Static and dynamic light scattering.

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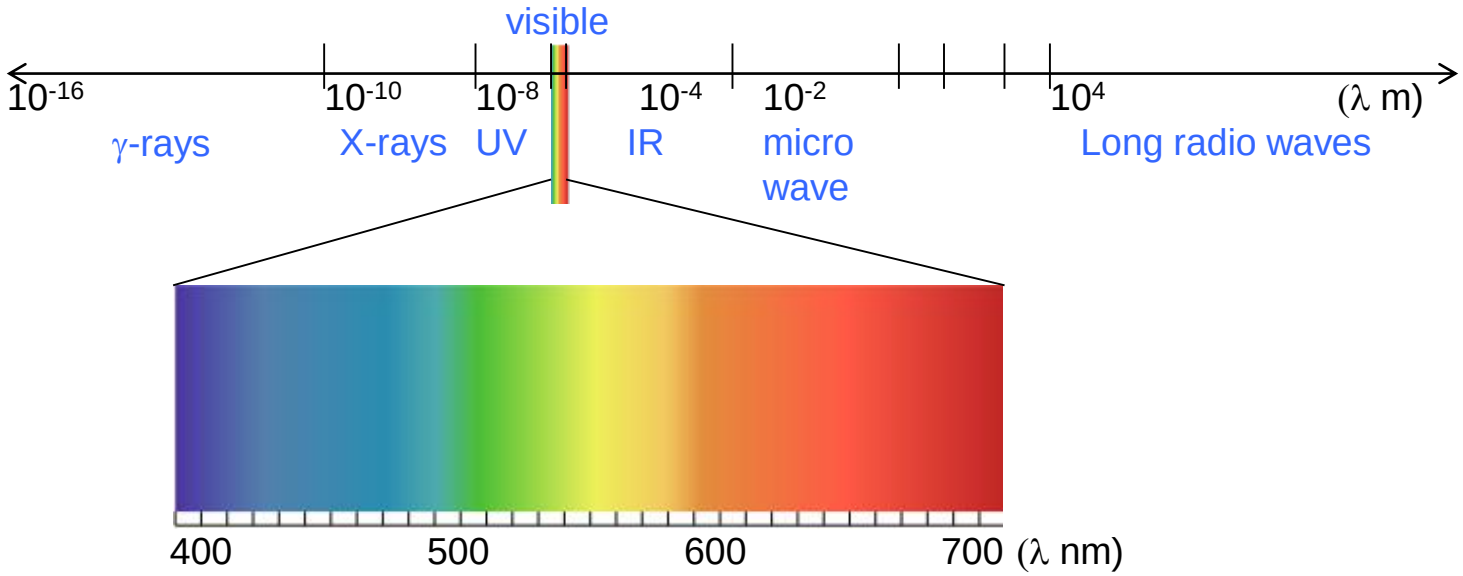
EMBL Hamburg

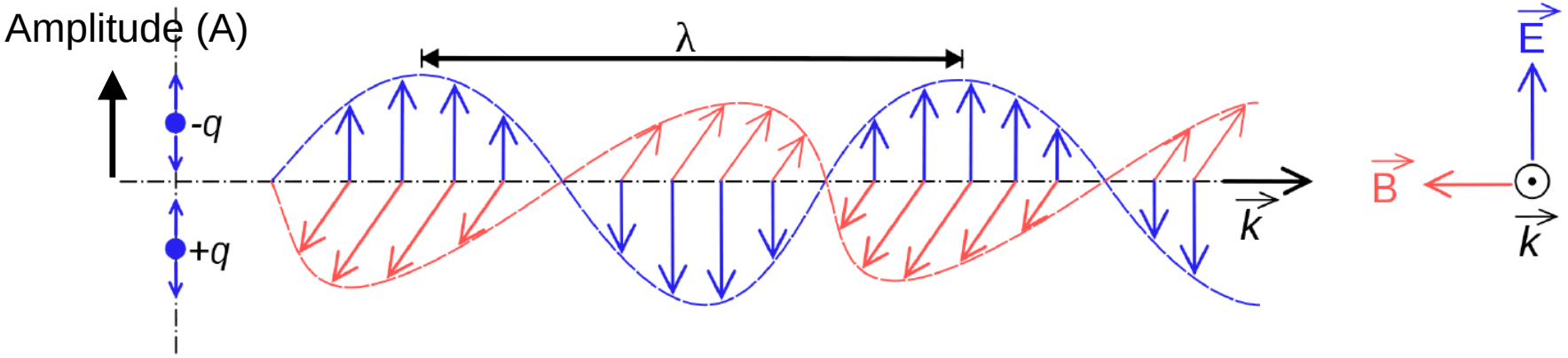
EMBL 40 YEARS | 1974–2014

Introduction.

- The electromagnetic spectrum.



Introduction.



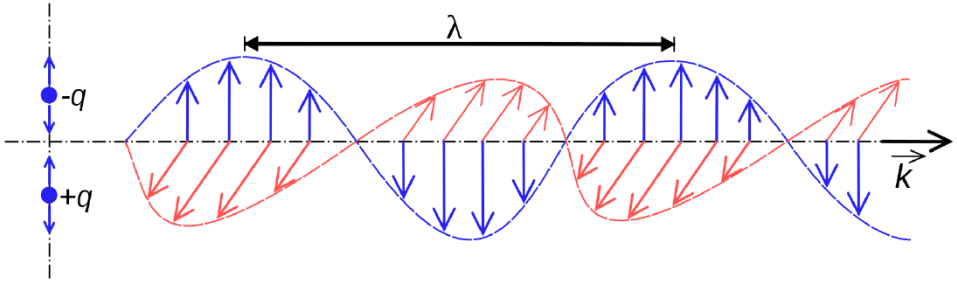
- *Four main outcomes when illuminating a sample with EM radiation*
- 1) Nothing happens – straight through (= Transmission)
- 2) Get absorbed.
- 3) Get absorbed and re-emitted at a different wavelength: Fluorescence.
- 3) ***Scatter***. Elastic scattering = NO CHANGE IN ENERGY.

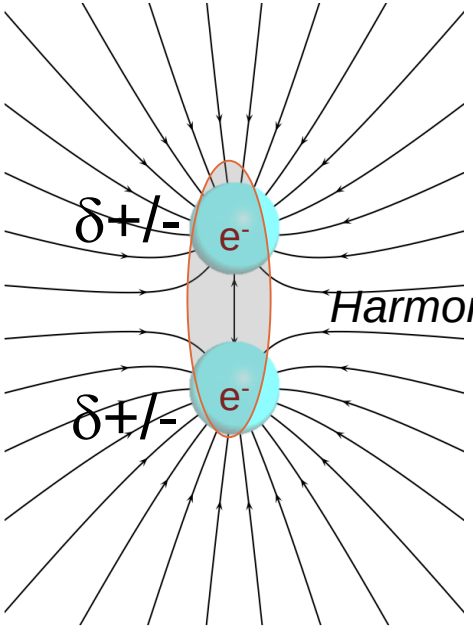
  Inelastic scattering = CHANGE in energy.

Now I take an electron…

And put it in an electromagnetic field…

…the electron begins to oscillate in the applied EM field at the frequency of the incident wave.





Harmonic dipole

Option 1:

- The radiation is transmitted.

