MRC | Laboratory of Molecular Biology

Light Scattering Techniques

Basic Theory
Static Light Scattering (SLS) in Structural Biology
Dynamic Light Scattering (DLS) in Structural
BiologyOther uses of scattering

Chris Johnson

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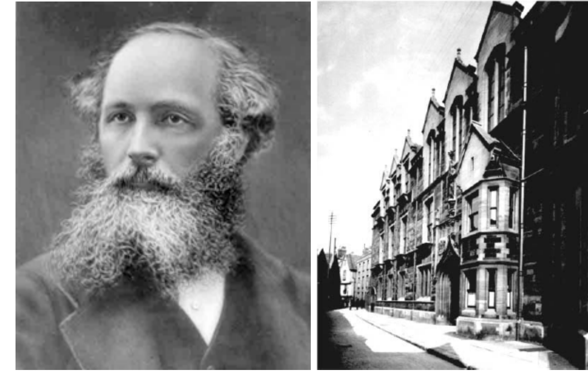
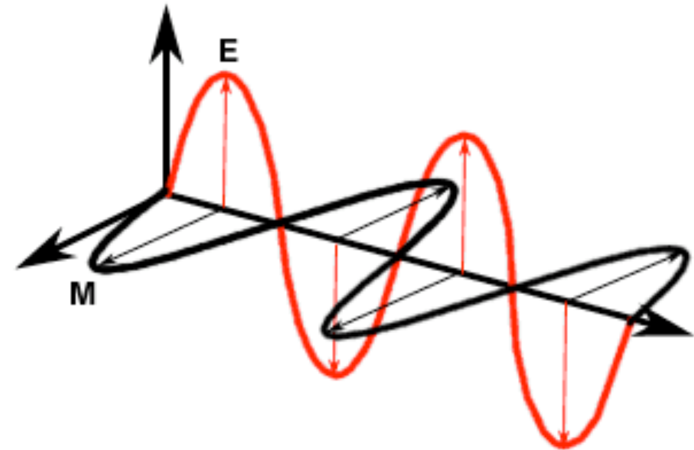
Light as Electromagnetic Radiation



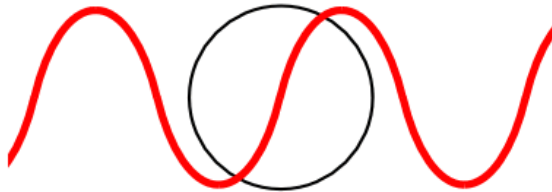
James Clerk Maxwell (1831–1879)
1st Cavendish Professor of Experimental Physics

- Light has oscillating electric and orthogonal magnetic vectors (Maxwell's equations and theory of electromagnetism).
- Polarised light has a single electric field oscillation $|E|$.
- The intensity of light (I) is proportional to the square of the amplitude of the electric vector $I \propto |E|^2$
- The interaction of this electric field with matter is the major contributor to light scattering

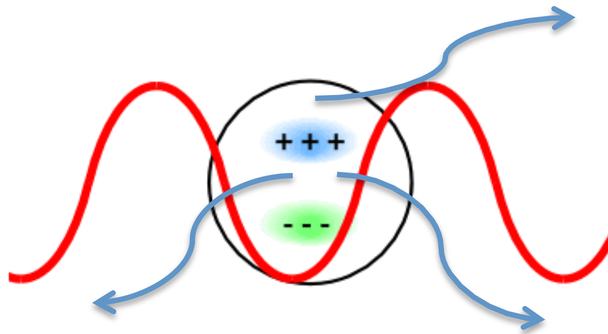


Rayleigh Scattering

- The electric vector |E| induces oscillating dipoles in matter (nuclei) that is polarisable, i.e. has +ve and –ve charges that can be separated



- These oscillating dipoles reemit light at the same frequency: so called "Rayleigh scattering". For polarised light the scattering is predominantly in a plane perpendicular to that of the incident light



John William Strutt
aka Lord Rayleigh (1842-1919)
2nd Cavendish Professor of Experimental Physics

- Intensity of scattering reflects polarisability which is proportional to the square of the refractive index increment of a substance:

$$I \propto \text{polarisability} \propto (dn/dc)^2$$

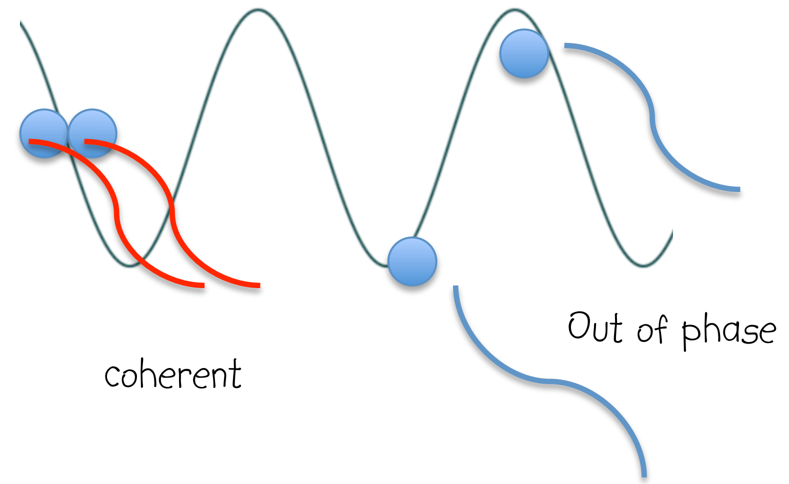
- Intensity of scattering is proportional to $1/\lambda^4$ for particles less than 5% of wavelength

Scattering and Mass

- The many dipoles in a single particle emit light coherently whereas those in different particles are out of phase.
- Intensity of coherent (in phase) light from dipoles

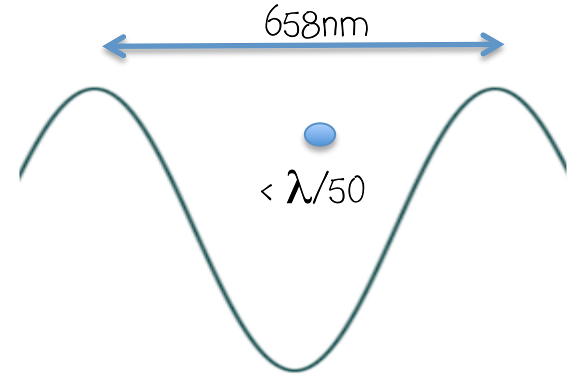
  $|E+E+....|^2 = (nE)^2$ (square of the sum)
- Intensity of incoherent light from dipoles on different particles (out of phase)

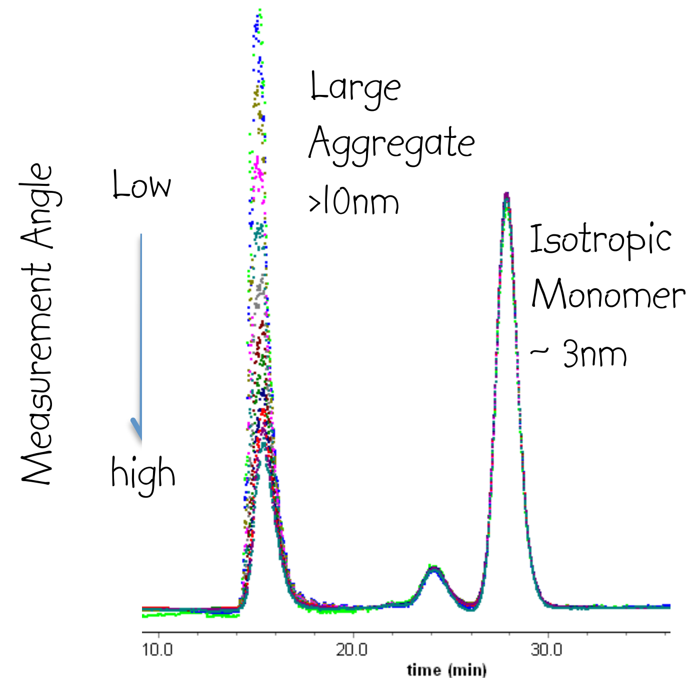
  $|E|^2+|E|^2+... = n(E)^2$ (sum of the squares)
- Thus, for any given level of polarisability ($(dn/dc)^2$) the intensity of scattered light scales directly with particle mass
- SLS + DLS instrumentsation uses polarised laser light source and measure the intensity of perpendicular polarised light that is scattered

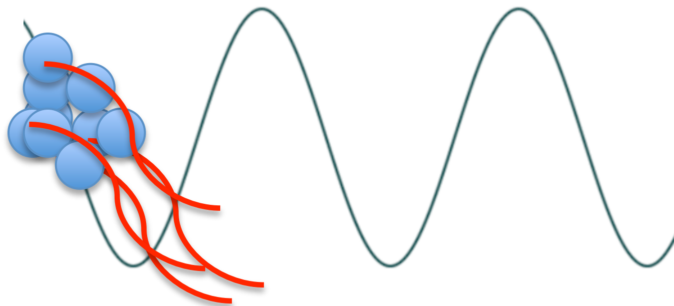


Scattering and Particle Size

- Small particles (< λ/50 nm) scatter light equally in all angles; so called 'isotropic' point scattering
- Eventually as particle size increases some of the coherence of scattered light is lost and there is increasing attenuation of the scattered intensity at angles > 0° due to the intramolecular interference (phase shifts)

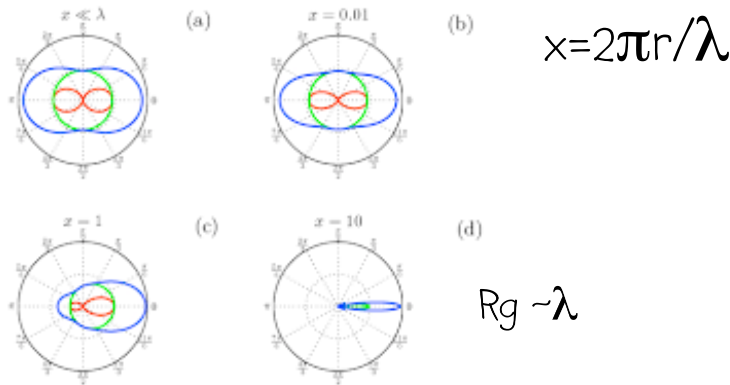






Scattering and Particle Size (Radius)

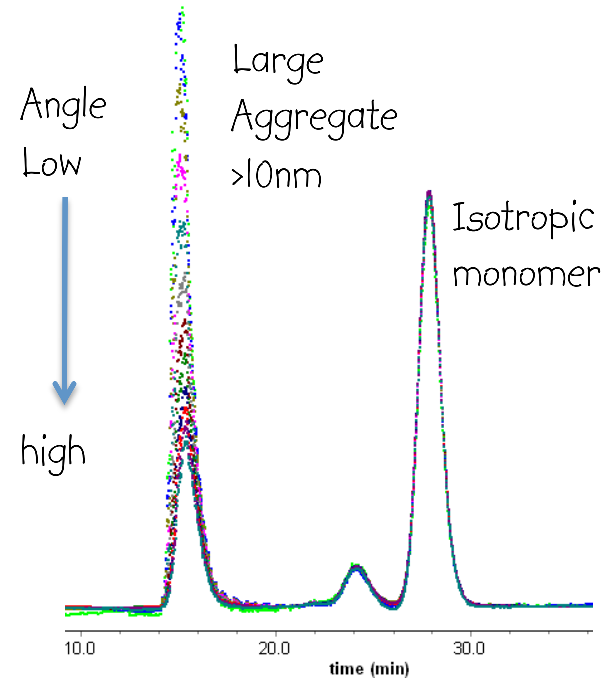
- Mathematical description of this attenuation in the scattering phase function* or 'form factor' includes the wavelength, angle (θ) and particle size



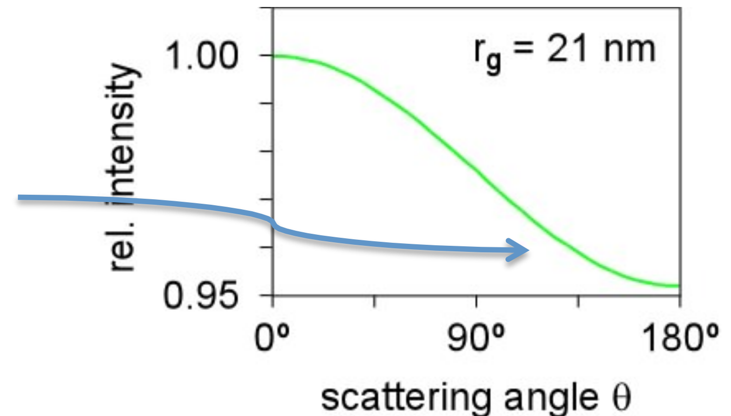
$x=2\pi r/\lambda$

Rg ~0.2λ

Rg ~λ



- By measuring and fitting the attenuation we can determine **root mean square radius** (rms radius) also known as the radius of gyration (**Rg**)
- Using 658 nm for particles > 10nm

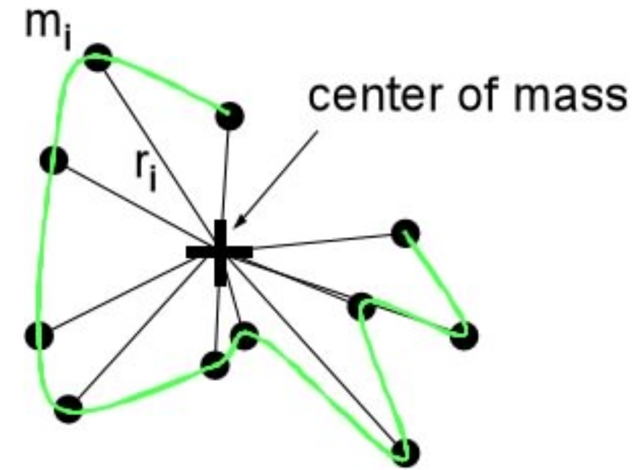


*Calculated using Mie Scattering theory

Root mean square radius (rms)

- Radius of gyration name is misleading since there is not a 'dynamic' component to the measurement
- Measured radius reflects the mass distribution around the centre of mass weighted by the square of the distance from the centre

- For a hollow sphere with all mass in the outer shell, Rg is the physical radius
- For a solid sphere of uniform density the physical radius, R= 1.29*Rg
- For a random coil polymer average length, L = 2.45*Rg
- For a rigid rod length L = 3.46*Rg



$$\langle r_g^2 \rangle = \frac{\sum r_i^2 m_i}{M}$$

Simple analytical description of Rayleigh scattering

$0.2 > x < 0.002$ $(x=2\pi r/\lambda)$

The Rayleigh-Gans-Debye Equation

$$R(\theta) = \frac{4\pi^2 n_0^2}{N_A \lambda_0^4}\left(\frac{dn}{dc}\right)^2 McP(\theta)\left[1 - 2A_2 McP(\theta)\right]$$

Excess Rayleigh scatter (above solvent) at angle θ

K* ; "optical constant", includes the **polarisability factor (dn/dc)^2** and refractive index of solvent $\mathbf{n_0}$ Note $1/\lambda^4$ giving strong wavelength dependence

