Sample Preparation for SAXS

Considerations prior to and during beamtime

Max Watkins — Ando Lab @ Cornell — CHESS HP Bio Workshop 2021 — April 29 2021
Sample quality: why is it important?

Ni-NTA, no SEC

- EFA Component elutions
- Aggregate
- Species of interest
- Buffer component

Cobalt + SEC

- EFA Component elutions
- Species of interest
- Aggregate
- Buffer component
Consequences of heterogeneous samples

For a homogenous sample, scattering in solution appears as a rotational average of a single protein

\[ I(q) \propto N \left\langle \left| F \left[ \rho(\vec{r}) \right] \right|^2 \right\rangle_{\Omega} \]

For a mixture of \( M \) species, the scattering can be represented as a linear combination of the scattering from each component

\[ I(q) = \sum_{m=1}^{M} \langle N_m \rangle \tilde{I}_{1,m}(q) \]
Types of heterogeneity

• Different types of heterogeneity have different effects on the data
  • Contaminant proteins
  • Aggregates
  • Oligomeric heterogeneity
  • Conformational heterogeneity

• Aggregates/oligomers can sometimes be separated by SEC-SAXS or AEX-SAXS

• However, math cannot solve all problems
  • Cleaner samples (less species) will be easier to deconvolute
  • Buffer mismatch can complicate analysis

• Conformational heterogeneity can be difficult to parse
Consequences of heterogeneous samples

• Mixtures of species can sometimes be separated mathematically
  • Singular value decomposition (SVD)$^1$
  • Evolving factor analysis (EFA)$^1$
  • Regularized alternating least squares (REGALS)$^2$

• Aggregates can be a major issue
  • $I(q) \propto MW^2$ - small amounts of aggregate can dominate signal
  • Centrifuging samples (5-10 mins @ 10,000 x g) immediately prior can help

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1. Meisberger et. al., JACS 2016
2. Meisberger, Xu and Ando, IUCrJ 2021
Types of heterogeneity: contaminants

Large MW contaminants have a much greater effect than small ones ($I(q) \propto MW^2$)

- Aggregates and large contaminant proteins have a major effect

Jeffries et. al., Nature Protocols 2015
Conformational heterogeneity can be more difficult to parse

- Differences in scattering profiles may be much more subtle
- Harder to separate by chromatography

Jeffries et. al., Nature Protocols 2015
Sample preparation: pre-beamtime

- Protein purification requirements for SAXS are more stringent than for many other techniques
  - Anion-exchange/Size-exclusion cleanup steps are almost always necessary
  - Be sure to choose a high enough resolution column (Superdex 200 increase, Mono-Q/S HR, etc.) to separate species well

Separation of BSA on different SEC columns, from Jeffries et. al., Nature Protocols 2015
Sample preparation: pre-beamtime

- Know your protein concentration as accurately as possible
  - Extinction coefficient (usually $A_{280}$) is often accurate to ~10%
  - Other methods (i.e. Bradford assay) may be necessary for proteins with absorbing cofactors or that lack aromatic residues

- Know behavior of protein upon freeze-thaw cycles
  - Does it precipitate out of solution?
  - Does it significantly increase aggregates?

- Preparing a 10x buffer stock for on-site sample-prep and buffer exchange is often helpful

- Prepare additive stocks in experimental buffer
What constitutes a "sample"?

• One experimental condition = one sample

• Generally, each condition of interest will require at least one prepared aliquot and matching buffer
  • >20 μL protein at desired experimental concentration (at least 1 mg/mL, higher for small proteins)
    • More for HP-SAXS
  • Plan on having duplicates of each condition
  • Good idea to prepare small aliquots at higher concentrations to dilute and prepare at the beamline
  • More details on this later
Basic sample analysis: pre-beamtime

- *A priori* knowledge: the more, the better!
  - Purity (gel filtration, analytical chromatography),
  - Activity/foldedness, available structural information

- Standard SDS-PAGE cannot distinguish oligomers or aggregates, only contaminant proteins
  - Native PAGE gels or analytical chromatography (SEC or AEX) prior to beamtime

Jeffries et. al., Nature Protocols 2015
Advanced sample analysis: pre-beamtime

- Other, more advanced techniques can be helpful in predicting behavior at the beamline
  - Analytical Ultracentrifugation (AUC)
  - Dynamic light scattering (DLS) or Multi-angle light scattering (MALSS)
  - SEC-MALS sometimes available as in-line validation for SEC-SAXS

Figure from Kushol Gupta
Transporting samples: checklist

• Crucial to know how well your protein handles freeze-thaw cycles

• If you can, store aliquots at -80°C and transport frozen (on dry ice)
  • Avoid introduction of air bubbles and centrifuge samples prior to freezing
  • Give samples enough time for CO2 from transport to diffuse
  • Thaw slowly (on ice) as needed at the beamline
  • If your protein is not frozen, transport on ice
  • Arrange for dry ice for return trip