- However during the oscillation the *phase velocity* of the EM wave (defined as λ/T, where λ is the wavelength and T the phase period) is momentarily “slowed” due to the interaction with the electric field of the electron. It may undergo a phase delay (e.g., 90 ^{o})

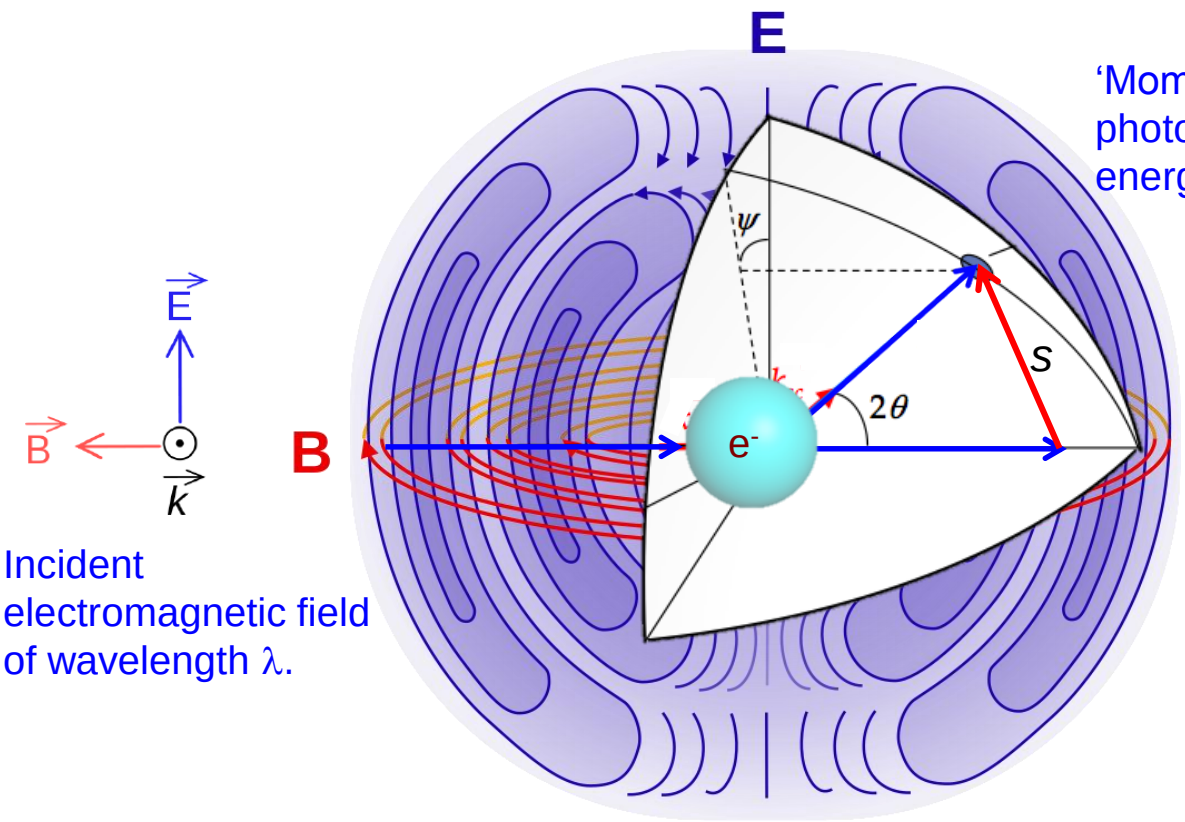
- For bulk materials, the intrinsic ability of a material to slow the phase velocity, *v*, relates to the *refractive index of the material, n,* whereby:

$$n = c/v$$

- The refractive index of water (using a laser at around 589 nm) is about 1.333 meaning that the light travels 1.333 times *slower* in water compared to light in a vacuum (*c*).

Option 2: Elastic scattering.

‘Harmonic oscillation’ of the electron in the EM field (di-pole oscillation).



‘Momentum transfer to the photon *without* a loss of energy= elastic scattering

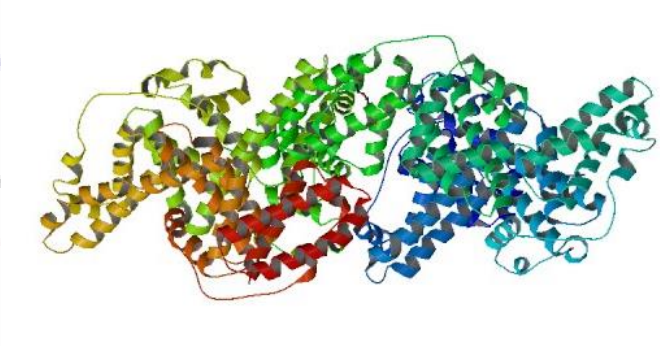
Spherical wave front is produced.

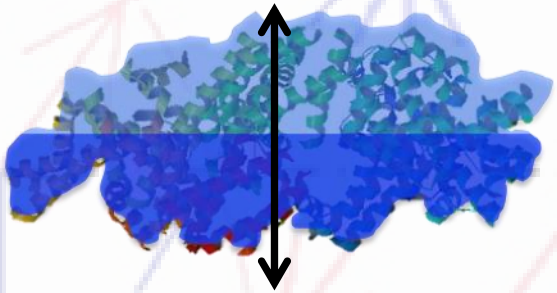
Incident electromagnetic field of wavelength λ.

By Original JPG (File:Felder um Dipol.jpg) due to Averse, SVG by Maschen. - Own work, CC0,

Now think of a protein as a collection of electrons…

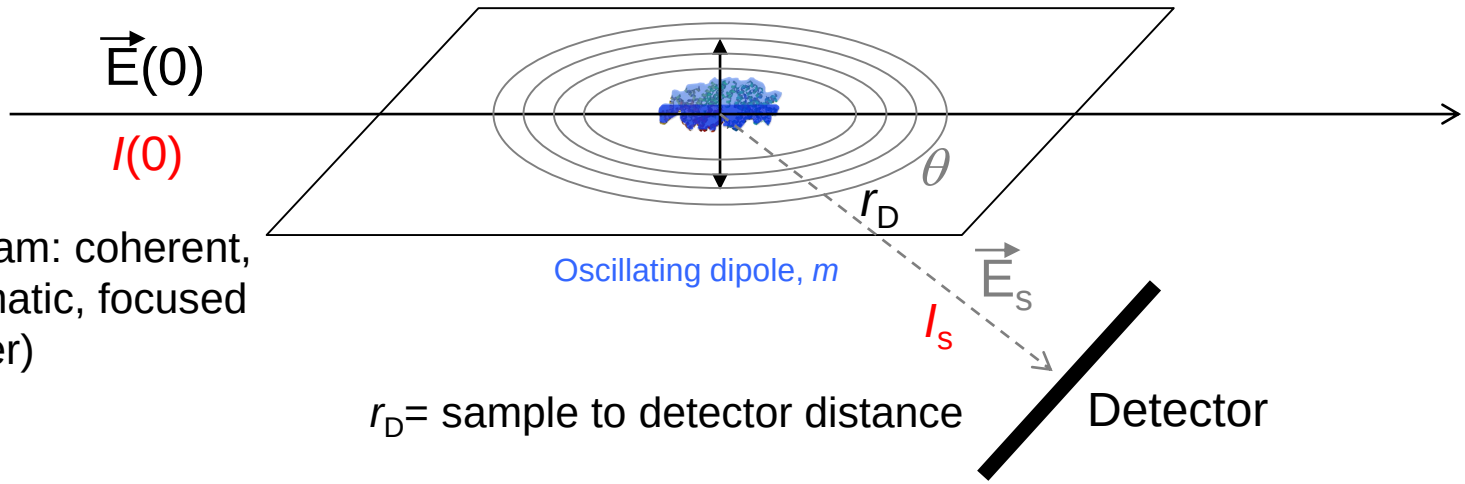
- If the protein is placed in an EM field with a wavelength MUCH LONGER that the overall size, the EM field will set up ‚protein-wide' dipole oscillation.