M, molar mass
c, concentration

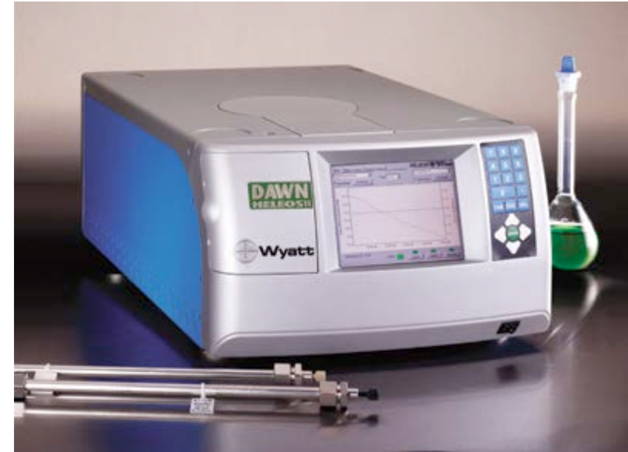
P(θ) "Form factor" Angular term reflecting 'size' Rg

Term accounting for the intermolecular interference terms in the second virial coefficient A_2 Normally this A2 function << 1 and can be ignored

Light Scattering in practice

LMB Instrumentation

- Multi (18) angle light scattering detector; R(θ) between 22.5° – 147° λ =658nm
- Differential refractometer allows measurement of $(dn/dc)^2$, the polarisability factor
- If dn/dc is already known (as for proteins), then refractive index measurement also allows the required concentration determination
- Can use any other concentration detector e.g., UV-VIS and dual concentration determination



LS

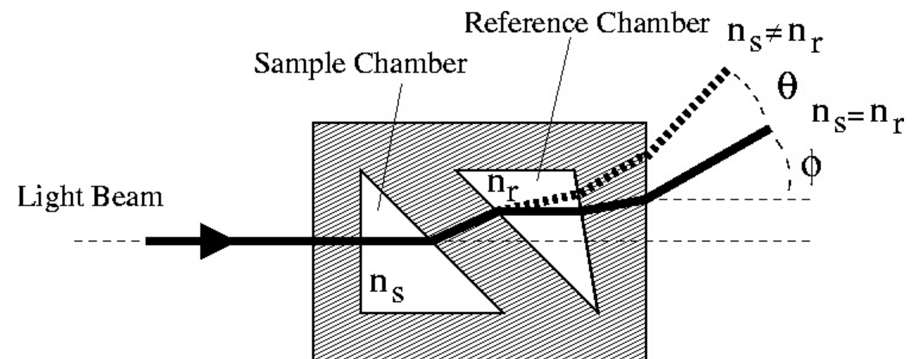
$$R(\theta) = \frac{4\pi^2 n_0^2}{N_A \lambda_0^4}\left(\frac{dn}{dc}\right)^2 McP(\theta)\left[1 - 2A_2 McP(\theta)\right]$$

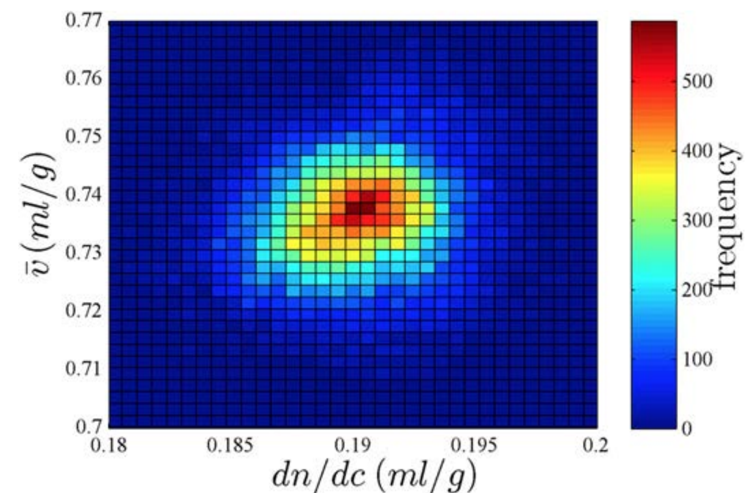
RI

http://www.wyatt.com/

Differential Refractive Index

- Differential (excess) refractive index (dRI) measured in a split cell with <u>solvent reference</u>.
- Proteins have a 'universal' dn/dc value of ~ 0.19 mL/g and so they can be quantified from dRI
- Useful if there are no aromatics or if the sequence and/or extinction coefficient is unknown
- Proteins are polarisable so scatter well; $I \propto (dn/dc)^2$
- Instrument is measuring dRI at $10^{-3} - 10^{-6}$ level for typical experiments on proteins (mg/ml)



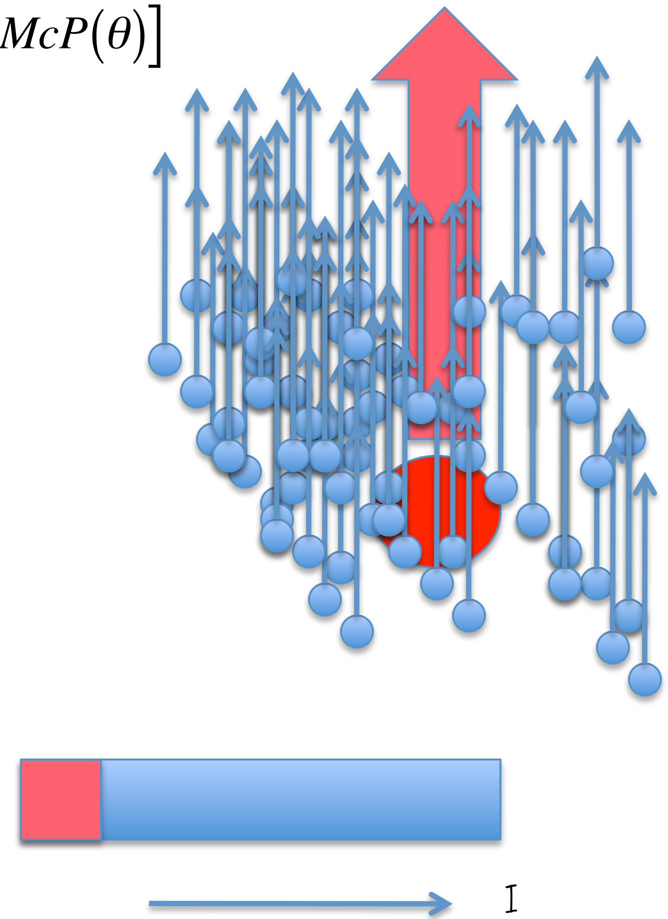


Calculated dn/dc for ~ 62000 protein sequences
Zhao, Brown and Schuck, 2011, Biophysical J. 2309-2317.

Batch Measurement

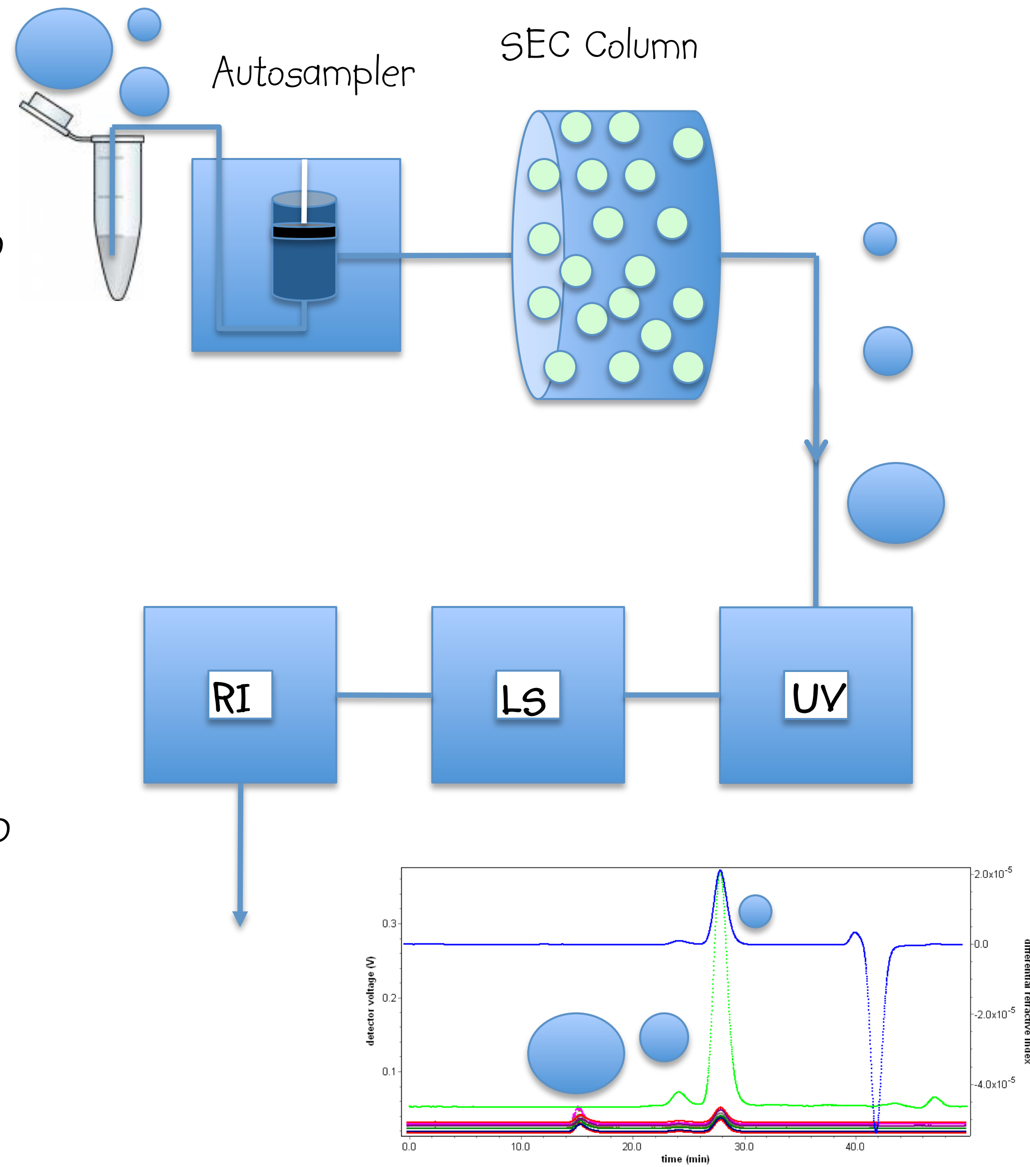
$$R(\theta) = \frac{4\pi^2 n_0^2}{N_A \lambda_0^4}\left(\frac{dn}{dc}\right)^2 McP(\theta)\left[1 - 2A_2 McP(\theta)\right]$$

- Scattered intensity α mass so low concentrations of large particles can significantly bias measurement to higher masses
- Material for batch measurements must be highly monodisperse and stable
- Filtering with very fine membranes (0.01um) may help, but 'hides' any polydispersity
- Fractionation prior to measurement would be optimal........

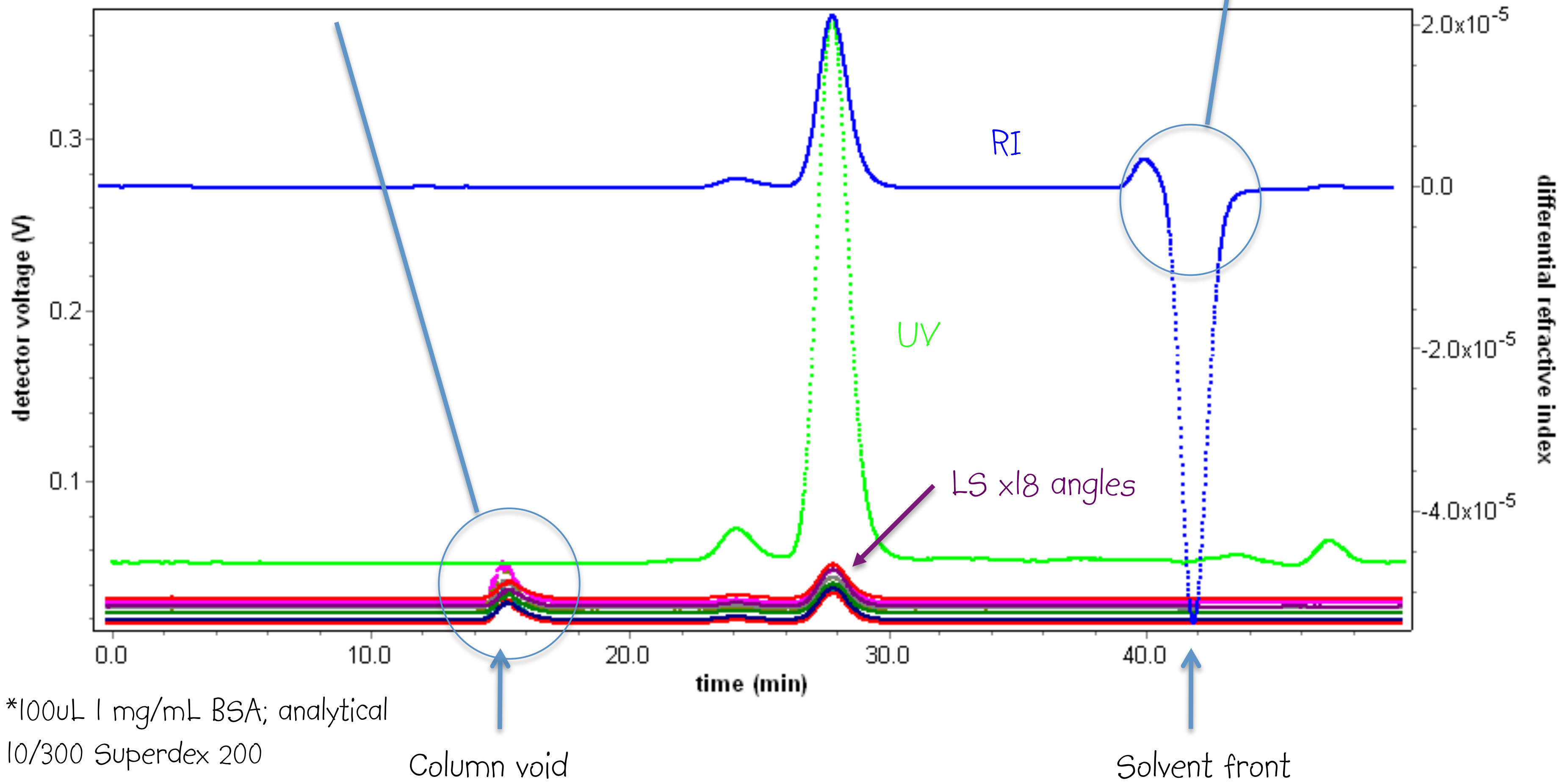


On line Size Exclusion Chromatography with MALS: 'SEC-MALS'

- For biological systems light scattering is commonly coupled to a SEC fractionation system to separate 'mixtures' of different mass components.
- Samples injected, separated (analytical gel filtration) and detected sequentially by UV, LS and RI flow cells
- Each point in the SEC MALS chromatogram can be analysed to give mass, Rg, Rh.



