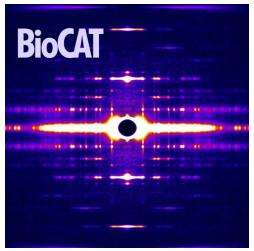

Basic data validation and analysis

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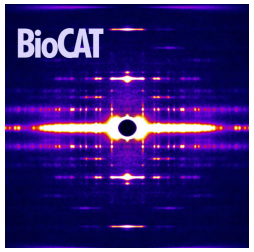
ILLINOIS INSTITUTE OF TECHNOLOGY

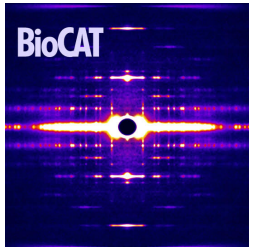
February 28, 2023

CSRRI



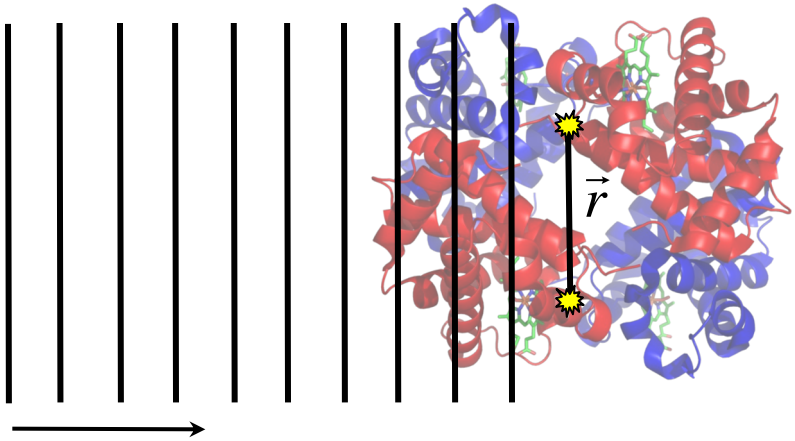
Overview

- The scattering profile
- What can go wrong with your data
- Guinier analysis
- Molecular weight analysis
- Porod and Kratky analysis
- Indirect Fourier Transforms
- Summary



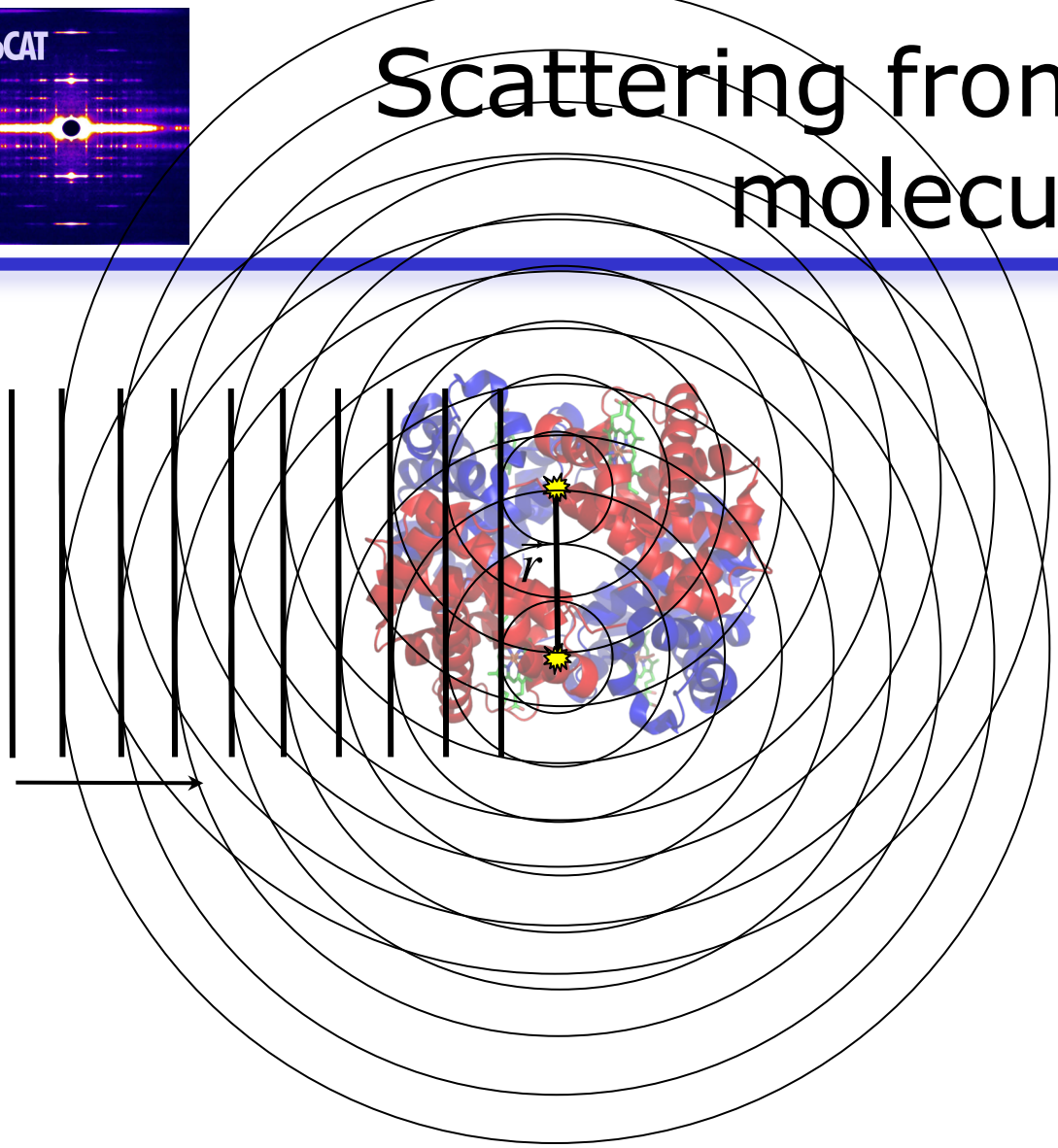
The scattering profile

Scattering from a single molecule

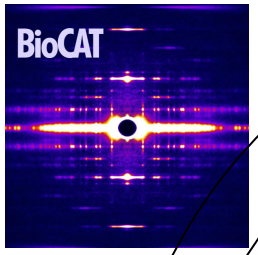


Images from Richard Gillilan's BioSAXS Essentials presentation

Scattering from a single molecule

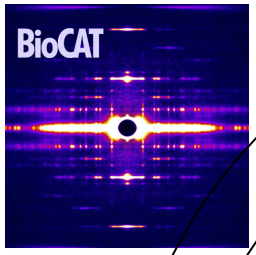


Images from Richard Gillilan's BioSAXS Essentials presentation

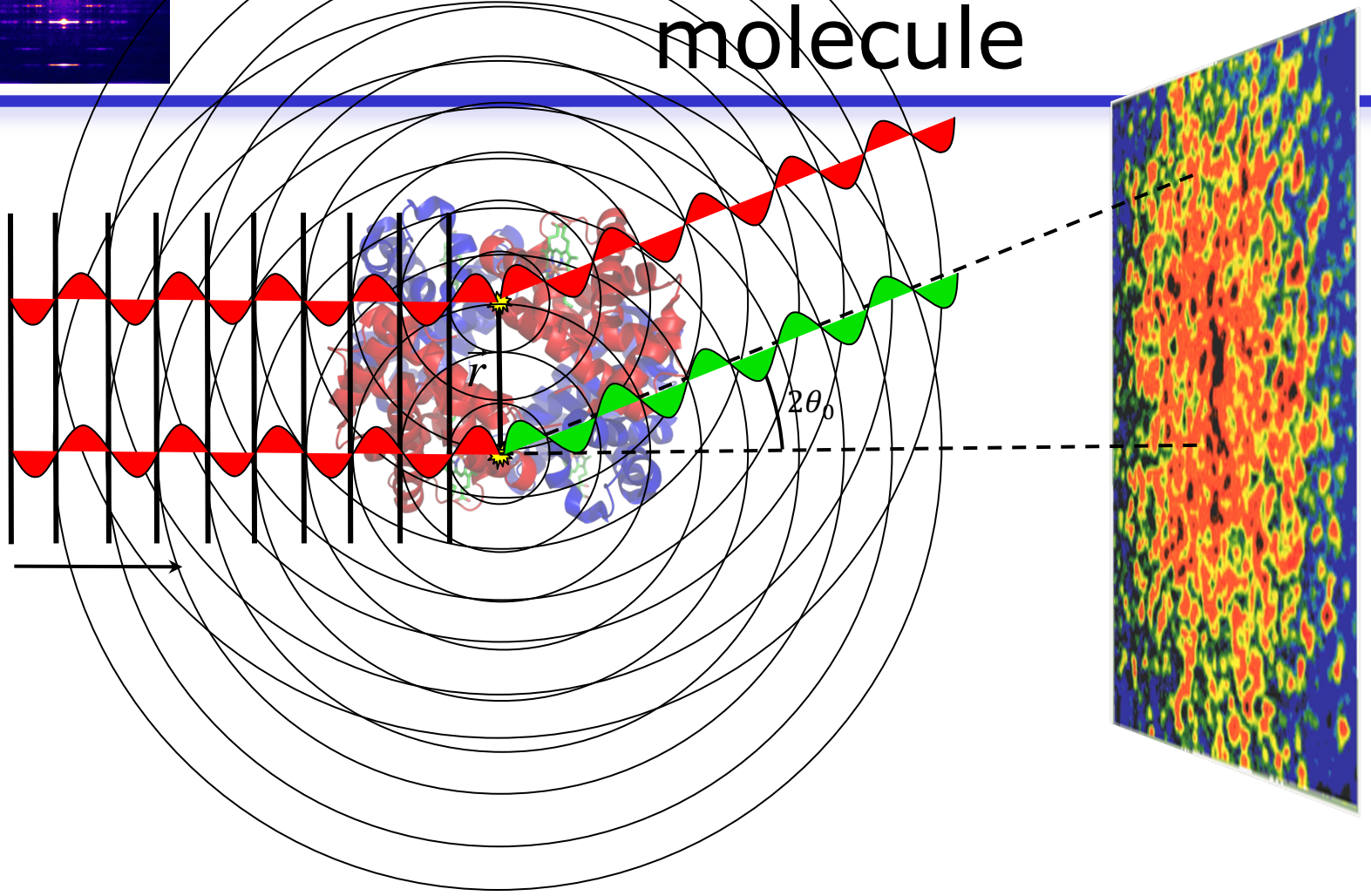


Scattering from a single molecule

Images from Richard Gillilan's BioSAXS Essentials presentation

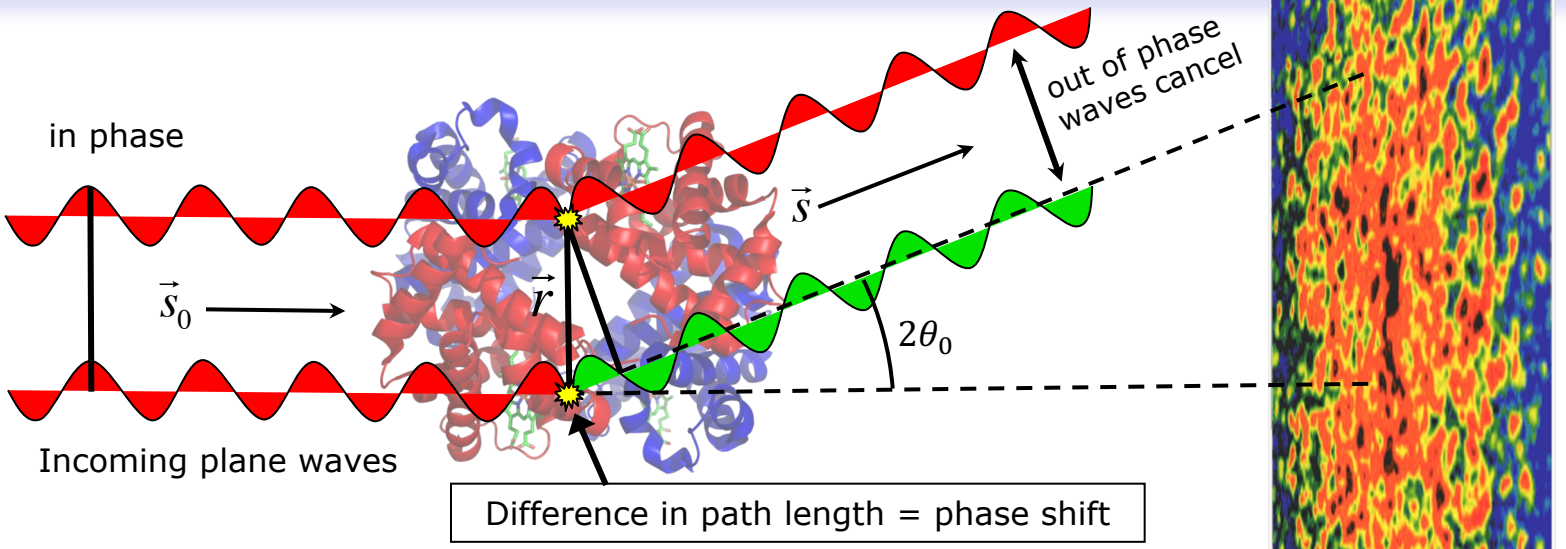


Scattering from a single molecule



Images from Richard Gillilan's BioSAXS Essentials presentation

Scattering from a single molecule

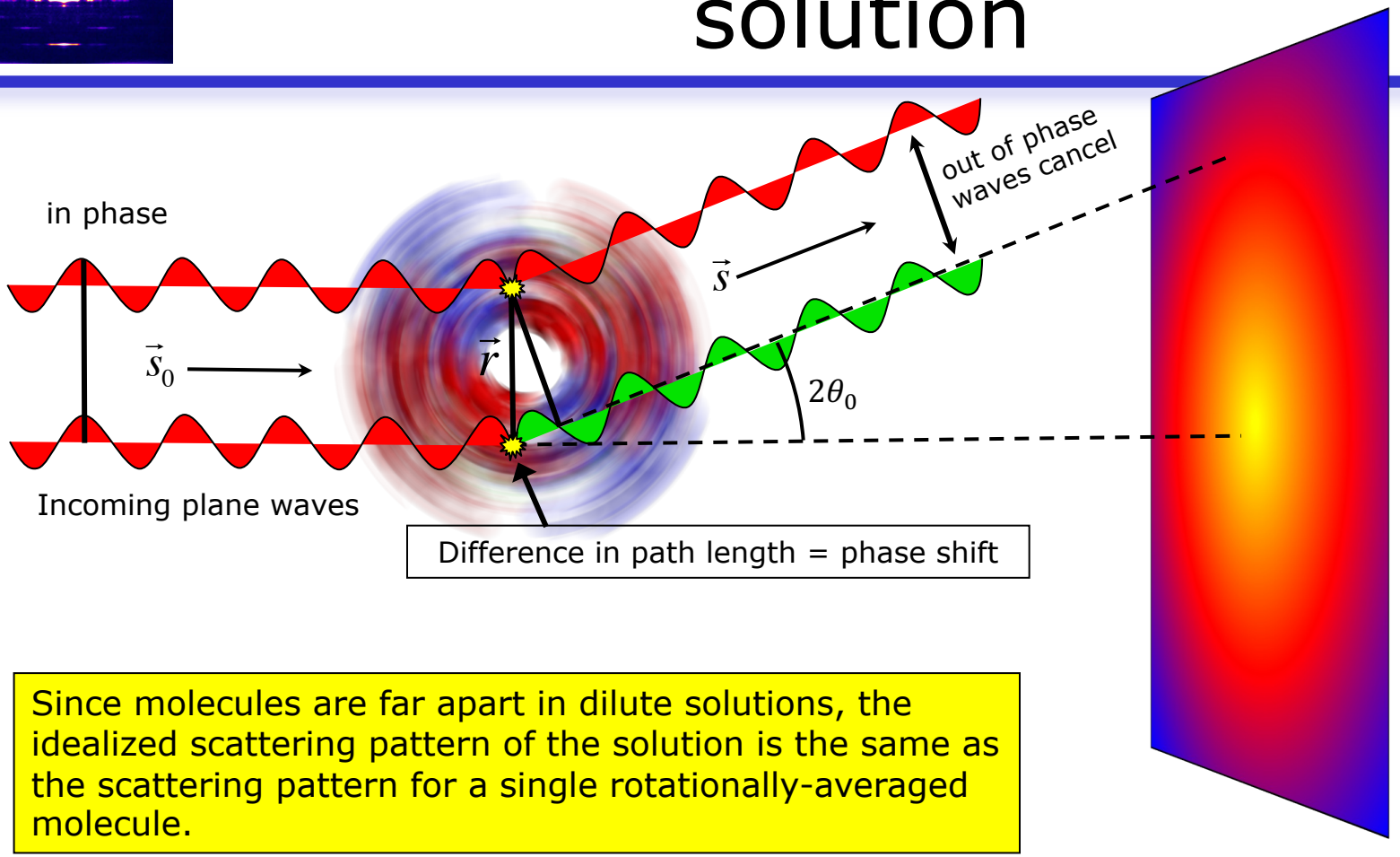


Far-field diffraction pattern

Waves scattered from different parts of the molecule result in phase shifts – a speckled intensity pattern on detector

