CHESS
CORNELL HIGH ENERGY SYNCHROTRON SOURCE

Extreme Biophysics RCN

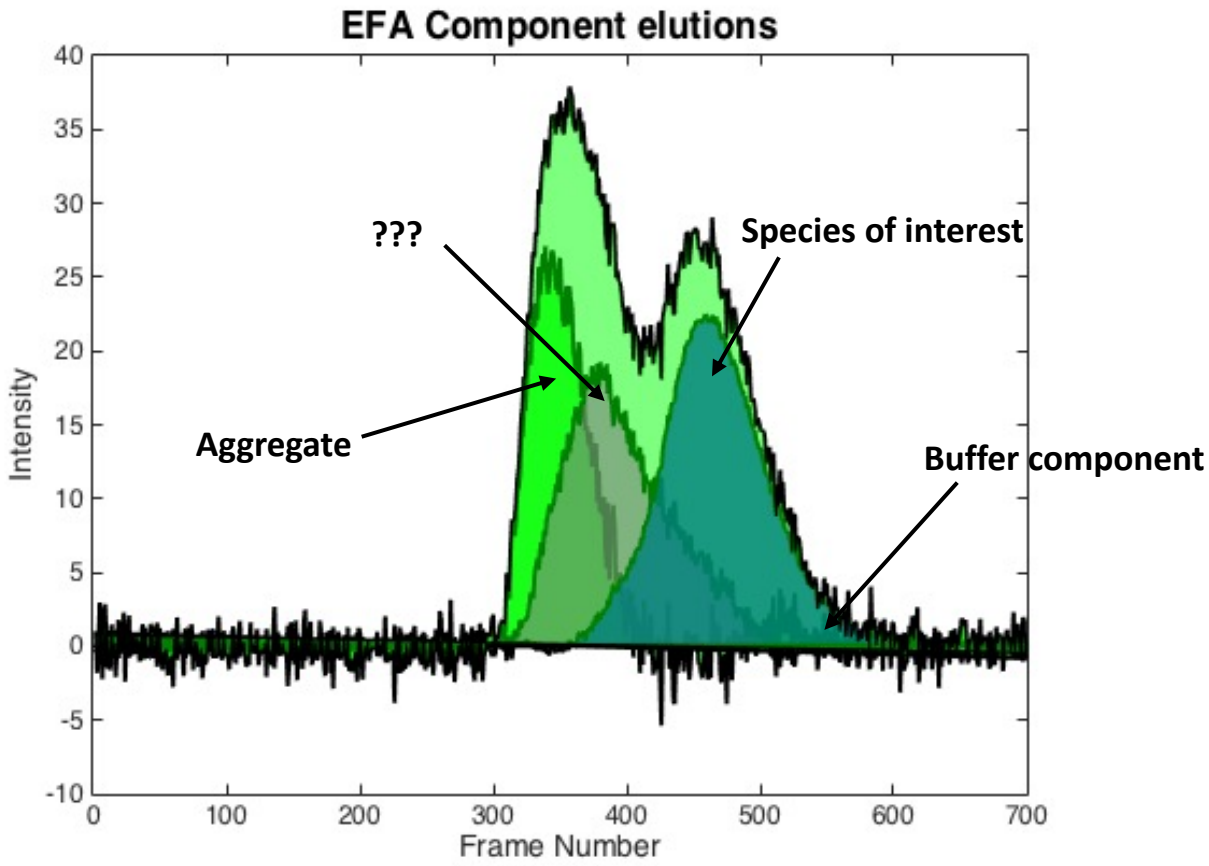
Sample Preparation for SAXS

Considerations prior to and during beamtime

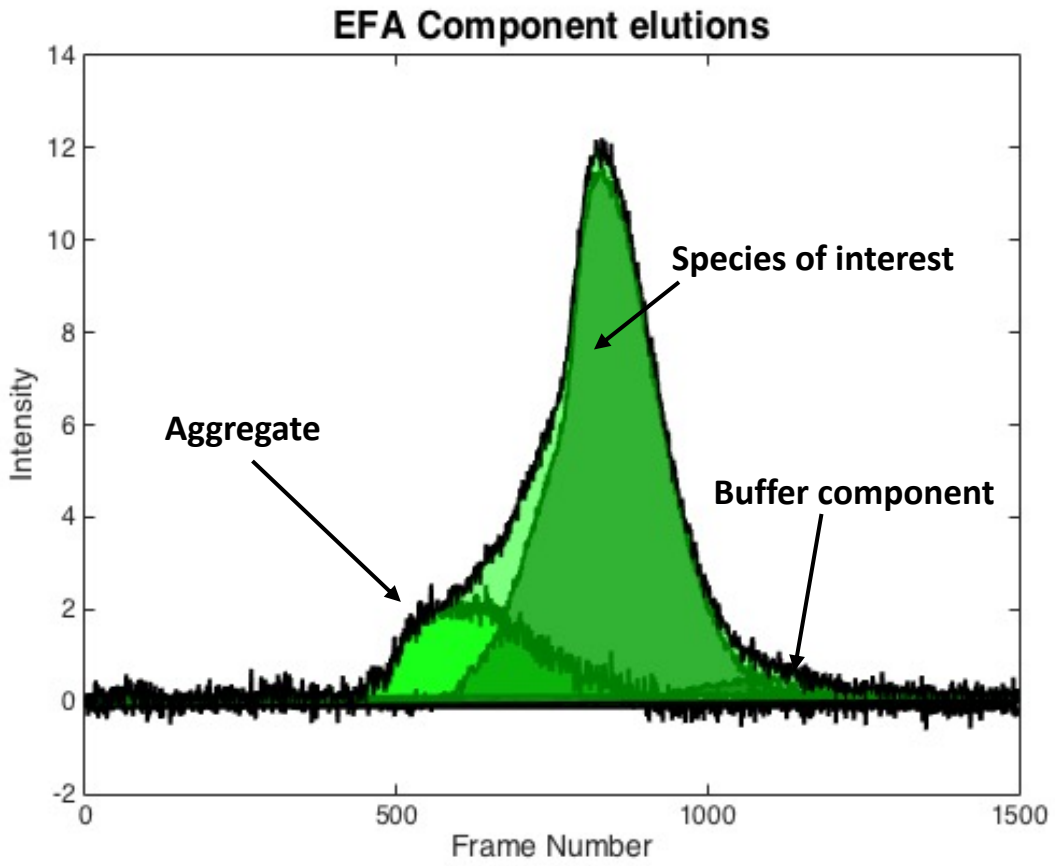
Max Watkins— Ando Lab @ Cornell — CHESS HP Bio Workshop 2021 — April 29 2021

Sample quality: why is it important?