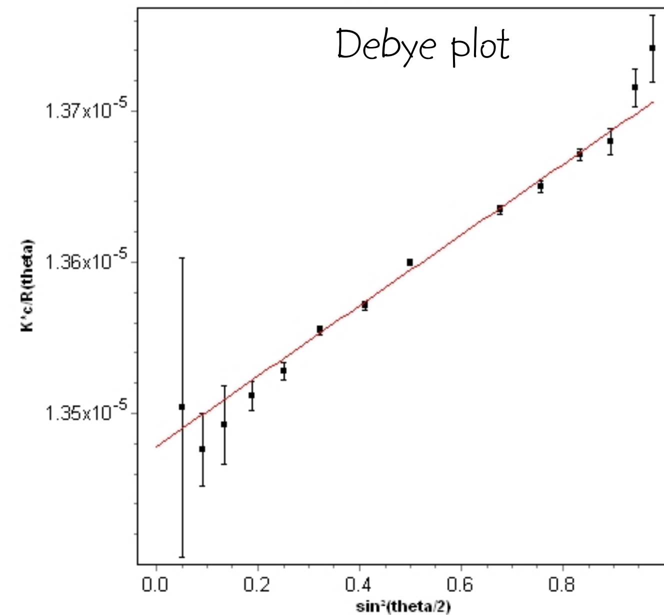
Typical* SEC MALS Chromatogram

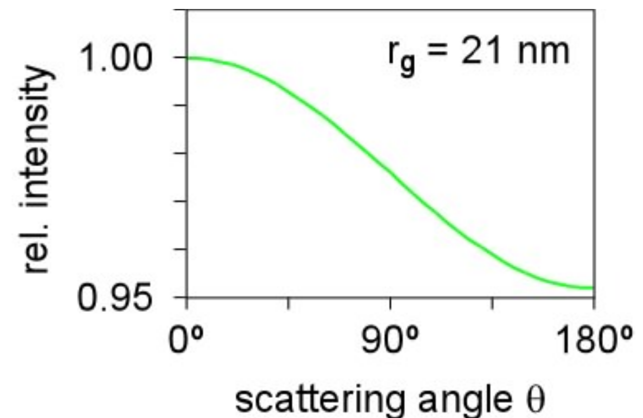


*100uL 1 mg/mL BSA; analytical 10/300 Superdex 200

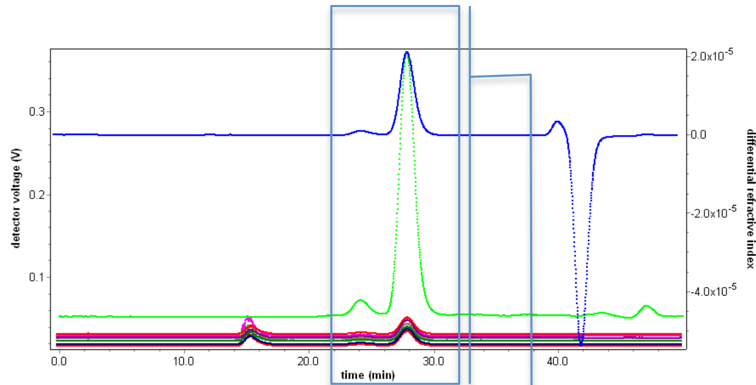
Graphical Analysis of LS data

- At each collection point in the chromatogram the data can be analysed in a Debye plot using a Zimm formalism
- K*c / R(θ) plotted against $\sin^2(\theta/2)$
- Y-axis intercept (zero angle) is used to evaluate mass
- Slope (which has meaning when positive since y-axis is 1 / R(θ) and scattering is attenuated at higher angles) can be used to evaluate Rg for particles > 10nm
- A Debye plot is produced at each data collection point in a defined region of the chromatogram and the values (intercept and slope) averaged
- Other methods for graphing the data and fitting (Berry, Debye and Random coil formalisms) may be appropriate when particles are much larger





Graphical display of mass calculations



calculated masses across peaks monomer + dimer

Since there is LS and RI data collected every second or so we can calculate mass at each point

Single LS angle

RI

molar mass (g/mol)

1.0×10^5

5.0×10^4

0.0

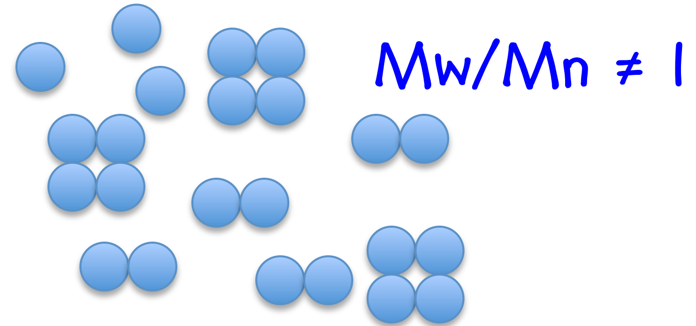
10.0 11.0 12.0 13.0 14.0 15.0 16.0

volume (mL)

Statistical Analysis of mass calculations

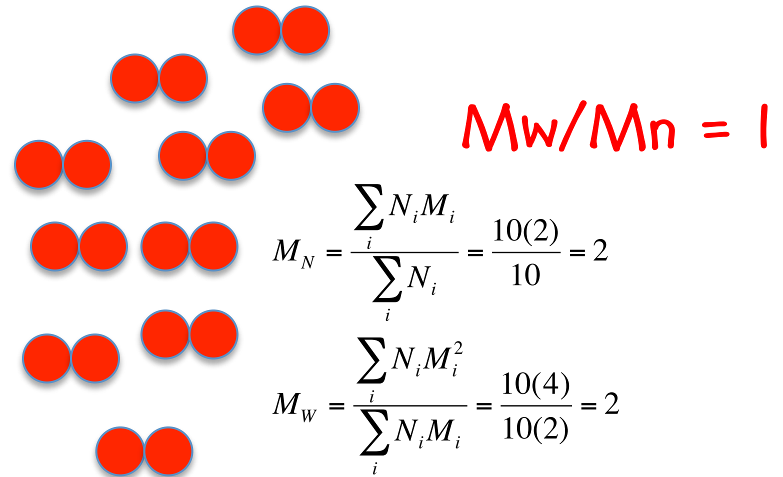
- Processing time: Wednesday March 04, 2009 02:24 PM GMT Standard Time
- Collection time: Friday September 05, 2008 10:24 AM GMT Standard Time
- Detectors used: 3 4 5 6 7 8 9 10 11 12 13 14 15 16
- **Concentration detector: RI**
- Mass results fitting: none (fit degree: n/a)
- Radius results fitting: none (fit degree: n/a)
- Peak 1
- Peak limits (min) 27.505 - 28.195
- **dn/dc (mL/g) 0.190**
- A_2 (mol mL/g^2) 0.000
- UV ext. (mL/(g cm)) 0.000
- Model Zimm
- Fit degree 1
- Injected mass (g)0.0000
- **Calc. mass (g) 3.5582e-5**
- RESULTS
- Peak 1
- **Polydispersity**
- **Mw/Mn 1.000(0.4%)**
- Mz/Mn 1.000(0.6%)
- **Molar mass moments (g/mol)**
- **Mn 6.571e+4(0.3%)**
- Mp 6.581e+4(0.3%)
- Mv n/a
- **Mw 6.571e+4(0.3%)**
- Mz 6.572e+4(0.6%)
- rms radius moments (nm)
- Rn n/a
- Rw n/a
- Rz n/a

- Masses can be averaged across the SEC peak
- Mn is number averaged, Mw is weight averaged.
- Their ratio Mw/Mn is thus an index of the **DISPERSITY** of material



$$M_N = \frac{\sum_i N_i M_i}{\sum_i N_i} = \frac{3(1)+4(2)+3(4)}{10} = 2.30$$

$$M_W = \frac{\sum_i N_i M_i^2}{\sum_i N_i M_i} = \frac{3(1)+4(4)+3(16)}{3(1)+4(2)+3(4)} = 3.17$$



$$M_N = \frac{\sum_i N_i M_i}{\sum_i N_i} = \frac{10(2)}{10} = 2$$

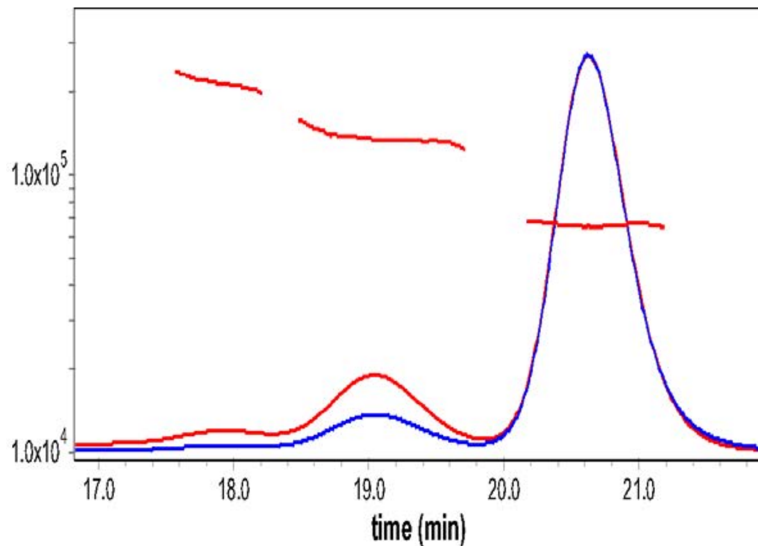
$$M_W = \frac{\sum_i N_i M_i^2}{\sum_i N_i M_i} = \frac{10(4)}{10(2)} = 2$$

Polydispersity

- Polydispersity indicated from Mw/Mn ratio or it can be seen visually from the mass calculation across the peak

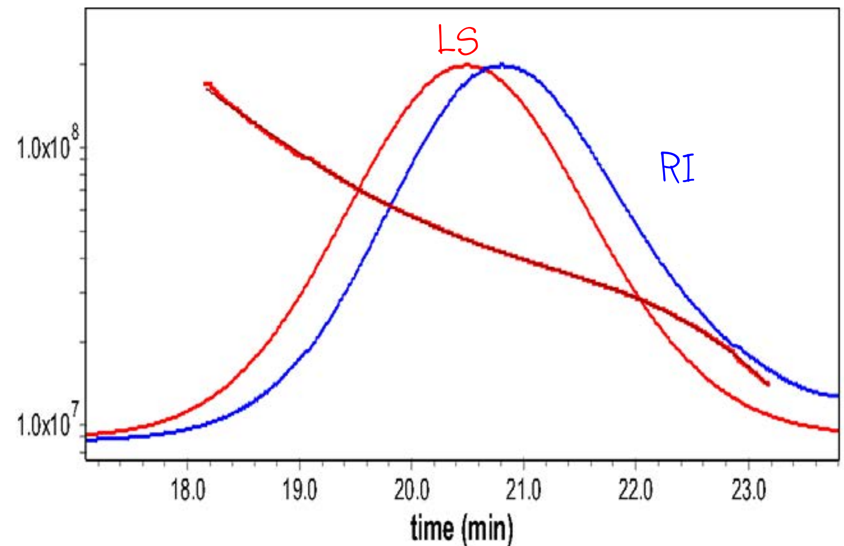
Monodisperse

Mw/Mn =1



Polydisperse

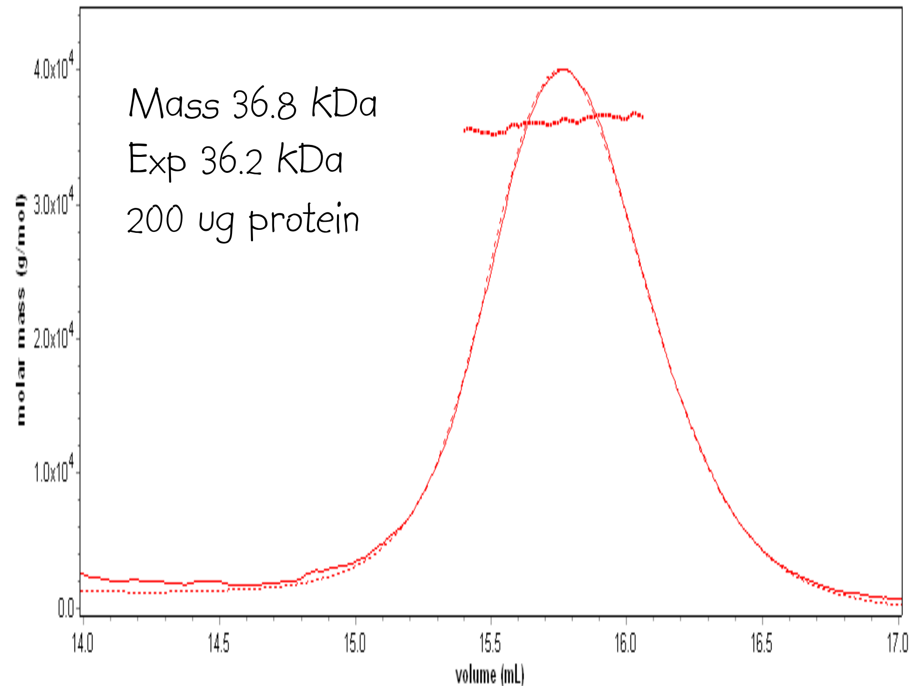
Mw/Mn ≠1

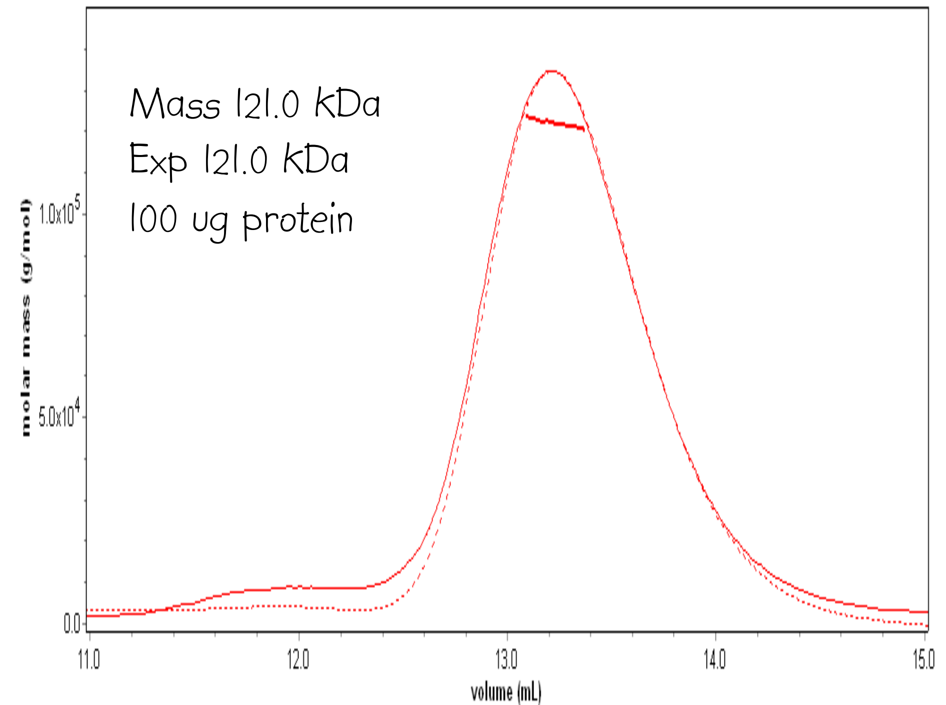


Applications

Applications of SEC MALS; Mass in solution

- 'Accurate and quick' * mass determination in solution 500 – 50 x 10^6 Da
- Mass independent of SEC elution volume