Images from Richard Gillilan's BioSAXS Essentials presentation

Scattering from molecules in solution

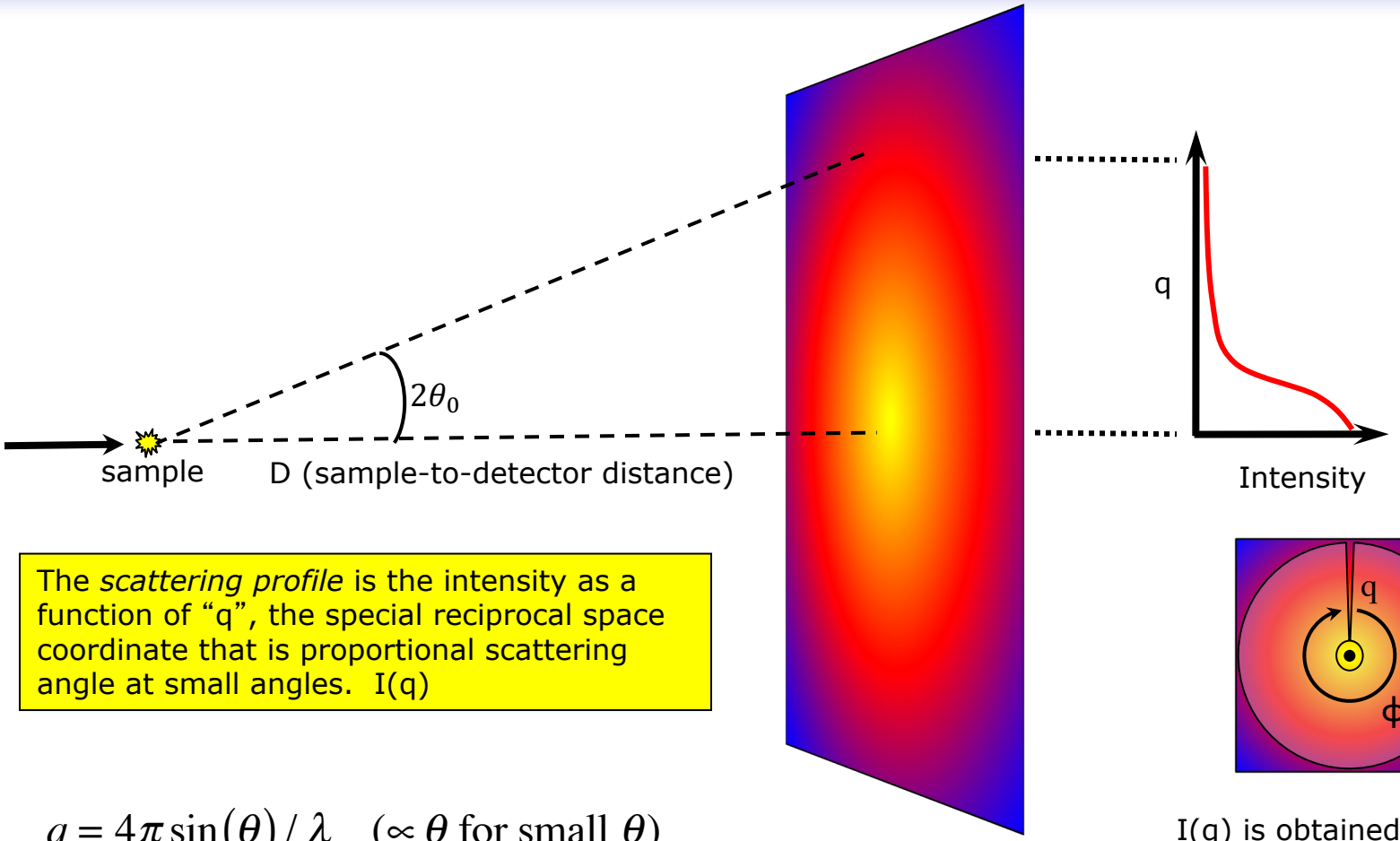


Since molecules are far apart in dilute solutions, the idealized scattering pattern of the solution is the same as the scattering pattern for a single rotationally-averaged molecule.

Far-field diffraction pattern

Images from Richard Gillilan's BioSAXS Essentials presentation

The scattering profile

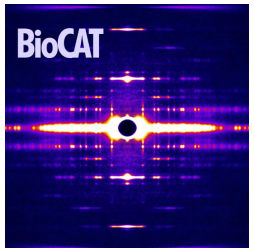


The *scattering profile* is the intensity as a function of "q", the special reciprocal space coordinate that is proportional scattering angle at small angles. I(q)

$$q = 4\pi \sin(\theta) / \lambda \quad (\propto \theta \text{ for small } \theta)$$

I(q) is obtained by integrating around the circle. For detectors, the standard deviation of signal σ(q) is also calculated.

Slide from Richard Gillilan's BioSAXS Essentials presentation



The scattering profile

$$I(q) \propto Mc(\rho_1 - \rho_2)^2 |F(q)|^2 S(q)$$

$I(q)$ – Experimental intensity

M – molecular weight

c – concentration

ρ – scattering density (electrons per unit volume)

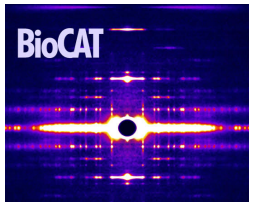
ρ_1 - particle

ρ_2 - solvent

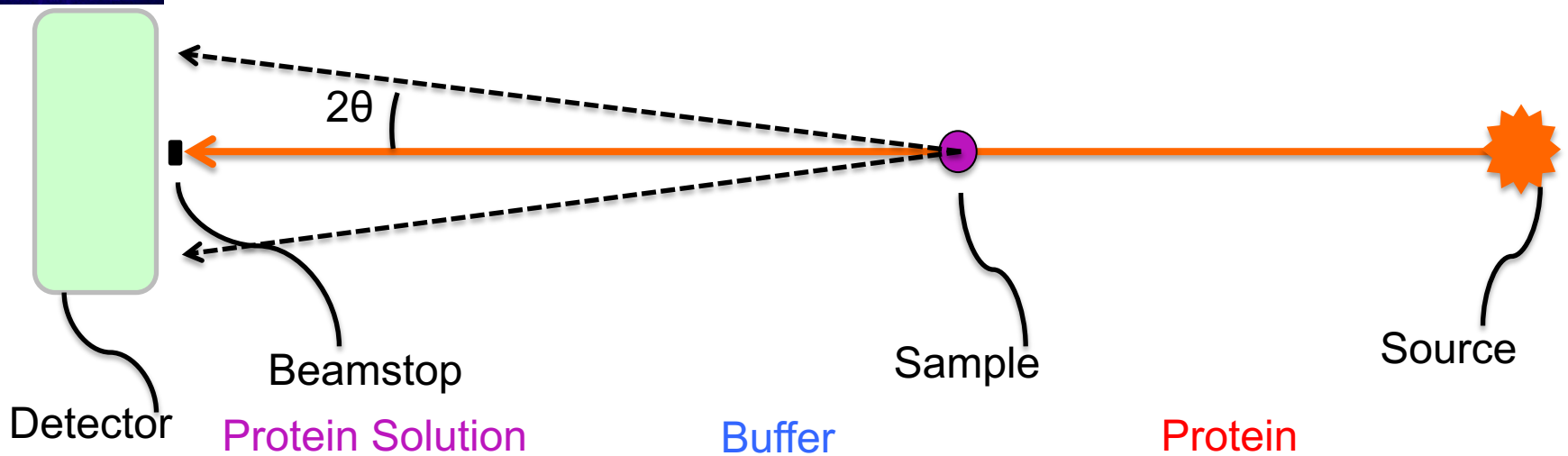
$F(q)$ – Form factor, i.e. molecular shape

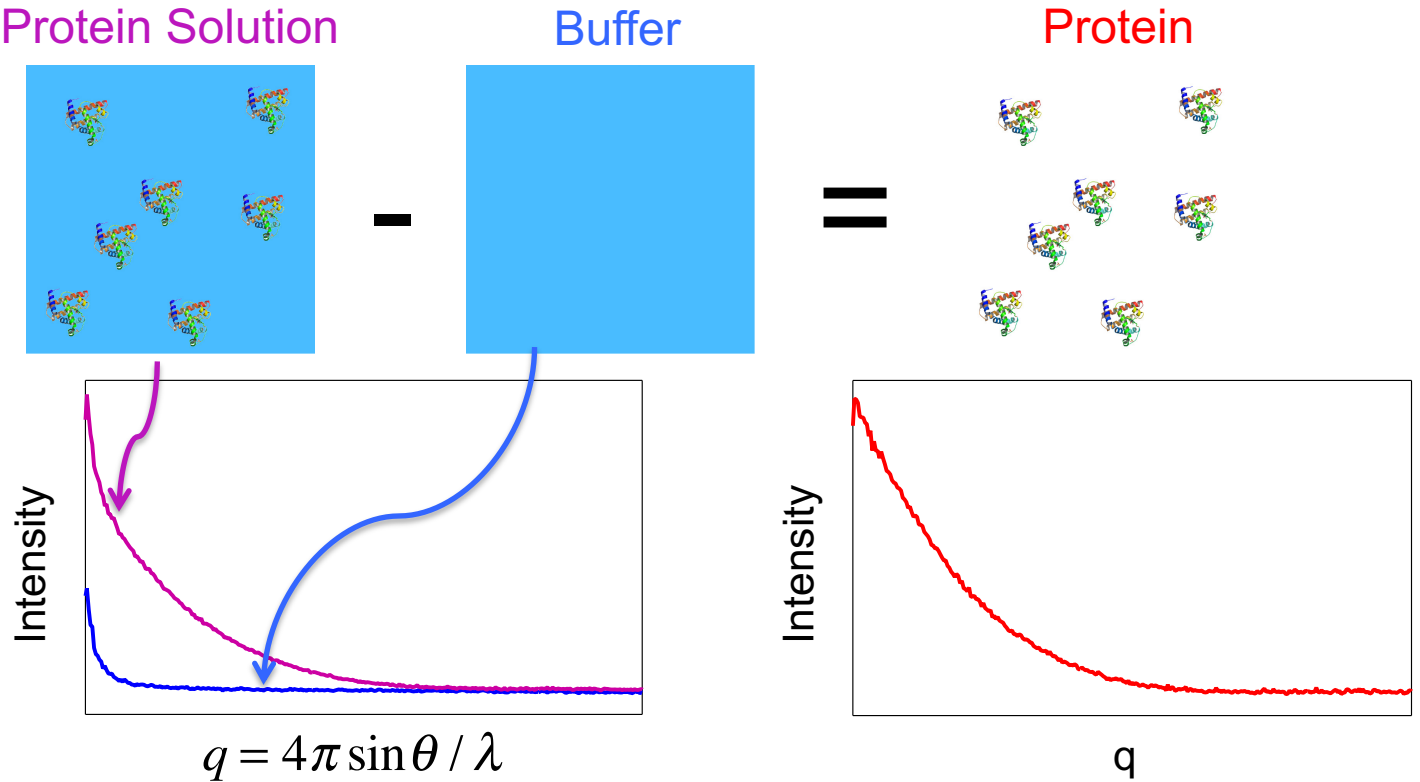
$S(q)$ – Structure factor, i.e. inter-molecular interaction

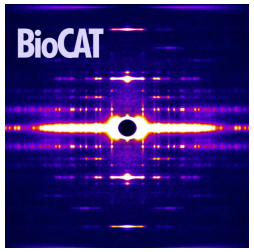
≈ 1 for dilute solutions



A typical SAXS experiment

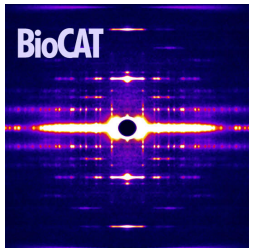






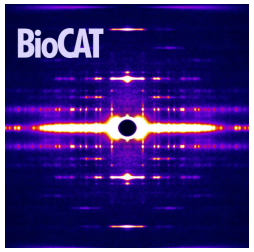
Summary of basic reduction

- Data is collected as 2D images
- 2D images are radially averaged to 1D scattering profiles, I(q)
- Multiple profiles collected for both sample and buffer are averaged
- Averaged buffer profile is subtracted from averaged sample profile to create a single subtracted scattering profile
- This subtracted scattering profile is the basic data form in SAXS



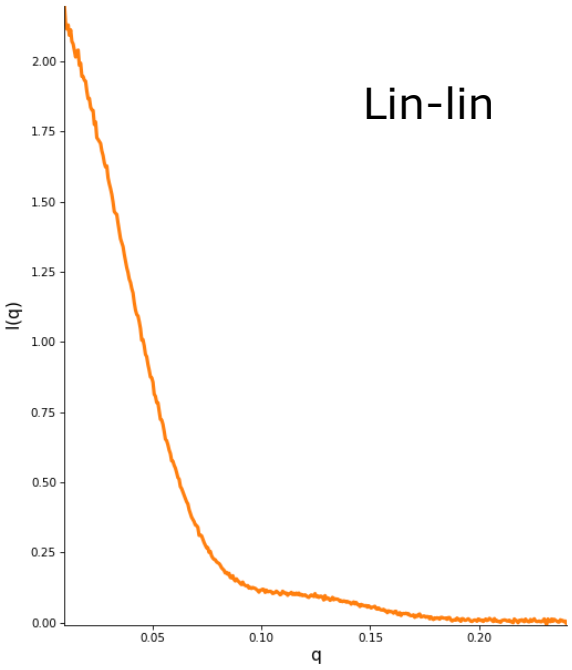
Summary of the scattering profile

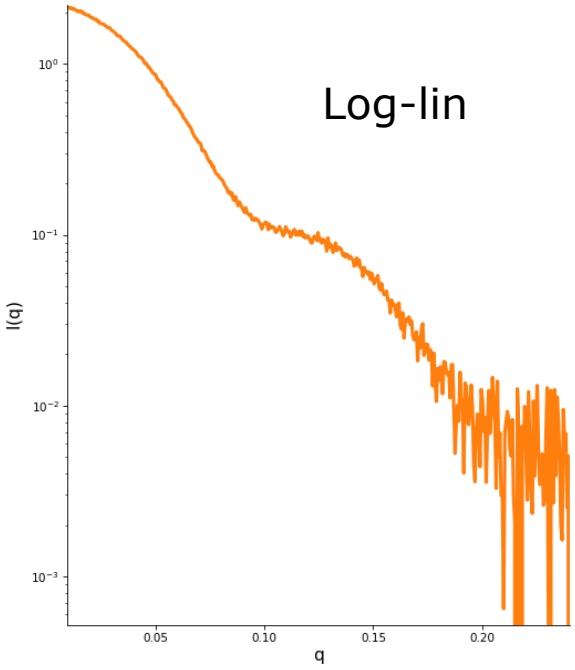
- I(q)
- Scattering profile exists in reciprocal space, q has units of 1/distance (usually 1/Å or 1/nm)
  - Big things scatter more at low q, small things more at high q
  - Low q contains overall size and shape, mid to high q contain finer structure (tertiary, secondary structure)
- Scattering profile represents the scattering from a rotationally averaged macromolecule in solution
- q dependence in scattering profile comes from molecular shape and molecular interactions
  - Important to eliminate interactions to learn about shape

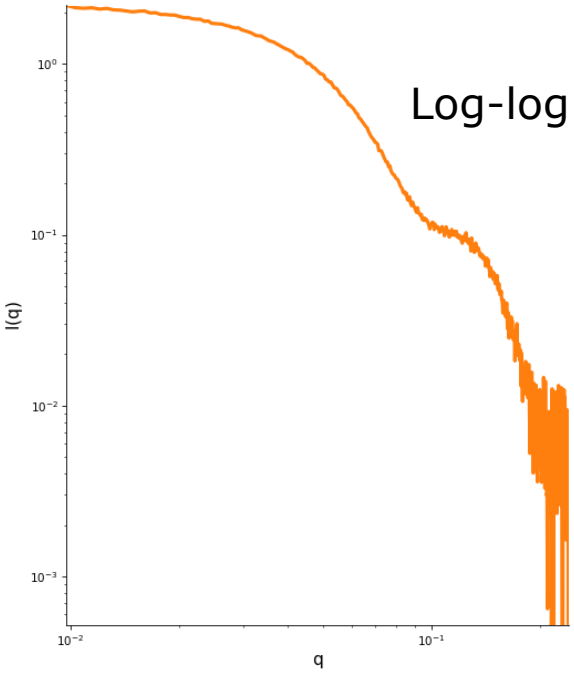


Plotting the scattering profile

Same profile, three different plots

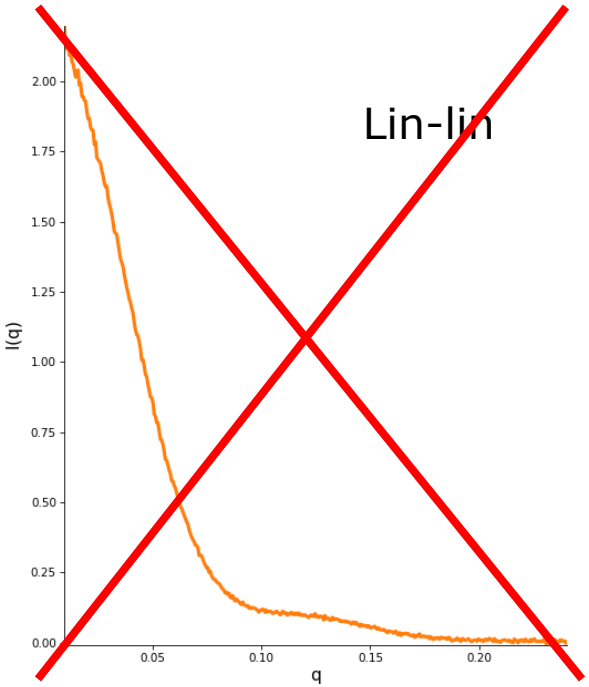


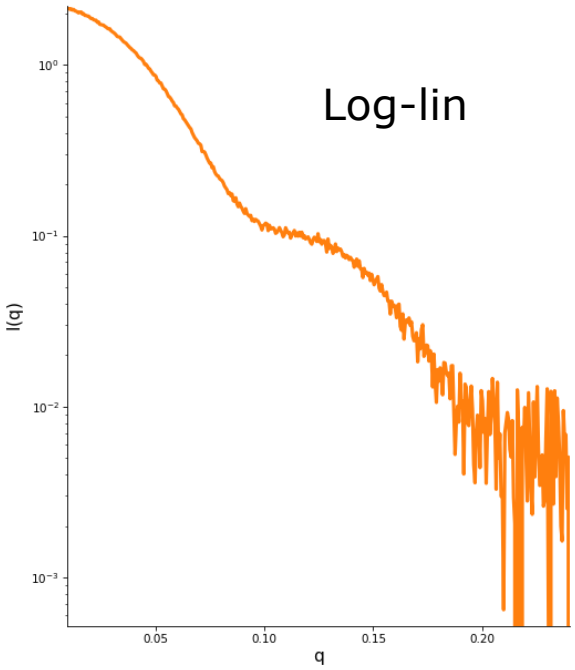


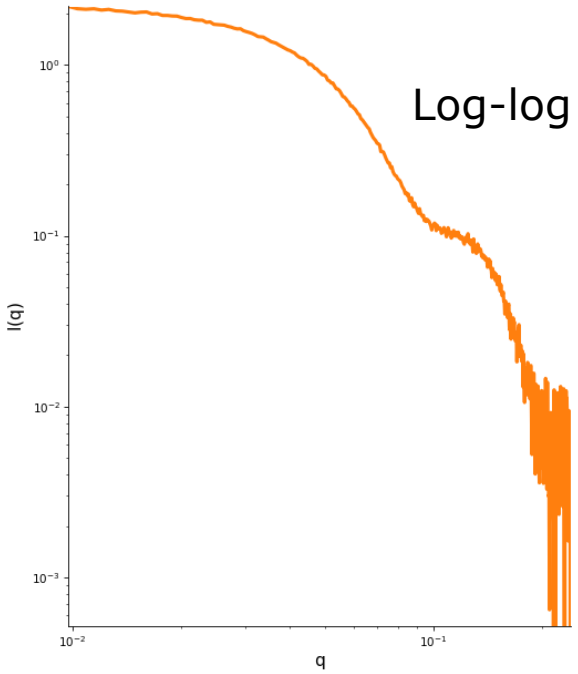


Plotting the scattering profile

Same profile, three different plots

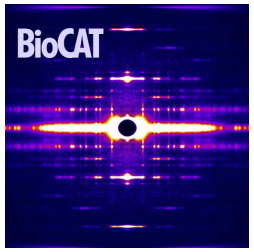




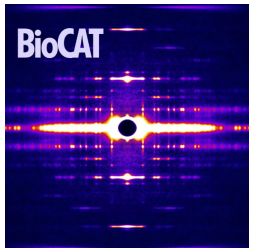


Profile covers 3-4 orders of magnitude. A linear y axis hides significant features