- *Intuitively, the larger the protein, the more electrons…*
- The more electrons = higher probability of scattering = higher scattering intensity.
- So, if you can measure the intensity of the scattered radiation and know the protein concentration, you can obtain a molecular weight estimate.

In effect the macromolecule acts as a point source emitter of ‘scattered wavelets’ with the same wavelength as the incident beam (elastic scattering).



Incident beam: coherent, monochromatic, focused (e.g., a laser)

r_D= sample to detector distance

The magnitude of the scattering amplitudes.

$$\vec{E}_s = \left(\frac{\partial^2 m}{\partial t^2} \right) \frac{1}{r_D c^2}$$

Inversely proportional to the sample-to-detector distance

Differential cross section (basically the probability of the volume occupied by the dipole, *m*, through a certain time, *t*, to scatter).

Just like SAXS, we cannot access the amplitudes.

…we measure the intensities.

Compare to SAS

$$I_s = \left\langle E_s E_s^* \right\rangle \qquad I(s) = \left\langle A(s) A(s)^* \right\rangle$$

There is less of an angular dependence in the intensities for *visible* light scattering.

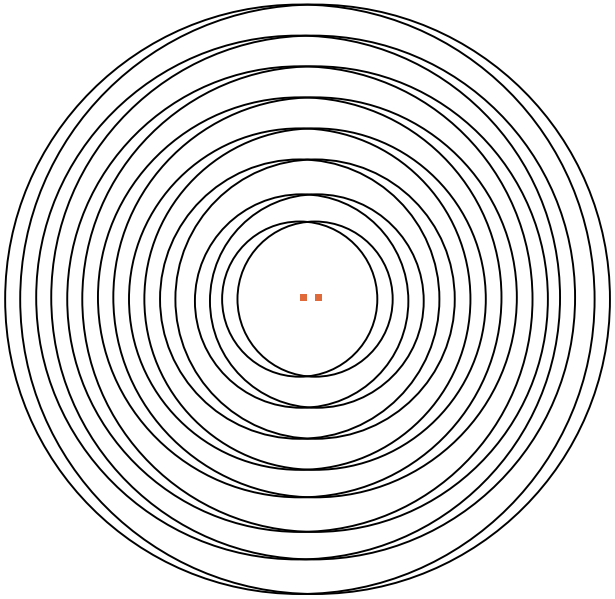
Why?

The wavelength!

Light scattering experiments are typically performed at, for example, 589 nm (the sodium D-line), compared to 0.1 nm for SAXS!

λ

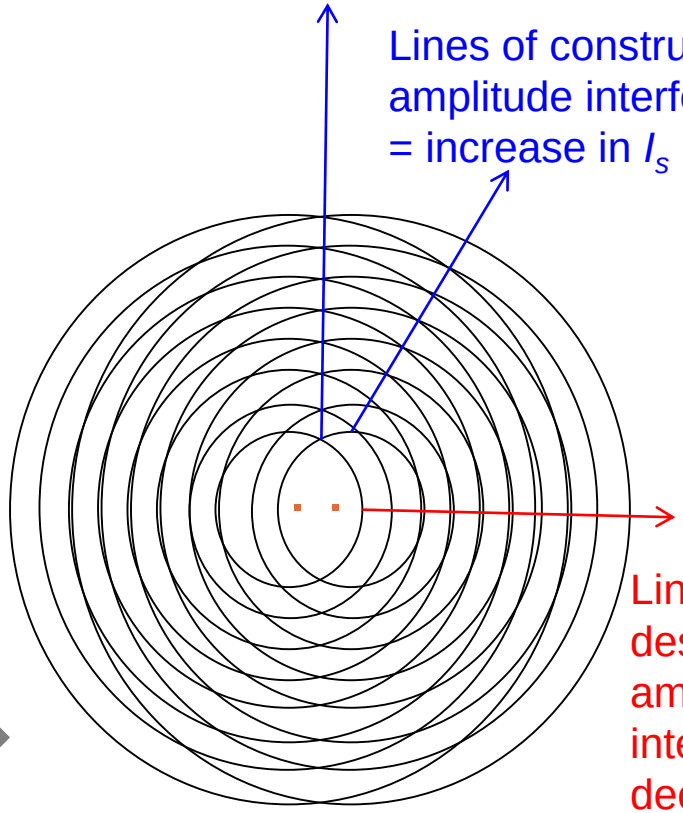
Particles smaller than λ



Isotropic scattering (low angular dependence of I_s)

Particles larger than λ

- Several dipoles set up within the sample macromolecule.
- I_s = Size and shape dependent (form factor $P(q)$).



- If the wavelength is really small, like in SAXS, then multiple diploes that sample small distances generate a significant angular dependence in I_s

Main issues

- Particles are in solution.
    - Therefore there has to be a difference between the refractive index of the solvent and the refractive index of the sample (analogous to contrast in SAS).
- Particles are moving in solution
    - Brownian motion.
- Particles may interact with the solvent (or each other)
    - Therefore interparticle interactions affect the scattering. The magnitude of the interaction is quantified by the second virial coefficient (analogous to, but not the same as, $S(q)$ in SAS.)

The intensity

Proportionate to the **concentration**

$$I: \; b^2 kT \frac{c}{\left(\frac{\partial \pi}{\partial c}\right)_{T,N}}$$

Essentially a **'contrast' term**: The refractive index of the solute must be different to the solvent ($n_{D,0}$)!

$$b^2 = \frac{4\pi^2}{\lambda_0^4 N_L} n_{D,0}^2 \left(\frac{\partial n_D}{\partial c}\right)^2 = K$$

Taking into account the differential **refractive index increment of the macromolecule**, *dn*/*dc* (mL/g)

And the wavelength

For an ideal solution:

$$\frac{\delta \pi}{\delta c} = \frac{kT}{M}$$

Where for an *real* solution:

$$\frac{\delta \pi}{\delta c} = kT\left(\frac{1}{M} + 2A_2 c + \cdots\right)$$

Concentration dependent interparticle interactions caused by enthalpic solvent-solute effects

Reformulating relative to a known standard, e.g., *toluene*, we express the intensity in terms of the Rayleigh ratio, *R*, which in effect is excess scattering intensity (normalised intensity of scattered light per solid angle per unit of illuminated scattering volume ΔV). Here *Kc* is short hand for the 'contrast' term above, also taking into account instrument constants.

$$\frac{Kc}{R} = \frac{1}{M} \qquad \frac{K_c}{R} = \frac{1}{M} + 2A_2 c + \ldots$$

…but, of course, in real solutions

- There is always some angular dependence on the scattering intensities. The magnitude of I_s at a given angle can be described by the momentum transfer, q:

Refractive index term

$$q = \frac{4\pi n_d \sin\left(\frac{\theta}{2}\right)}{\lambda}$$

Such that: $$\frac{Kc}{R} = \frac{1}{MP(q)} + 2A_2c + \ldots$$

where $P(q)$ is the form factor

That can be additionally expressed as (here R_g^2 is the mean squared R_g):

$$\frac{K_c}{R} = \frac{1}{M}\left(1 + \frac{R_g^2}{3}q^2\right) + 2A_2c + \ldots$$

Compare to SAS

No refractive index term…why?