Ni-NTA, no SEC



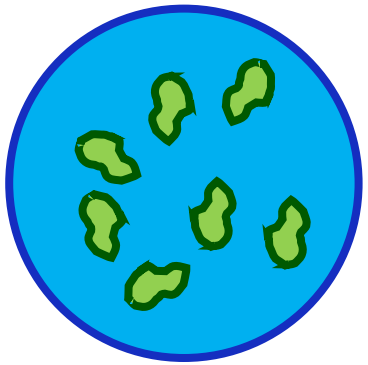
Cobalt + SEC



Consequences of heterogeneous samples

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

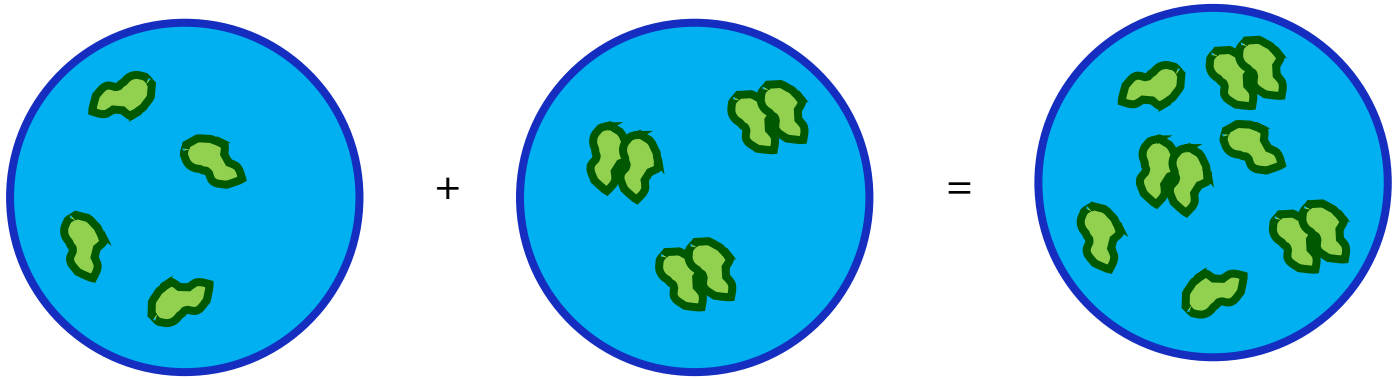
$$I(q) \propto N \left\langle \left| F\left[\rho(\vec{r})\right] \right|^2 \right\rangle_{\Omega}$$



Randomly oriented, homogenous particles

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

$$I(q) = \sum_{m=1}^{M} \langle N_m \rangle \tilde{I}_{1,m}(q)$$

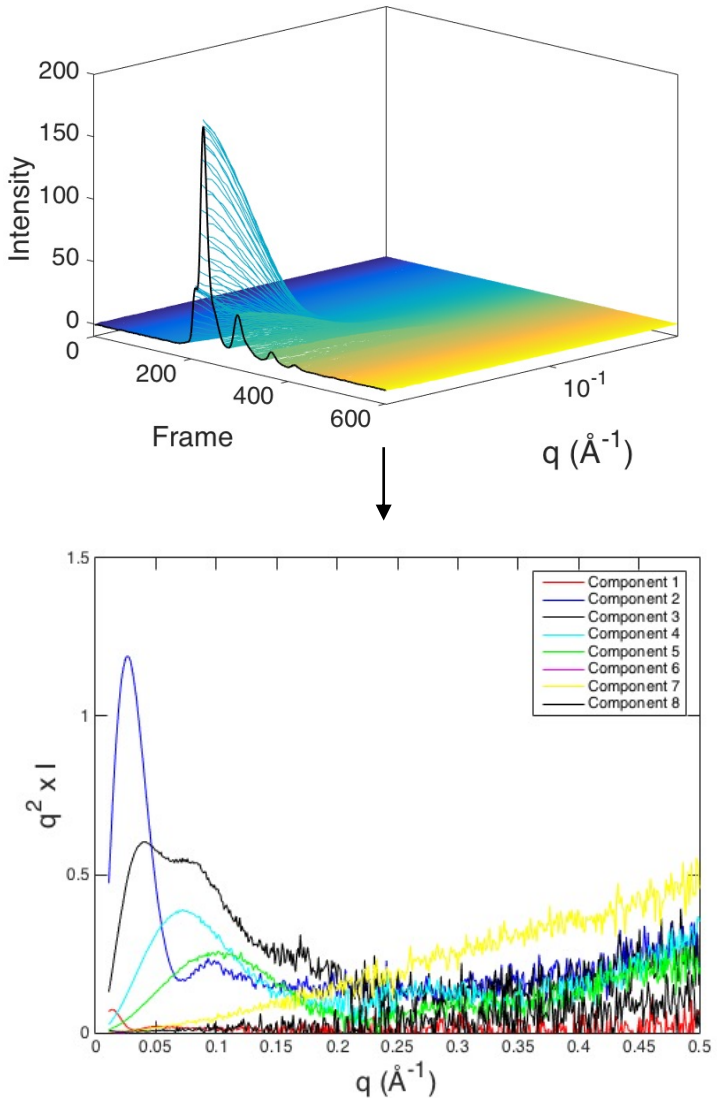


Types of heterogeneity

- 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



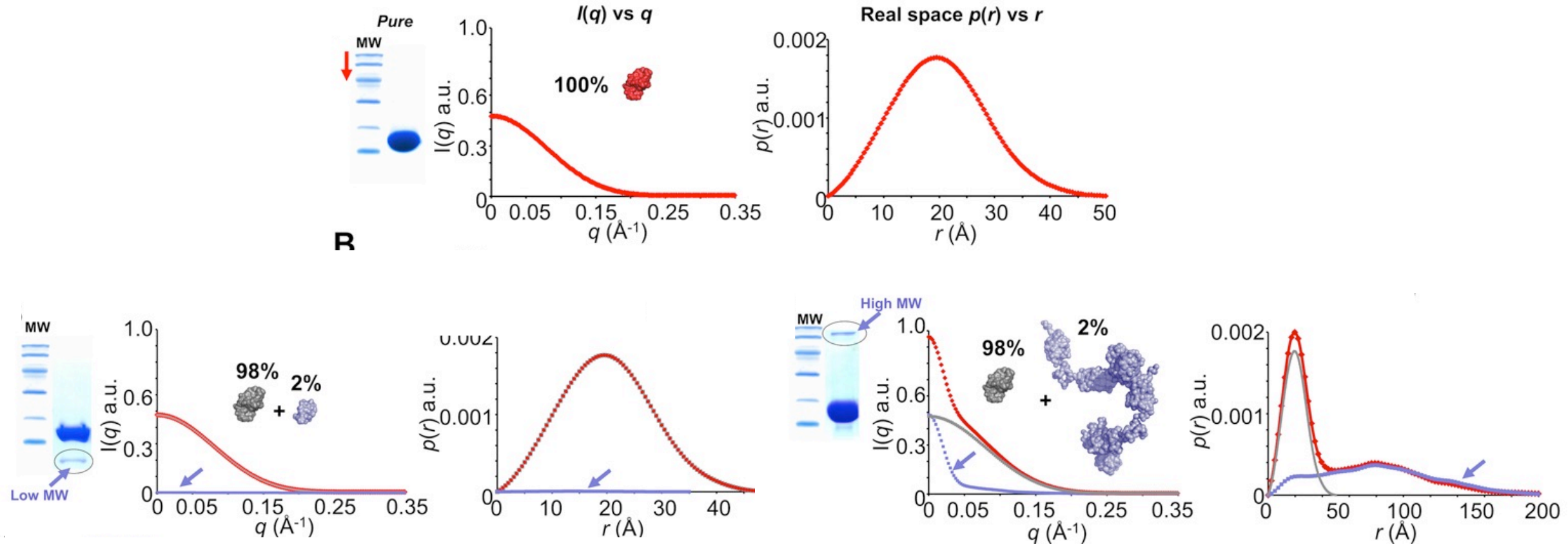
EFA separation of Bio-rad chromatography standards

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) ∝ MW²**)

