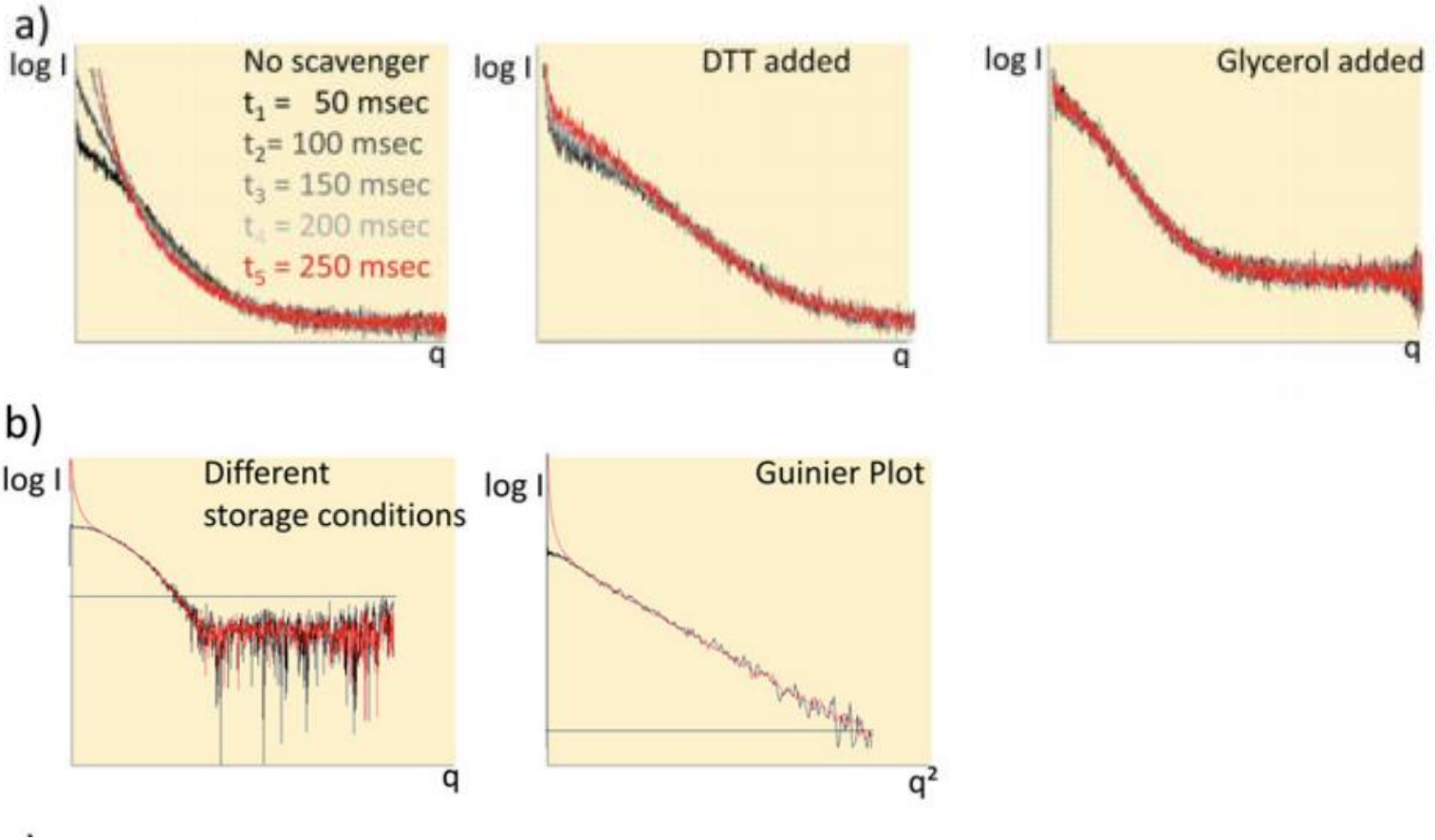
As a rule of thumb, you will get good SAXS data if your protein concentration in mg/ml in the SAXS cell is ~60/MW in kDa. For example, for a 20 kDa protein you would want a concentration of ~60/20 = 3 mg/ml whereas for a 150 kDa protein you would want a concentration of ~60/150 = 0.4 mg/ml. RNA and DNA samples scatter ~2.5 times more strongly than protein, so you can use a concentration of ~24/MW.