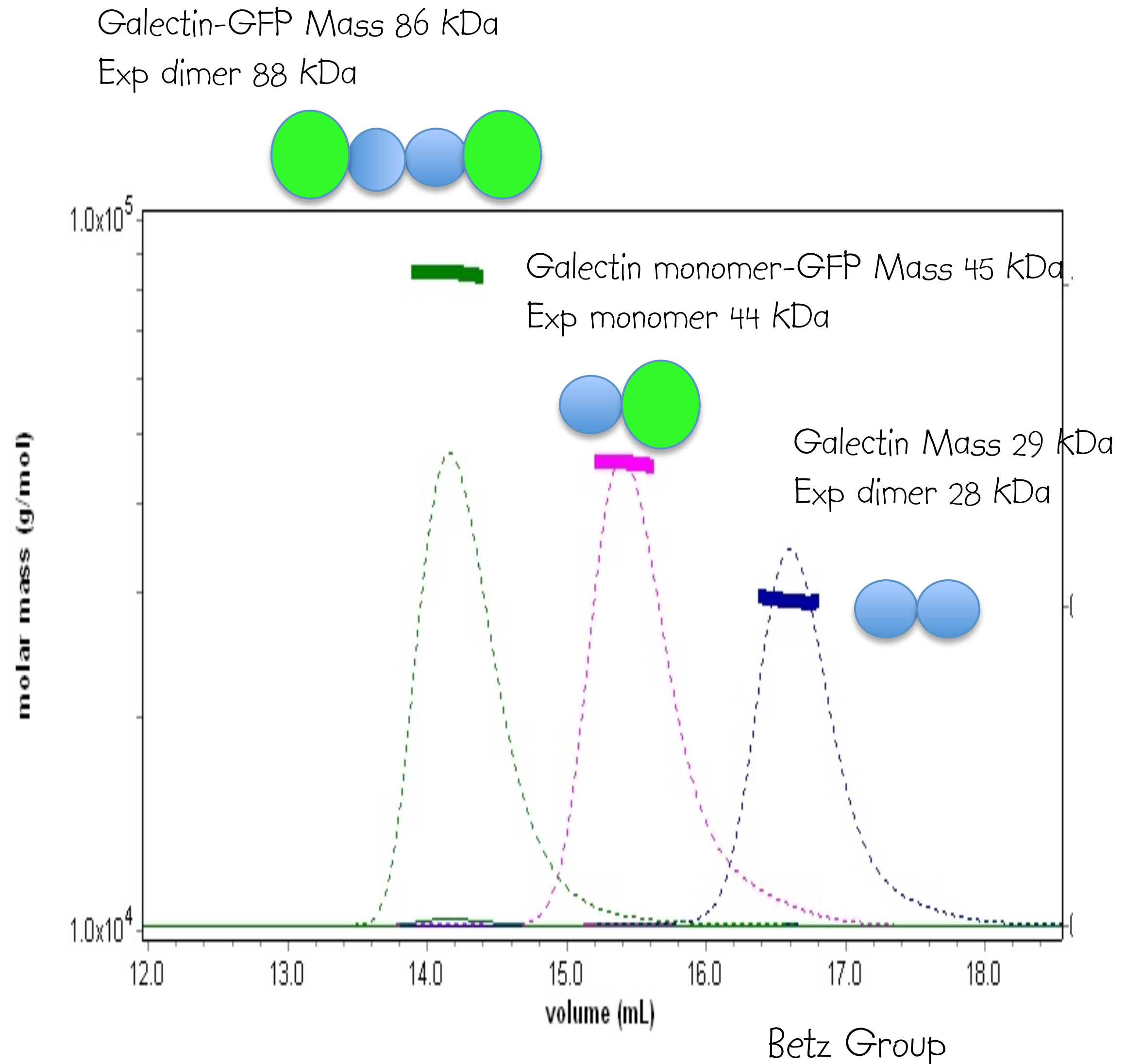


(*45min runs at 0.5 ml/min, higher flow rates possible)

Williams Group

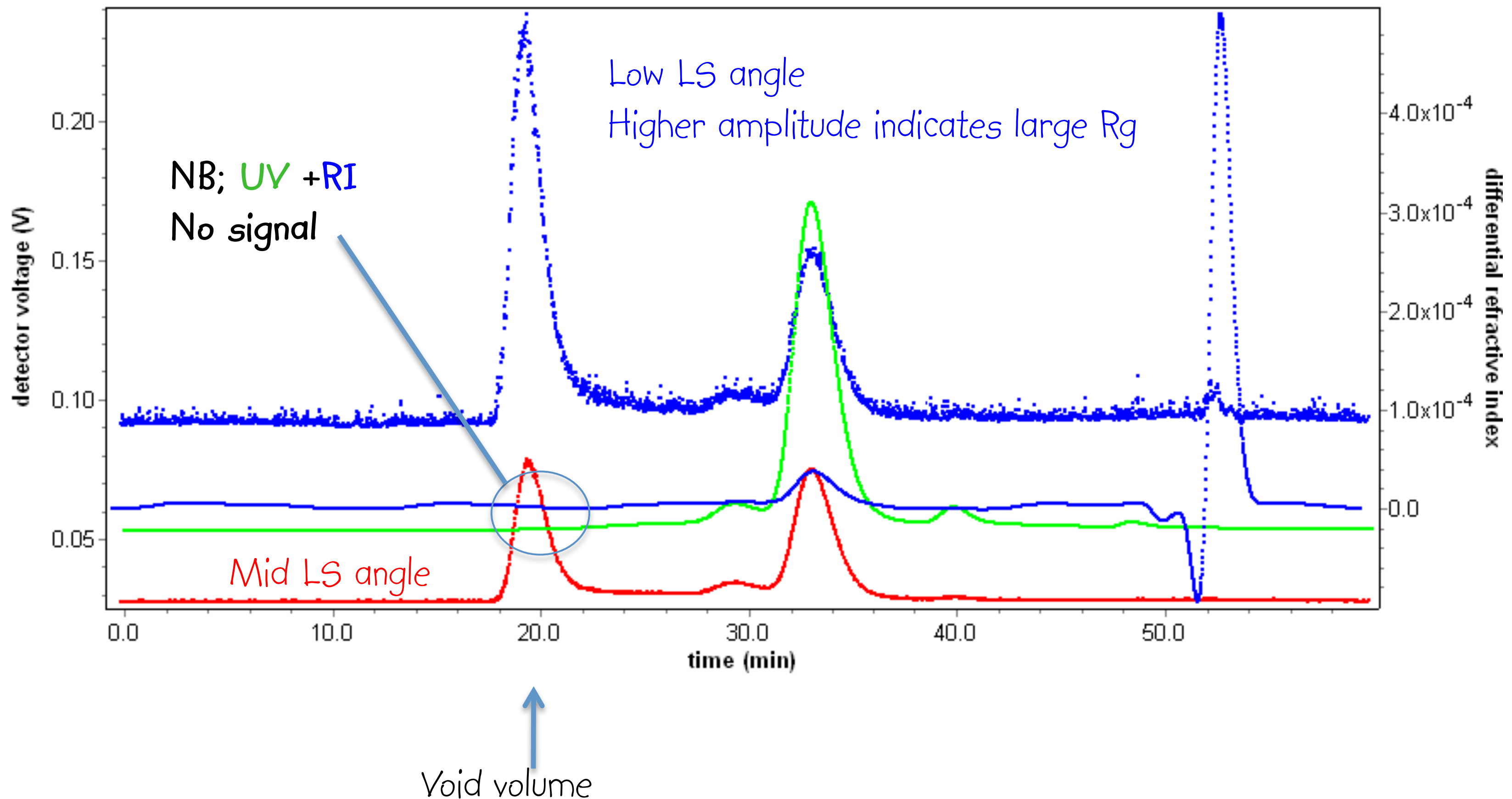
Applications of SEC MALS; Mass in solution

- Works for all proteins universal dn/dc
- Concentration does not need to be known before
- No UV absorbance (aromatics) required
- Can use less than 100% pure samples (because of SEC step)
- Labels tags etc fine (eg GFP). Laser is 658nm.



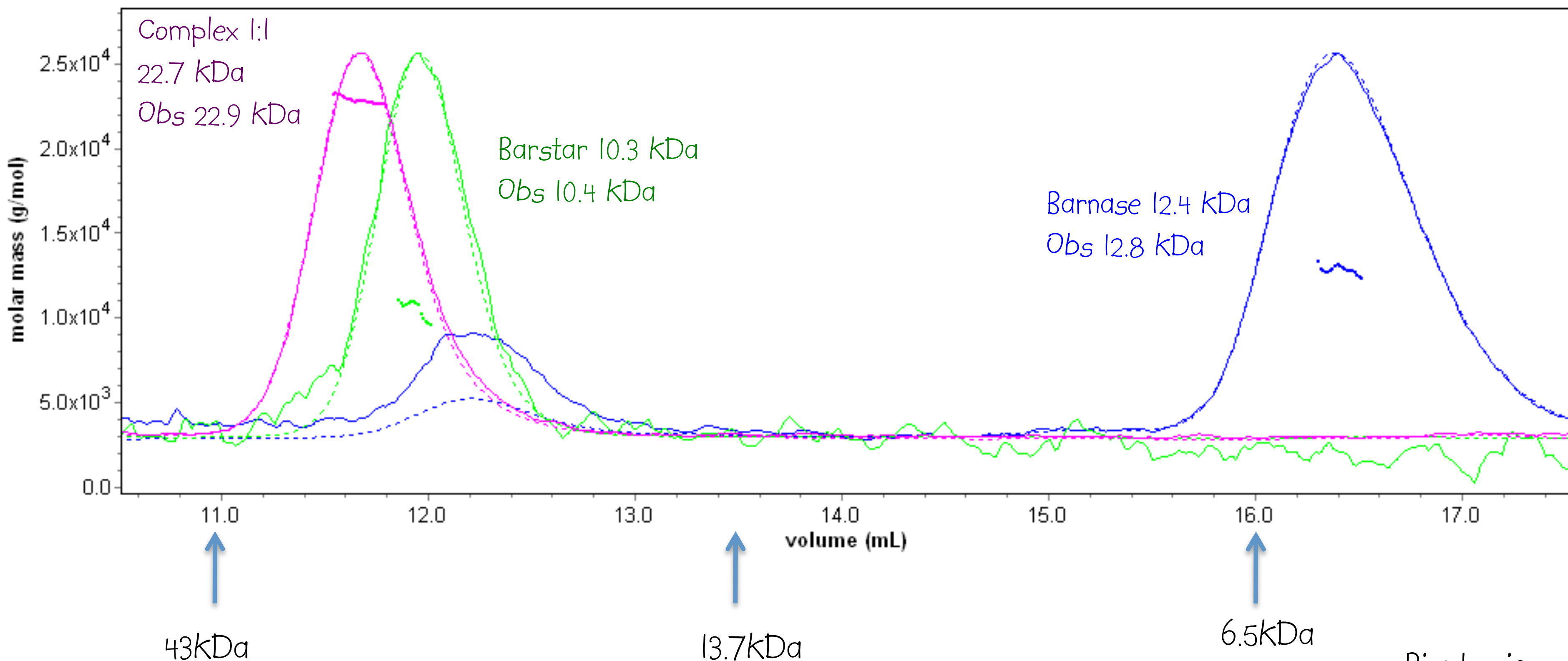
Applications of SEC MALS

- Highly sensitivity to low levels of high mass material; may be important for crystalisation.



Applications of SEC MALS; Tight Binding and stoichiometry

- Sample concentrations <u>above</u> Kd during SEC produce stable complex
- Note anomalous elution volumes of both free proteins; mass **independent** of SEC elution volume

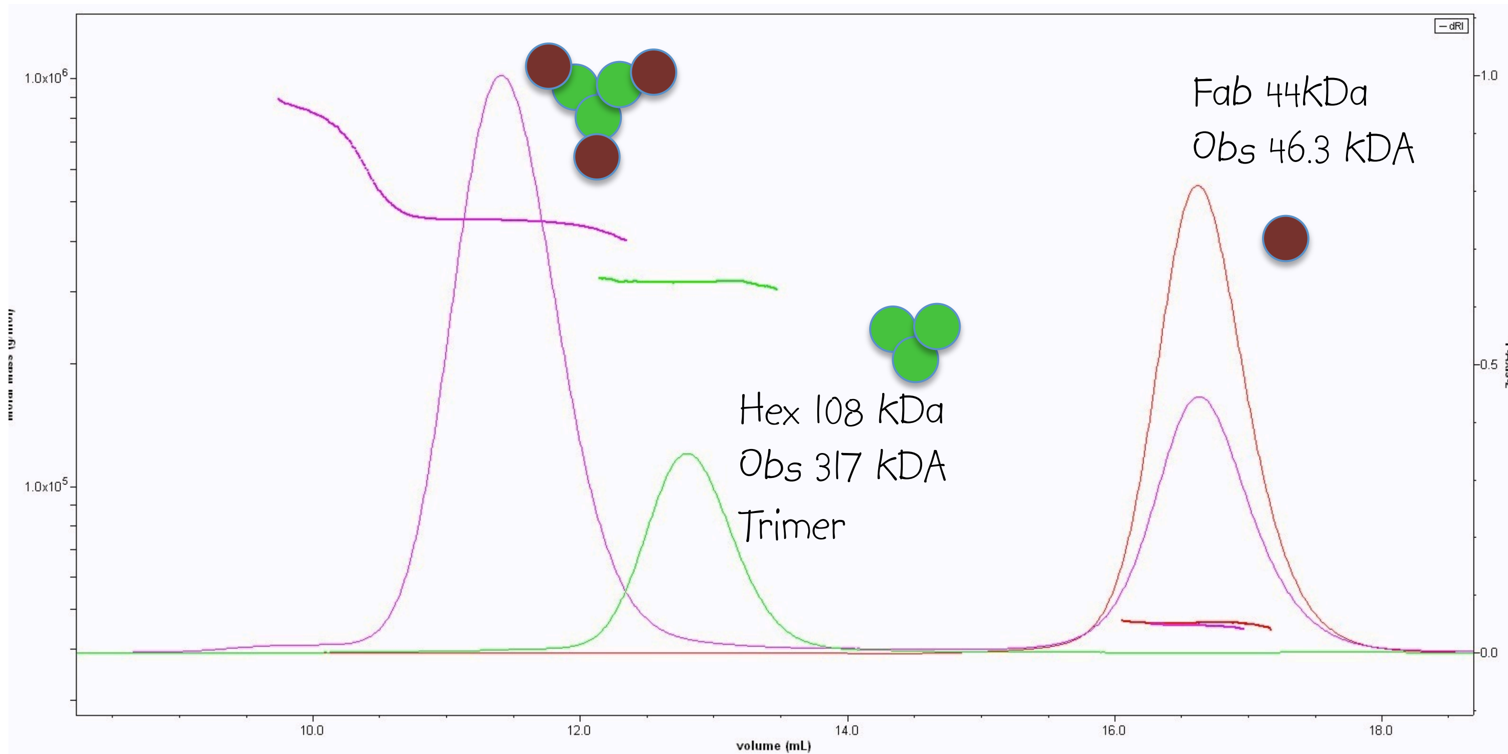


Biophysics

Applications of SEC MALS; Tight Binding and stoichiometry

Sample concentrations above Kd and excess Fab during SEC produce a stable complex