$$s = \frac{4\pi \sin\theta}{\lambda}$$

Compare to SAS

$$I(q) = N(\Delta\rho V)^2 P(q)$$

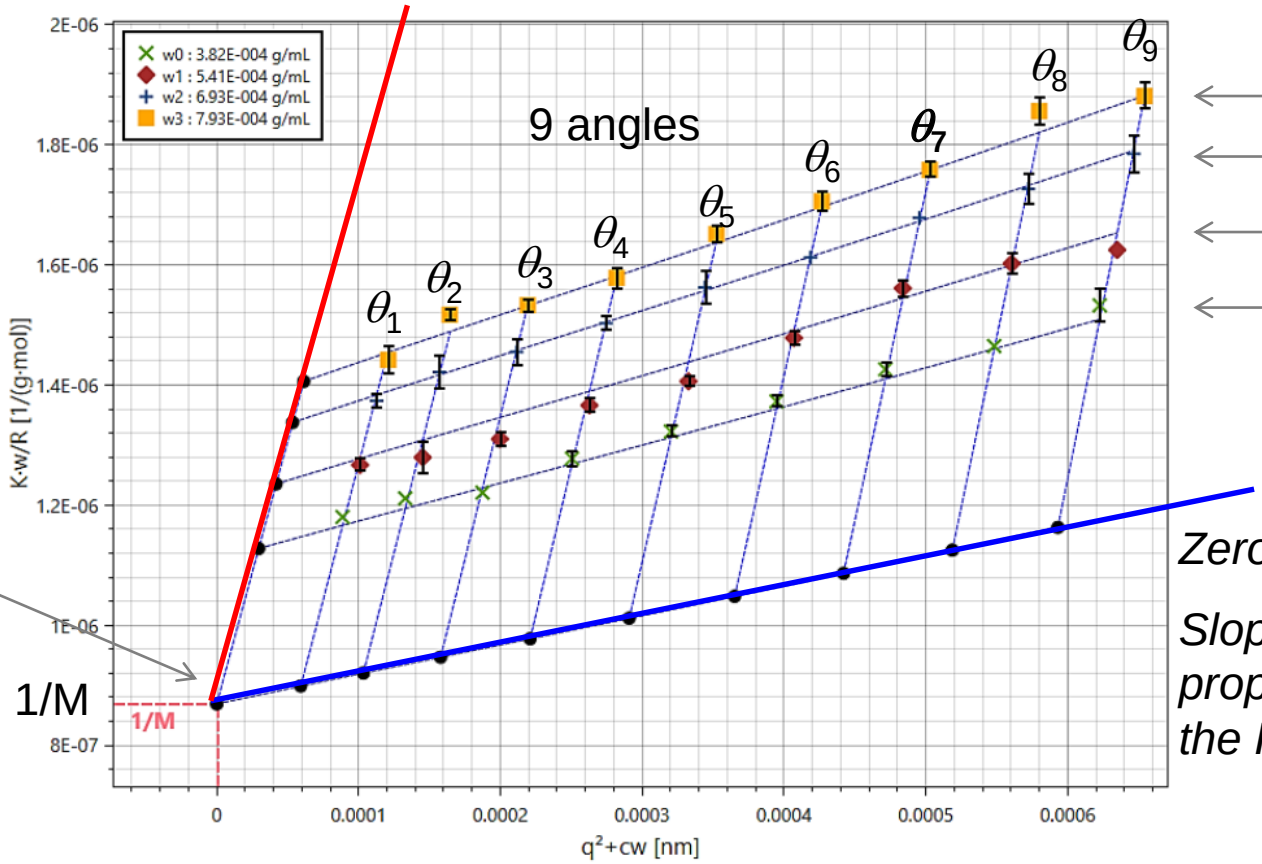
Compare to SAS

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

If the scattering intensities are measured at multiple angles as a function of concentration?

- **Zimm Plot: Kc/R vs (q^2 + calibration constant)**

Zero angle: Slope proportionate to second virial coefficient.



Extrapolate to zero angle, i.e., q = 0 and zero concentration and you obtain 1/M

c_1 (highest)

c_2

c_3

c_4

Zero concentration

Slope is proportionate to the R_g

From LS Instruments: https://www.lsinstruments.ch

R_g? Don't we use SAXS or SANS for that?

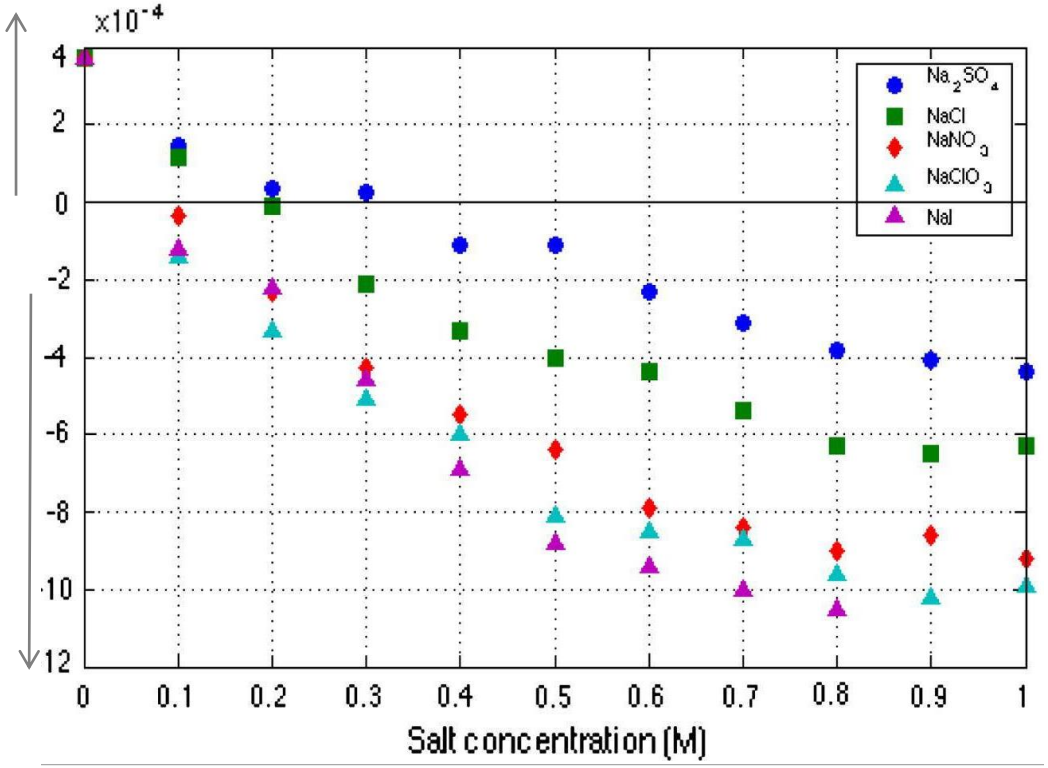
- **Yes!**
- Reason = for macromolecules of approximately 50-70 kDa the MALLS signals are pretty much equivalent/isotropic, i.e., there is no angular dependence, so you cannot extrapolate R_g from Zimm.
- 50-100 kDa, you have to be extremely careful *in terms of accurate protein concentration evaluation*. For SAXS and SANS, R_g is independent of concentration.
- The instrument must be exceptionally well-calibrated (detector responses have to be perfect). Pretty much technically difficult – i.e., annoying.
- In principle, you *migh*t get good results for particles with D_{max} between $\lambda/20$ and as you approach λ (λ is in the order of 600 nm, so for particles with D_{max} > 30 nm).

Example of second virial coefficient.