- Aggregates and large contaminant proteins have a major effect

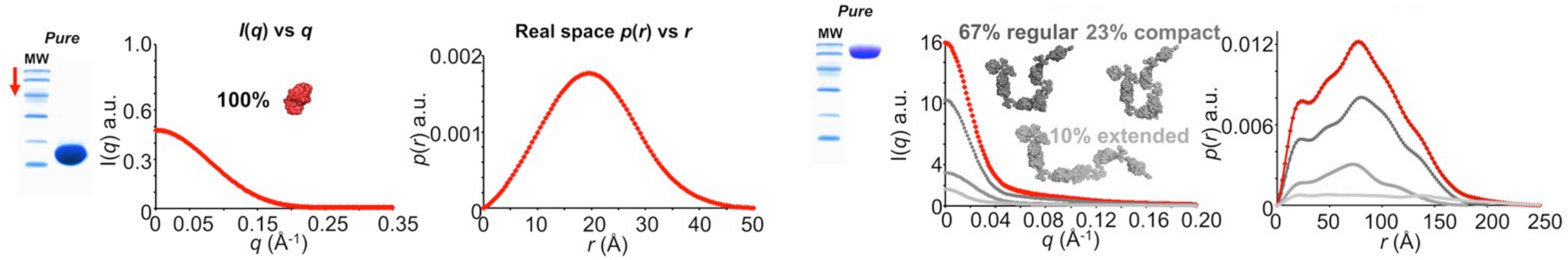


Jeffries *et. al.,* Nature Protocols 2015

Types of heterogeneity: conformational heterogeneity

Conformational heterogeneity can be more difficult to parse

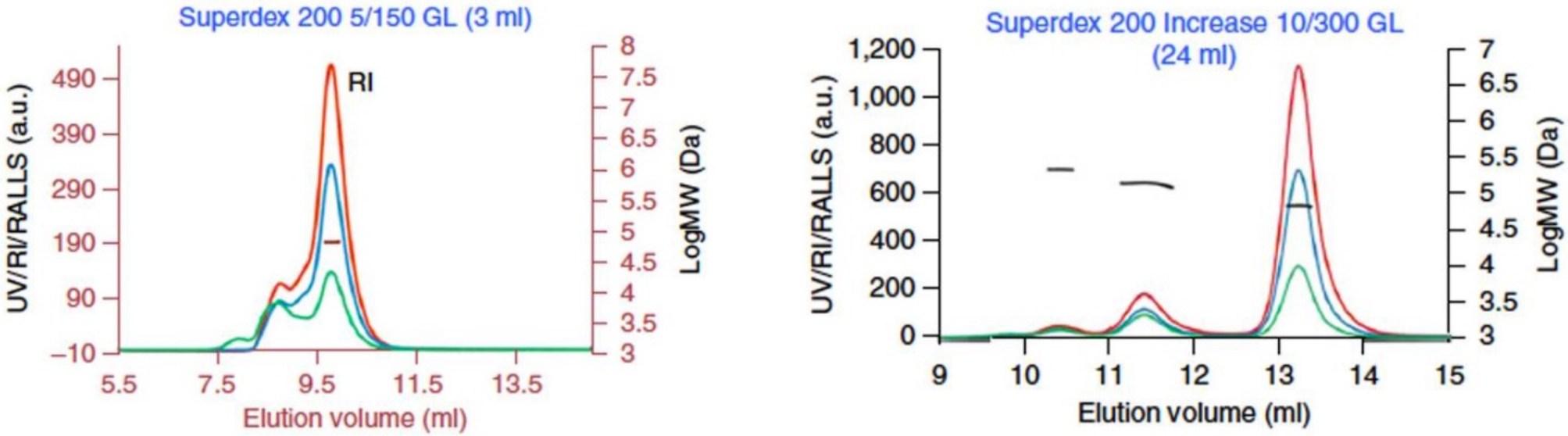
- Differences in scattering profiles may be much more subtle
- Harder to separate by chromatography



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

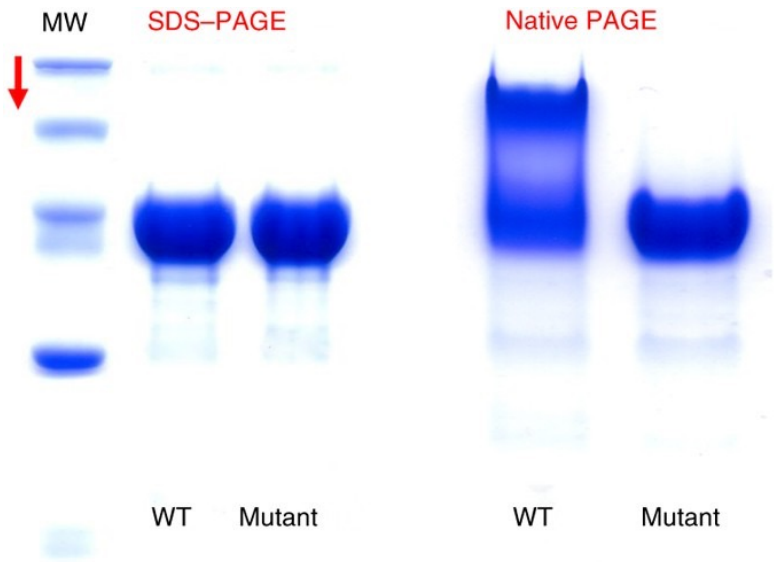
- Know your protein concentration as accurately as possible
  - Extinction coefficient (usually A_{280}) is often accurate to ~10%
  - Other methods (i.e. Bradford assay) may be necessary for proteins with absorbing cofactors or that lack aromatic residues
- Know behavior of protein upon freeze-thaw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

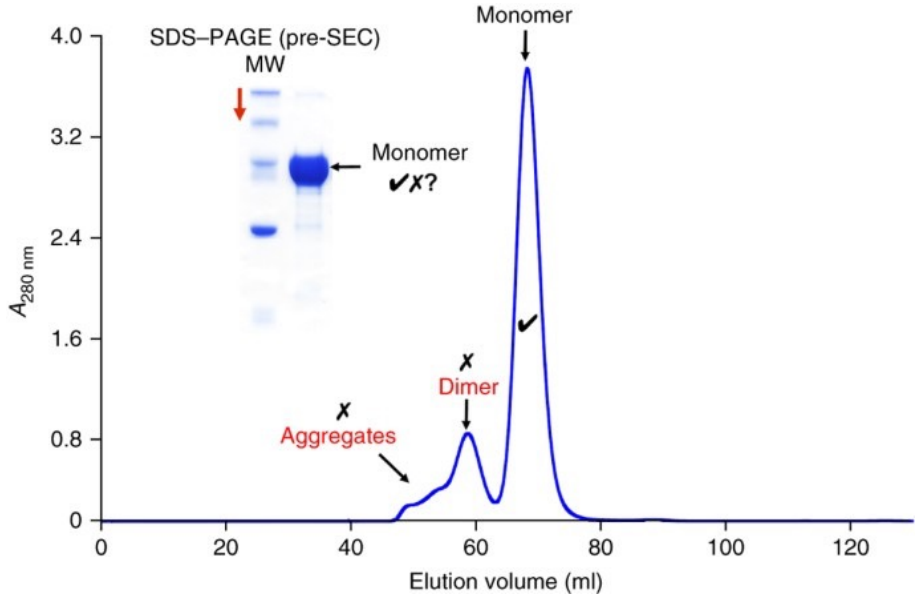
What constitutes a ”sample”?