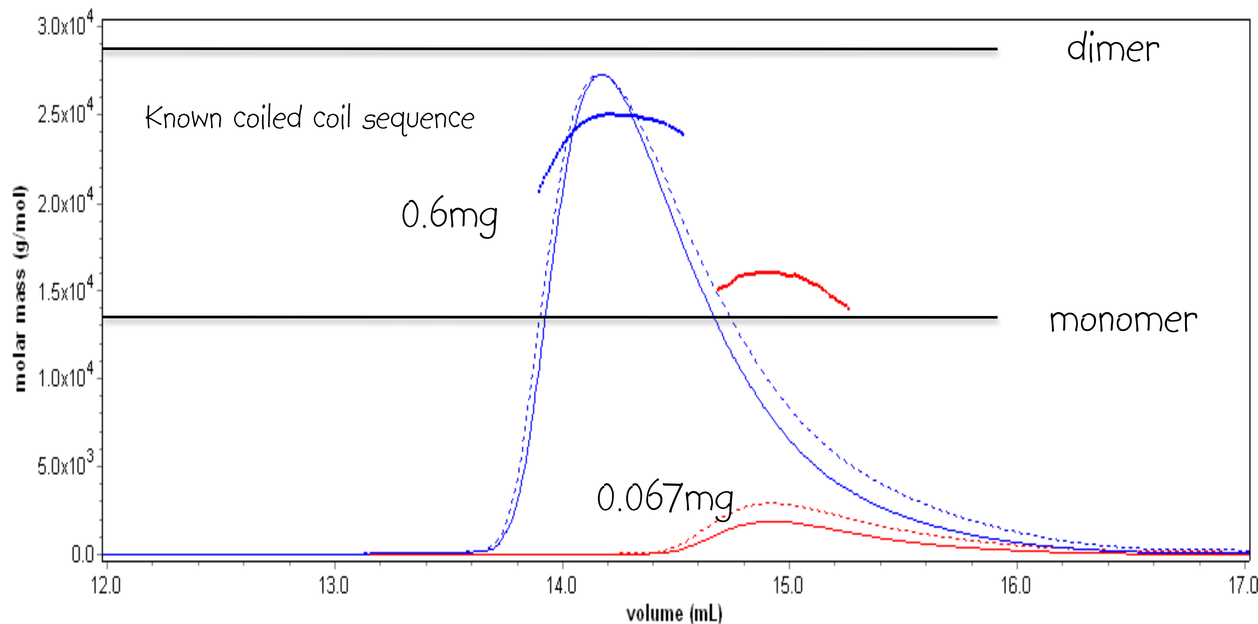
Hex+Fab 1:1 456 kDa
Obs 449 KDA



James Group

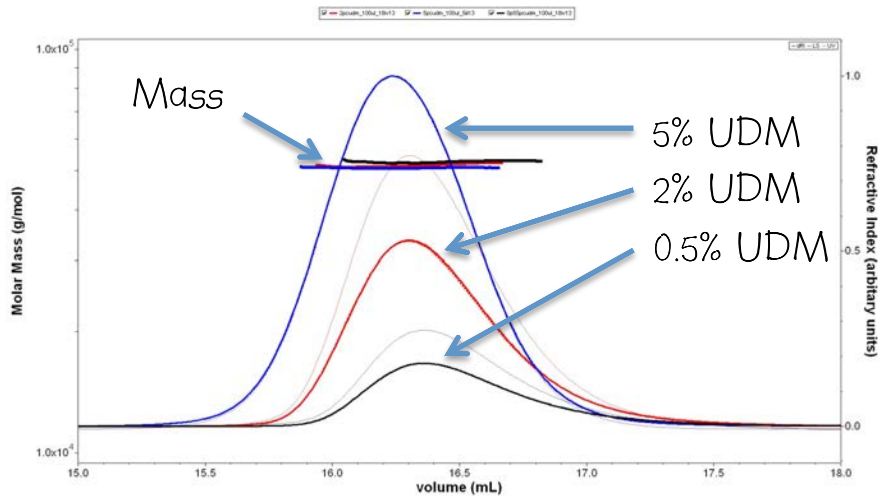
Applications of SEC MALS; Weaker interactions

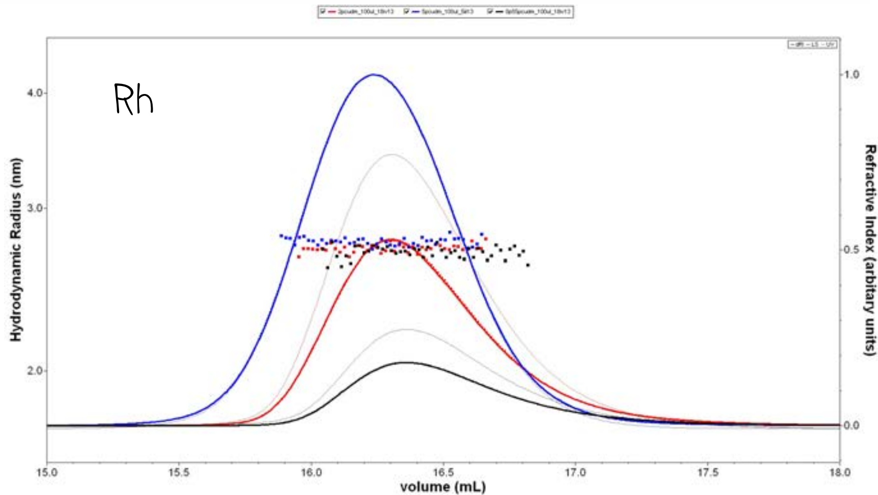
- Sample concentration varies through Kd across the peak. Kinetics of equilibria compete with the physical separation of the chromatography
- Mass distribution across the peak in this pattern is a hallmark for self or hetro association
- Ball park idea of Kd from concentrations
- Could be possible to extract Kd? (Calypso batch CG MALS technique)



James Group

Beyond Proteins....detergent micelles

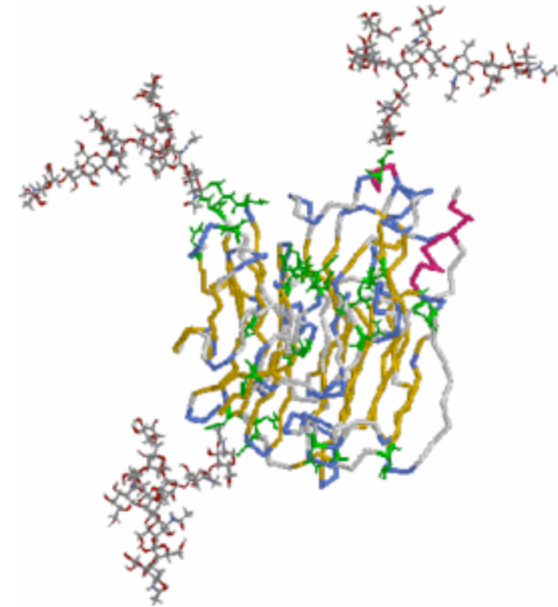


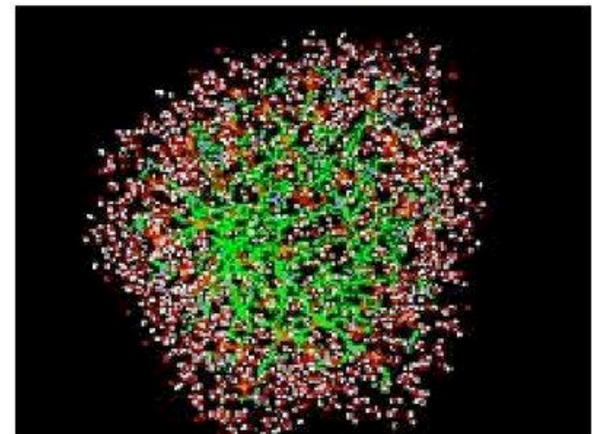


- Excess UDM micelles run in buffer above UDM CMC (0.03% w/v). Mass (52KDa) and Rh (2.7 nm) are accurately determined over a range of concentrations (0.55 – 5% w/v)
- Final concentration of excess detergent produced during protein concentration procedures can be determined from such standard curves

Applications of SEC MALS; Conjugate Analysis

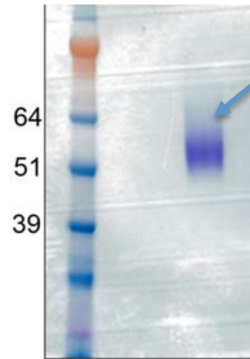
- In SEC MALS there are two signals, RI and UV, capable of yielding concentration
- In a simple (single component) system, the same mass will be calculated using either signal.
- For modified proteins, glcosylated, PEGylated, etc., or protein detergent complexes the mass of protein, the modifier and thus the complex can be determined simultaneously.
- dn/dc and ε_{280} for all components must be known and best accuracy obtained where these differ between the modifier and protein.

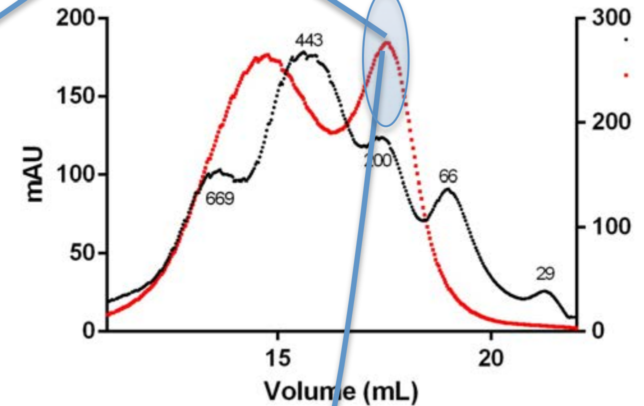




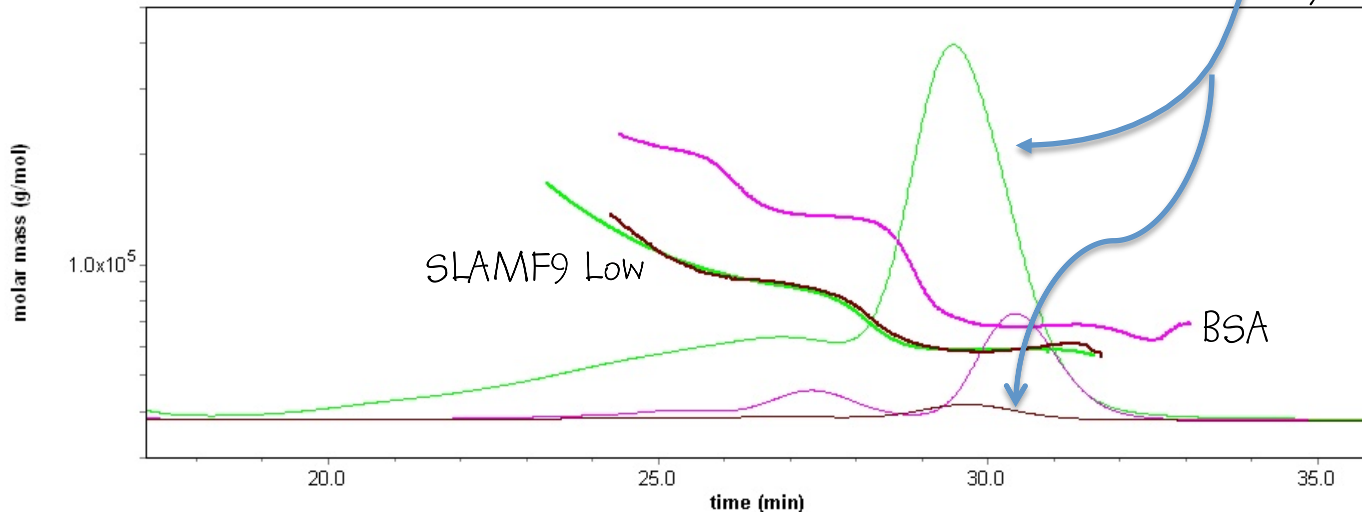
Conjugate Analysis Glycosylation

- Sequence (46KDa) and non reducing gel consistent with monomer in solution
- SEC shows two peaks between 200-500KDa based on elution of SEC standards
- SEC MALS indicates mass around 60 kDa consistent with glycosylated monomer based on predicted sites of modification and size of carbohydrate.
- Lack of concentration dependence supports monomer



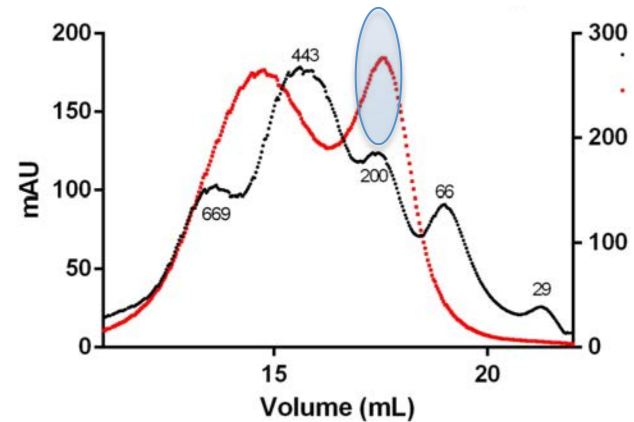


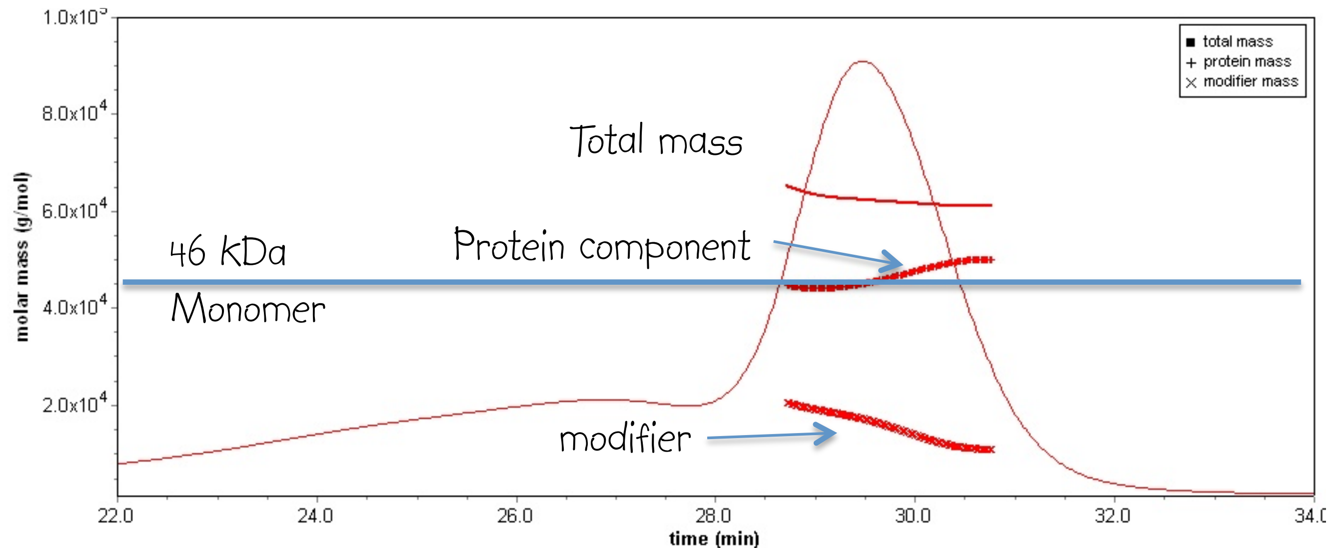
Run this fraction by SEC-MALS



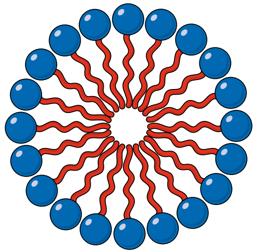
Conjugate Analysis Glycosylation

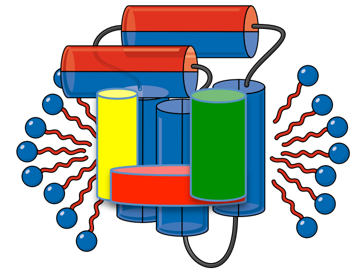
- 60 KDa Mass higher than monomer
- Protein has larger hydrodynamic properties than expected
- Protein has glycosylation sites and is expressed in eukaryotic vector
- Use UV extinction of protein and literature dn/dc and UV for carbohydrate