Protein in buffer with different salts

Repulsive interactions (positive A_2)

Attractive interactions (negative A_2)



All of this boils down to:

$$I_s \sim c\,(dn/dc)^2\,M$$

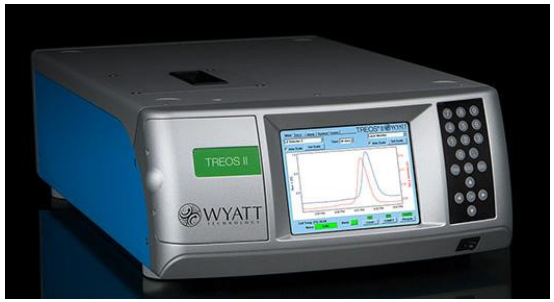
at different angles. It is possible to measure the concentration of the solute using a refractive index instrument, such that:

$$RI \sim c\,dn/dc$$

From these values it is possible to obtain the M of a protein in solution.

MALLS: Multi-angle laser light scattering

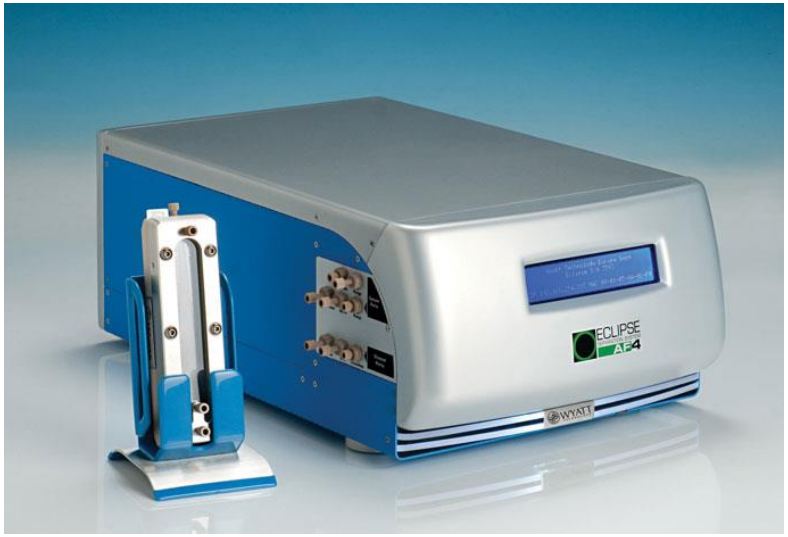
Size exclusion chromatography (SEC)

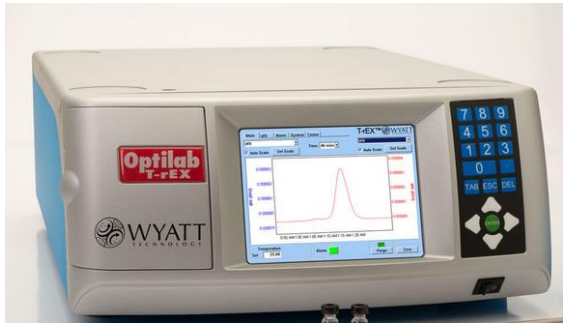


3-angle MALLS = 200 Da – 10 Mda

18-angle MALLS = 200 Da – 1 GDa

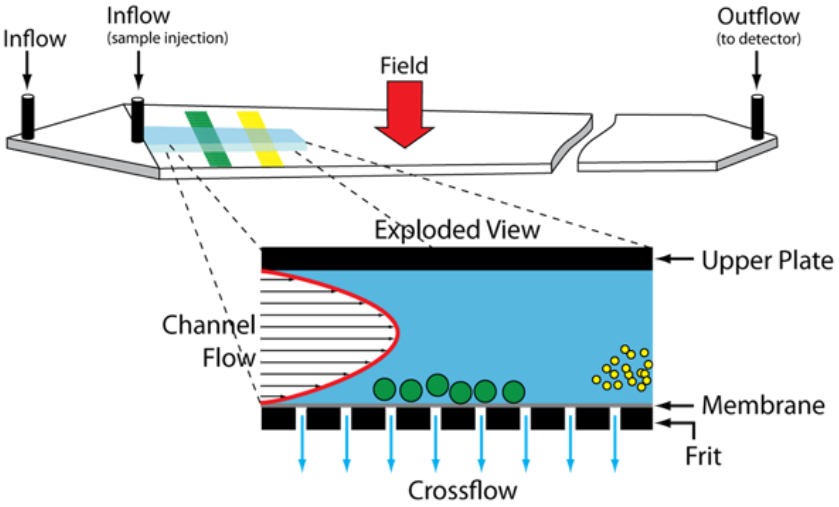
Asymmetric Flow Field-Flow Fractionation