- 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 (MALS)
  - SEC-MALS sometimes available as in-line validation for SEC-SAXS

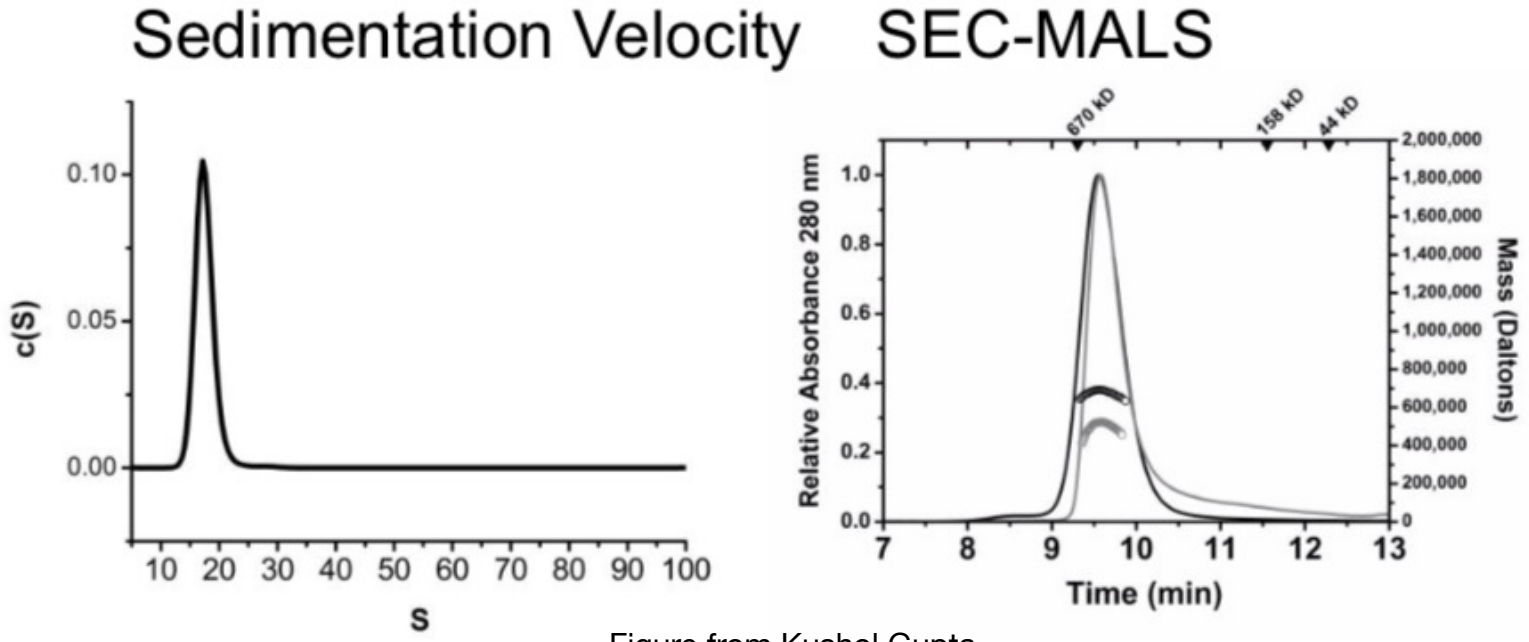


Figure from Kushol Gupta

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

Concentration effects: aggregation and repulsion

Ideally, scattering can be described as:

$$I(q) = F(q)\, x\, S(q)$$

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

$S(0) \approx 1$

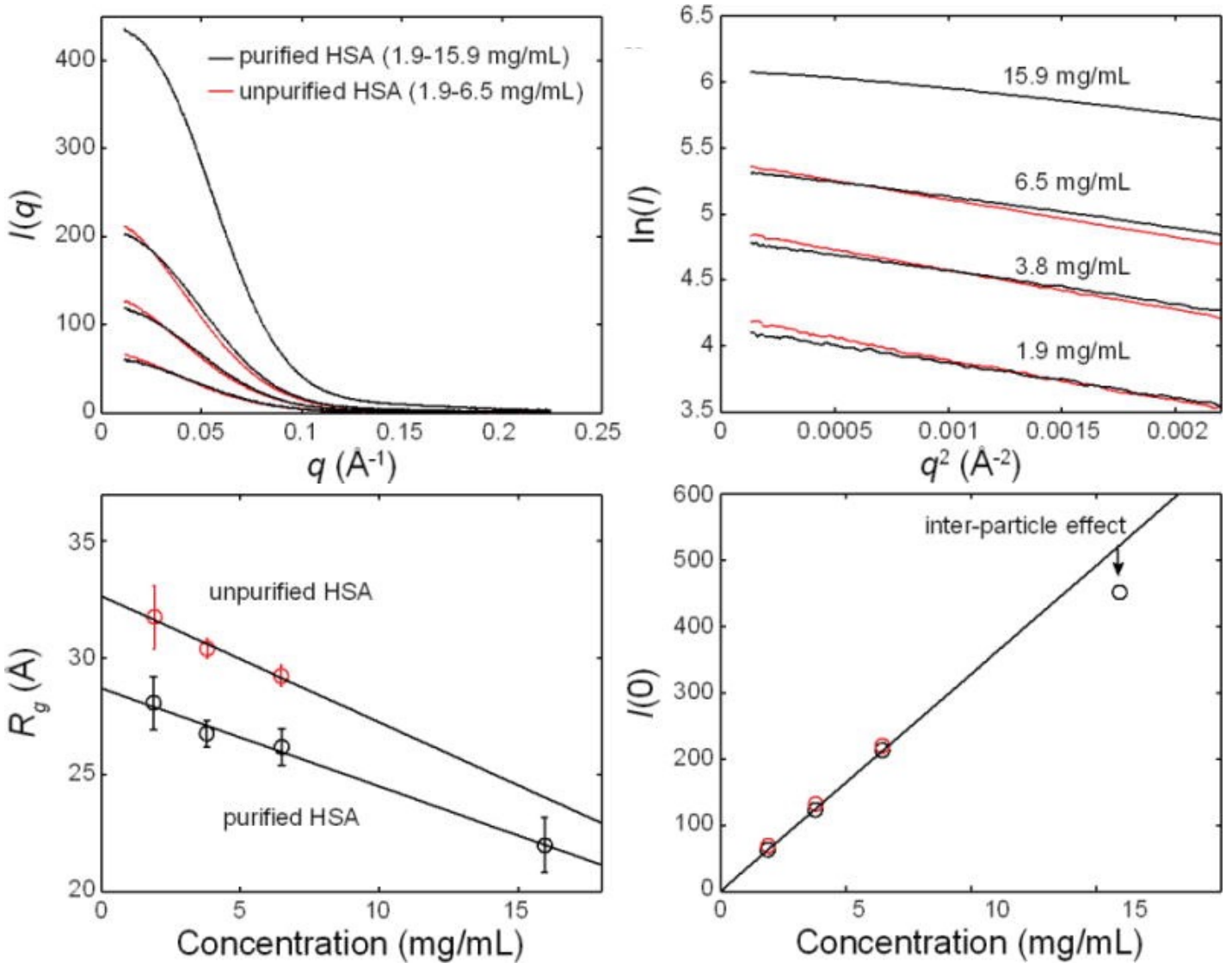
Aggregation

$S(0) > 1$

Repulsion

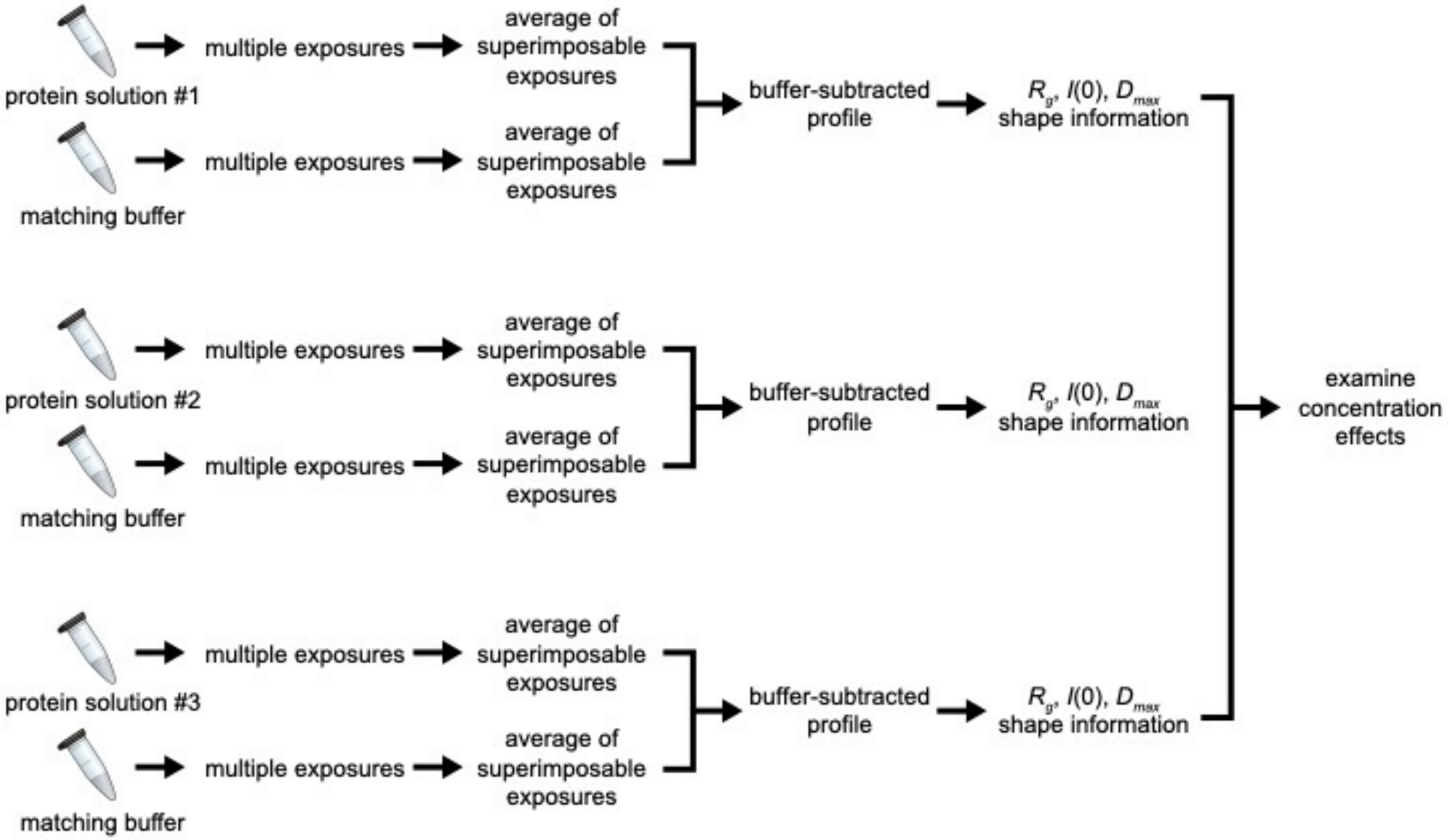
$S(0) < 1$

Examining concentration effects: concentration series



Skou *et al.,* Nature Protocols (2014)

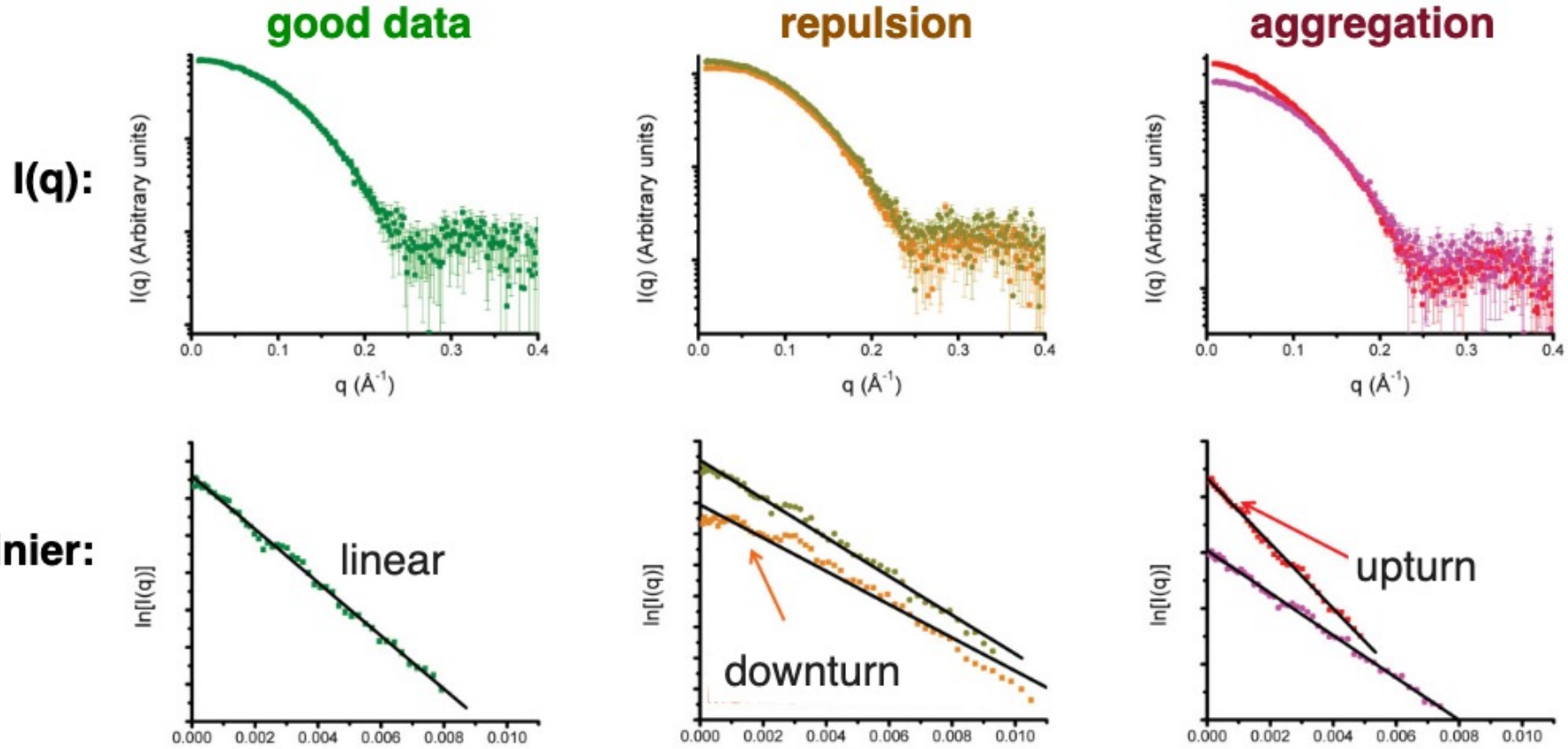
Planning a concentration series experiment