Log-lin emphasizes mid to high q (shape), log-log emphasizes low q (size)



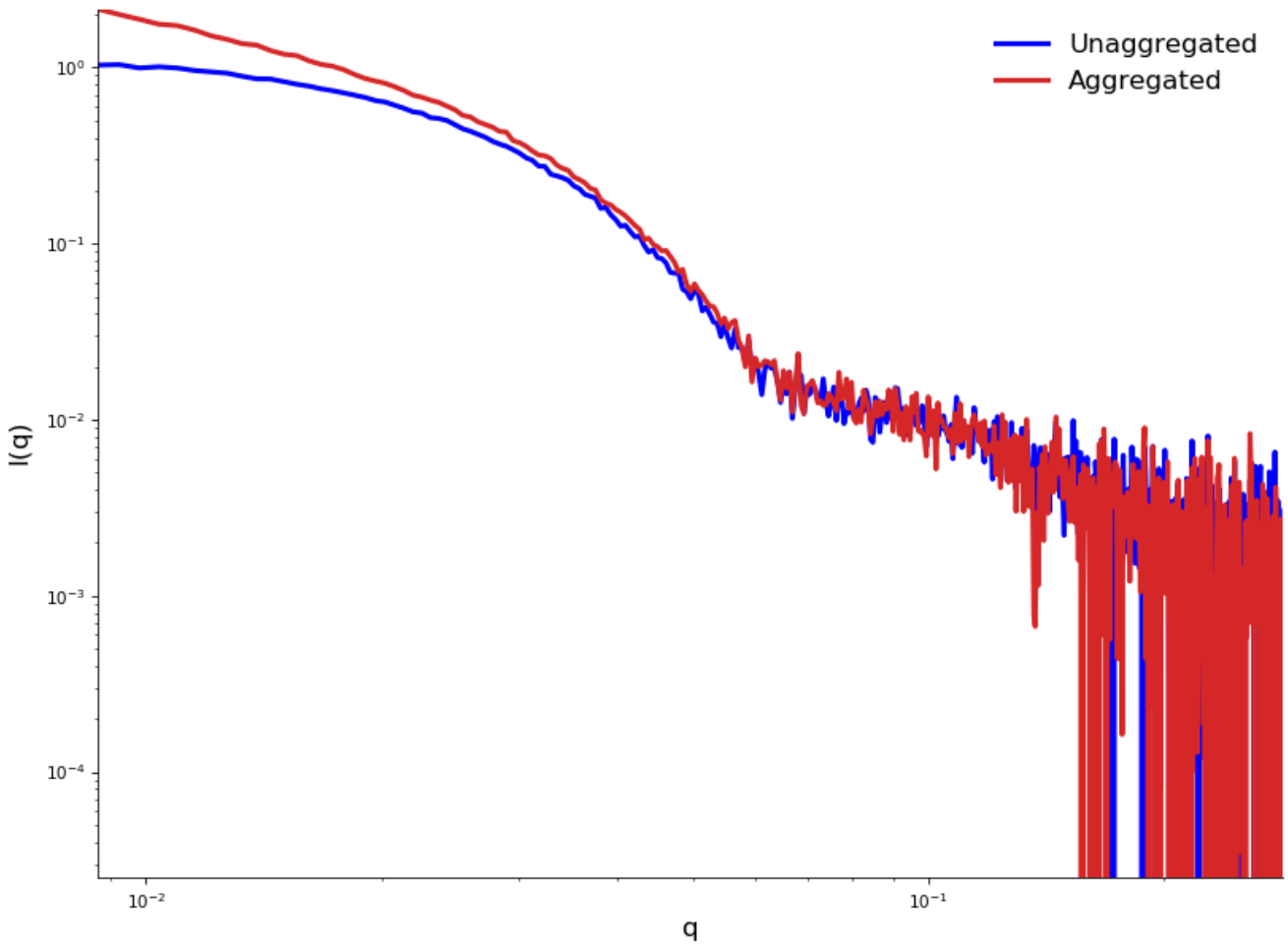
What can go wrong with your data



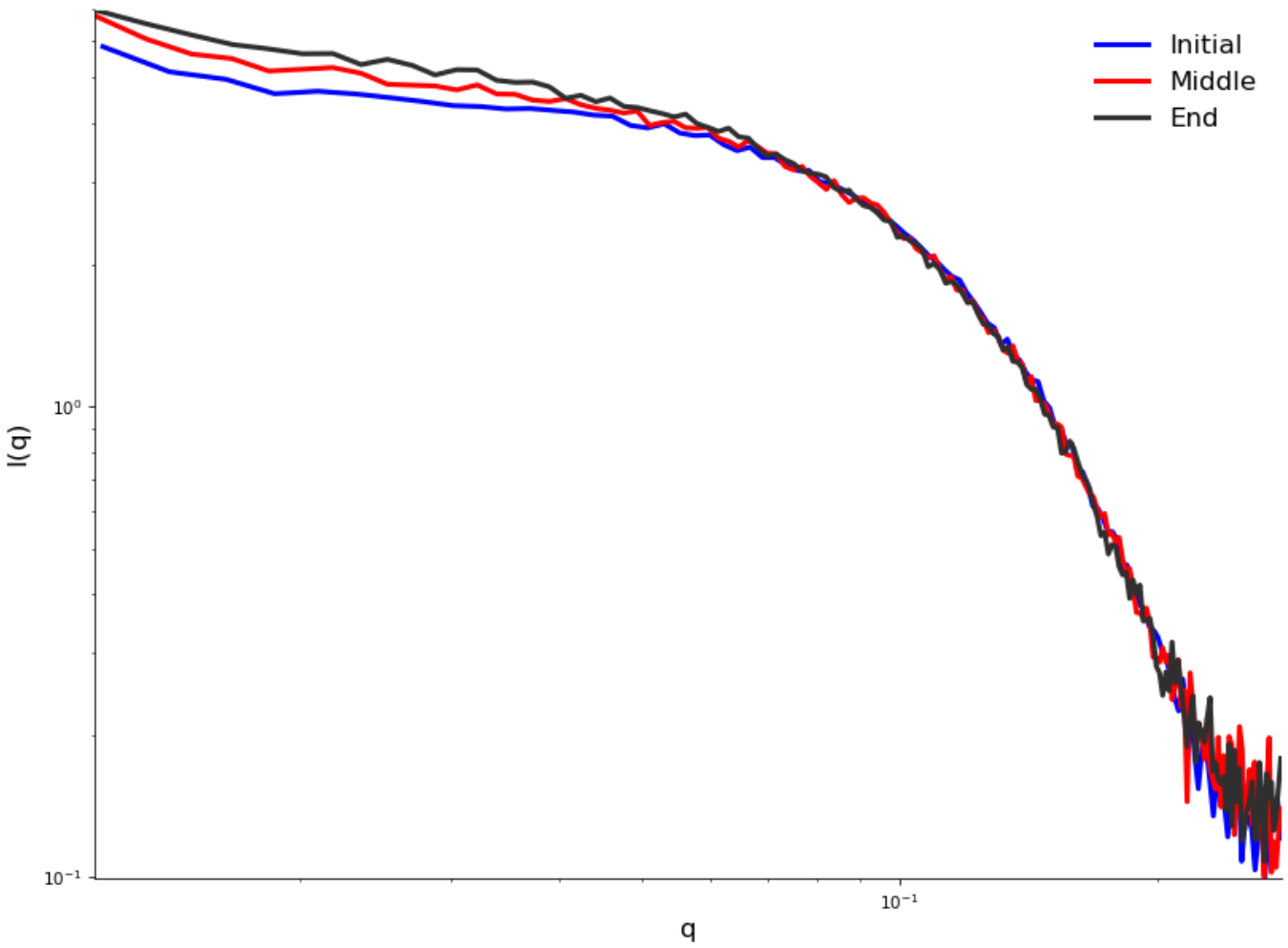
What can go wrong with your data

- Poor quality sample
  - Aggregates or unexpected oligomers in solution
  - Oligomeric state doesn’t match expectations
  - Complex didn’t form
- Radiation damage
  - Unexpected time dependent changes in the measured profile
  - Usually manifests as time dependent aggregation
- Concentration effects (structure factor)
  - Concentration dependent changes in the measured profile
  - Upturn (attraction) or downturn (repulsion) at low q
- Bad buffer subtraction
  - Bad buffer match
  - Capillary fouling
  - Profile going negative at high q or low q (over subtraction)
  - Profile offset at high q, uptick at low q (under subtraction)