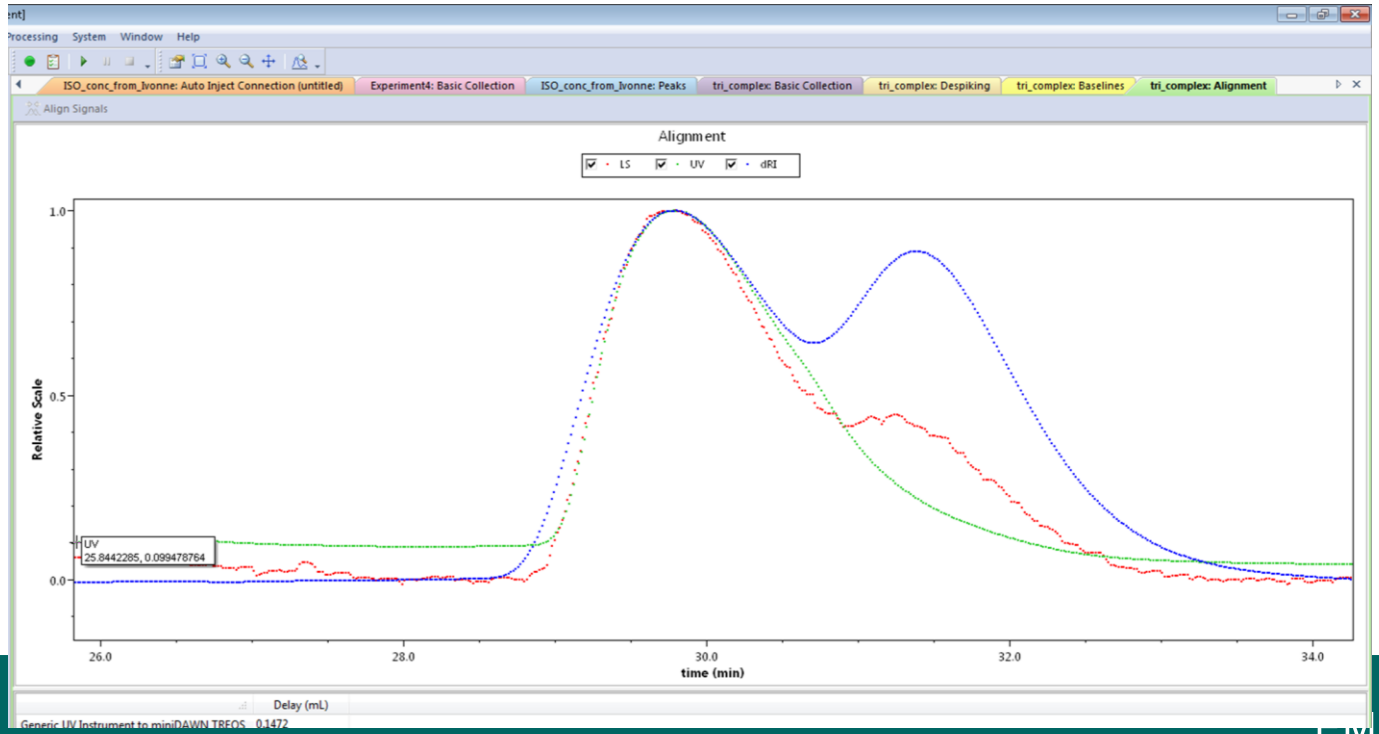


Differential refractometer

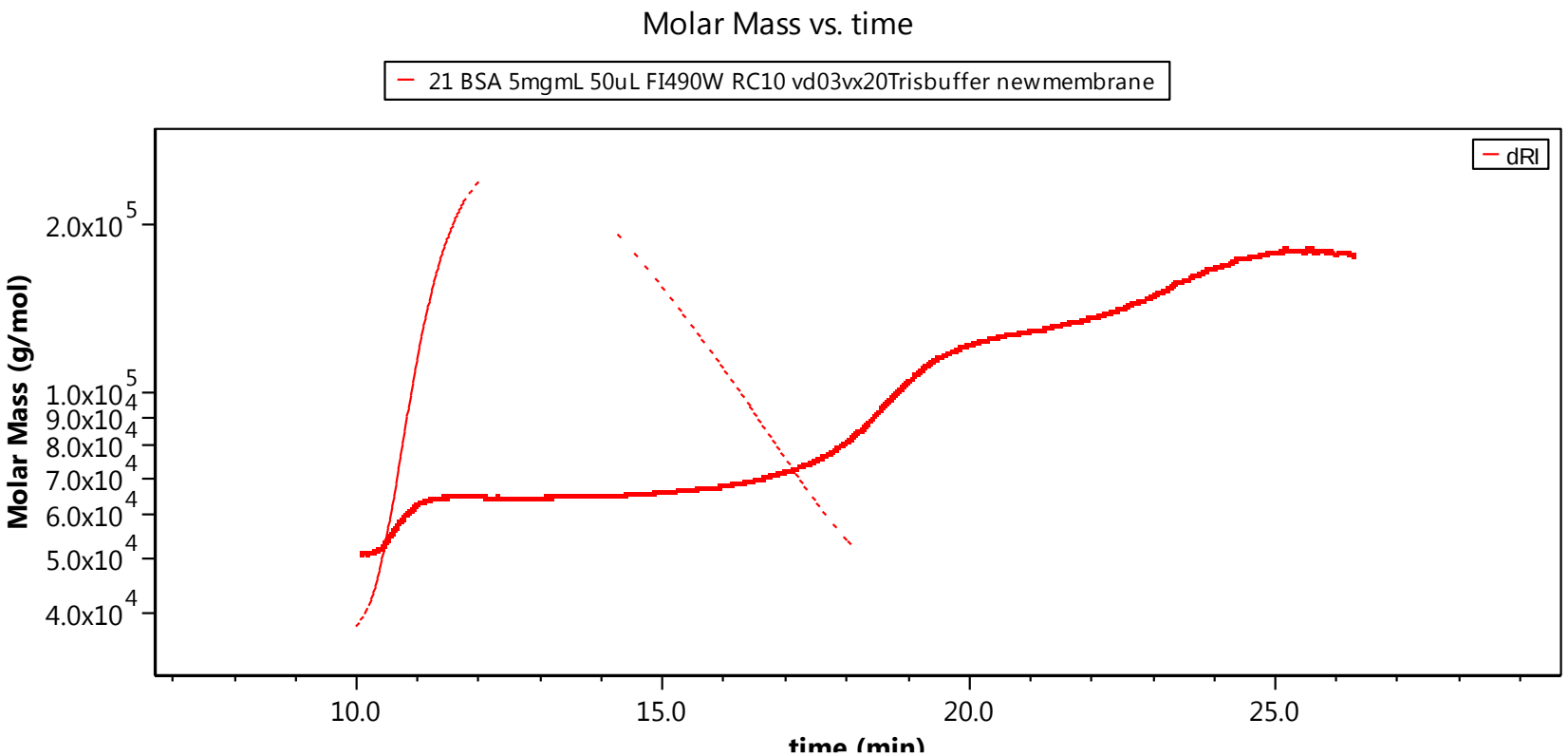
Asymmetric Flow Field-Flow Fractionation



Light scattering and RI measurements (continuous flow operation)



With AFFFF, smaller particles elute first (reverse of SEC)

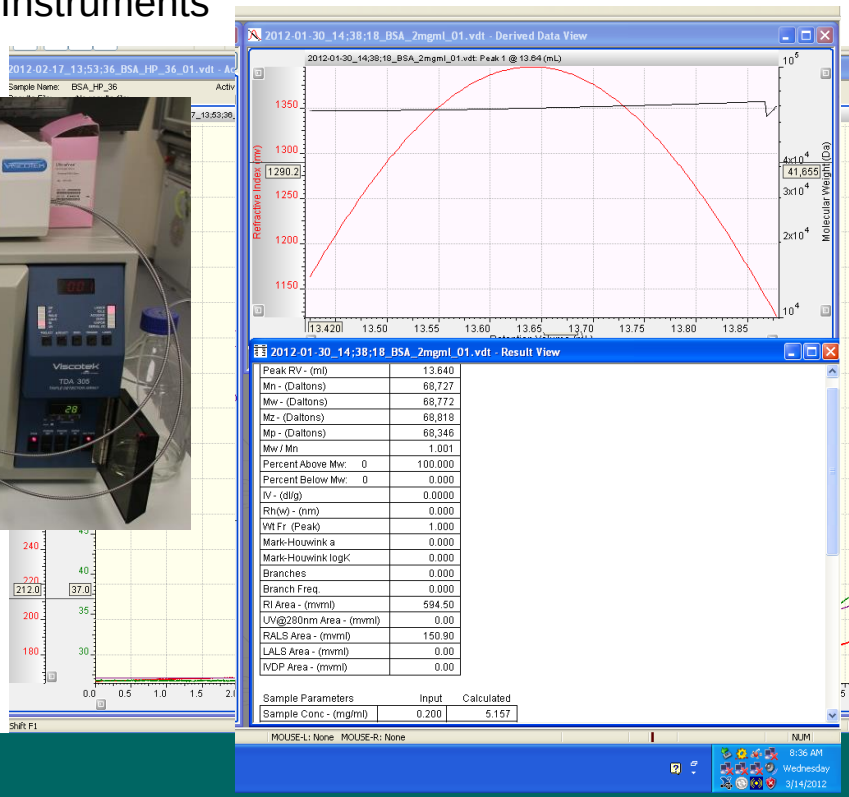


Plot of Molar Mass vs elution time. The DRI Signal is shown as a overlay. Experiment was performed with Vd=0.3ml/min Vx=2.0 Loading: 1mg BSA

Right-angle laser light scattering (RALLS)

- Only one angle.
- Becomes increasingly inaccurate for larger particles (due to anisotropic scattering contributions, i.e., $P(q)$).

Viscotek TDA 305 - Malvern Instruments

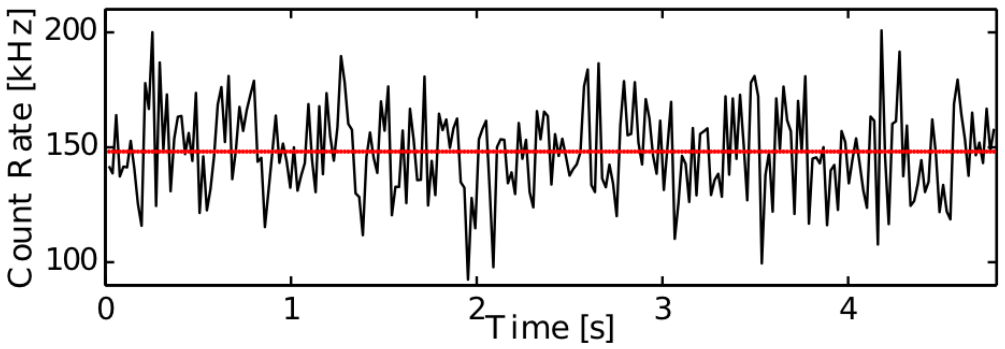


Why is it called static light scattering?