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



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

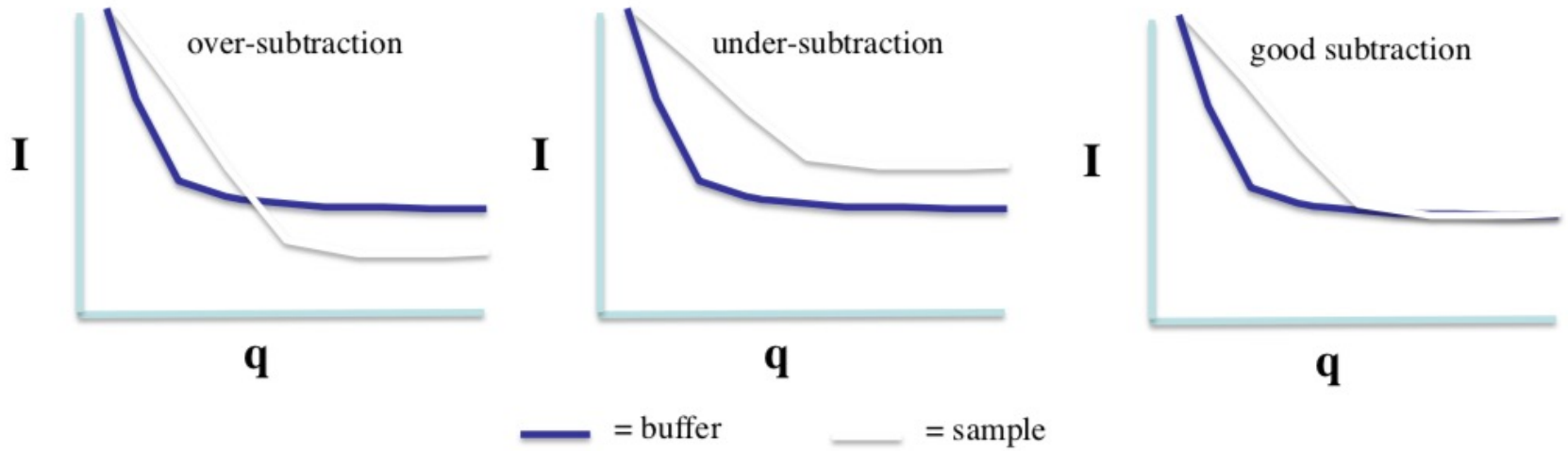
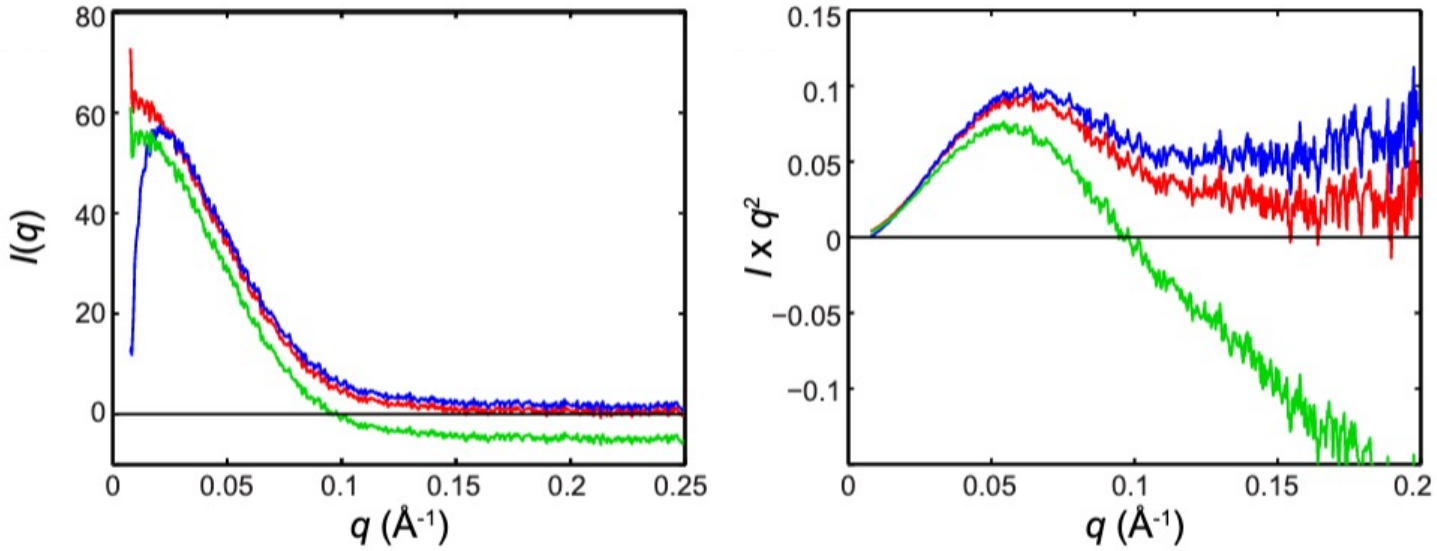


Figure from Richard Gillilan

Kratky plots as diagnostic tools

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

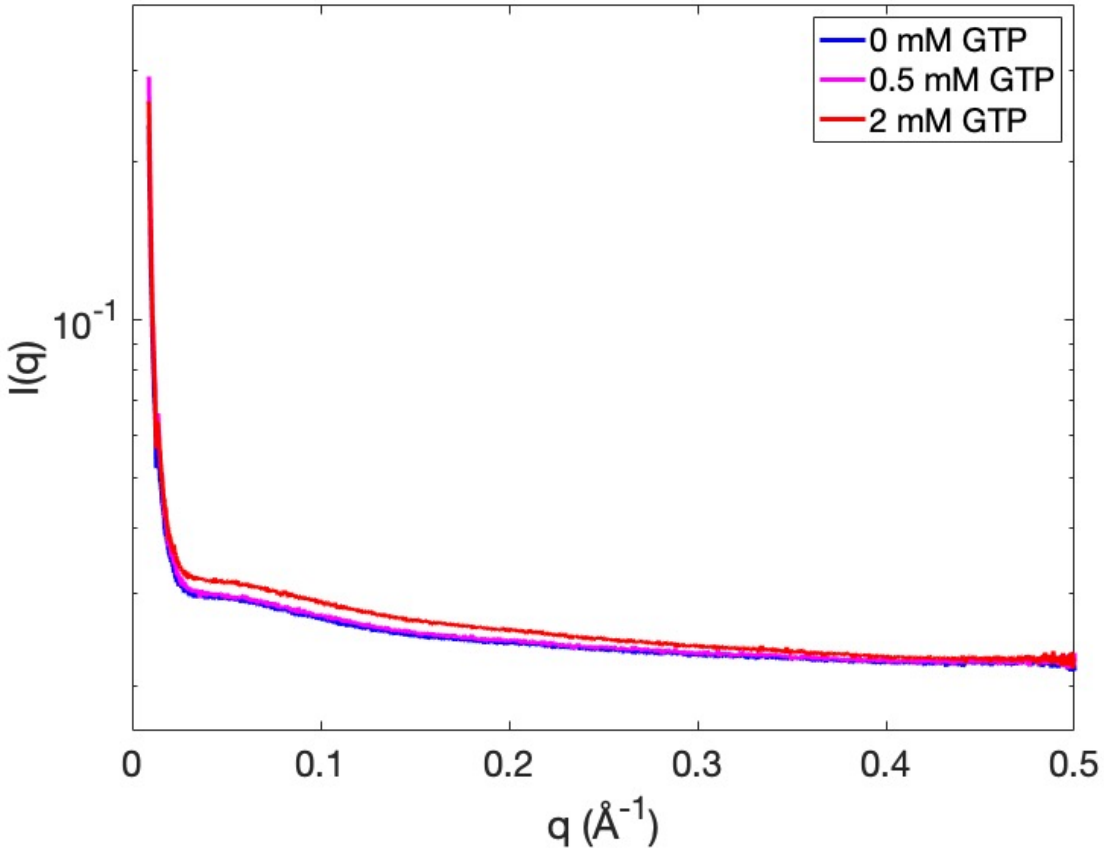


Exact buffer match
Buffer + glycerol (undersubtracted)
Completely different buffer (oversubtracted)

Skou *et al.,* Nature Protocols (2014)