• Additional things to transport:
  • Concentrated buffer solutions
  • Concentrated stock solutions of additives (reductants, substrates), preferably in experimental buffer
  • Equipment for buffer exchange (i.e. micro-spin columns)
Benefits of CHESS in the winter
Ideally, scattering can be described as:

$$I(q) = F(q) \times S(q)$$

where $F(q)$ is the form factor and $S(q)$ is the structure factor ($S(0) = 1$ under the dilute limit)

- Increasing concentration leads to increased probability of particle interaction
- $S(0)$ will deviate from unity
  - Aggregation ($S(0) > 1$)
  - Interparticle repulsion ($S(0) < 1$)
Interparticle effects

Minimal interparticle effects

Aggregation

Repulsion

$S(0) \approx 1$

$S(0) > 1$

$S(0) < 1$
Examining concentration effects: concentration series

Skou et al., Nature Protocols (2014)
Planning a concentration series experiment

- Protein solution #1
  - Multiple exposures
  - Average of superimposable exposures
  - Buffer-subtracted profile
  - $R_g$, $l(0)$, $D_{max}$ shape information

- Protein solution #2
  - Multiple exposures
  - Average of superimposable exposures
  - Buffer-subtracted profile
  - $R_g$, $l(0)$, $D_{max}$ shape information

- Protein solution #3
  - Multiple exposures
  - Average of superimposable exposures
  - Buffer-subtracted profile
  - $R_g$, $l(0)$, $D_{max}$ shape information

Examine concentration effects

Skou et al., Nature Protocols (2014)
Diagnosing concentration effects

Guinier plots are particularly sensitive to concentration effects, since they manifest largely in the low-q region.

![Graphs showing Good Data, Repulsion, Aggregation, Linear, Downturn, Upturn]
Importance of good buffer subtraction

- Exact buffer match is crucial to good-quality SAXS data
  - Scattering intensity is sensitive to small differences in salt/buffer/additive concentrations
- On-site buffer exchange (gel filtration, micro-spin columns) is best practice
  - In-line SEC (SEC-SAXS) should also give you a correct buffer match

Figure from Richard Gillilan
Viewing data as a Kratky transformation ($I(q)^2$ vs $q$) can be helpful in diagnosing mismatches.

- Exact buffer match
- Buffer + glycerol (undersubtracted)
- Completely different buffer (oversubtracted)

Buffer subtraction: Addition of ligands

• Ligand/substrate titrations are common practice for verifying conformational changes

• Any changes in solution composition (i.e. addition of ligands) can significantly alter the scattering profile

Buffer profiles with different GTP concentrations
(data courtesy of Will Thomas)
Buffer subtraction: Addition of ligands

• You need an exact buffer match for each condition in a titration
  • Careful pipetting is key!
  • Good to prepare matched buffer simultaneously with protein sample

• With good buffer subtraction, small conformational changes can be visualized!

Titration of dATP into ribonucleotide reductase

Ando et. al., Biochemistry 2015
Pipetting Tips: Increasing accuracy

• Maintain vertical angle when aspirating
• Touch tip to side of tube when dispensing
• Proper immersion depth


For weak acids and bases, the dissociation constant is dependent on temperature and pressure.

This can be described by the Planck equation:

\[
\left( \frac{\delta \ln K_a}{\delta P} \right)_T = -\frac{\Delta V^o}{RT}
\]

- \( P \) is pressure, \( T \) is temperature, \( K_a \) is the dissociation constant and \( \Delta V^o \) is the reaction volume (difference in partial molar volumes of the acid and ionized molecules).
Buffer considerations: Temperature dependence

- Generally, pH decreases with temperature
  - Increased propensity for ionization
- The effect of temperature varies considerably depending on the buffer
  - Tris is known to be especially poor
  - Amine buffers generally less T-dependent
  - Many purifications are done at 4°C, which may not be ideal for SAXS experiments that probe biological activity

<table>
<thead>
<tr>
<th>Buffer System</th>
<th>pKₐ/20°C</th>
<th>ΔpKₐ/10°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>MES</td>
<td>6.15</td>
<td>-0.110</td>
</tr>
<tr>
<td>ADA</td>
<td>6.60</td>
<td>-0.110</td>
</tr>
<tr>
<td>PIPES</td>
<td>6.80</td>
<td>-0.085</td>
</tr>
<tr>
<td>ACES</td>
<td>6.90</td>
<td>-0.200</td>
</tr>
<tr>
<td>BES</td>
<td>7.15</td>
<td>-0.160</td>
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<tr>
<td>MOPS</td>
<td>7.20</td>
<td>-0.013</td>
</tr>
<tr>
<td>TES</td>
<td>7.50</td>
<td>-0.200</td>
</tr>
<tr>
<td>HEPES</td>
<td>7.55</td>
<td>-0.140</td>
</tr>
<tr>
<td>Tricine</td>
<td>8.15</td>
<td>-0.210</td>
</tr>
<tr>
<td>Tris</td>
<td>8.30</td>
<td>-0.310</td>
</tr>
<tr>
<td>Bicine</td>
<td>8.35</td>
<td>-0.180</td>
</tr>
<tr>
<td>Glycyglycine</td>
<td>8.40</td>
<td>-0.280</td>
</tr>
</tbody>
</table>