- Measure the scattering intensity per unit time, i.e., the absolute mean intensity.

What happens if we increase the sampling time?

- We begin to observe fluctuations in the intensity around the mean.

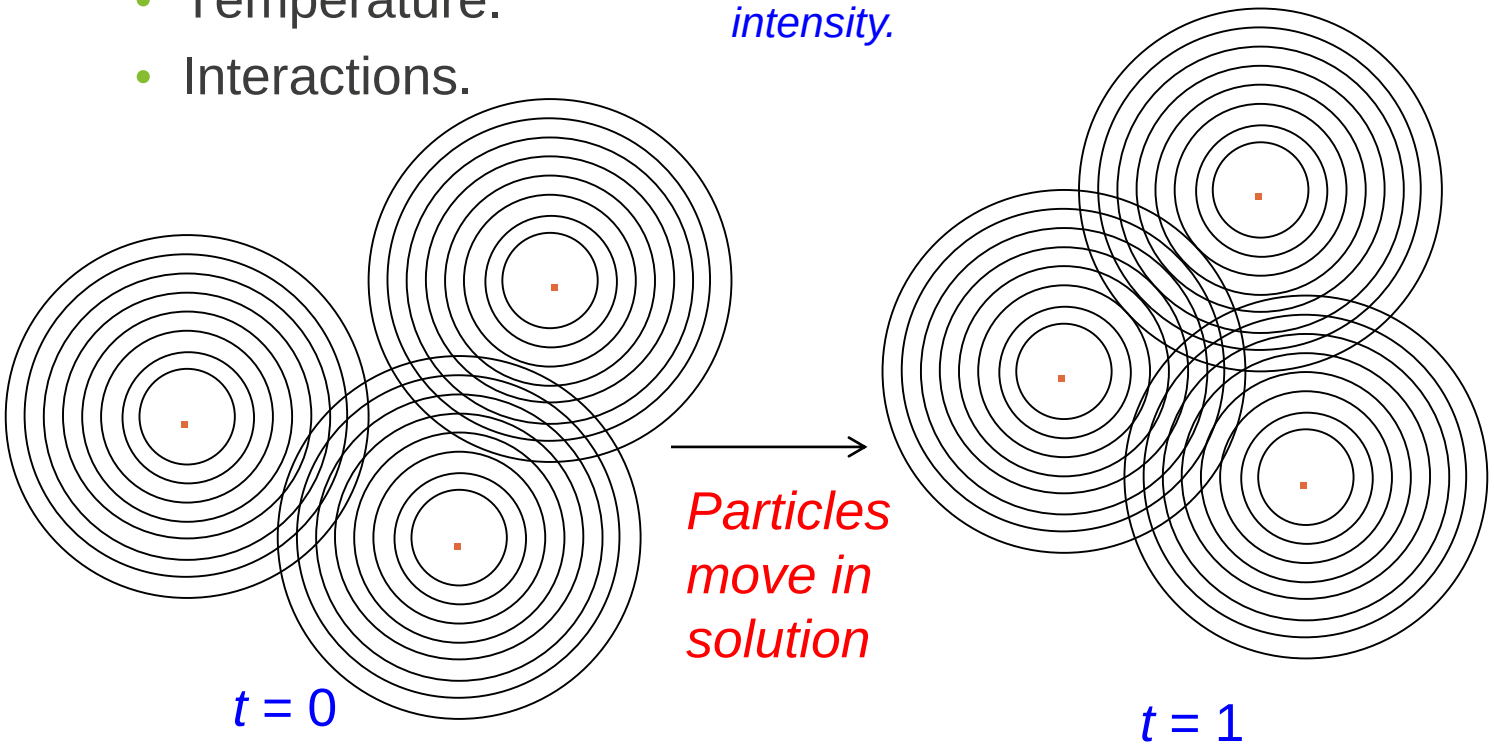


Absolute mean intensity

Dynamic Light Scattering

- Essentially the intensity fluctuations are caused by the motions of particles in solution.
  - Particle size.
  - Solution viscosity.
  - Temperature.
  - Interactions.

Different lines of constructive and destructive amplitude interference develop through time and cause the fluctuations around the absolute mean intensity.

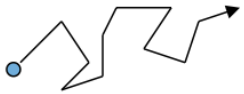


Dynamic Light Scattering: Brownian motion

Robert Brown (1773-1858) observed the 'jiggling' of pollen grains in solution. We now call this 'Brownian Motion.'

Albert Einstein published a paper in 1905:

- a mean square displacement given by $\langle r^2 \rangle = 6D\tau_c$



Distance, **r**
Diffusion coefficient, **D**
Correlation time, τ_c

- Diffusion coefficient: $D = kT/(6\pi\eta R_h)$ (for a sphere with a hydrodynamic radius R_h, k=Boltzman constant, T=Temperature, η=viscosity)

'Stokes-Einstein relation'

What Einstein showed was that the diffusion of an object undergoing Brownian motion will diffuse at a particular rate (known as the mean squared displacement) and that this rate depended upon the number of atoms or molecules in a mole of the fluid in which the object is suspended (Avogadro's number). From this one could determine the size of molecules.

Dynamic Light Scattering: *Hydrodynamic* properties of biomolecules in solution

- The fluctuations in intensity are evaluated via what is known as an autocorrelation function.

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

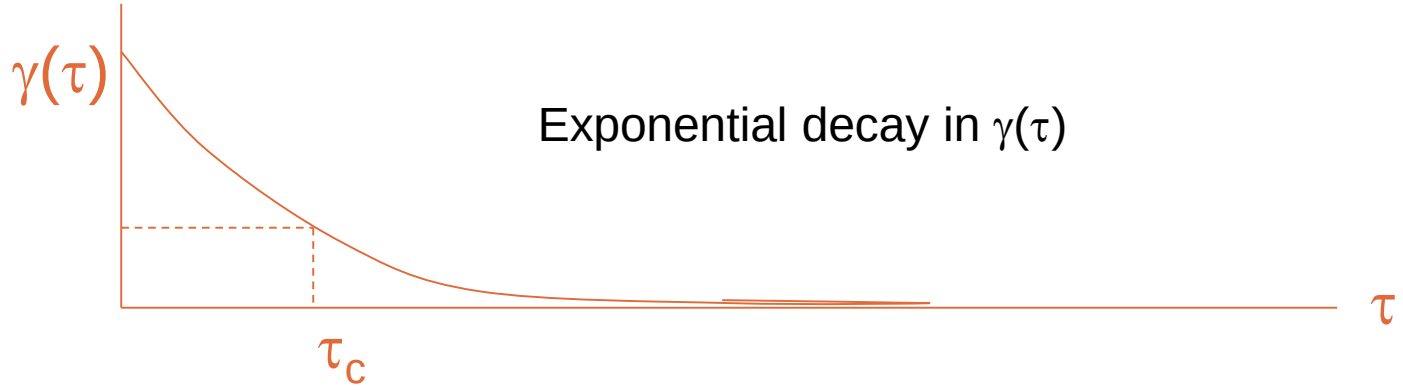
τ is the delay time

$$\gamma(\tau) = Ae^{-2\Gamma\tau} + B$$

A and *B* are machine constants

where $\Gamma = q^2 D$ $D = \frac{k_b T}{6\pi\eta R_h}$ $q = \frac{4\pi n_d \sin\left(\frac{\theta}{2}\right)}{\lambda}$

What does all this mean?