The above rule of thumb applies to the concentration in the SAXS cell. In the SEC-SAXS and SEC-MALS-SAXS experiments the sample is diluted by the column, and may elute in several

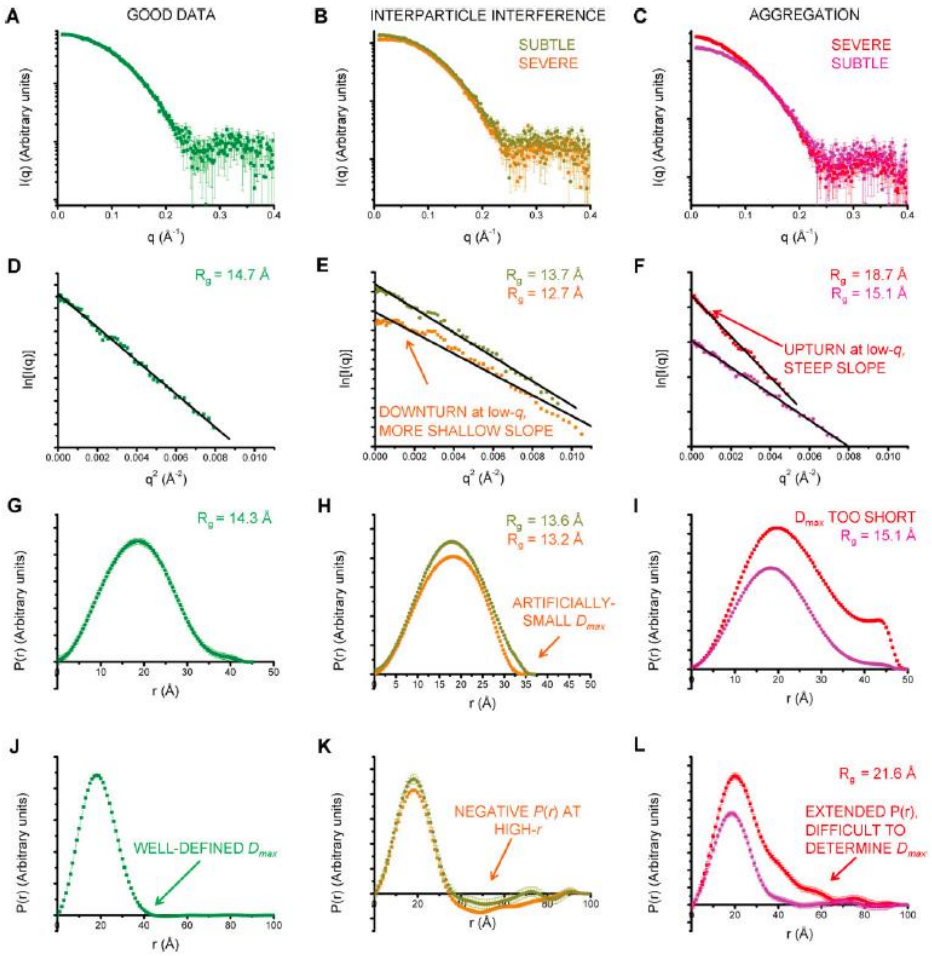
At the Beamline

- High-speed Spin immediately before your experiment
- Take a Nanodrop reading
- Talk to the Beamline Scientists
  - Recommended dosage and attenuation
  - How to analyze your data as you go
- Perform many different concentrations to assess concentration-dependent behavior
- Assess Radiation Damage

At the Beamline



At the Beamline



Jacques and Trewhella, 2010 *Protein Science* Review

At the Beamline

- Additives that can affect contrast
  - High Glycerol (>5%)
  - High Salt (> 1M)
    - Nucleic Acid vs Protein & Interparticle Interference
  - Detergents
    - Complex phase behavior – micelles scatter!
  - Carbohydrates (ie: Sucrose)
  - High Z elements

- Additives that can help guard against radiation damage (Synchrotron SAXS):
  - Low Glycerol (~1-3%)
  - Reducing Agent (ie: 1 mM+ DTT, 10 mM BME, 0.1 mM TCEP)
  - Tris, HEPES versus Phosphate buffers