Reference

Buffer considerations: pressure dependence

- Water molecules pack more closely around free ions, resulting in a net reduction of system volume
- Generally, pressure favors ionization of weak acids
- Anionic buffers (phosphate, DMG) are more sensitive than cationic/zwitterionic buffers (HEPES, MES, Tris)

<table>
<thead>
<tr>
<th>Buffer</th>
<th>$pK_a^d$ (25°C)</th>
<th>$\Delta V^\circ$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrophosphate (3rd)</td>
<td>6.70</td>
<td>-20.7</td>
</tr>
<tr>
<td>HEPES (2nd)</td>
<td>7.50</td>
<td>4.8</td>
</tr>
<tr>
<td>MES</td>
<td>6.10</td>
<td>3.9</td>
</tr>
<tr>
<td>Bis-tris</td>
<td>6.50</td>
<td>3.1</td>
</tr>
<tr>
<td>MOPS</td>
<td>7.15</td>
<td>4.7</td>
</tr>
<tr>
<td>Tris</td>
<td>8.10</td>
<td>4.3</td>
</tr>
<tr>
<td>Bicine</td>
<td>8.30</td>
<td>-0.2</td>
</tr>
<tr>
<td>DMG (2nd)</td>
<td>6.35</td>
<td>-25.0</td>
</tr>
<tr>
<td>Bis-tris propane</td>
<td>6.75</td>
<td>10.5</td>
</tr>
</tbody>
</table>

• Continued exposure to X-rays will degrade sample quality

• X-rays interact with water and generate free radicals (particularly HO•)
  • Can then cause specific structural disruption in individual residues

• Most commonly manifests as aggregation in SAXS
  • Radiation tolerance generally much lower than cryo-MX (~1000x)

• In most cases damage is a strict function of dose

How to check for radiation damage

• Radiation damage varies widely by sample

• Radiation sensitivity can be quantified

• In practice, usually sufficient to plot $R_g$ or $P(r) D_{\text{max}}$ as a function of dose (i.e. $R_g$ vs. frame number)
  • Cut off exposures where $R_g$ starts to increase considerably or Guinier behavior becomes poor

SAXS data parameters vs. dose for lysozyme (4.1 mg/mL)

Protecting against radiation damage

- Optimization of buffer conditions (salt, pH)
- Decrease exposure or flux
- Many additives are effective at mitigating radiation damage
  - Reductants (DTT, TCEP)
  - Other small molecules often help (glycerol, sucrose, ascorbate)
  - Be careful, may decrease contrast at high concentrations (>10%)

Jeffries et. al., Journal of Synchrotron Radiation 2015
1. Run a concentration series, if sample is new to SAXS
   • Minimum 3 points
   • Check for concentration effects
2. Vary buffer salt concentration and assess radiation damage, if needed
3. Experiment with various additives (DTT, glycerol, etc.) to mitigate radiation damage
4. It is a good idea to characterize a sample with ambient-pressure SAXS prior to a high-pressure experiment, as pressure effects are often subtle.
Sample amount considerations: Regular BioSAXS

• There are no absolute rules for sample concentrations or amounts
  • Every protein behaves differently*check website

• Some general considerations:
  • Scattering intensity is $\propto M^{2}$, so small proteins may require considerably higher concentrations or more exposures
  • Studying proteins at or near physiological concentrations is ideal, if possible

• For most proteins, absolute minimum consumption per measurement $\sim$20 $\mu$L at 1 mg/mL for standard flow cell
  • Plan on having more per sample
  • Running duplicates is common (expect to use $\sim$50 $\mu$L sample per measurement)

• Need 5-10x more for SEC-SAXS experiments
• Higher concentrations may be necessary for small (< 50 Kda) proteins

• [https://www.chess.cornell.edu/macchess/biosaxs/visit](https://www.chess.cornell.edu/macchess/biosaxs/visit)
Additional considerations for HP-BioSAXS

• At CHESS, batch mode samples (60 μL each) are loaded into a diamond-windowed cell
  • Again, expect to use duplicates for each condition

• Sample-to-buffer contrast decreases significantly with pressure
  • May need 2-3 times higher concentration than for regular BioSAXS
  • Recommended rule of thumb: minimum concentration = 150/MW (kDa)

• In batch mode, radiation damage can be significant (static sample)
  • Less of an issue for HP SEC-SAXS

• Pressure series (constant T) are much less time-consuming than temperature series (constant P)
Workflow of a basic SAXS experiment

1. Plan experiments carefully (pre-plan recipes for experimental solutions) and have an order of priority
2. Thaw protein on ice, slowly, as needed. Do not vortex or shake!
3. Dilute buffer stock and add fresh reductant to the buffer solution, if needed
4. Buffer exchange on-site into freshly prepared buffer
5. Prepare experimental solution (and matching buffer) carefully: avoid introducing air bubbles, use proper pipetting techniques
6. Incubate with ligand/substrate at desired experimental temperature
7. Centrifuge samples (5-10 minutes @ 10,000 x g, refrigerated centrifuge)
8. Load sample! Avoid air bubbles