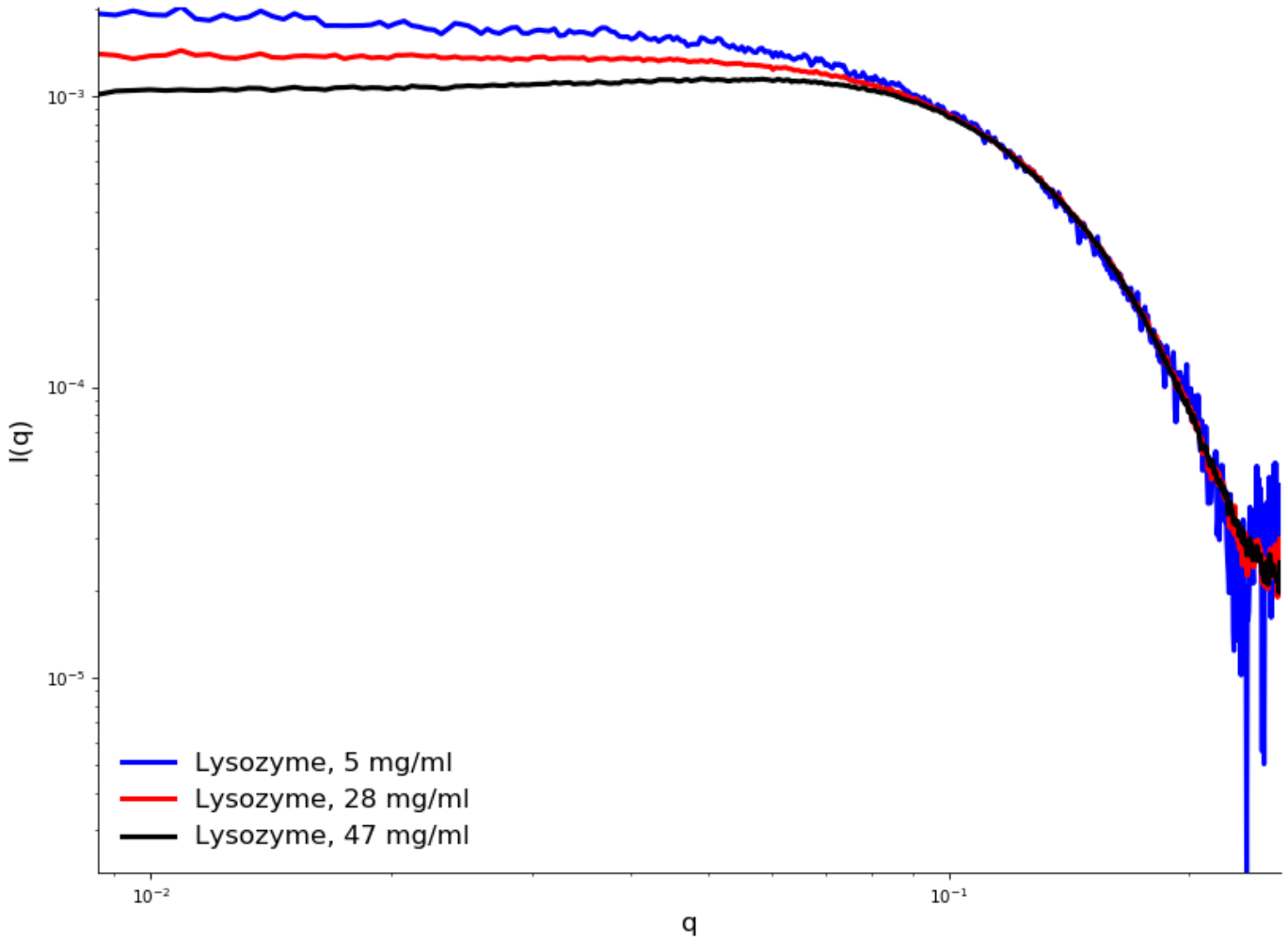
Aggregation

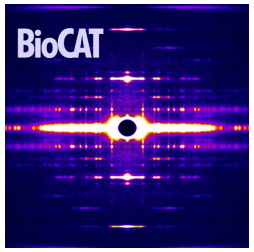


Radiation damage



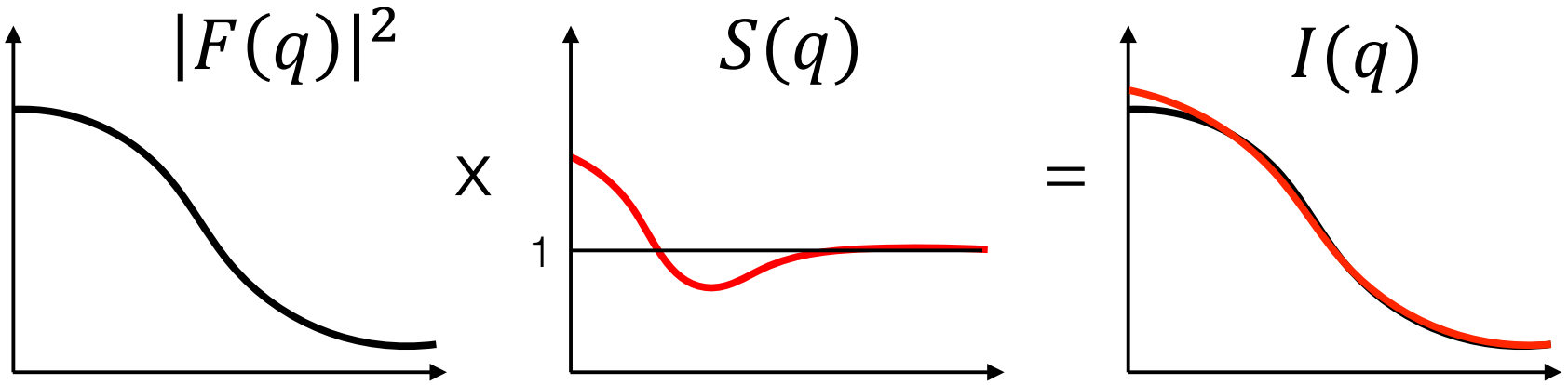
Interparticle Interaction





Interparticle Interactions

Attraction



Repulsion

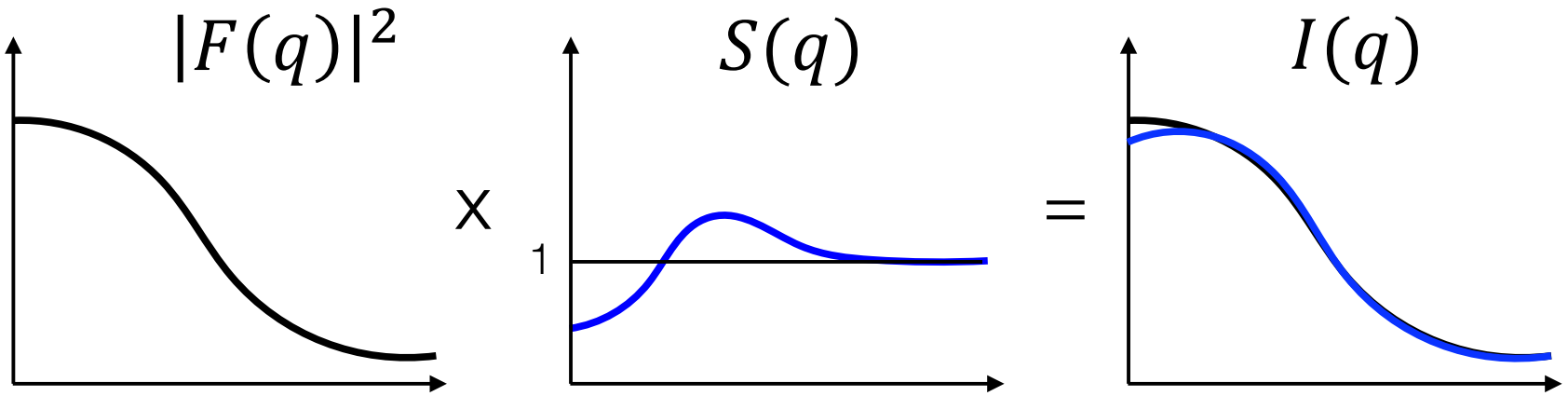
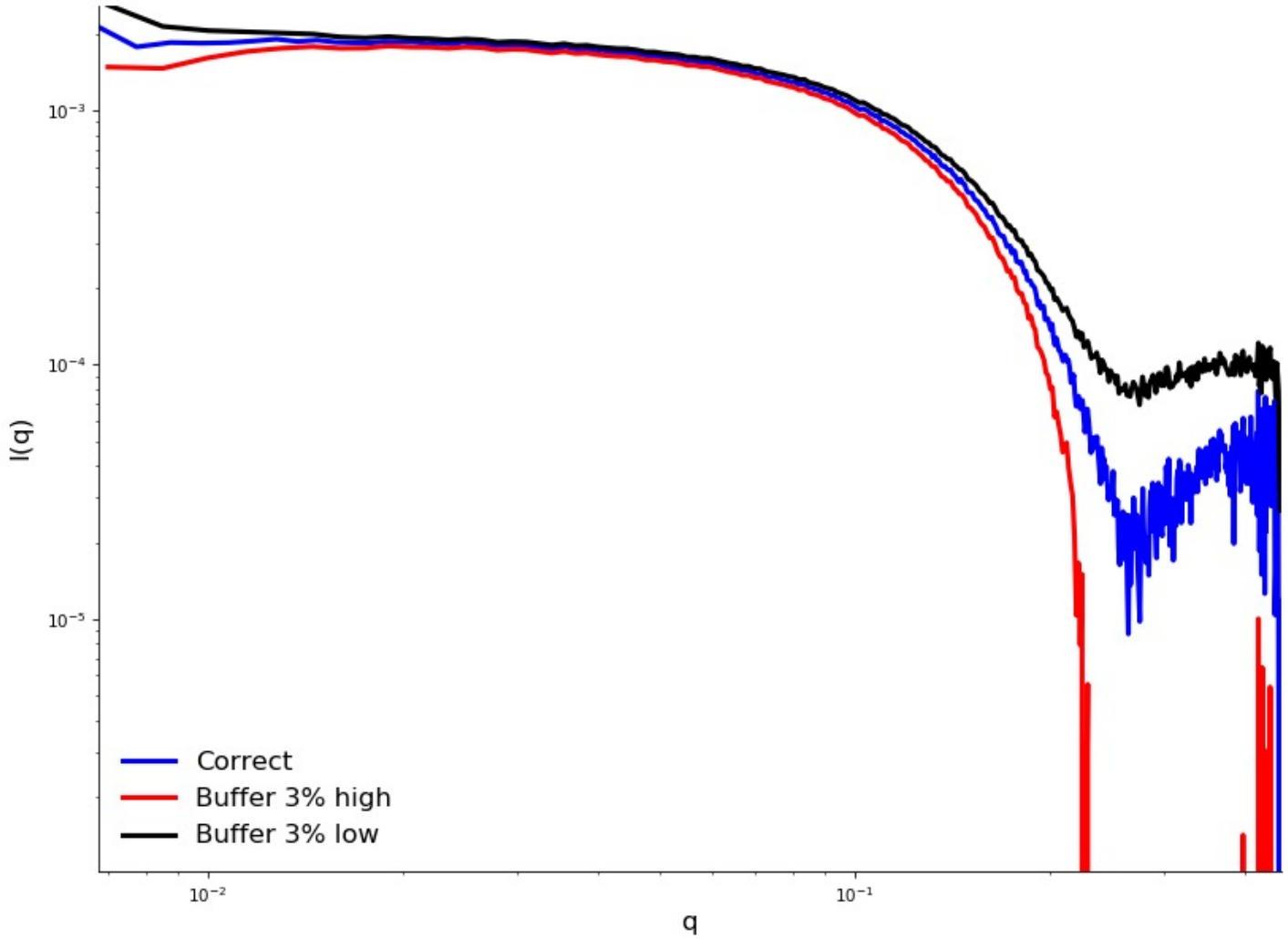
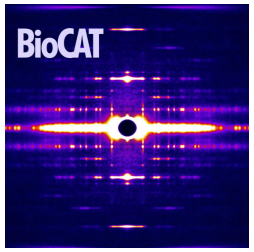


Image from a talk by Thomas Grant

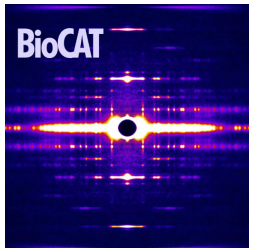
Subtraction errors



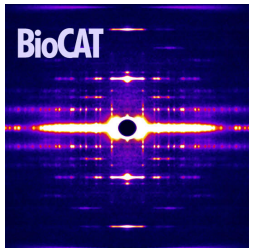


Importance of data validation

- SAXS is a low information content measurement
- Even ‘bad’ SAXS samples provide signal
  - SAXS samples should be homogeneous and monodisperse
- Because you always get a result, and because it’s easy to overfit SAXS data, validating that you have measured good data is extremely important
- Basic analysis methods for SAXS both verify data quality and provide useful information on the system
  - Guinier fit
  - M.W. calculation
  - Porod/Krakty plots
  - IFT/P(r) function



Guinier analysis

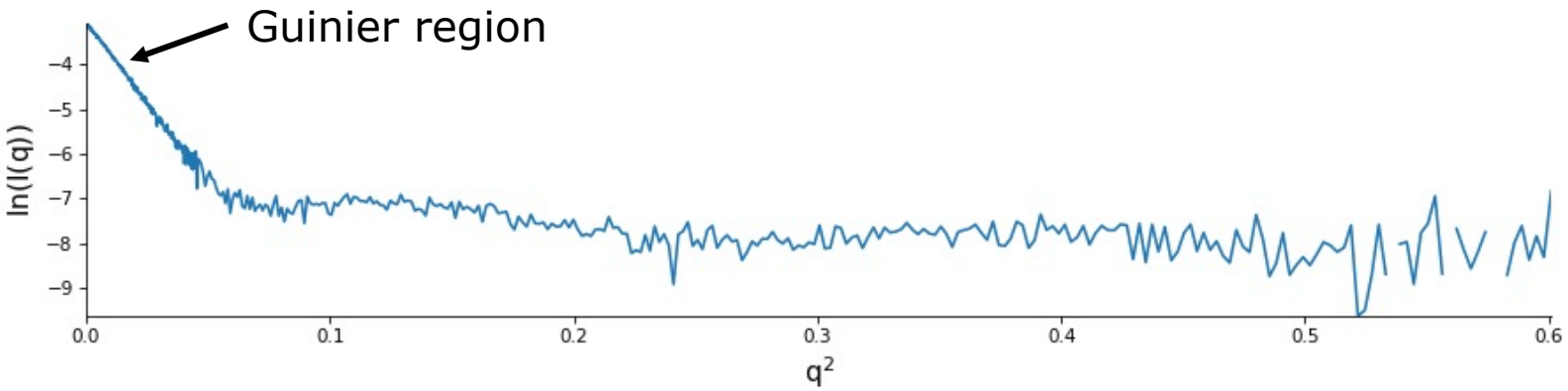


Guinier analysis

- As $q \to 0$, intensity can be approximated by:

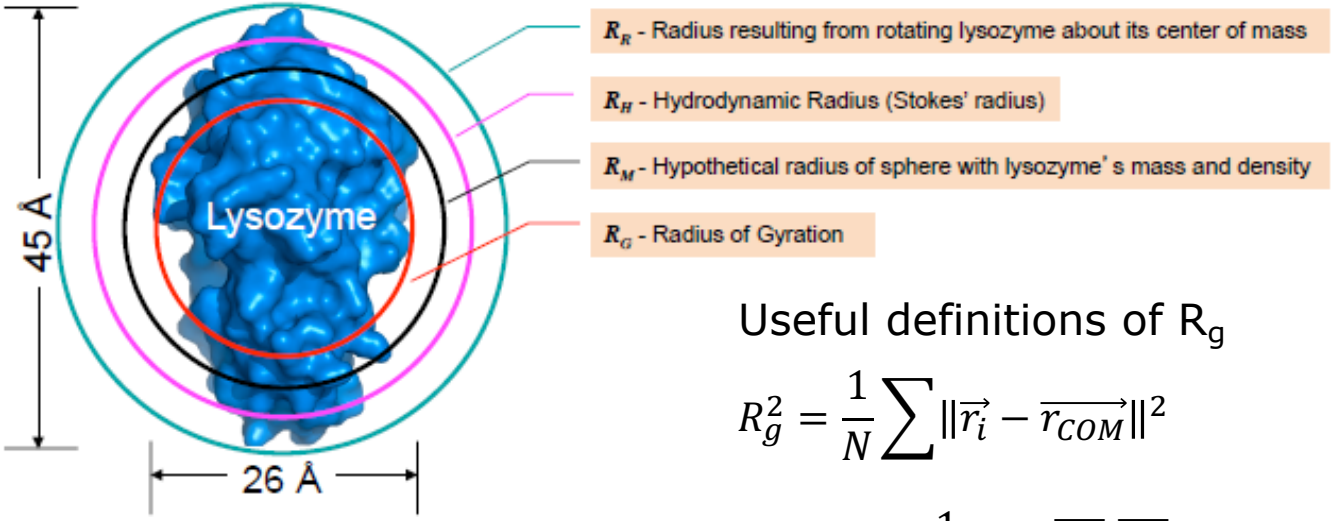
$$I(q) = I(0)e^{-q^2 R_g^2/3}$$

  - R_g - radius of gyration (size)
  - I(0) - scattering at zero angle (M.W related)
- Plot $\log(I)$ vs. q^2: slope = $-R_g^2/3$, intercept = $\log(I(0))$
  - Fit the Guinier region to find these parameters



Guinier analysis

- Radius of gyration:
  - RMS distance from center of mass



Useful definitions of R_g

$$R_g^2 = \frac{1}{N}\sum \|\vec{r_i} - \overrightarrow{r_{COM}}\|^2$$ by atoms

$$R_g^2 = \frac{1}{2N(N-1)}\sum_i \sum_j \|\vec{r_i} - \vec{r_j}\|^2$$ by atom pairs

$$R_g^2 = \int_V r^2\rho(r)dr \Big/ \int_V \rho(r)dr$$ by electron density

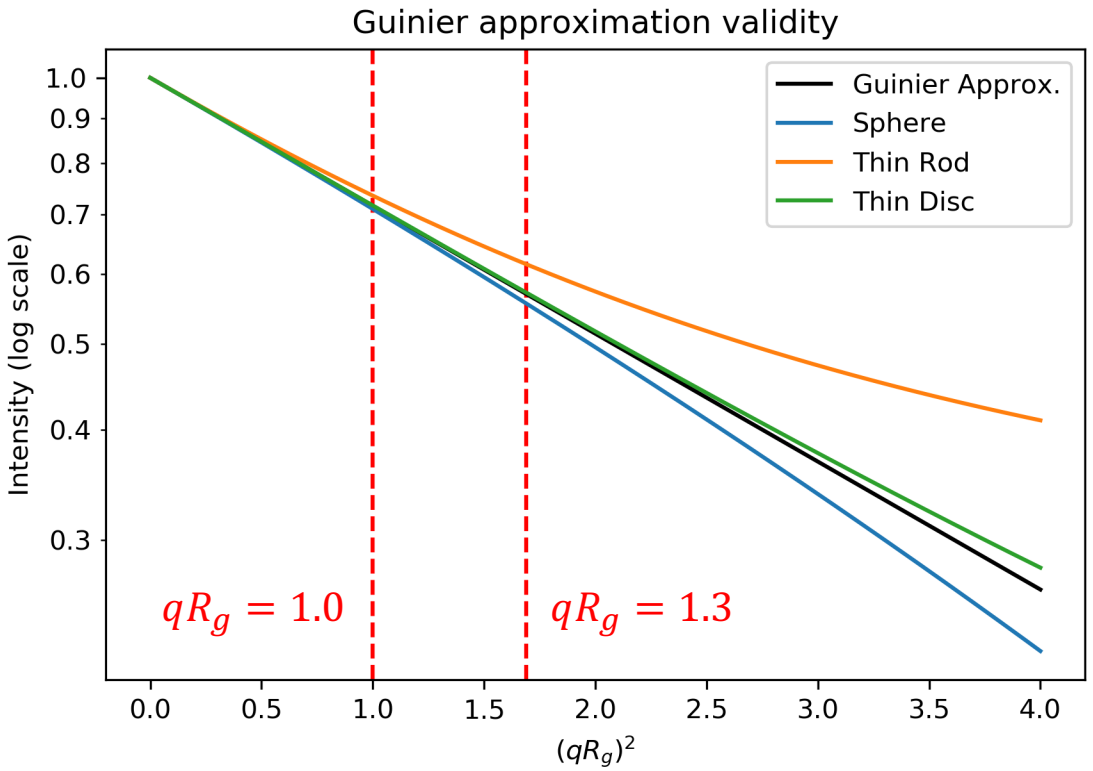
$$R_g^2 = \frac{1}{2}\int_V r^2 p(r)dr \Big/ \int_V p(r)dr$$ by pair distribution

Guinier analysis

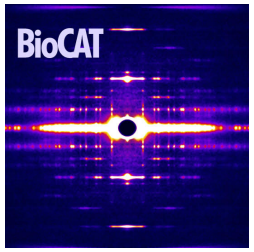
- The Guinier approximation is only accurate at low q. How do you pick you fit endpoints?
  - It depends on particle shape and size!

$$I(q) = I_0 e^{-q^2 R_g^2/3}$$

Need qR_g sufficiently small that this approximation holds

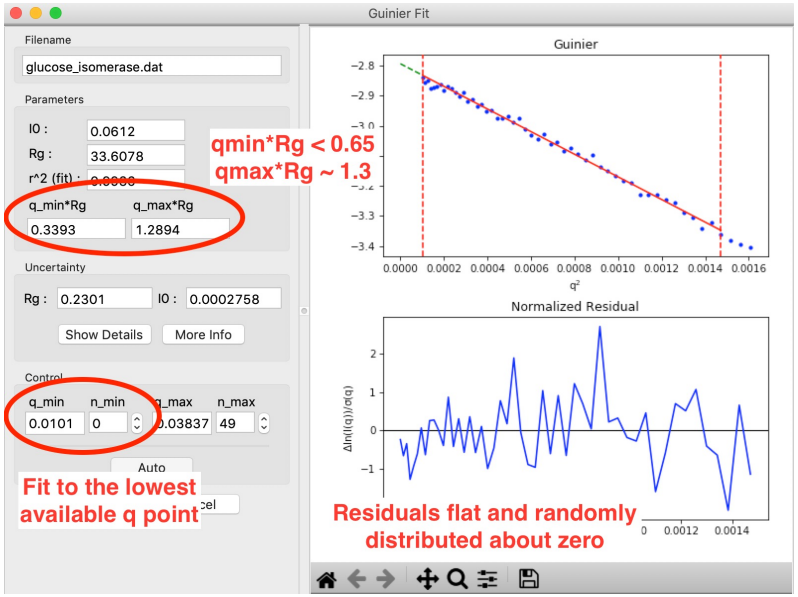


- Conventionally, we fit the Guinier region to $qR_g \approx 1.3$
  - This works for globular molecules
- Rods are fit to $qR_g \approx 1.0$
- Guinier region should be fit to as low q as your data goes
  - Excluding more than 3-5 points at start may mean data is bad
- Need $q_{min}R_g < 1.0$, preferably $q_{min}R_g < 0.65$



Guinier analysis

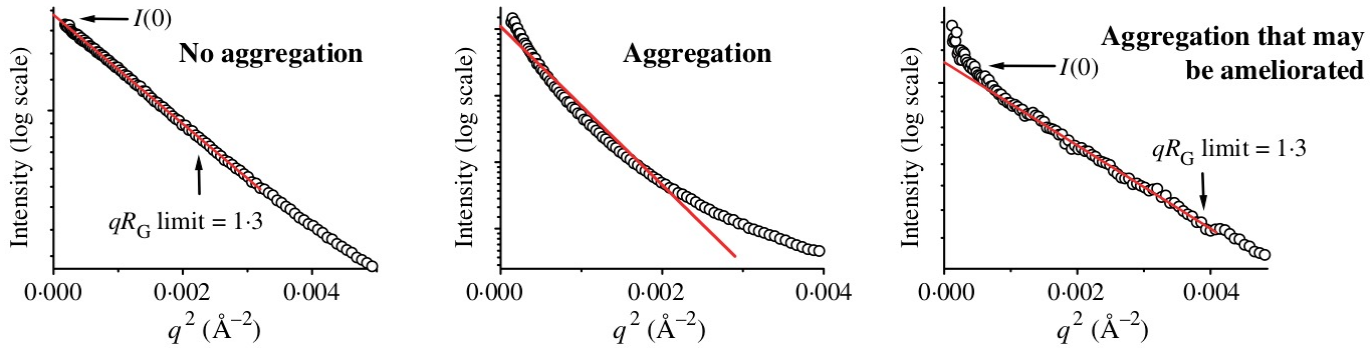
- How do we do a Guinier fit?
  1. Guess a starting maximum q value for fit
  2. Calculate Guinier fit and get R_g
  3. If $q_{max}R_g > 1.3$ (or 1.0) reduce maximum q. If $q_{max}R_g < 1.3$ (or 1.0) increase the maximum q
  4. Repeat steps 2 and 3 until you converge on a final maximum q
- Minimum q should be lowest available q point
- Criteria for a good Guinier fit:
  - $q_{min}R_g < 0.65$
  - $q_{max}R_g \approx 1.3$ (globular/disc) or $q_{max}R_g \approx 1.0$ (extended)
  - Guinier fit residuals are flat and randomly distributed
  - Fit extends to lowest available q point (or very close)
- If unsure about particle shape, start with $q_{max}R_g \approx 1.3$, decrease to 1.0 if residuals not flat