Buffer subtraction: Addition of ligands

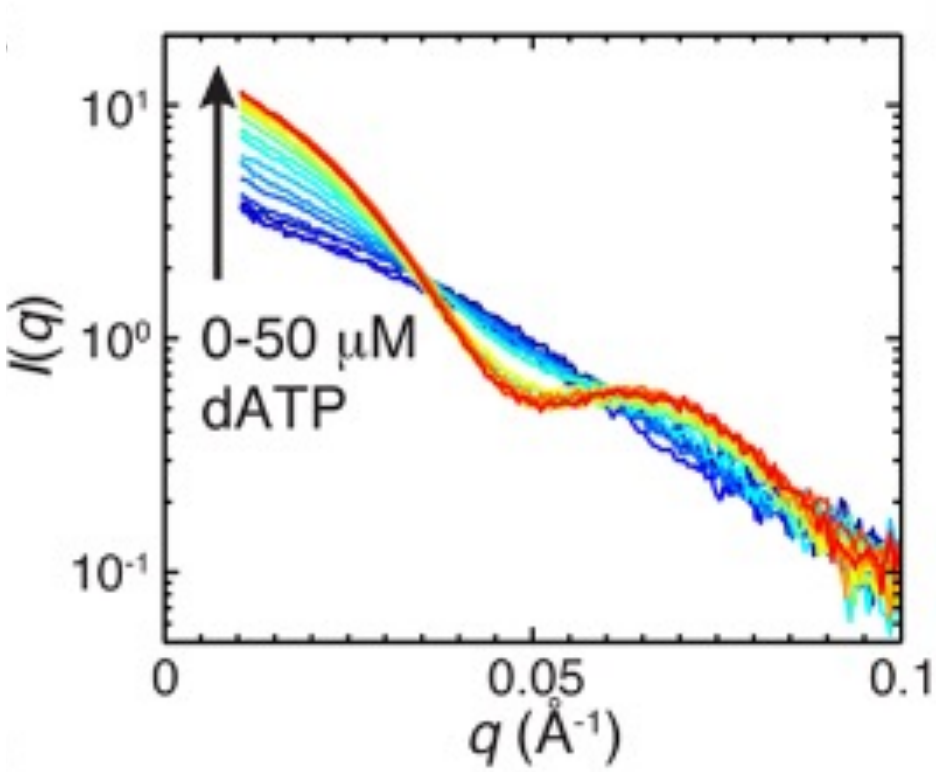
- Ligand/substrate titrations are common practice for verifying conformational changes
- Any changes in solution composition (i.e. addition of ligands) can significantly alter the scattering profile



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

Proper immersion depth

Improper immersion depth

https://www.mt.com/de/en/home/library/videos/rainin-pipettes/pipette_tip_immersion.html

Buffer considerations: Effects of T and P

- For weak acids and bases, the dissociation constant is dependent on temperature and pressure
- This can be described by the Planck equation:

$$\left(\frac{\delta \ln K_a}{\delta P}\right)_T = -\frac{\Delta V^{\circ}}{RT}$$

- ***P*** is pressure, ***T*** is temperature, $\boldsymbol{K_a}$ is the dissociation constant and $\boldsymbol{\Delta V^{\circ}}$ 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

Temperature Dependence of pH for Commonly Used Buffers.

Buffer System	pK_a/20°C	ΔpK_a/10°C
MES	6.15	–0.110
ADA	6.60	–0.110
PIPES	6.80	–0.085
ACES	6.90	–0.200
BES	7.15	–0.160
MOPS	7.20	–0.013
TES	7.50	–0.200
HEPES	7.55	–0.140
Tricine	8.15	–0.210
Tris	8.30	–0.310
Bicine	8.35	–0.180
Glycylglycine	8.40	–0.280

Reference

Good, N.E. (1986) *Biochemistry* **5**, 467.

Goldberg, RN et al. J. Phys. Chm. Ref. Data. (2002)

Buffer considerations: pressure dependence

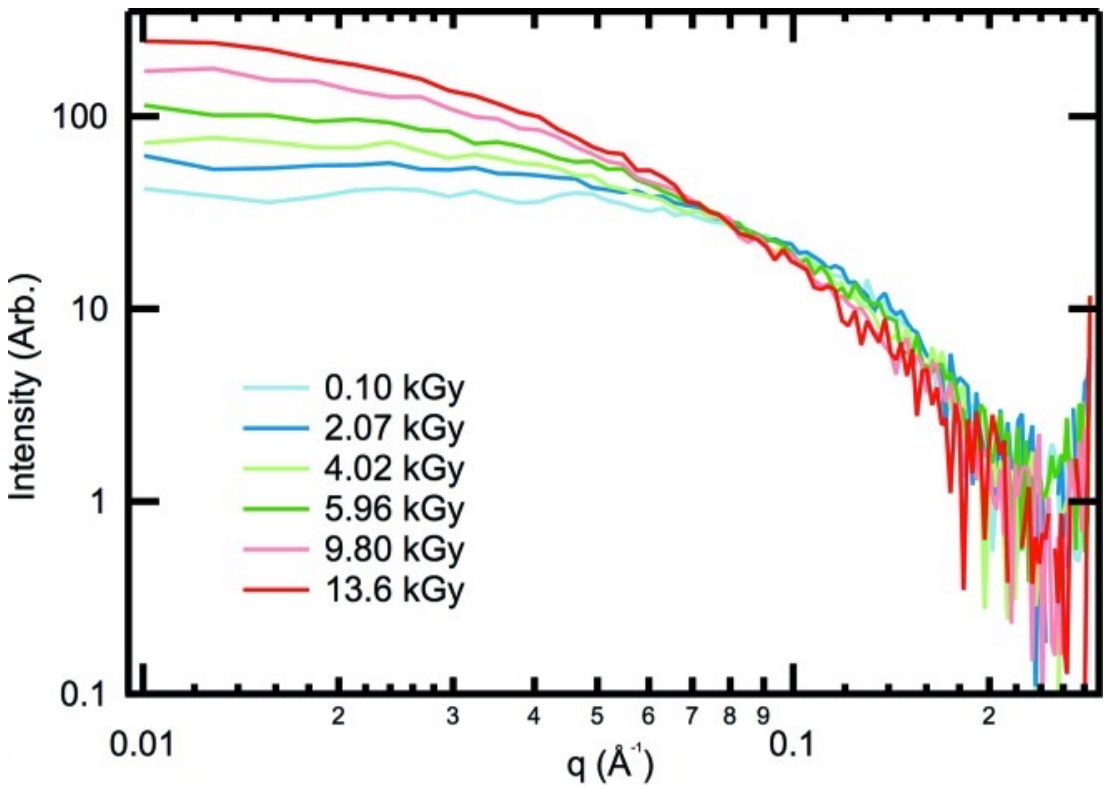
- Water molecules pack more closely around free ions, resulting in a net reduction of system volume
- Generally, pressure favors ionization of weak acids
- Anionic buffers (phosphate, DMG) are more sensitive than cationic/zwitterionic buffers (HEPES, MES, Tris)

Buffer	pK_a^d (25°C)	ΔV°
Pyrophosphate (3rd)	6.70	-20.7
HEPES (2nd)	7.50	4.8
MES	6.10	3.9
Bis-tris	6.50	3.1
MOPS	7.15	4.7
Tris	8.10	4.3
Bicine	8.30	-0.2
DMG (2nd)	6.35	-25.0
Bis-tris propane	6.75	10.5

Kitamura, Y. and Itoh, T. Journal of Solution Chemistry (1987)