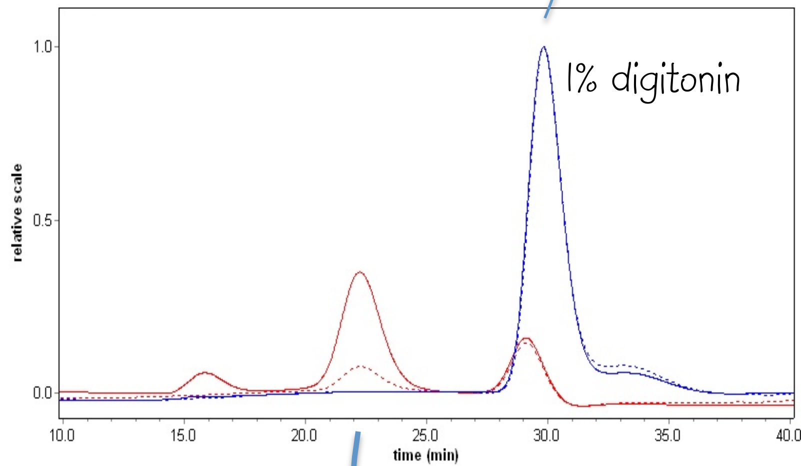


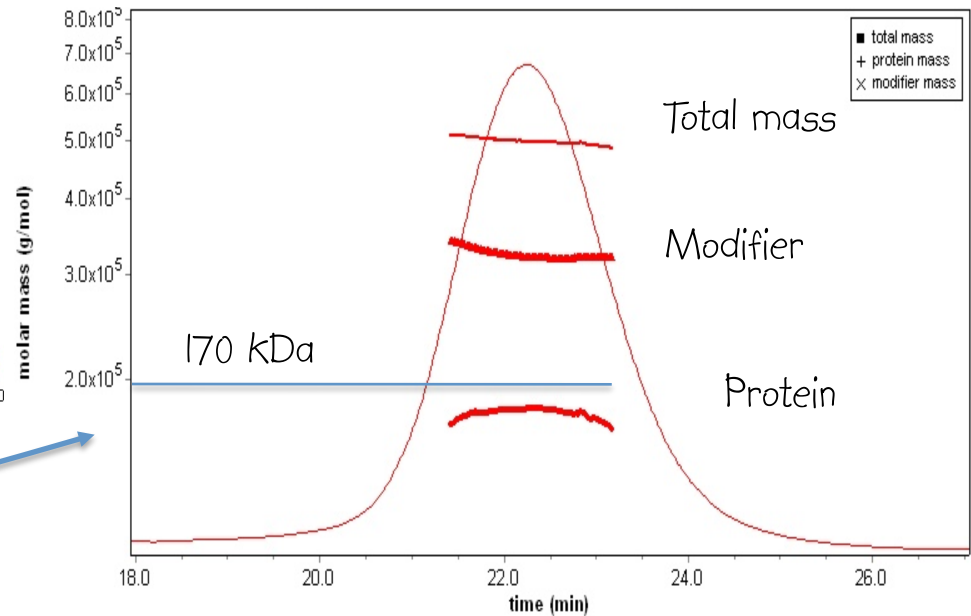


Conjugate Analysis of Detergent









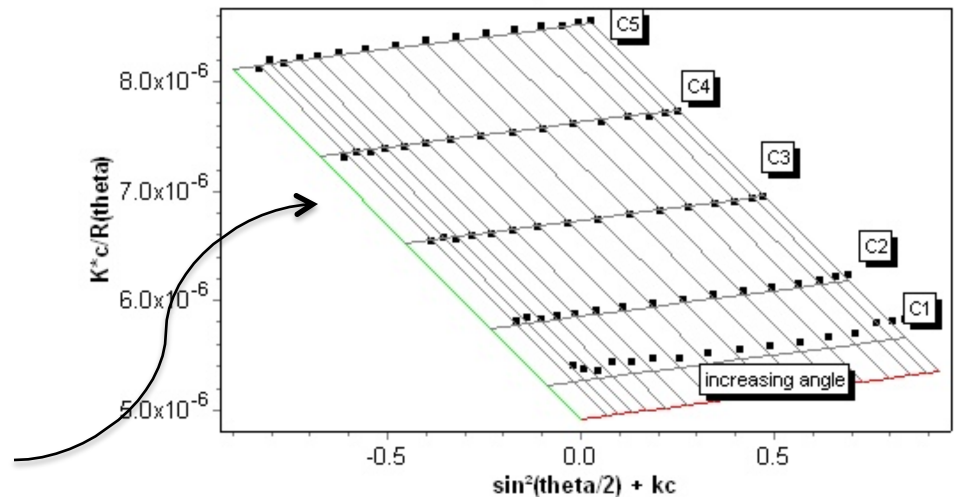
5 protein hetero complex in 0.1% digitonin a glycoside detergent from digitalis

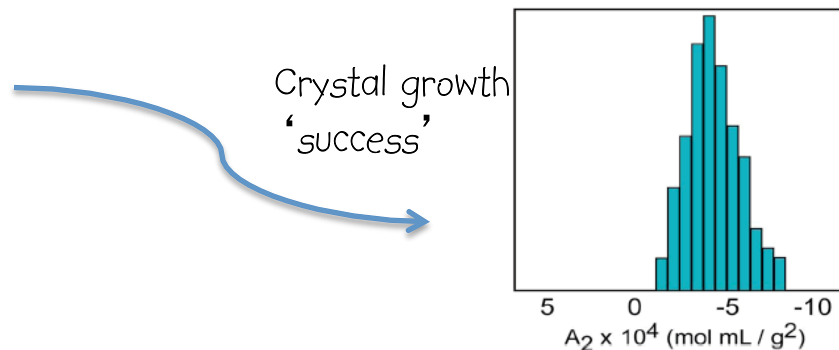
Mass of 1:1:1:1:1, 170 KDa

St James-Hyslop Group, CIMR

What about the second Virial Coefficient; A_2 ?

- A thermodynamic property of proteins being the second virial expansion of osmotic pressure with respect to protein concentration
- If A_2 is positive proteins prefer interacting with the solvent rather than other protein molecules and vice-versa for a negative value
- It can be measured from the slope of the concentration dependence of scattering in a Zimm plot. (A 3-d Debye plot)
- Conditions giving a negative A_2 are considered good for crystal growth

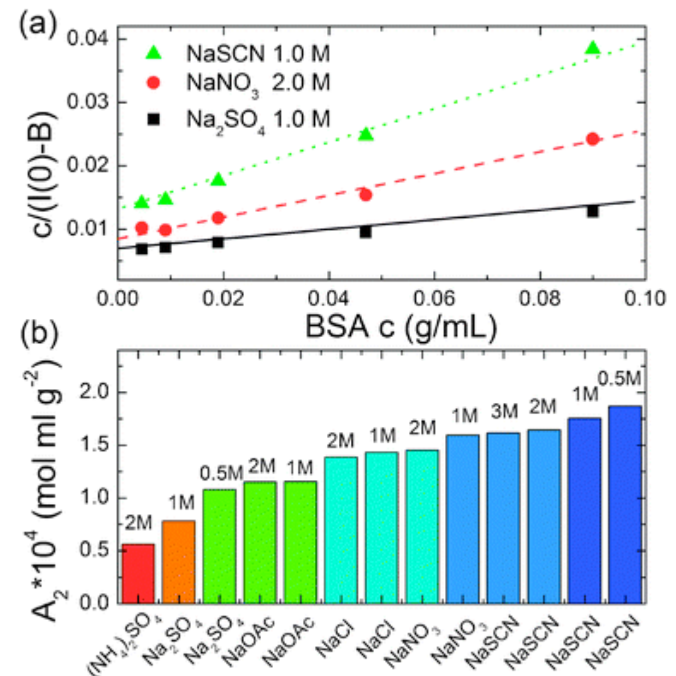




What if A_2 is not Known?

$$R(\theta) = \frac{4\pi^2 n_0^2}{N_A \lambda_0^4}\left(\frac{dn}{dc}\right)^2 McP(\theta)\left[1 - 2A_2McP(\theta)\right]$$

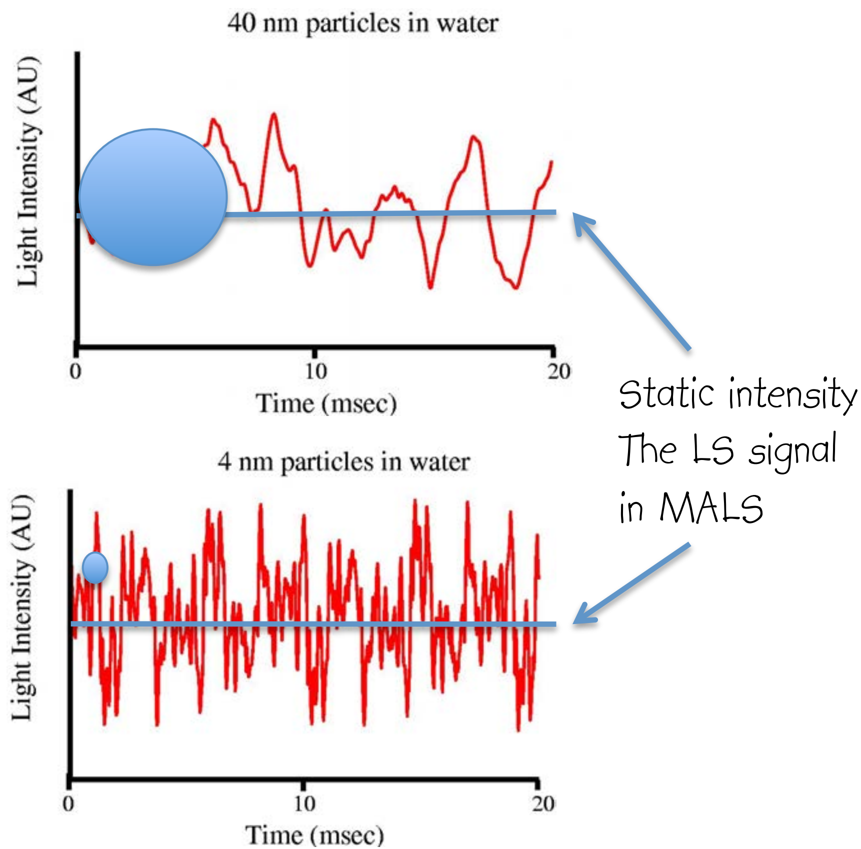
- At concentrations used in SEC MALS the second virial term is << 1 and so can be dropped from the above expression e.g., A_2 ~ 1 x 10^{-4} mol mL/g^2, c = 0.1 mg/mL, M = 50 kDa, A_2 containing term ~0.001
- At higher concentrations (batch experiments) or for large masses the A_2 expansion can be significant. A_2 can be determined by varying concentration and then be included in analysis.



Zhang et al., Phys. Chem. Chem. Phys., 2012, 14, 2483-2493

DLS (dynamic light scattering)

DLS aka, Quasi elastic light scattering (QELS) or Autocorrelation spectroscopy (ACS)



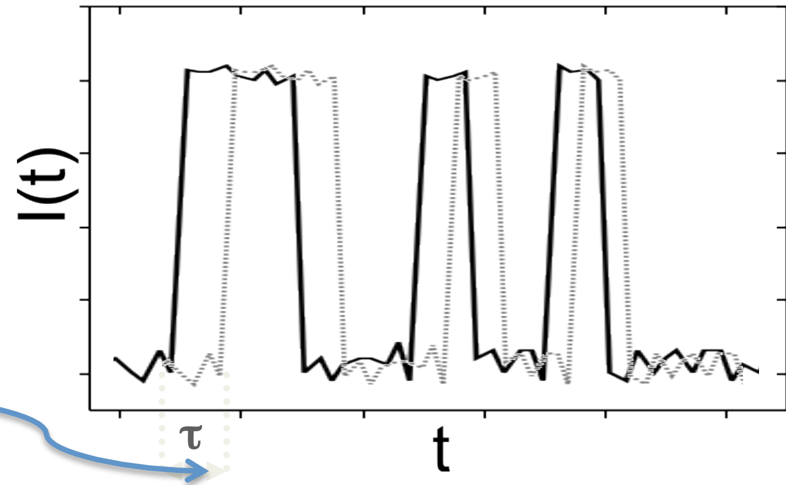
- Particle diffusion causes fluctuations in scattering intensity which are averaged by integration in static light scattering measurements
- Frequency of fluctuations reflects the particle size

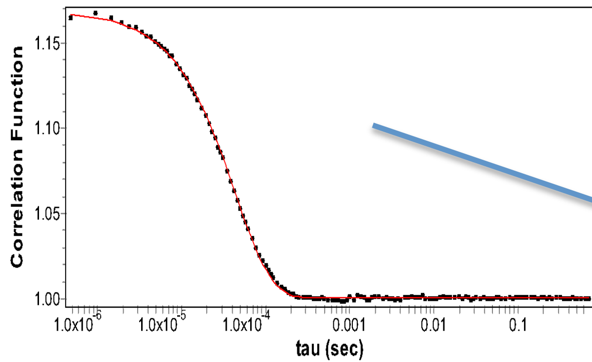
Analysis of intensity fluctuations

FCS: statistical analysis via the autocorrelation function:

$$G(\tau) = \frac{\langle I(t)I(t+\tau)\rangle}{\langle I(t)\rangle^2}$$

Offset τ





$$g^{(2)}(\tau) = 1 + \beta e^{-2D_t q^2 \tau}$$

Decay in the autocorrelation function is related to the translational coefficient Dt

Hydrodynamic Radius (Rh) from diffusion coefficient

- Stokes-Einstein equation

$$D_t = \frac{kT}{6\pi\eta R_h}$$

- K Boltsman constant
- T temperature
- η solvent viscosity (also strongly temperature dependent)

- Rh is the equivalent radius of a sphere that would diffuse with the same translational coefficient. It does not mean the particle is a sphere nor is it a physical dimension of the particle