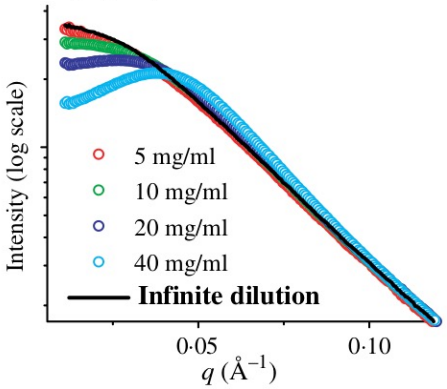
Guinier analysis

- Non-linearities in Guinier analysis are indicative of problems with your sample

Aggregation causes a characteristic upturn at low q
- Could be caused by aggregates in the sample, or by radiation induced aggregation (radiation damage)



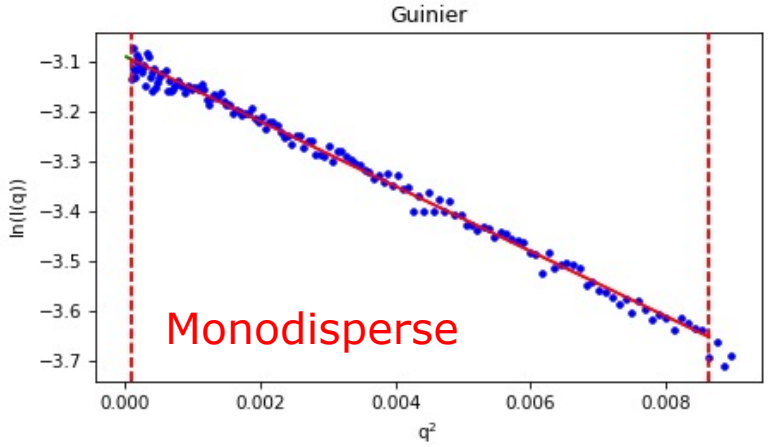
Downturns at low q are characteristic of structure factor, also show up in a Guinier fit

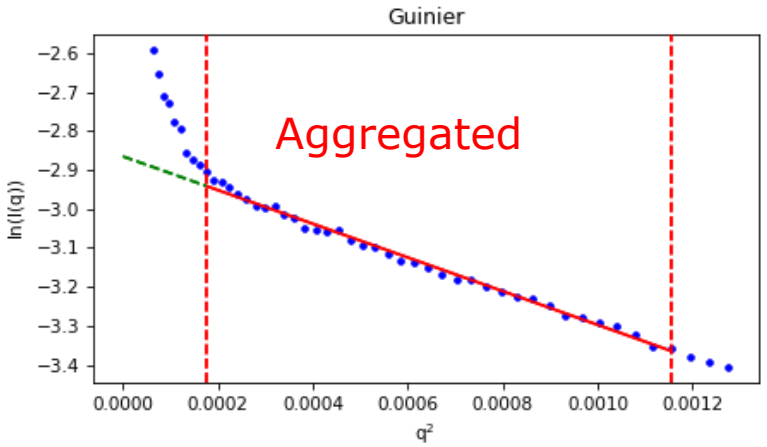


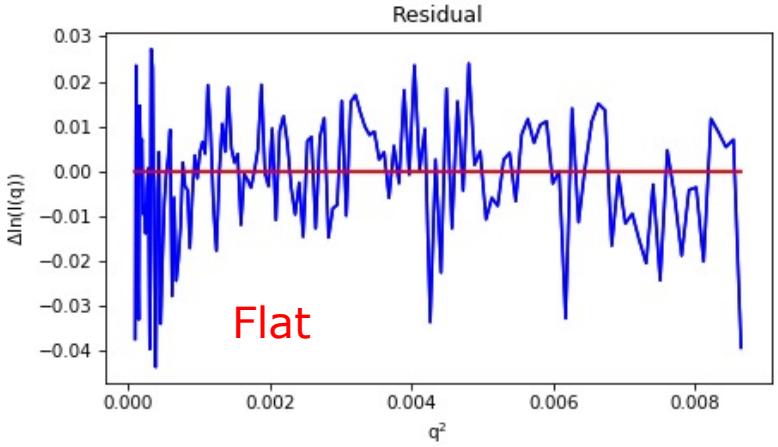
Images from Putnam et al. Quarterly reviews of biophysics, 40(3) 2007.

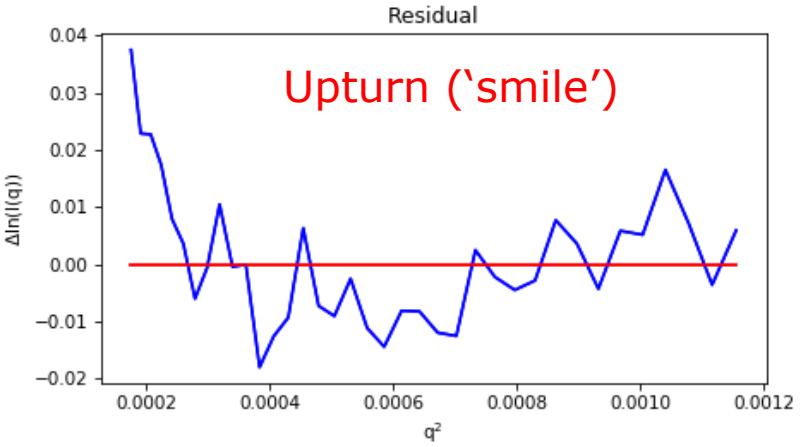
Guinier analysis

- Fit residual can help you see problems



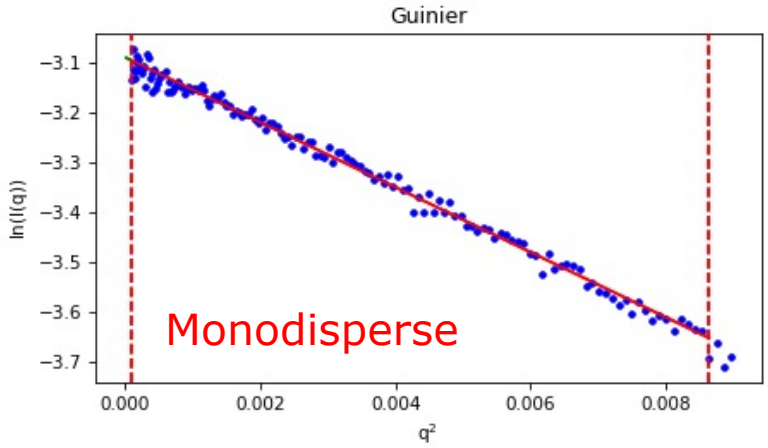


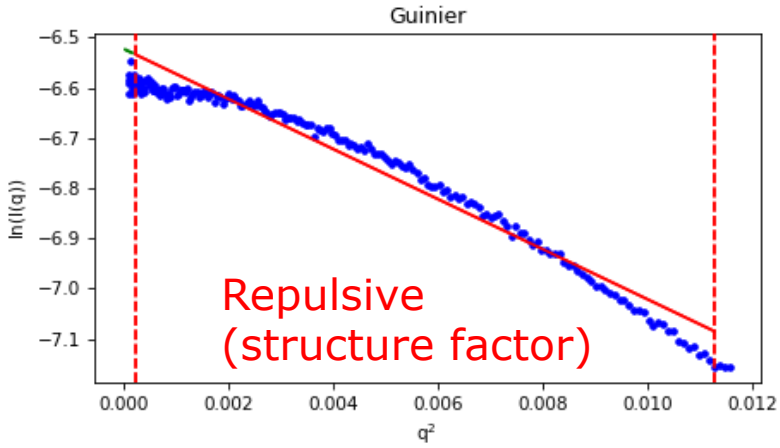


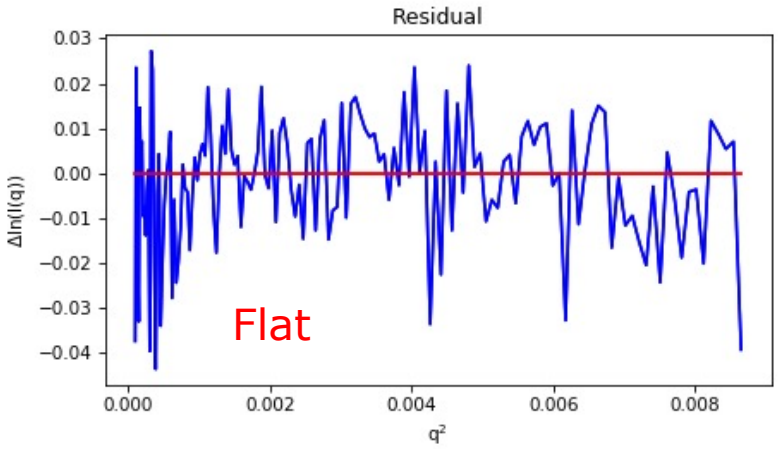


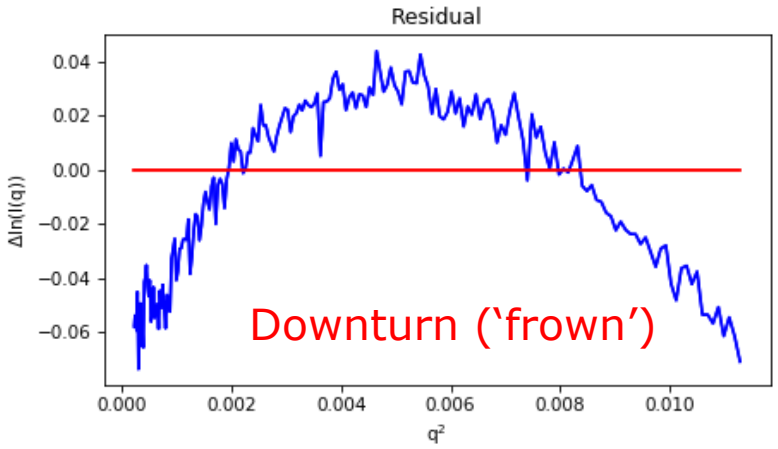
Guinier analysis

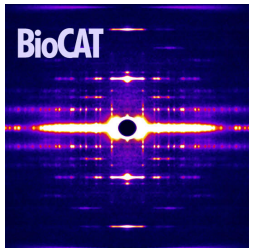
- Fit residual can help you see problems





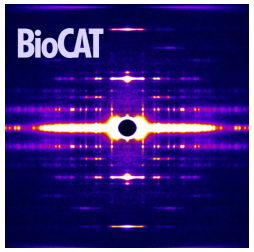




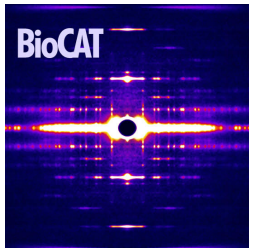


Guinier analysis summary

- Guinier analysis sensitive to low q
- Most problems with your data will show up here!
  - Aggregation
  - Radiation damage
  - Interparticle interactions
  - Some buffer subtraction issues
- Guinier region should be linear, with flat fit residuals
  - Upturn in profile or residuals usually aggregation
  - Downturn in profile or residuals usually repulsion
- Gives R_g, informs on particle size
- Gives $I(0)$, informs on particle mass

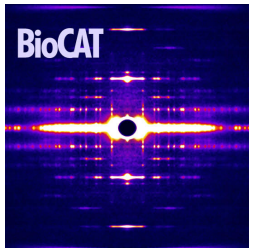


Molecular weight analysis



Molecular weight from SAXS

- Molecular weight estimates from SAXS are ~10% accurate at best
  - Don’t rely on SAXS to determine MW of system, use another approach (e.g. MALS)
- Use SAXS MW to verify state of macromolecule in solution
  - Oligomeric state
  - Is the sample intact
  - Is the complex formed
  - Important to verify what’s in solution is what you’re expecting
- Six different methods supported for calculating MW
  - Two concentration dependent methods (not useful for SEC-SAXS)
  - Four concentration independent methods



Molecular weight from SAXS

1. **I(0) in absolute units (water/glassy carbon standard)**
   Scattering intensity actually has "absolute" units of cm^{-1} when properly calibrated with a known standard such as water. *Once I(0) is expressed in absolute units*,

$$\text{Mol. Wt.} = \frac{N_A I(0)/c}{(\Delta\rho_M)^2}$$

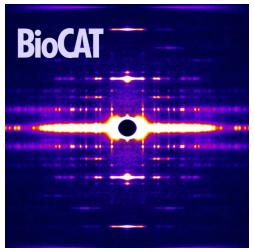
$N_A = 6.02 * 10^{23}$ c = concentration $\Delta\rho_M$ = "scattering contrast"

   Reference: Mylonas, E. & Svergun, D. I. (2007). *J. Appl. Cryst. 40, S245-S249*

2. **Protein standards**
   Unknown molecular weights can be determined by comparison with known protein standards such as lysozyme or glucose isomerase:

$$\text{Mol. Wt.} = \frac{I(0)/c}{I(0)_{std}/c_{std}}(MW_{std})$$

   Reference: Mylonas, E. & Svergun, D. I. (2007). *J. Appl. Cryst. 40, S245-S249*



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Refere… *S245-*…

Both of these methods require accurate concentration measurements!

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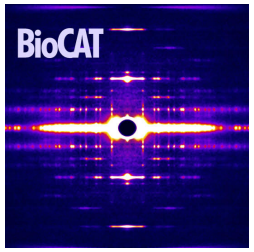


Molecular weight from SAXS

3. Porod volume methods

Mass (in kDa) can calculated as the density times the volume of the particle. The Porod volume of the particle is used, and is calculated:

$$V = 2\pi^2 I(0) / \int_0^\infty q^2 I(q) dq$$

The density used is typically 0.83*10^{-3} kDa/Å^3, but can be adjusted for the particular application.

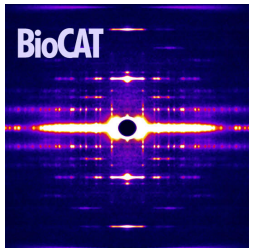
More advanced techniques based on this idea can be relatively accurate
Reference: Fischer et al. (2009). *J. Appl. Cryst.*, 43, 101-109

4. Volume of correlation method

Molecular weight can be estimated using the empirically relation:

$$\text{MW} = \left(\frac{Q_R}{c}\right)^{1/k} \quad \text{where} \quad Q_R = \frac{V_c^2}{R_g} \quad \text{and} \quad V_c = \frac{I(0)}{\int qI(q)dq}$$

The values of *k* and *c* depend on the type of macromolecule. For proteins *k* = 1 and *c* = 0.1231, for RNA *k* = 0.808 and *c* = 0.00934.
Reference: Rambo and Tainer (2013). *Nature*, 496, 477-481



Molecular weight from SAXS

5. Comparison to known structures method (Shape&Size)

A machine learning method that categorizes SAXS data into shape categories based on comparison to a catalog of known structures from the PDB. By finding the nearest structures in shape and size, it then estimates the MW of the sample. Implemented in the ATSAS package.

Reference: Franke, D., Jeffries, C. M. & Svergun, D. I. (2018). Biophys. J. 114, 2485–2492. DOI: 10.1016/j.bpj.2018.04.018

6. Bayesian inference method

A Bayesian inference method for calculating the M.W. The method calculated the M.W. using the Porod volume, volume of correlation, and comparison to known structures (methods 3-5) for a large set of theoretical scattering profiles. A probability distribution was created for each method that describes the probability of obtaining a particular calculated M.W. based on the true M.W.

For an input protein, the M.W. by methods 3-5 is calculated as evidence. Bayesian methods are then used to combine the prior probability distributions from the theoretical scattering profiles to calculate the most likely M.W. of the sample.

Reference: Hajizadeh, N. R., Franke, D., Jeffries, C. M. & Svergun, D. I. (2018). Sci. Rep. 8, 7204. DOI: 10.1038/s41598-018-25355-2

Molecular weight from SAXS

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Methods 3-6 do not rely on the concentration of the sample, making them useful as checks for methods 1 and 2, and in cases where the concentration may not be known (such as SEC-SAXS).

M.W. using the Porod volume, volume of correlation, and comparison to known structures (methods 3-5) for a large set of theoretical scattering profiles. A probability distribution was created for each method that describes the probability of obtaining a particular calculated M.W. based on the true M.W.