Radiation damage: cause and effect

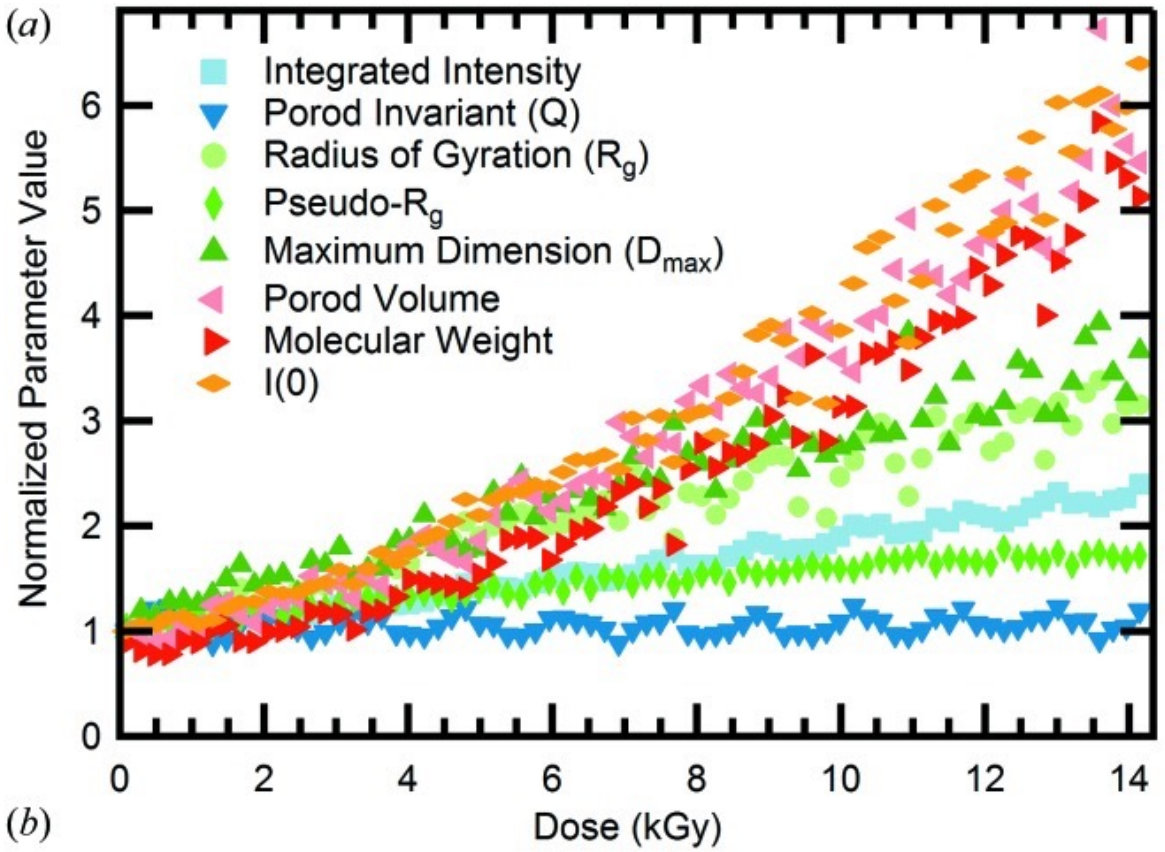
- Continued exposure to X-rays will degrade sample quality
- X-rays interact with water and generate free radicals (particularly HO·)
  - Can then cause specific structural disruption in individual residues
- Most commonly manifests as aggregation in SAXS
  - Radiation tolerance generally much lower than cryo-MX (~1000x)
- In most cases damage is a strict function of dose



Hopkins and Thorne, J. Appl. Cryst. 2016

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_{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

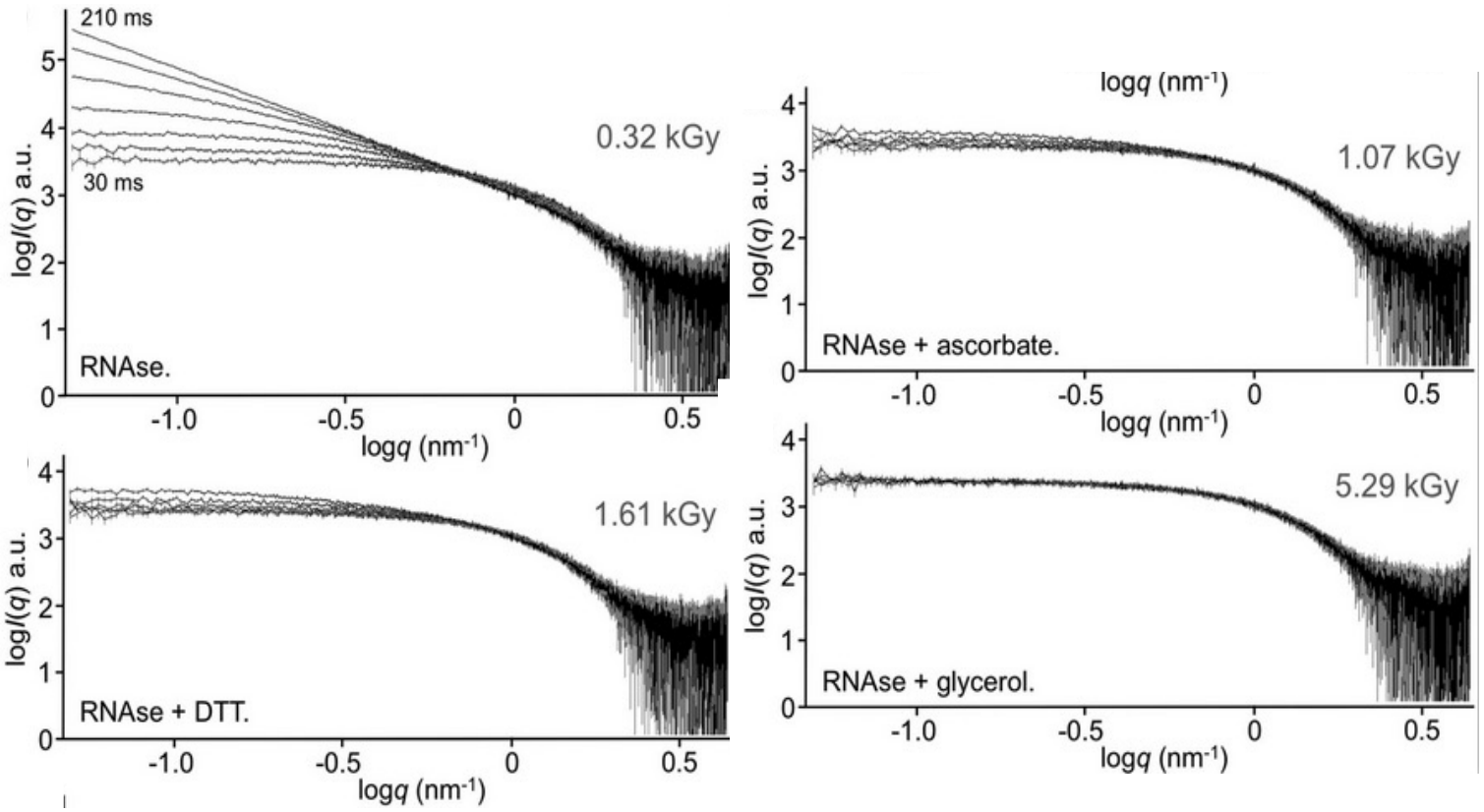


SAXS data parameters vs. dose for lysozyme (4.1 mg/mL)

Hopkins and Thorne, J. Appl. Cryst. 2016

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

Checklist for characterization of a new sample

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 $\propto MW^2$, 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 ~20 μL at 1 mg/mL for standard flow cell
  - Plan on having more per sample
  - Running duplicates is common (expect to use ~50 μL 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

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

Additional resources

- Jeffries, CM *et al*. Preparing Monodisperse Macromolecular Samples for Successful Biological Small-Angle X-ray and Neutron Scattering Experiments. Nature Protocols (2016)
- Skou, S. Gillilan, R and Ando, N. Synchrotron-based small-angle X-ray scattering (SAXS) or proteins in solution. Nature Protocols (2014)
- Goldberg, RN *et al*. Thermodynamic Quantities for the Ionization Reactions of Buffers, J. Phys. Chm. Ref. Data. (2002)
- Kitamura, Y. and Itoh, T. Reaction Volume of Protonic Ionization for Buffering Agents. Prediction of Pressure Dependence of pH and pOH. Journal of Solution Chemistry (1987)
- Jeffries, CM *et al.* Limiting radiation damage for high-brilliance biological solution scattering: practical experience at the EMBL P12 beamline PETRAIII. Journal of Synchrotron Radiation (2015)
- Hopkins, JB and Thorne, RE. Quantifying Radiation Damage in Biomolecular Small-angle X-ray scattering. J Appl Crystallogr (2016)