Batch measurement of DLS

Cuvette based batch measurement

Avid Nano and Malvern Zetasizer

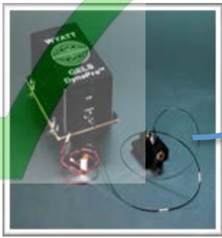
http://www.avidnano.com/products/products.htm

http://www.malvern.com/en/products/product-range/zetasizer-range/

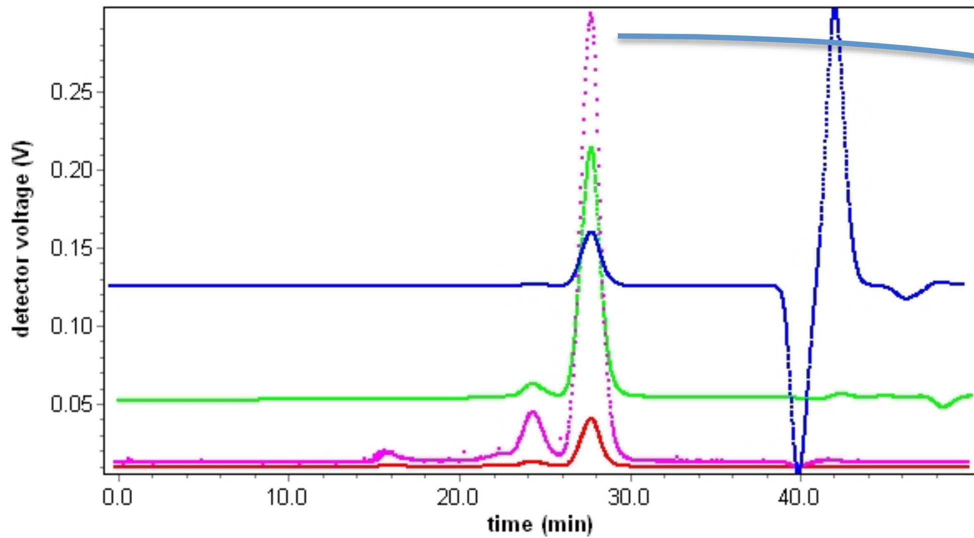
Wyatt Plate reader

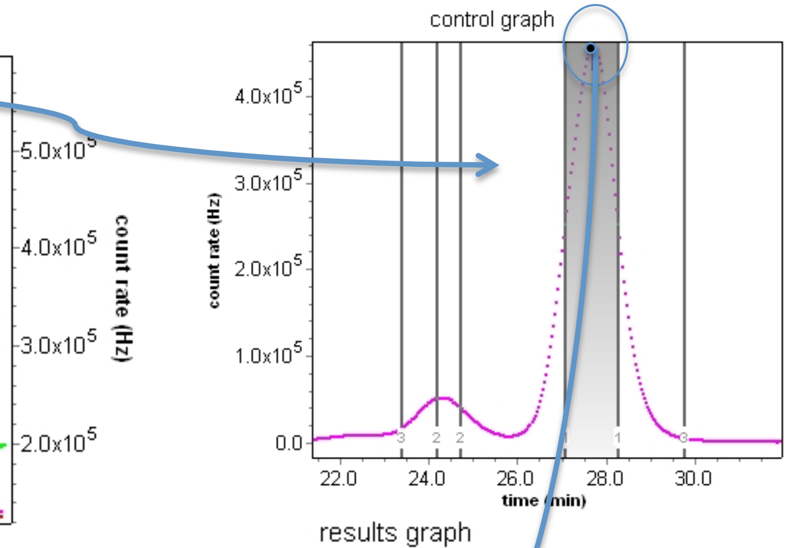
http://www.wyatt.com/products/instruments/dynapro-dynamic-light-scattering-plate-reader.html

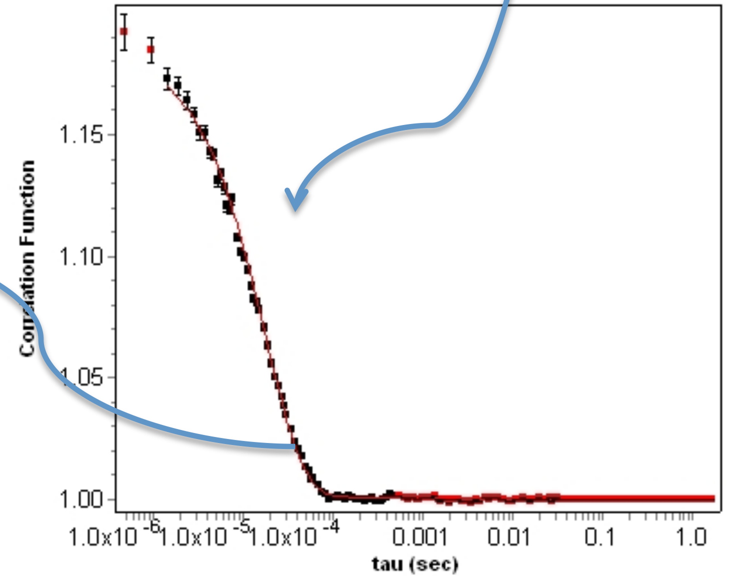
Batch methods are again sensitive to small amounts of large particles and complex analysis of ACF is required to deconvolve (Cumulants, Regularisation). Require 0.2 mg/ml and 5-100 uL



SEC MALS + QELS





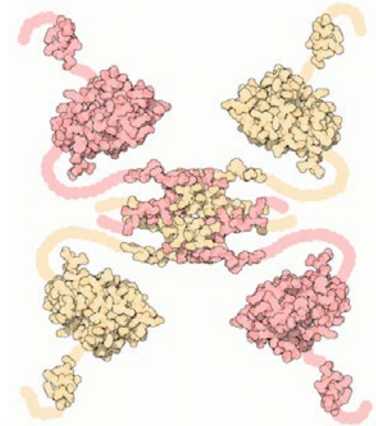


Replace one LS angle with QELS (DLS) detector

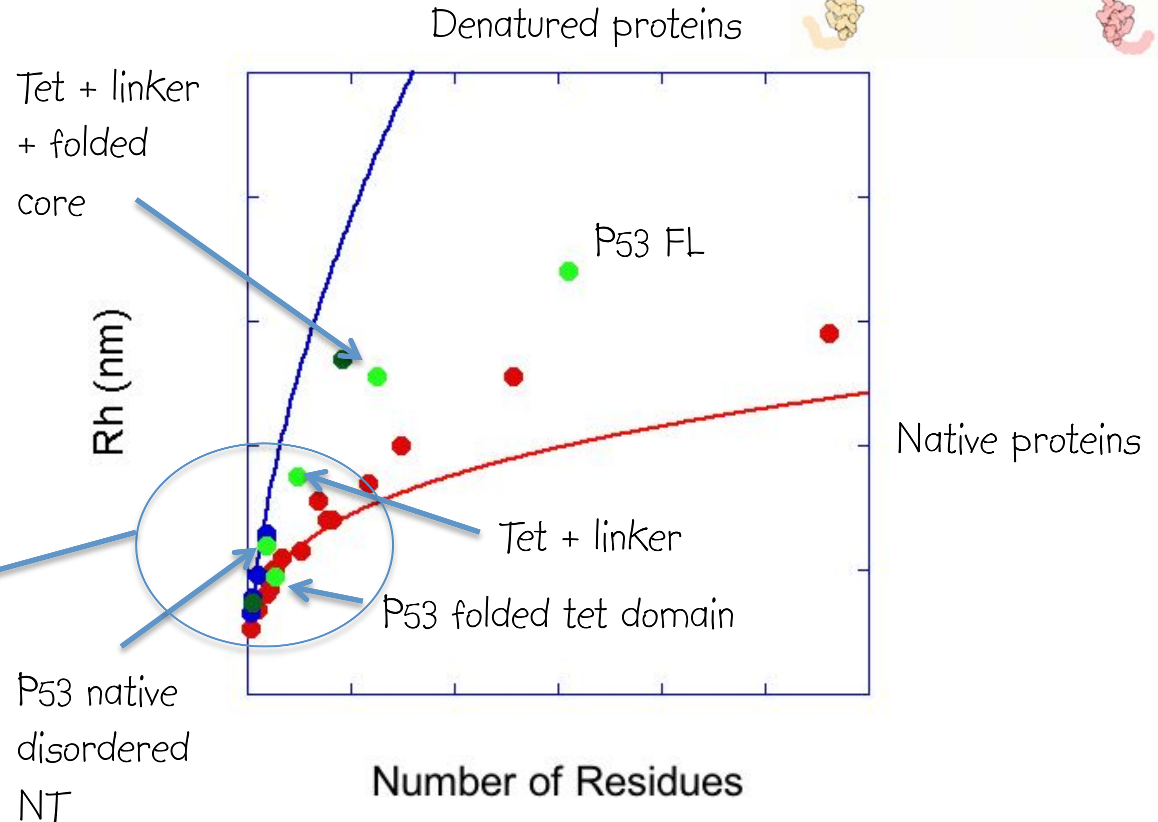
Dt and calculated Rh can be averaged across

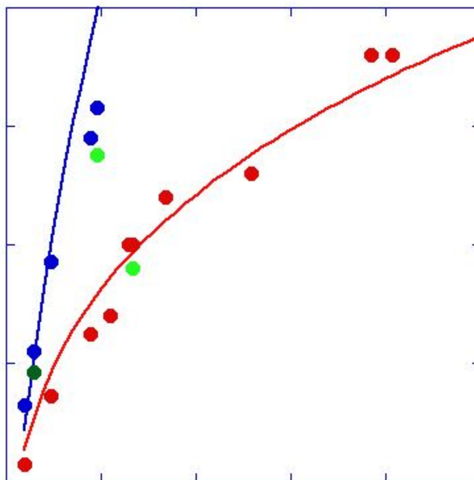
Chromatogram peak as with mass

QELS Applications, Rh Typical?



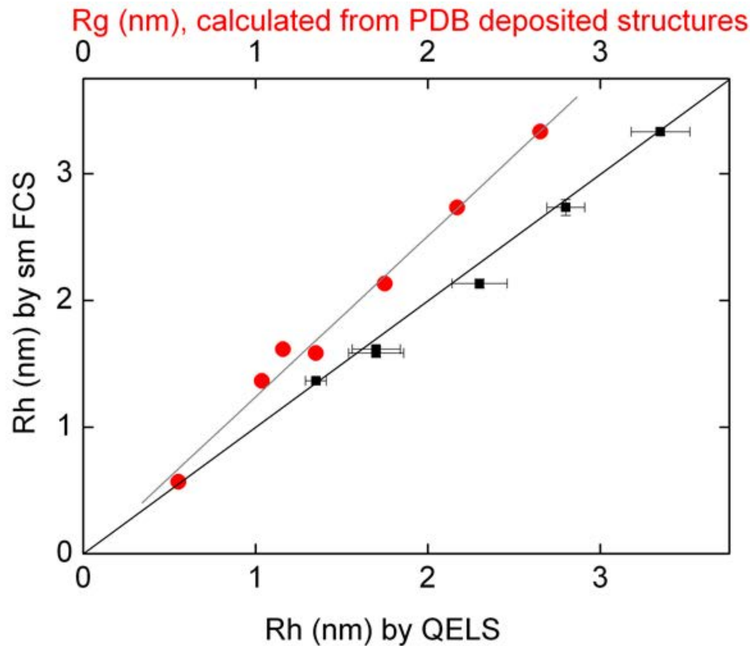
- Most proteins are compact globular spheres
- Rh scales with size
- Unfolded or partially disorderd segments increase the Rh as in p53

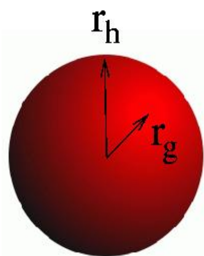




Fersht Group

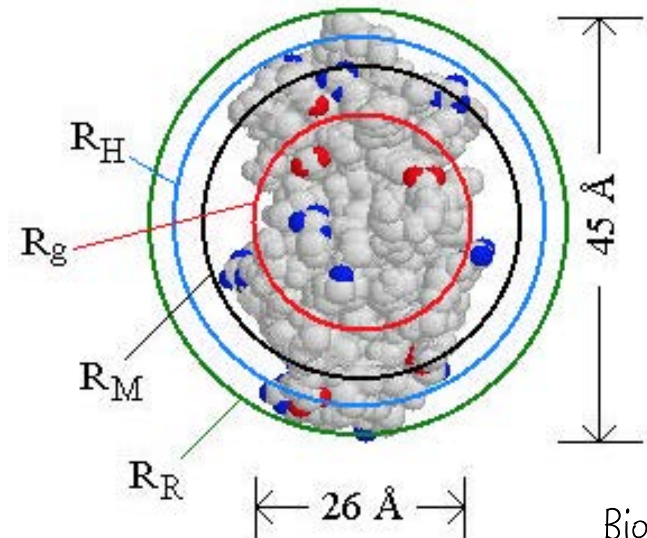
QELS Applications, Diffusion and Shape





$$\rho = \frac{R_g}{R_h} = 0.77$$

- Verifying Dt from other measurements, e.g., single molecule spectroscopy
- Comparison with Rg from structure or measured by MALS
- For compact spheres theory predicts Rg/Rh = 0.77* and this holds for most small proteins such as lysozyme;

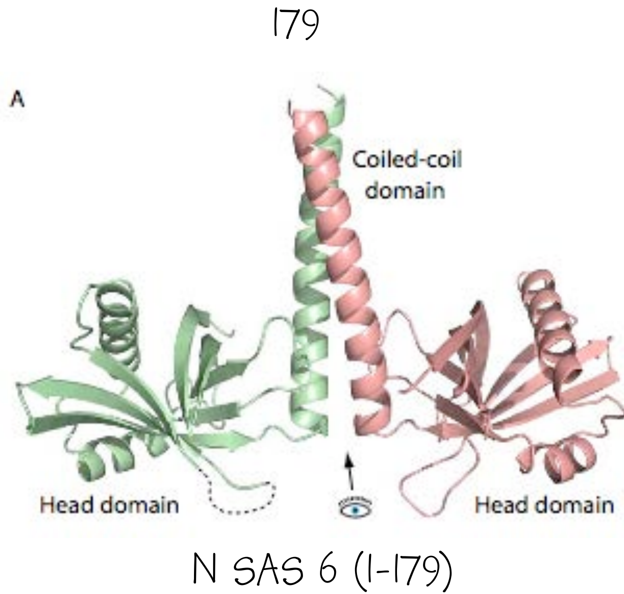


* Burchard et al. (1980) Macromolecules, 13, 1265-1272

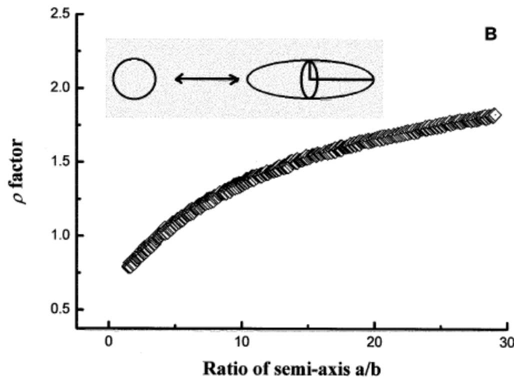
Rg/Rh ; ρ 'Shape factor'

Structures of SAS-6 suggest its organization in centrioles.
van Breugel et al. Science. (2011) 331,1196

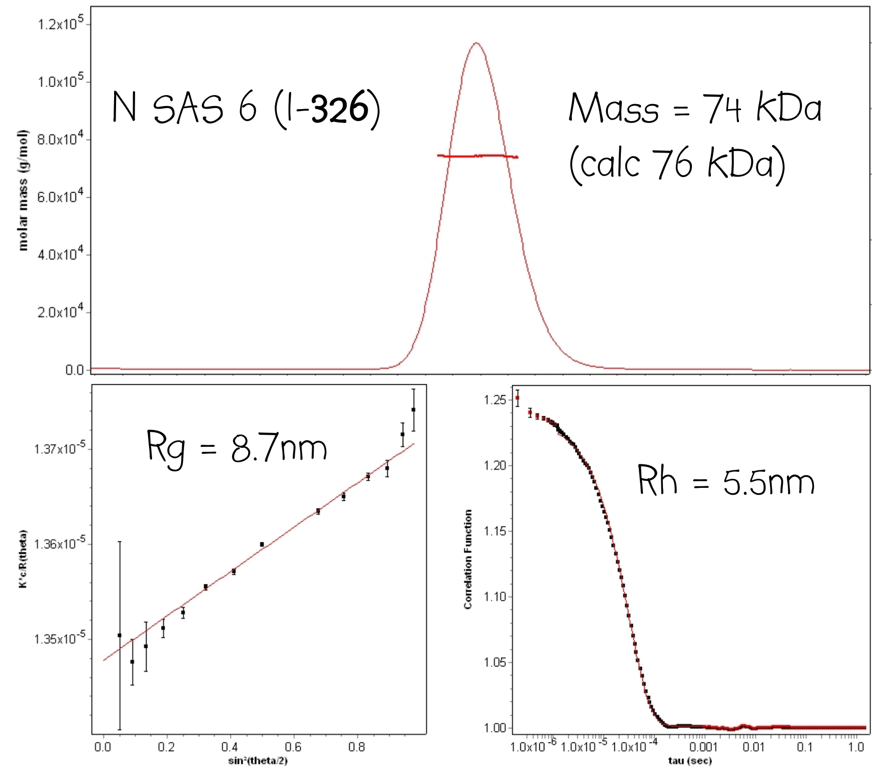
179



N SAS 6 (1-179)