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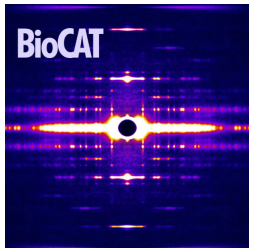
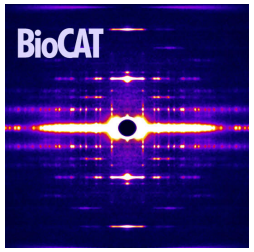


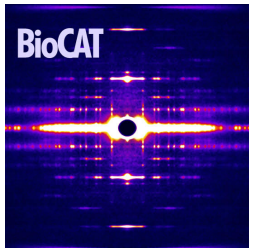
Molecular weight from SAXS

- Each method fails in different ways
  - Absolute scale – Requires accurate calculation of macromolecule contrast, partial specific volume. Depends on accuracy of concentration, absolute scale calibration
  - Reference to known standard: Reference standard must be in a buffer with similar contrast as your sample. Depends on accuracy of concentration for both reference and your sample
  - Porod volume: Works best for compact, globular, rigid molecules. Requires accurately knowing the macromolecule density.
  - Volume of correlation: Fails for protein-nucleic acid complexes. Requires the integral to converge. Sensitive to noisy high q data. Fails for molecules $\lesssim$ 20 kDa.
  - Comparison to known structures: Only works for proteins. Doesn't work for flexible systems.
  - Bayesian inference: Only works for proteins.
- All methods will fail if your Guinier fit is bad
- Integral methods are sensitive to accurate background subtraction



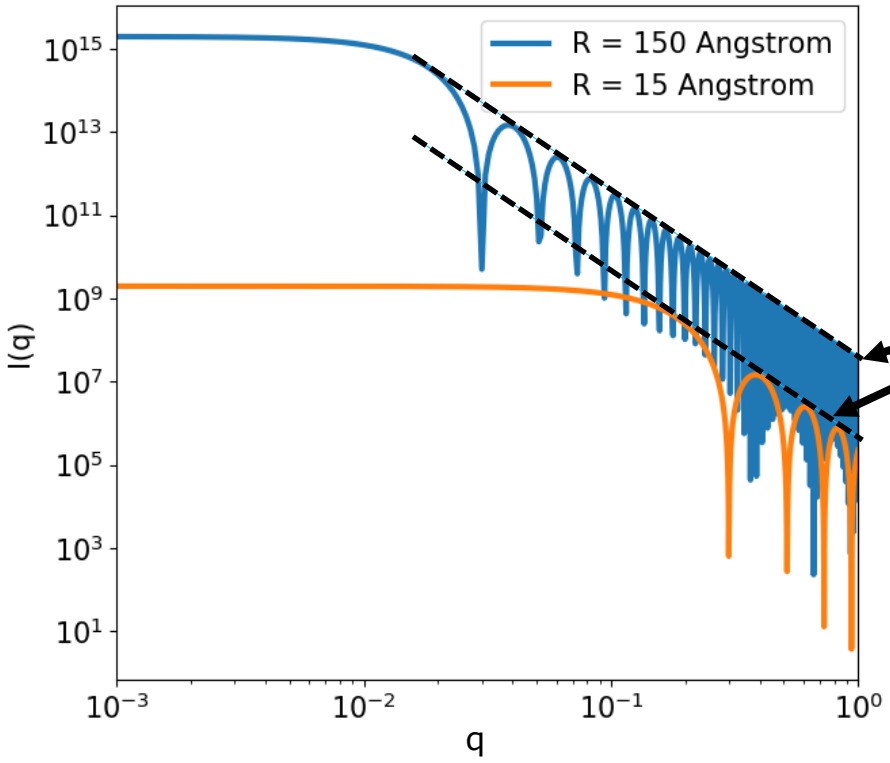
Molecular weight in SAXS

- Be aware of different failure modes
- Use the method(s) that should work best for your data, not the one that best matches your expectations
- Verify that MW matches expected oligomeric state
- If MW doesn't match expected, don't assume you know what's going on. Could be an error in MW calculation, could be a sample problem.
  - Test with another method (e.g. MALS)



Porod and Kratky analysis

Porod Analysis



Objects with sharp boundaries, like ideal spheres, have scattering that follow Porod's law at wide angles:

$$I(q) \propto q^{-4} \quad \text{with} \quad qR \gg 1$$

Slope = -4

More generally: $I(q) \propto q^{-D}$

D is the Porod exponent

Hard sphere:	$I(q) \propto q^{-4}$
Rod:	$I(q) \propto q^{-3}$
Disc:	$I(q) \propto q^{-2}$
Unfolded 'random walk' polymer:	$I(q) \propto q^{-2}$
Fully extended chain:	$I(q) \propto q^{-1}$

- The Porod exponent can be interpreted in terms of particle shape and porosity (usually for materials)
- Be careful: Law breaks down at higher q due to shape, hydration effects

Kratky analysis

Unfolded proteins have Porod exponents near 2, folded generally near 4 (if globular)

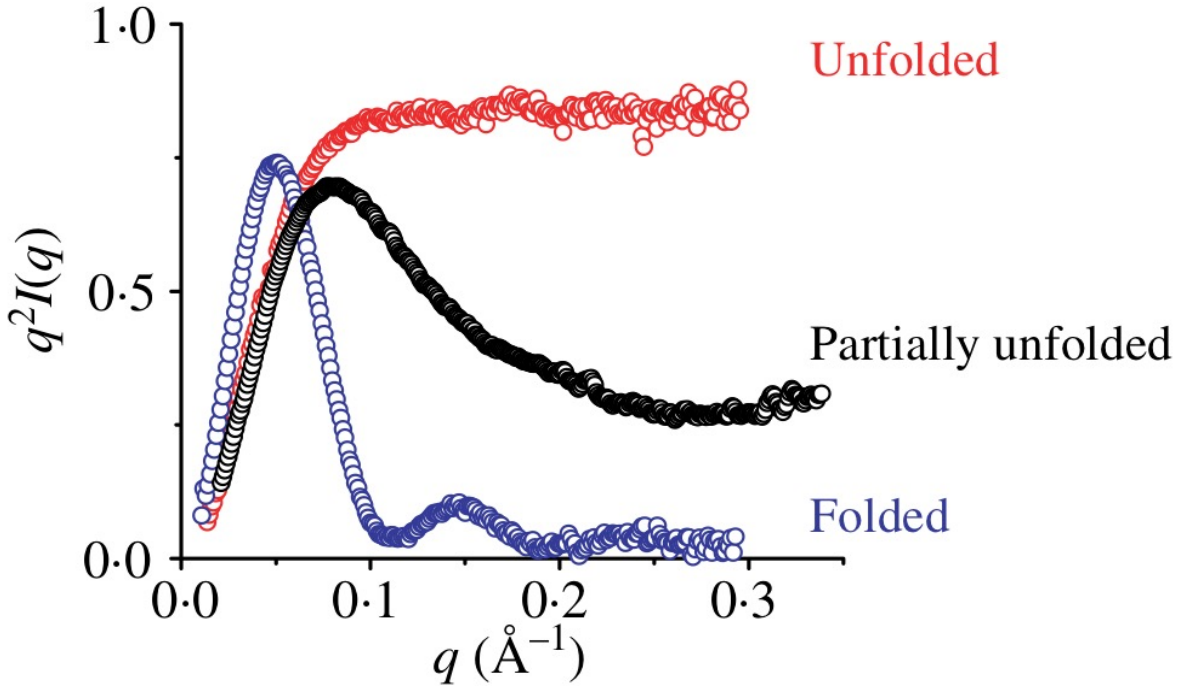
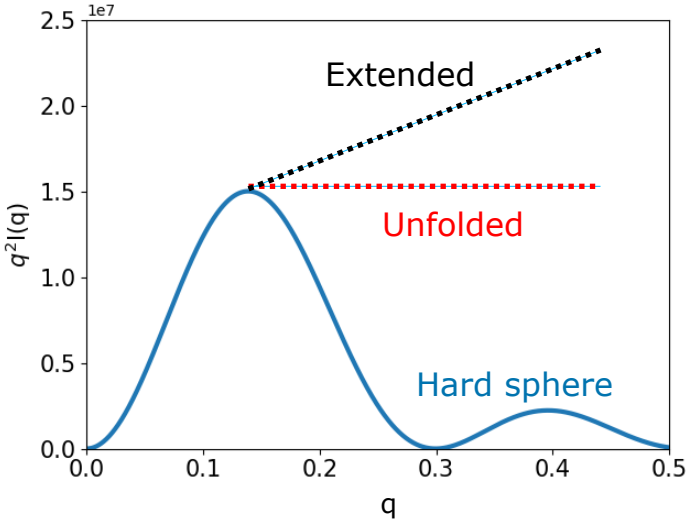


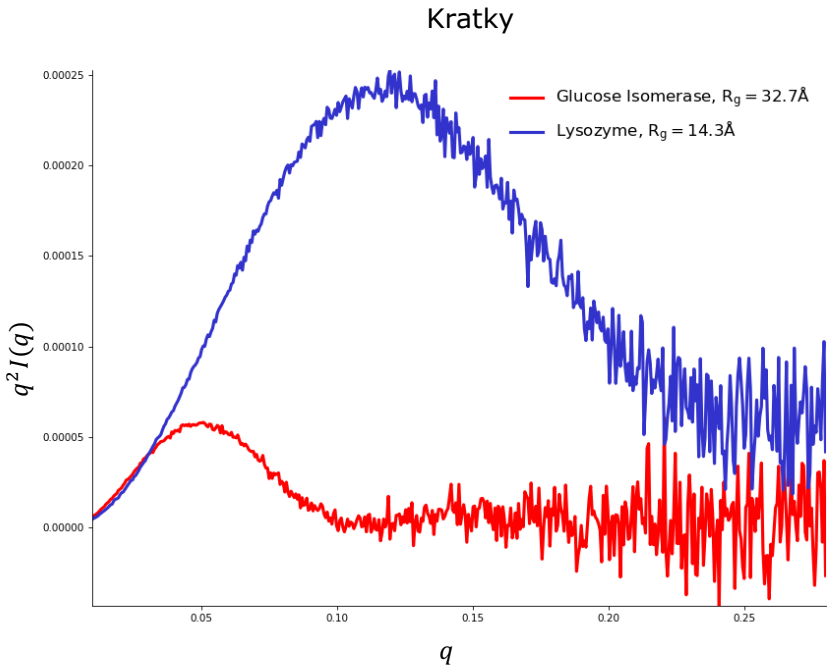
Image from Putnam et al. Quarterly reviews of biophysics, 40(3) 2007.

Kratky plot:
$q^2I(q)$ vs q



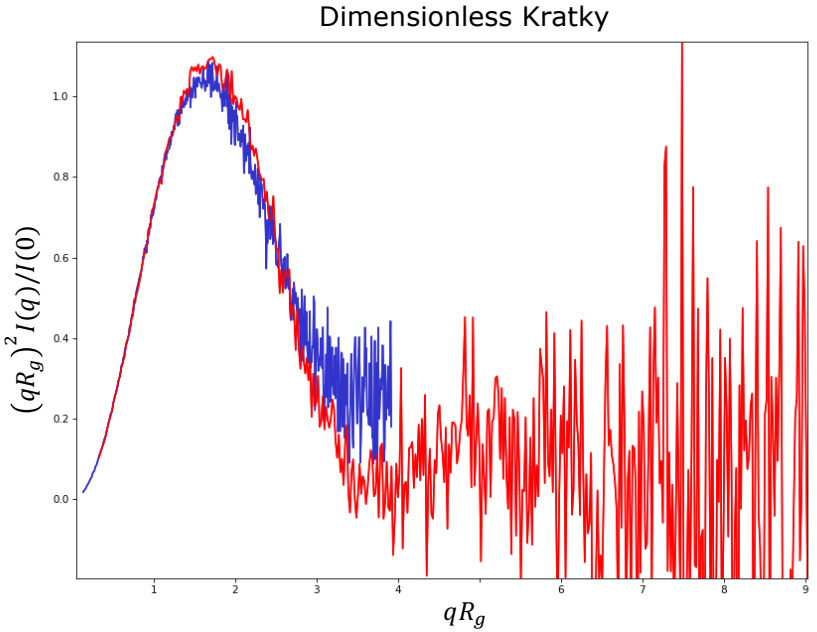
Kratky analysis

Problem: Kratky plot depends on size of an object, scaling of scattering profiles



Solution: normalize by R_g and $I(0)$

Dimensionless Kratky plot:
$(qR_g)^2 I(q)/I(0)$ vs. qR_g



Kratky analysis

Globular particles all have the same shape.
Deviations inform on flexibility/extendedness

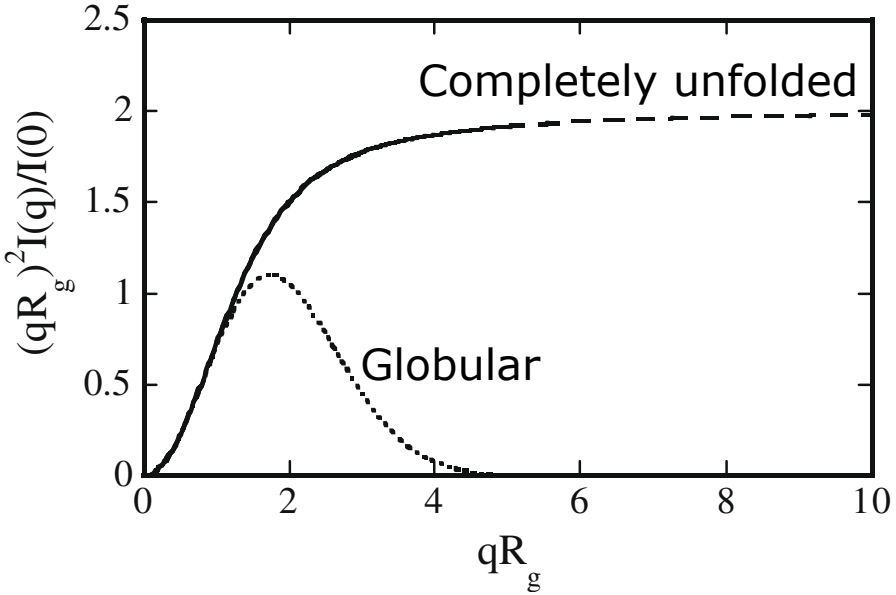


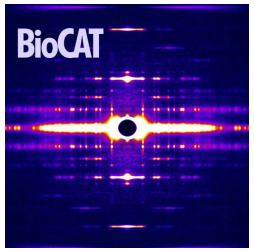
Image from Durand et al. J. Struct. Biol. 169, 2010

Globular particles have a maximum of 1.1 at $qR_g = \sqrt{3} \approx 1.73$

An ideal random chain rises to a plateau of 2

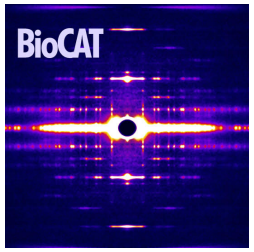
A fully extended chain continues to slope upward without a plateau (not shown)

Shifts in peak location to the right of 1.73, or a partial plateau, indicate more flexibility or extension in a system. Changes in shape are directly comparable because the curves are dimensionless (no size effects).



Kratky analysis

- Kratky plots inform on flexibility and shape
- Kratky plots are relatively insensitive to a small amount of aggregates or radiation damage
  - Dimensionless Kratky plots depends on R_g, I(0) from Guinier, very sensitive to aggregates
- Kratky plots are extremely sensitive to buffer subtraction issues
- Dimensionless Kratky plots can provide semi-quantitative assessment of flexibility and shape

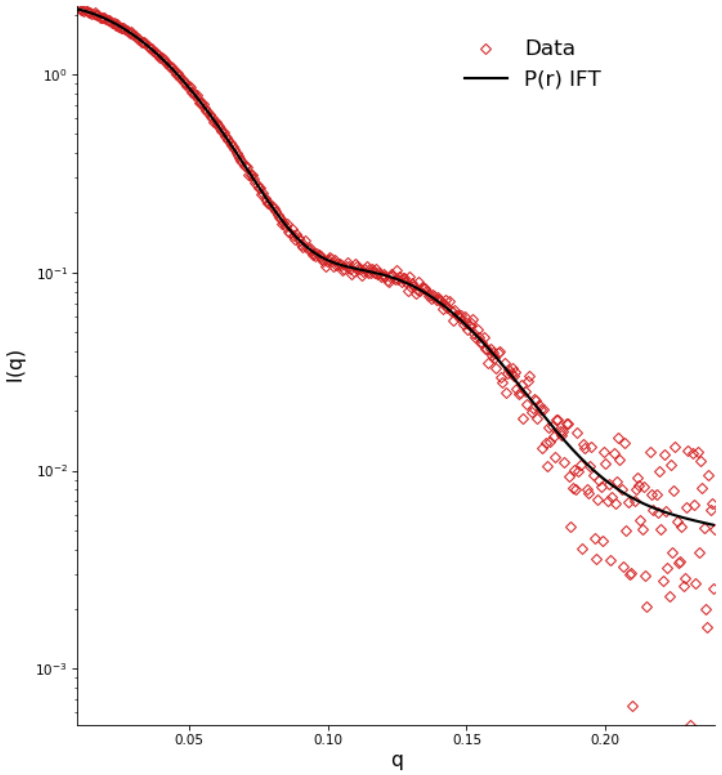


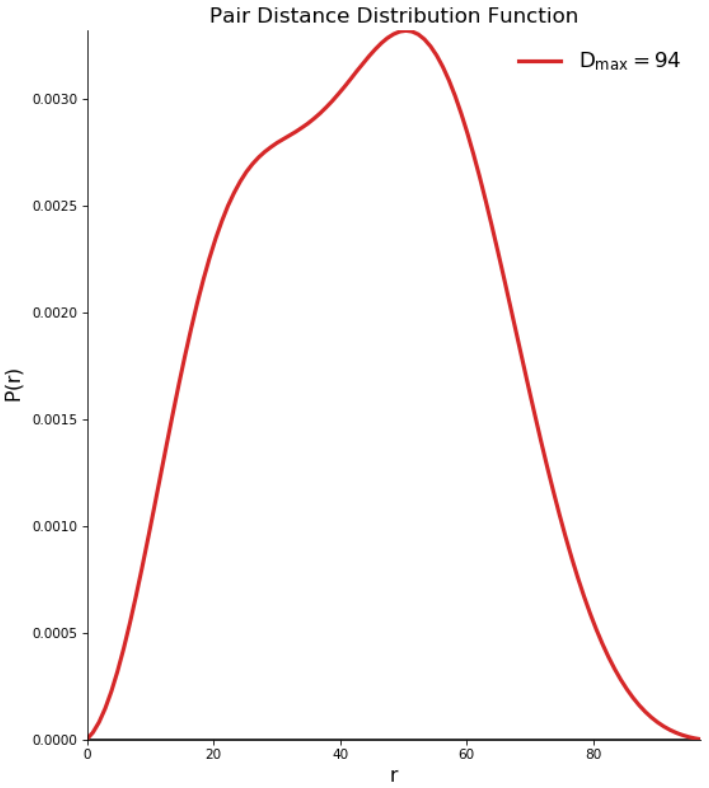
Indirect Fourier transforms

Indirect Fourier Transform (IFT)

- By performing a Fourier transform on the scattering profile we can obtain real space information about the macromolecule

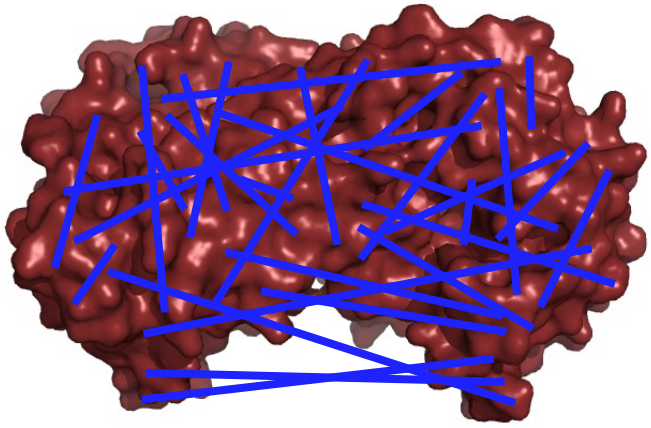
$$I(q) = 4\pi \int_0^{D_{max}} P(r) \frac{\sin(qr)}{qr} dr \quad \longleftrightarrow \quad P(r) = \frac{r^2}{2\pi^2} \int_0^{\infty} q^2 I(q) \frac{\sin(qr)}{qr} dq$$