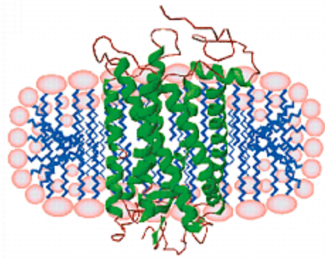
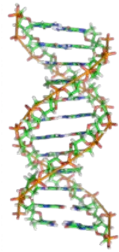


Coiled coil extended by 150 residues

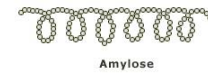


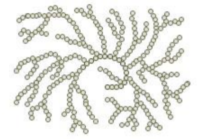
Rg/Rh = 1.6 consistent with an extended rod like shape in solution

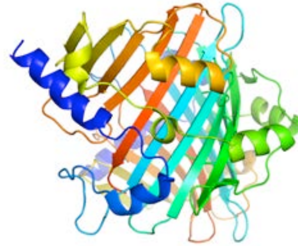
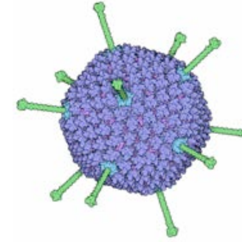
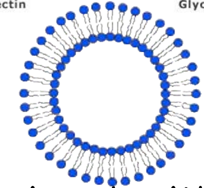
Van Breugal Group

Uses of MALS and DLS

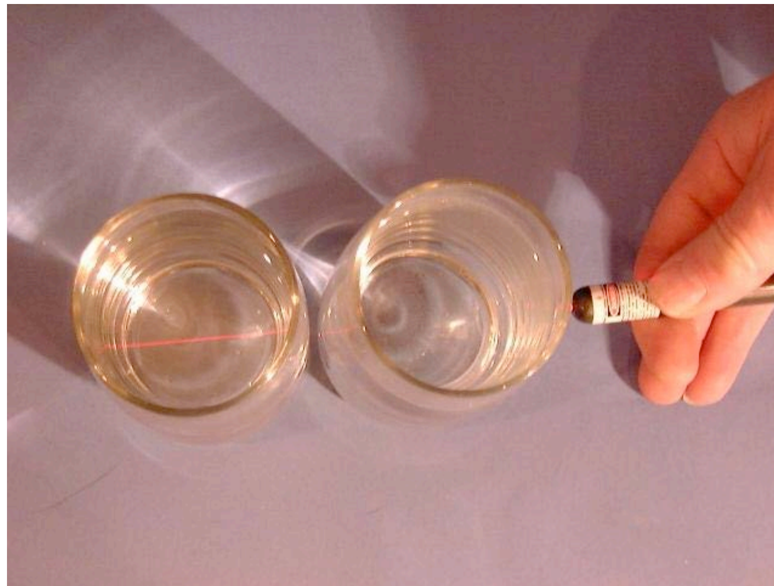




- Mass in solution An 'accurate' mass (can be ± a few %) determined within minutes using a model free method (shape and conformation independent). Range; 500 – 50 x 10^6 Da. SEC removes fractionates before measurement.
- Mass averaged radius (radius of gyration, Rg) for molecules > 10nm in diameter. 10 – 500 nm
- Second virial coefficient (A_2) from the concentration dependence of scattering. 'Measures' solvent-solute interactions; 'good' solvent vs 'bad' solvent
- Hydrodynamic radius (Rh). Autocorrelation analysis in DLS gives translational diffusion coefficient and thus Rh (assuming a sphere). 1-500nm
- Comparison of Rg and Rh indicates gross conformation (sphere, rod, branched etc)
- Solution based measurement. Quick, automated, easy and for proteins, as little as 10 μg per run

Other ways to improve your science using "Light scattering"

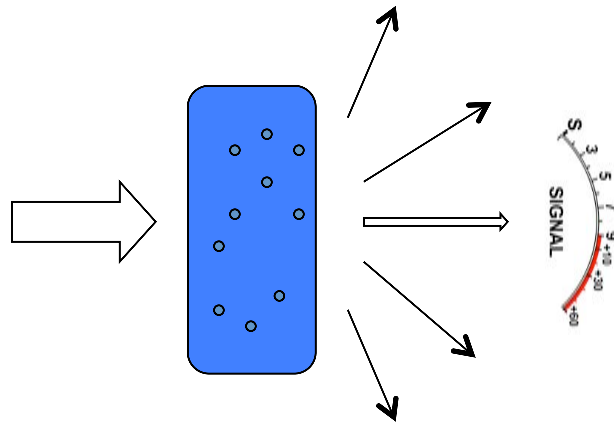


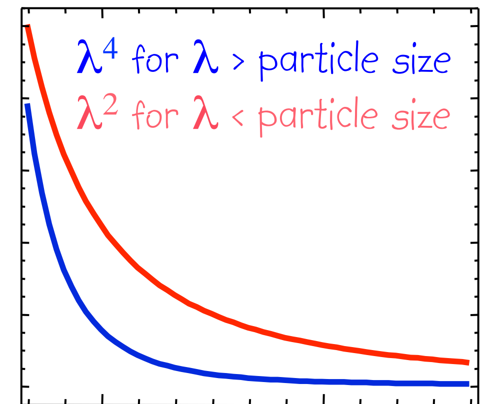
Wavelength dependence of scattering

(why the sky is blue(sometimes))

$$R(\theta) = \frac{4\pi^2 n_0^2}{N_A \lambda_0^4}\left(\frac{dn}{dc}\right)^2 McP(\theta)\left[1 - 2A_2McP(\theta)\right]$$

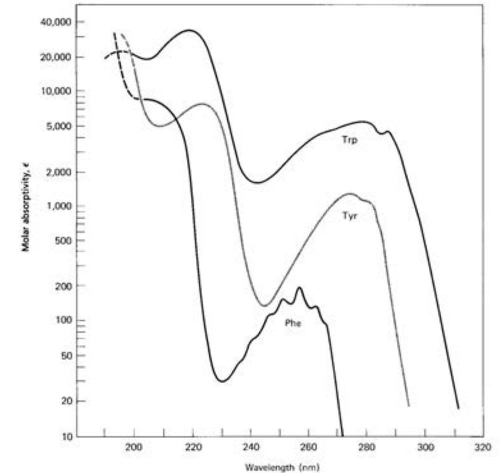
Scattering produces apparent absorbance in samples, particularly at low wavelengths such as where proteins are quantified (280nm)

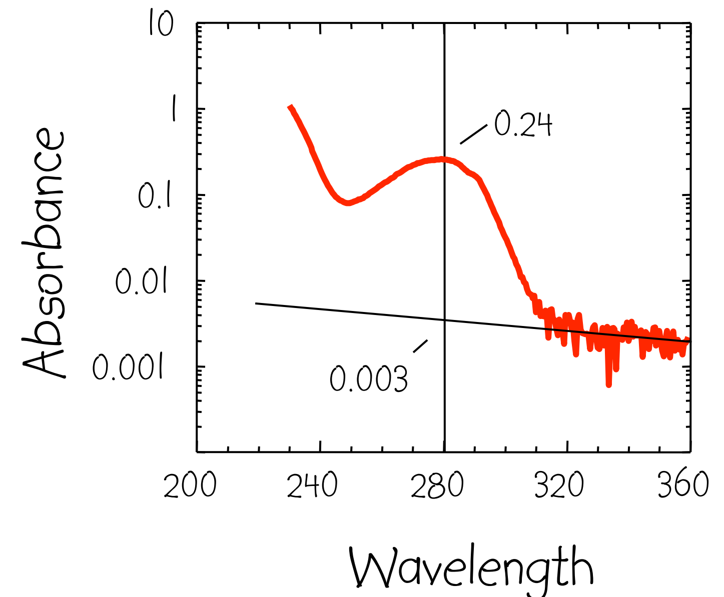




Correcting Protein Absorbance in Scattering Samples

- Measure protein *absorbance* <u>spectrum,</u> including wavelengths *above* 320 nm where there is no aromatic *absorbance*, e.g., 200 - 600 nm
- Scatter $\alpha\ 1/\lambda^n$ *so* log or ln *absorbance* is linear with wavelength
- Correct *observed* A for *scatter* from linear extrapolation; *should be* a few % of A for typical protein *solution*
- Gives the true [protein]
- Indicates problematic *solution* conditions



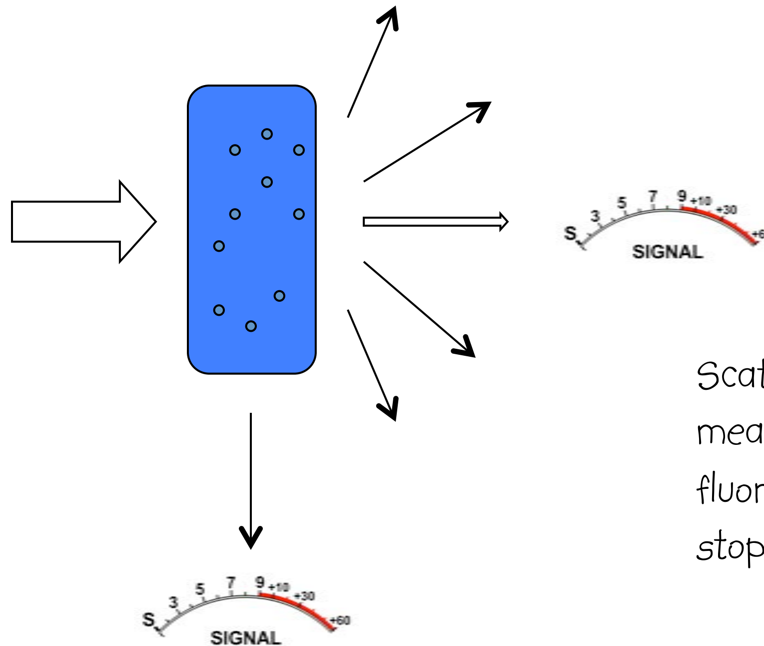


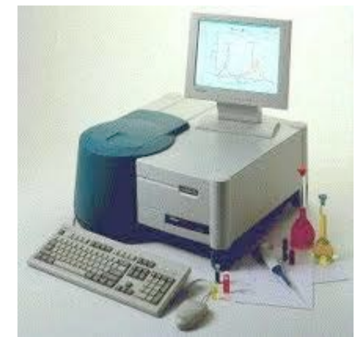
Mass dependence of scattering

The Rayleigh-Dans-Debye Equation

$$R(\theta) = \frac{4\pi^2 n_0^2}{N_A \lambda_0^4}\left(\frac{dn}{dc}\right)^2 McP(\theta)\left[1 - 2A_2 McP(\theta)\right]$$

Scattering is a useful probe where there is a significant change in mass and is useful for bulk cuvette based kinetic measurements

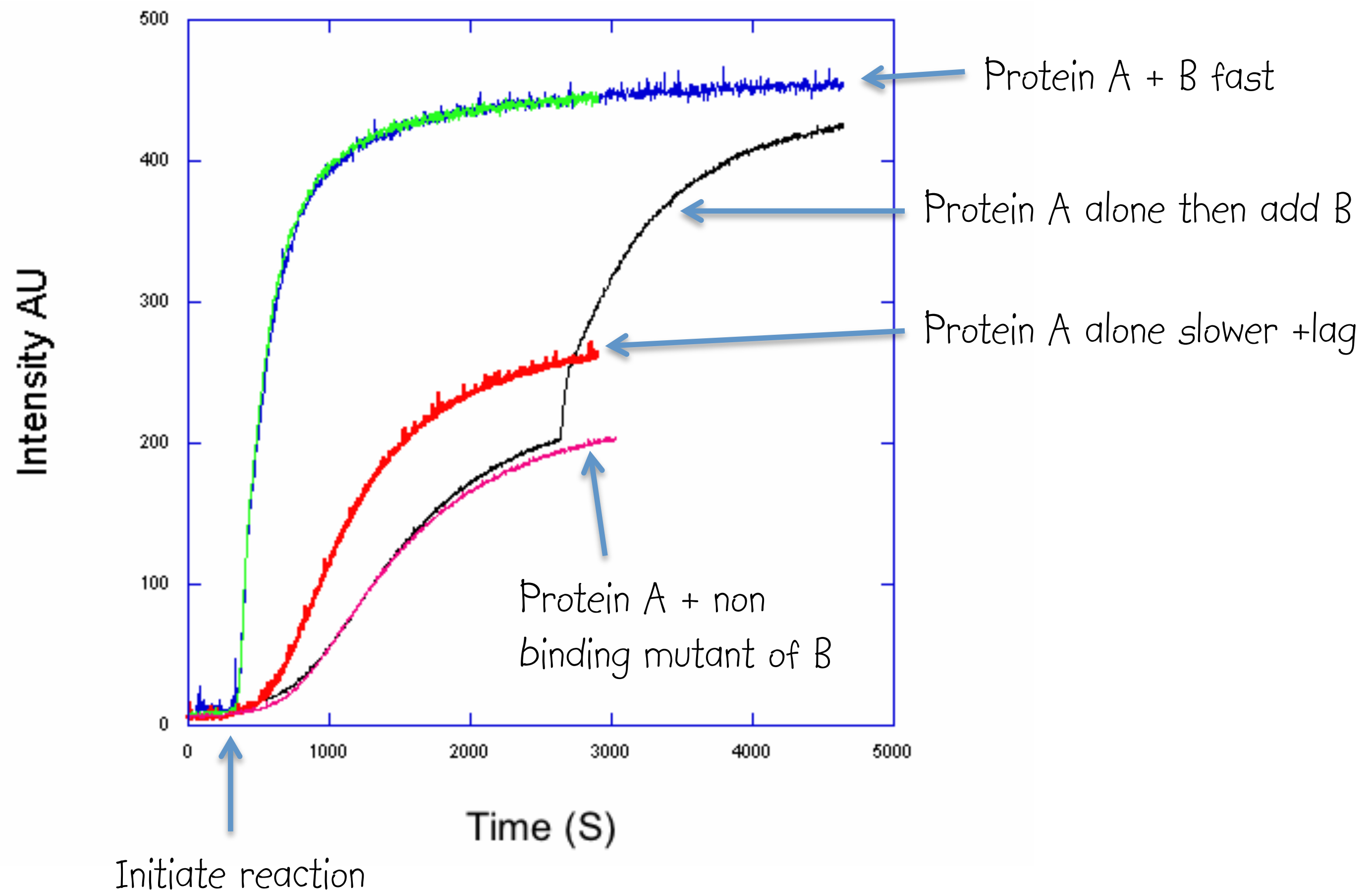




Scattering can be measured at 90 using a fluorimeter or in a stopped flow

Filament polymerisation kinetics

rates, endpoints and lags



Lowe Group

And not forgetting small angle x-ray scattering (SAXS) and x-ray crystallography...........

Advice, discussion, access, training, etc.

2S005

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