Physical interpretation of P(r)

P(r) is the histogram of all possible pairs of electrons: the pair distance distribution function



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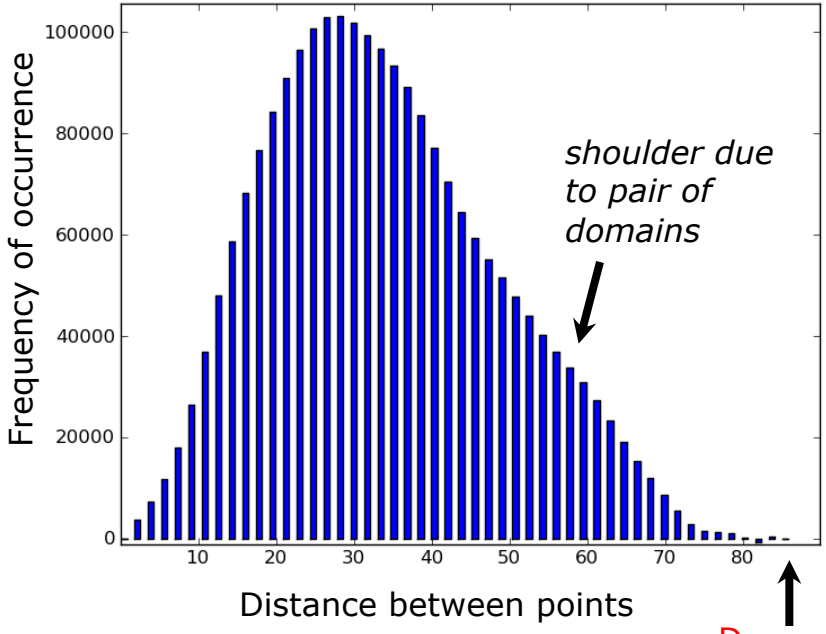
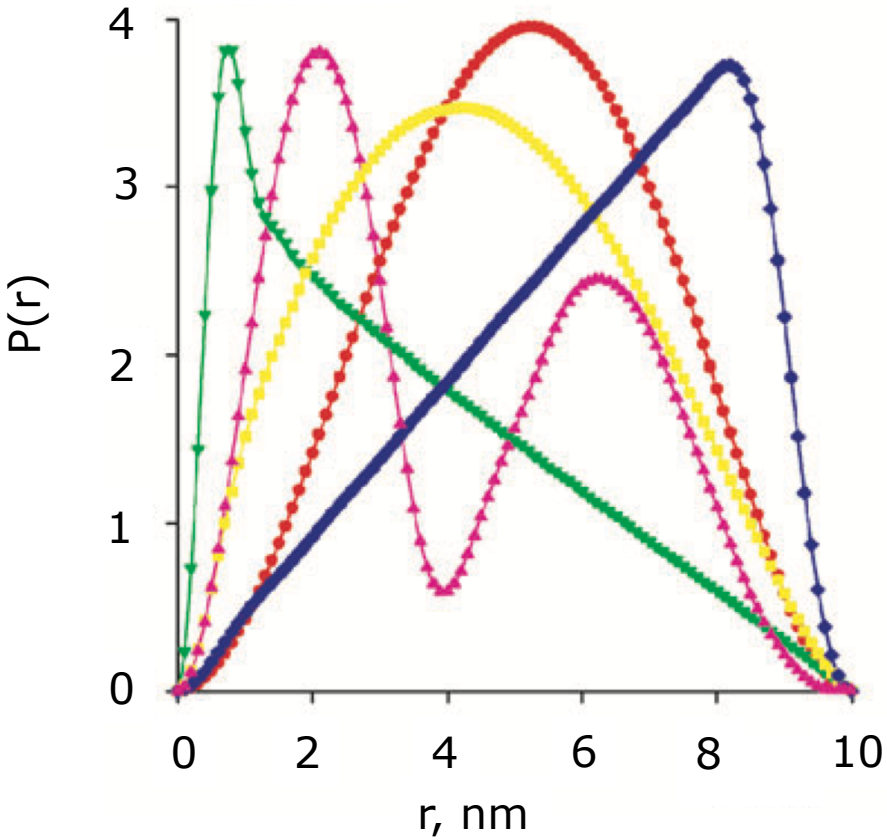


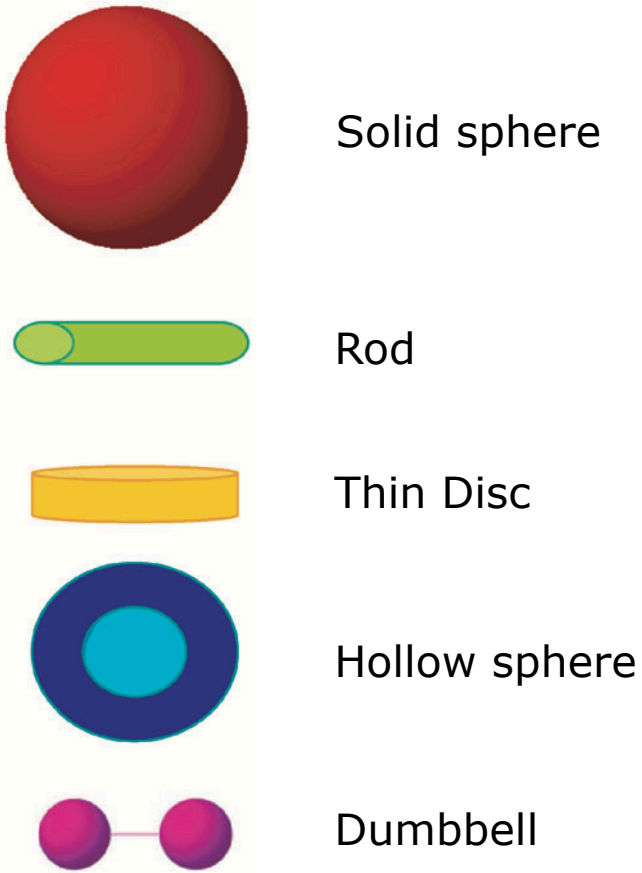
D_{max} maximum dimension of object

Images from Richard Gillilan's BioSAXS Essentials talk

Physical interpretation of P(r)

The shape of the P(r) function can tell you a lot about the shape of your particle





Images from Svergun & Koch, Reports on the progress of physics, 66, 2003.

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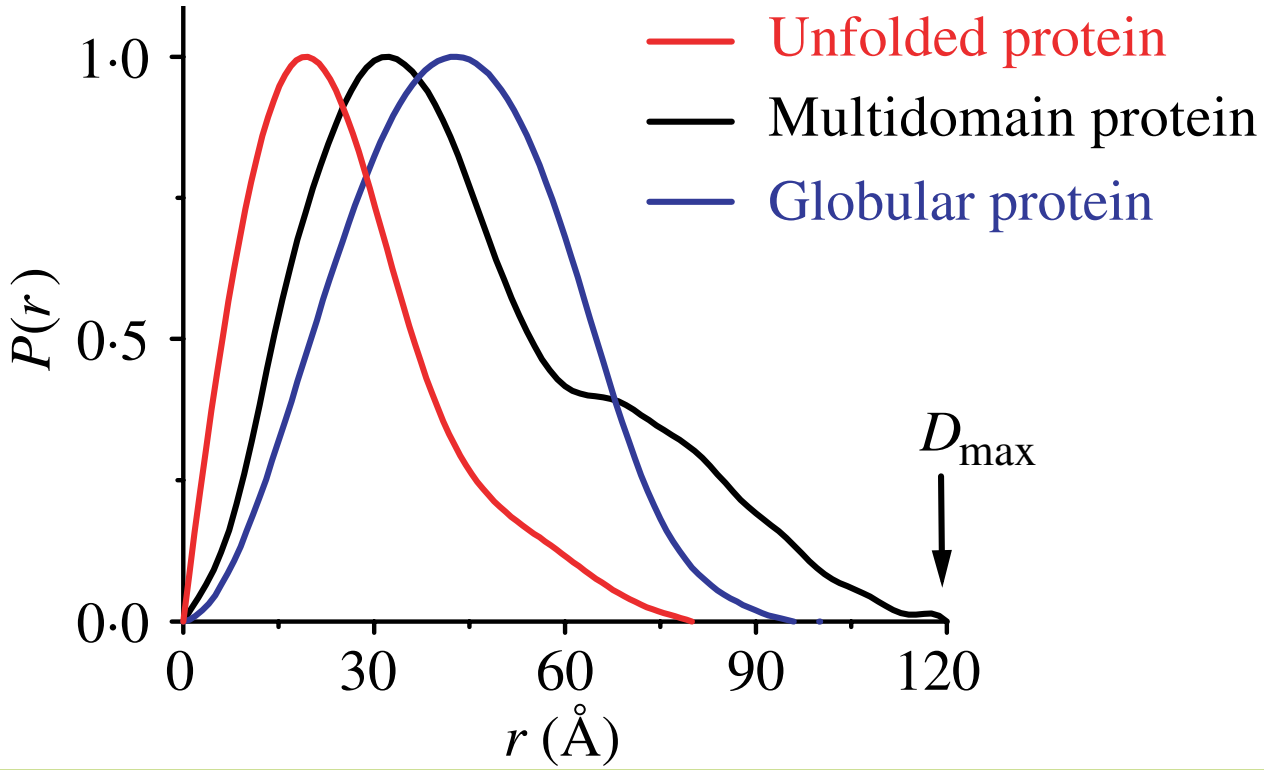

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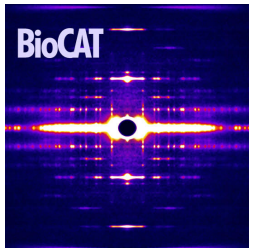


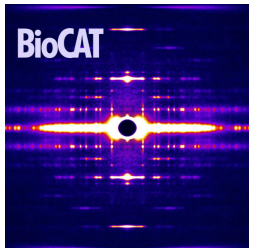
Physical interpretation of P(r)

The P(r) function can be used to calculate the R_g and $I(0)$ values of the curve.

- Uses entire curve
- Automatic extrapolation to $q = 0$
- Especially useful for large particles with small Guinier regions and for noisy data
- Good check against Guinier analysis

$$R_g^2 = \frac{\int_0^{D_{max}} r^2 P(r) dr}{2 \int_0^{D_{max}} P(r) dr}$$

$$I(0) = 4\pi \int_0^{D_{max}} P(r) dr$$



How to calculate a P(r) function

Why can't you directly do a Fourier transform (why the I in IFT)?

$$P(r) = \frac{r^2}{2\pi^2}\int_0^{\infty} q^2 I(q)\frac{\sin(qr)}{qr}\,dq$$

The finite extent of our measurement (and measurement noise) means that a direct Fourier transform distorts the true P(r) function. You get 'truncation artifacts'.

We calculate P(r) functions somewhat backward:

- Pick a D_{max}
- Calculate the best fit P(r) function with that D_{max}
- Check if the P(r) function makes sense

$$I(q) = 4\pi\int_0^{D_{max}} P(r)\frac{\sin(qr)}{qr}\,dr$$

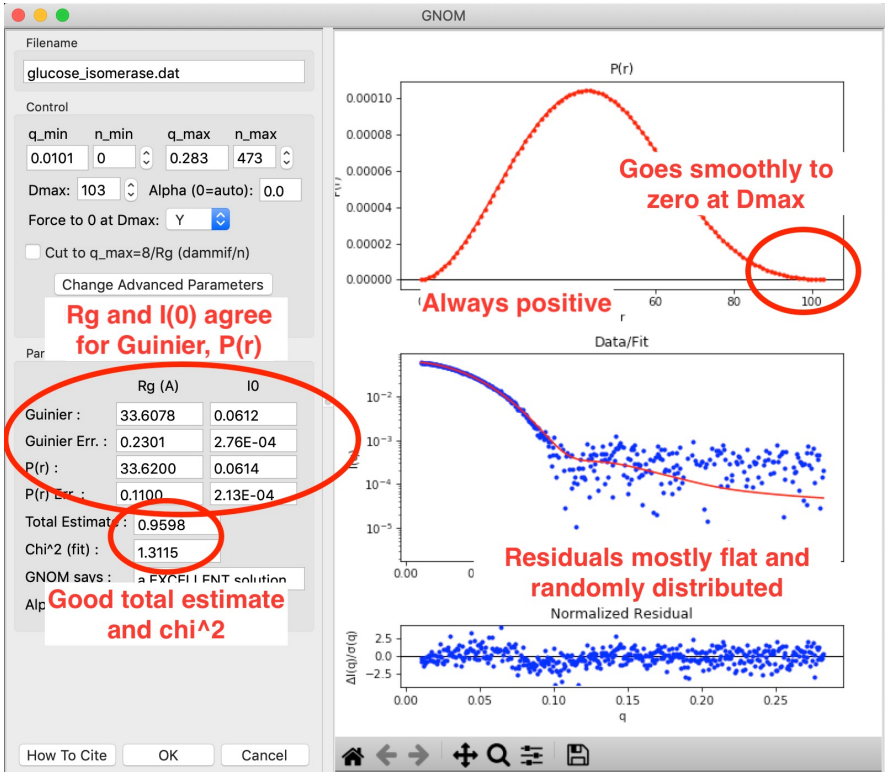
You generate a P(r) with a given D_{max} by fitting against the data

- Fitting criteria include both 'fit' (χ^2) and 'regularization' parameters
- Regularization include 'perceptual' criteria such as
  - Smoothness of the P(r)
  - Stability of the solution when changing parameter weighting
  - Positivity of the solution

How to calculate a P(r) function

Most commonly we use a program called GNOM to do the IFT, though others exist.

- Requires estimate of D_{max} for IFT
- Criteria for judging a good D_{max} based on P(r) function:
  - P(r) falls gradually to zero at D_{max}
  - Underestimated D_{max} has an abrupt descent
  - Overestimated D_{max} usually shows oscillation about zero
- Additional P(r) criteria:
  - P(r) goes to zero at r=0 and r=D_{max}
  - The transform of P(r) fits your data
- Usually true:
  - R_g and $I(0)$ from Guinier and P(r) should agree well (except for flexible systems)
  - P(r) function is always positive (except for proteins in lipid systems)
- Even for good data, uncertainty in determining D_{max} can be >10%



How to calculate a P(r) function

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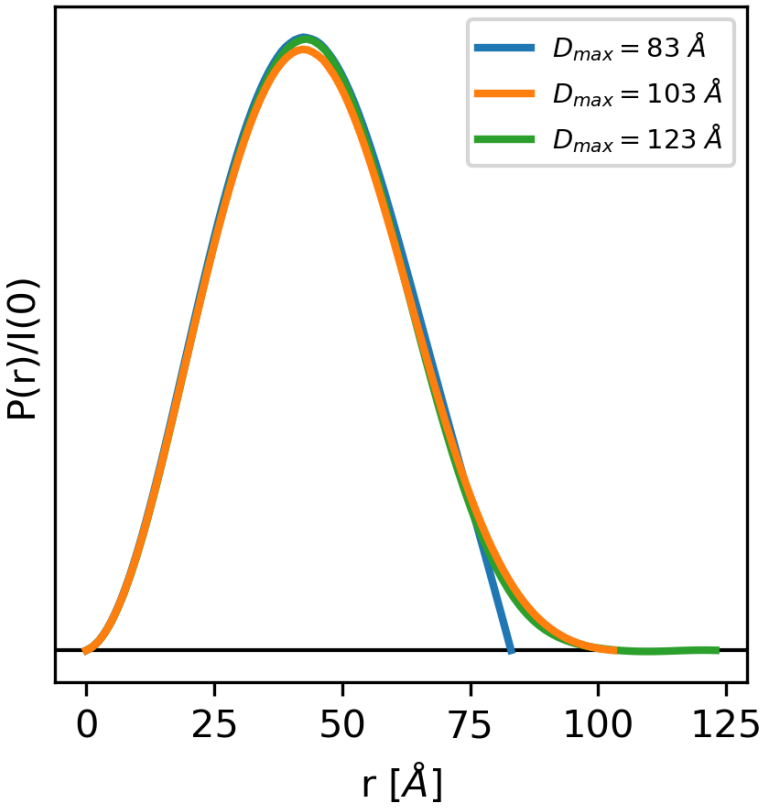
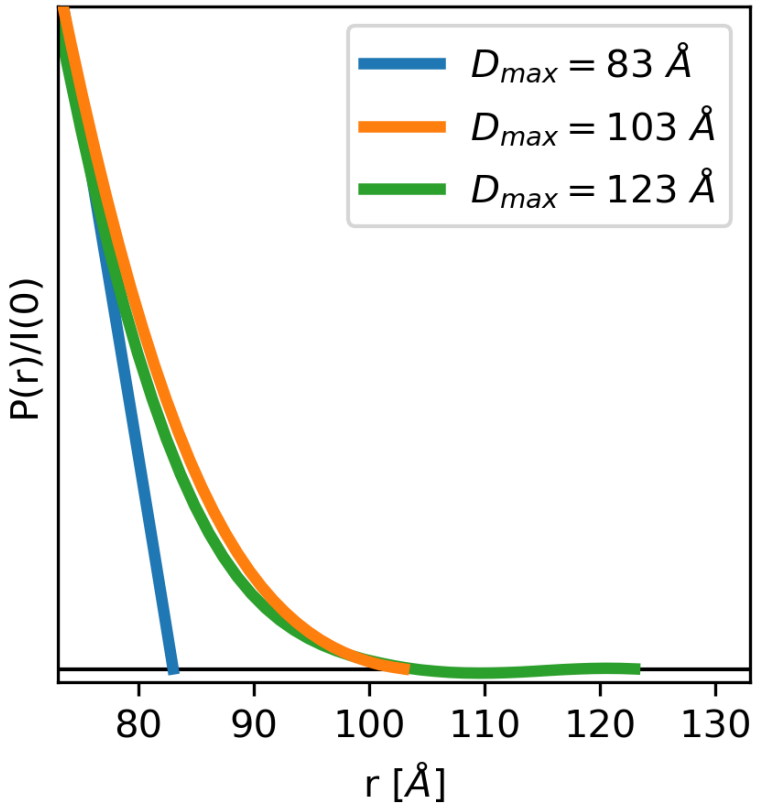
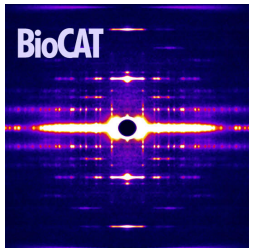




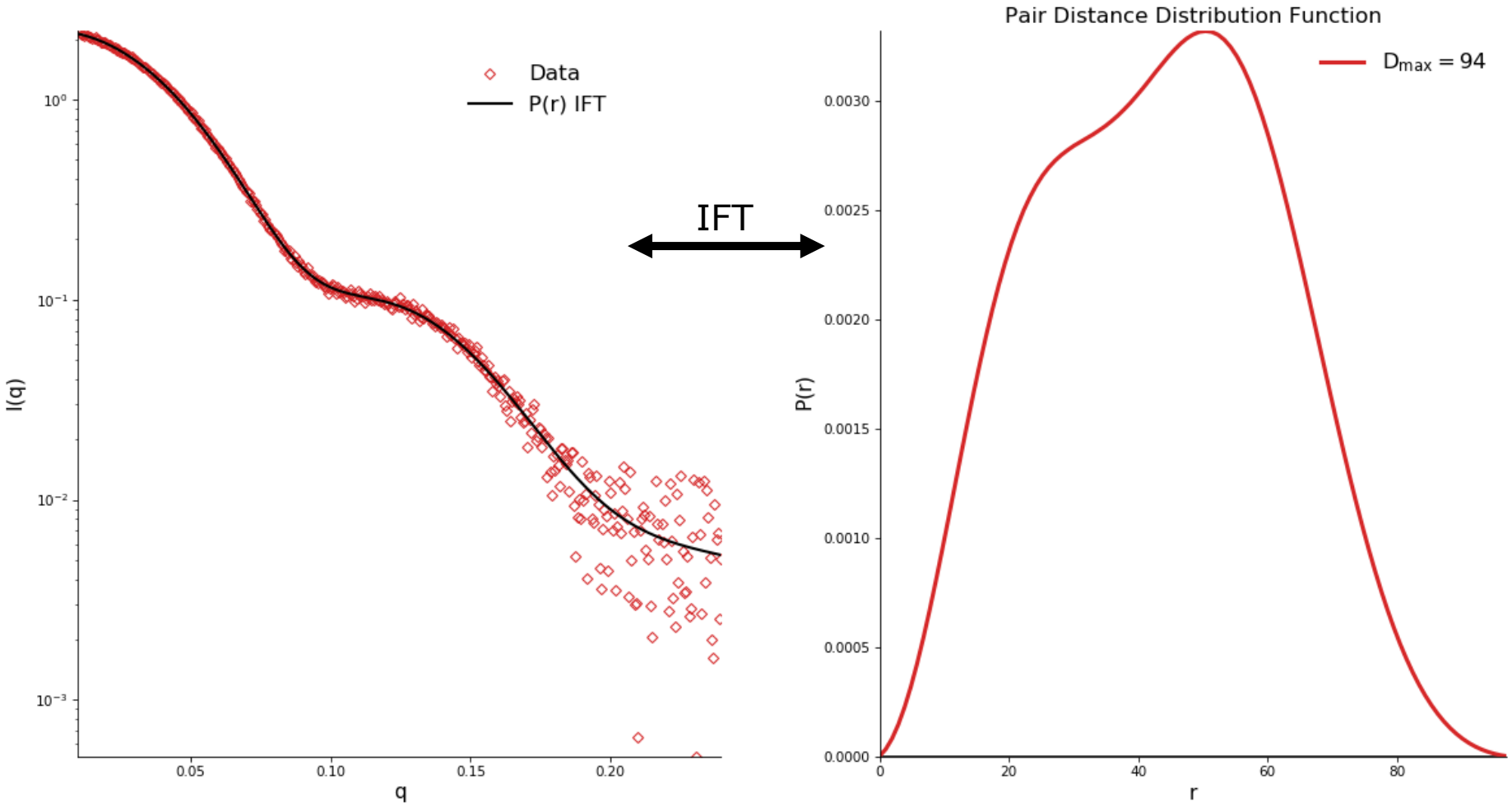


How to calculate a P(r) function

- My usual approach (using GNOM in RAW):
  1. Open the GNOM interface. It defaults to what RAW thinks is a reasonable D_{max}
  2. Set the D_{max} value to 2-3 times larger than the initial value
  3. Look for where the P(r) function drops to 0 naturally. Set the D_{max} value to this point
  4. Turn off the force to zero at D_{max} condition
  5. Tweak D_{max} up and down until it naturally goes to zero (with the force to zero turned off)
  6. Turn the force to zero at D_{max} condition back on

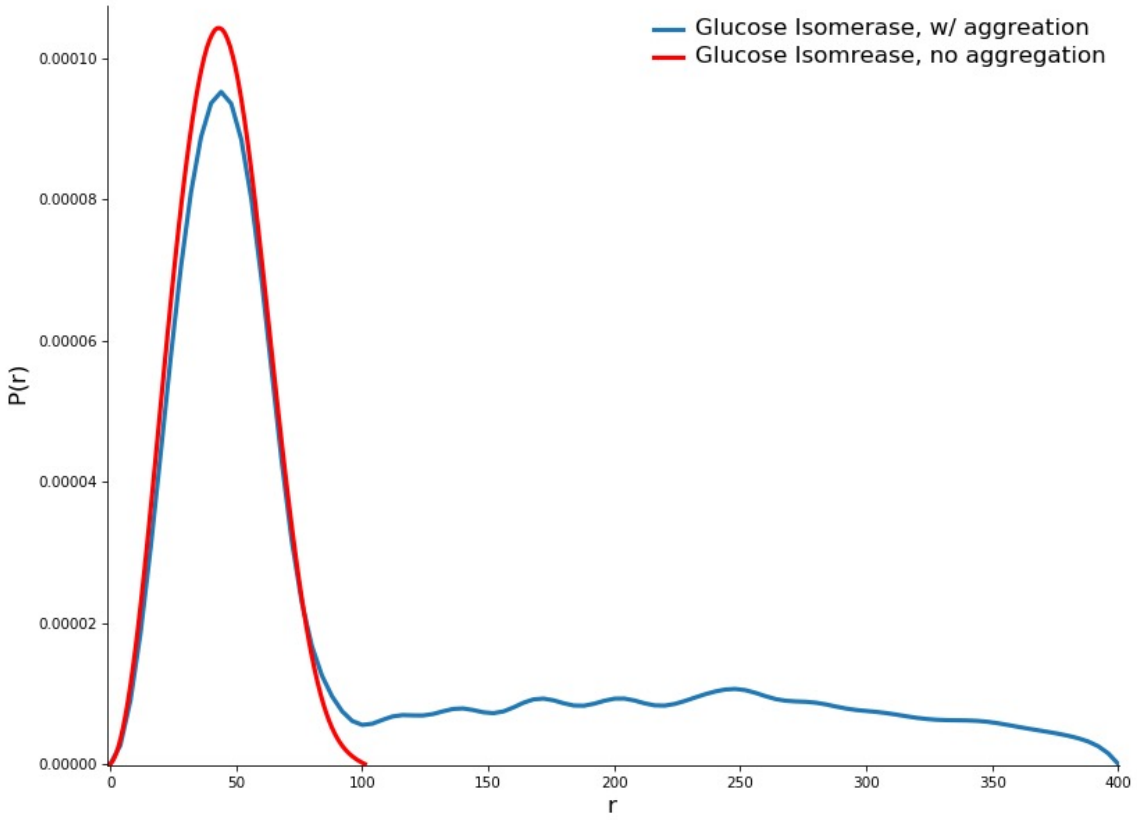
- If you have good quality data, this ought to produce a good P(r) function

How to calculate a P(r) function



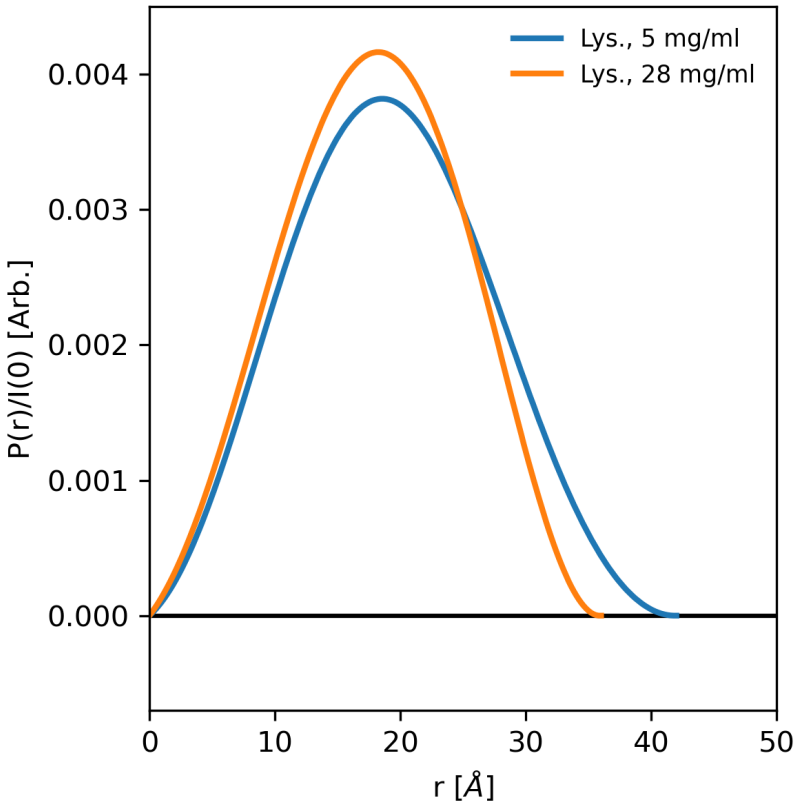
Aggregation and the P(r)

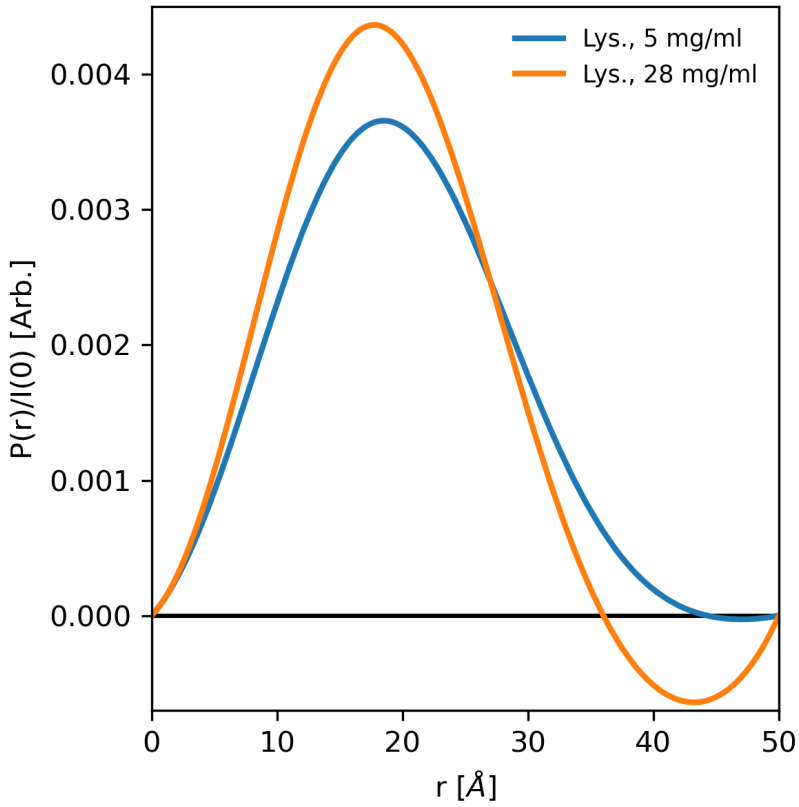
When doing an IFT, if you are unable to find a reasonable D_{max}, may indicate aggregation

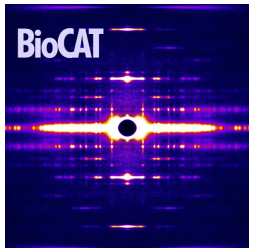


Interparticle interference and P(r)

- Interparticle interference that leads to a downturn in the low q (repulsion) leads to an artificially small D_{max}
- If extended, P(r) function will show characteristic dip below 0

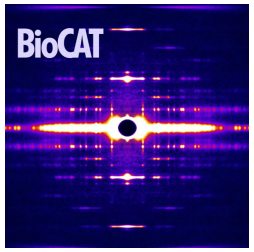






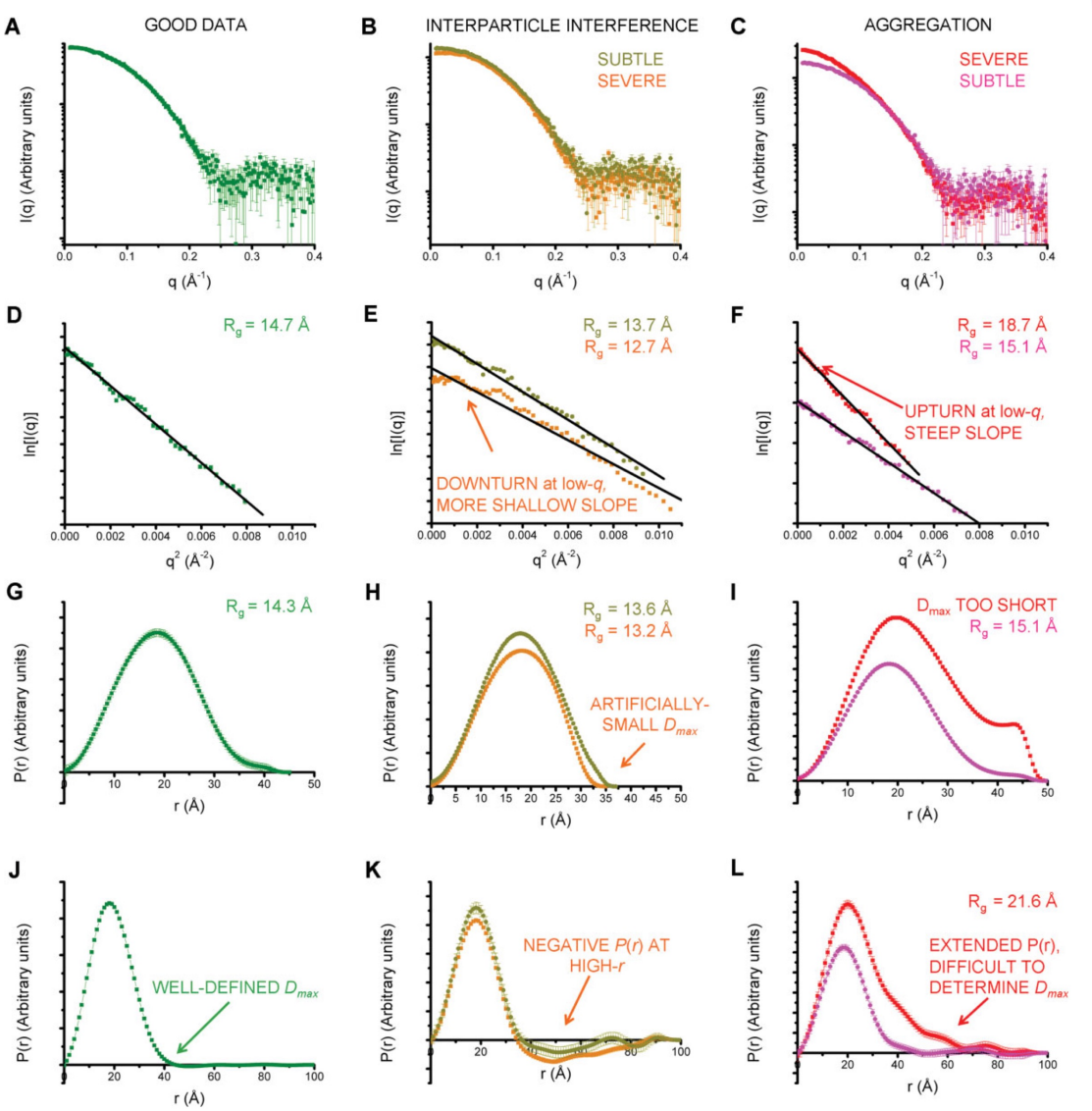
The P(r) function

- Provides real space structural information about the shape of the macromolecule
- Provides an estimate of D_{max}, and potentially more accurate determination of R_g and $I(0)$
- Sensitive to aggregation and interparticle interference
- Generally required before moving on to more advanced analysis

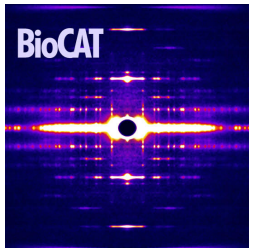


Summary

Summary of data validation

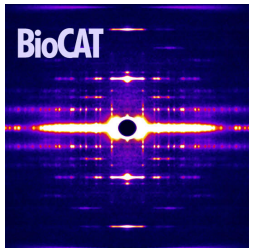


Jacques and Trewhella, Protein Science Review 2010.



Summary of data validation

- Guinier fit will show most issues
- P(r) function good for catching aggregation, interparticle interference
- MW validates what you have in solution
  - Use appropriate method(s)
- Kratky plot particularly sensitive to background subtraction
  - Dimensionless Kratky sensitive to issues with Guinier fit



Summary of data analysis

- Guinier plot gives estimates of R_g and $I(0)$
  - Sensitive to data quality issues
- MW is relatively unreliable from SAXS, but required to validate what state/sample you have in solution
  - Pick the right calculation method
- Kratky and dimensionless Kratky plots provide analysis of flexibility and shape
- P(r) function provides real space shape information, estimate of D_{max}, and potentially more accurate determination of R_g and $I(0)$
  - Also sensitive to data quality issues
- P(r) is generally required before moving to advanced analysis techniques