Monodisperse system:
Simple decay -- decay rate is proportional to the particle size and the diffusion coefficient (as well as wavelength, measurement angle, and refractive index.)

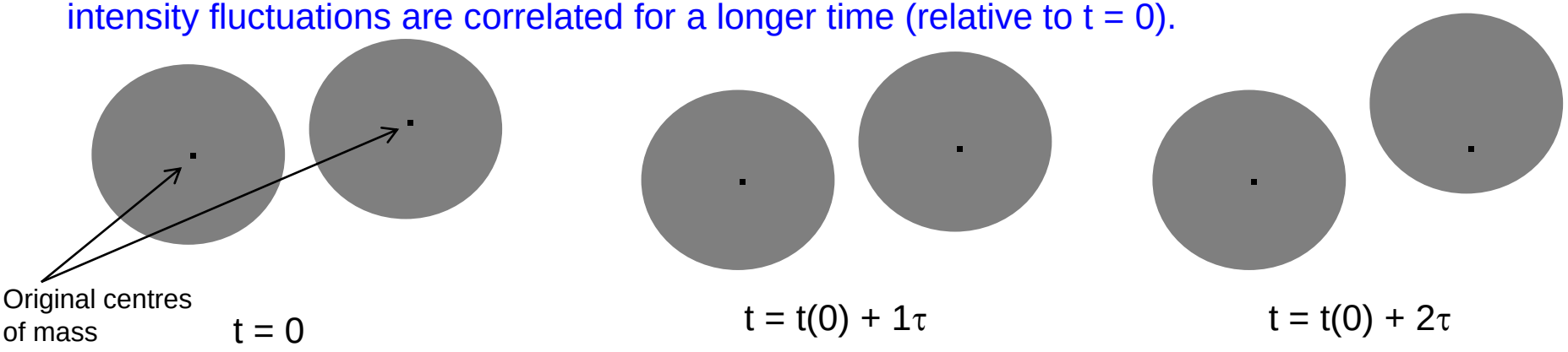
Polydisperse system:
Autocorrelation function is a ***sum*** of the exponential decays corresponding to each of the species in the population;

Importantly: the resolution for separating two different particle populations is approximately a factor of five x R_h or higher.

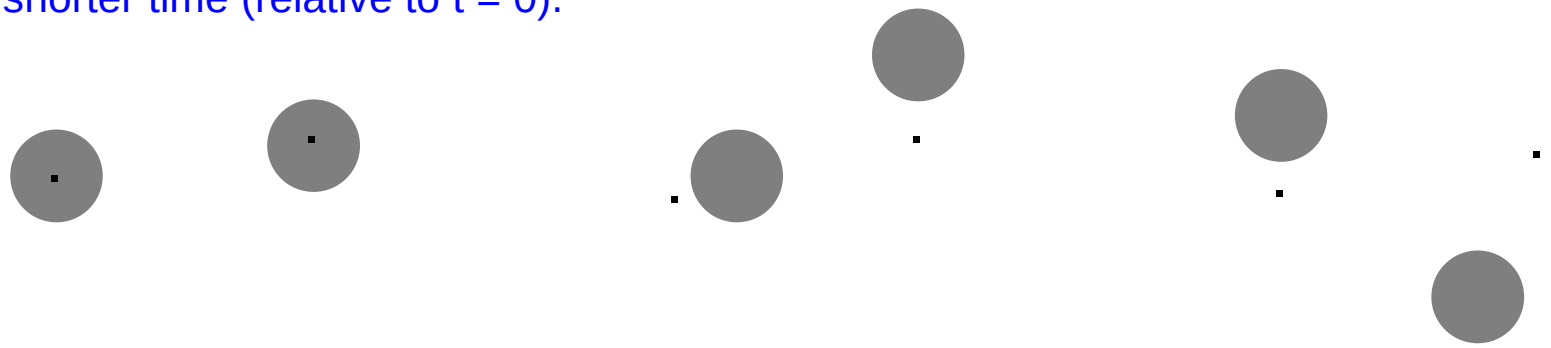
So what?

- We measure the correlation of the scattered intensity fluctuations over time, in the order of 10^{-6} to 10 seconds.

Large particles move slower in solution, i.e., take longer to shift position and hence the intensity fluctuations are correlated for a longer time (relative to t = 0).

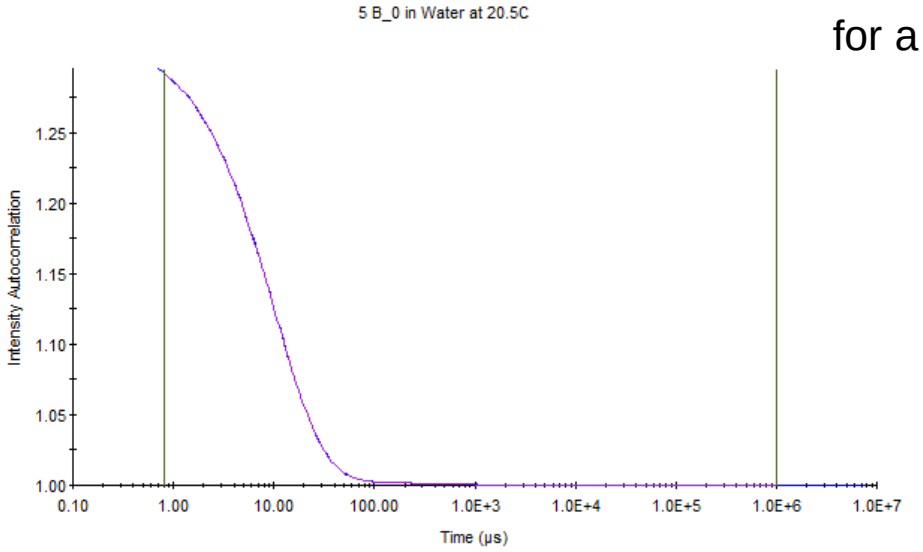


Smaller particles move faster in solution and hence the intensity fluctuations are correlated for a shorter time (relative to t = 0).



Assuming that the temperature and solvent viscosity are the same…

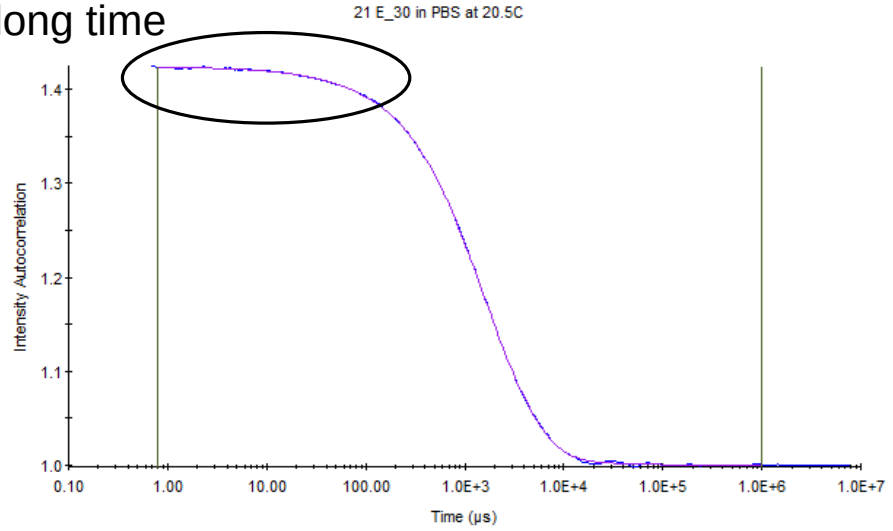
Small particle



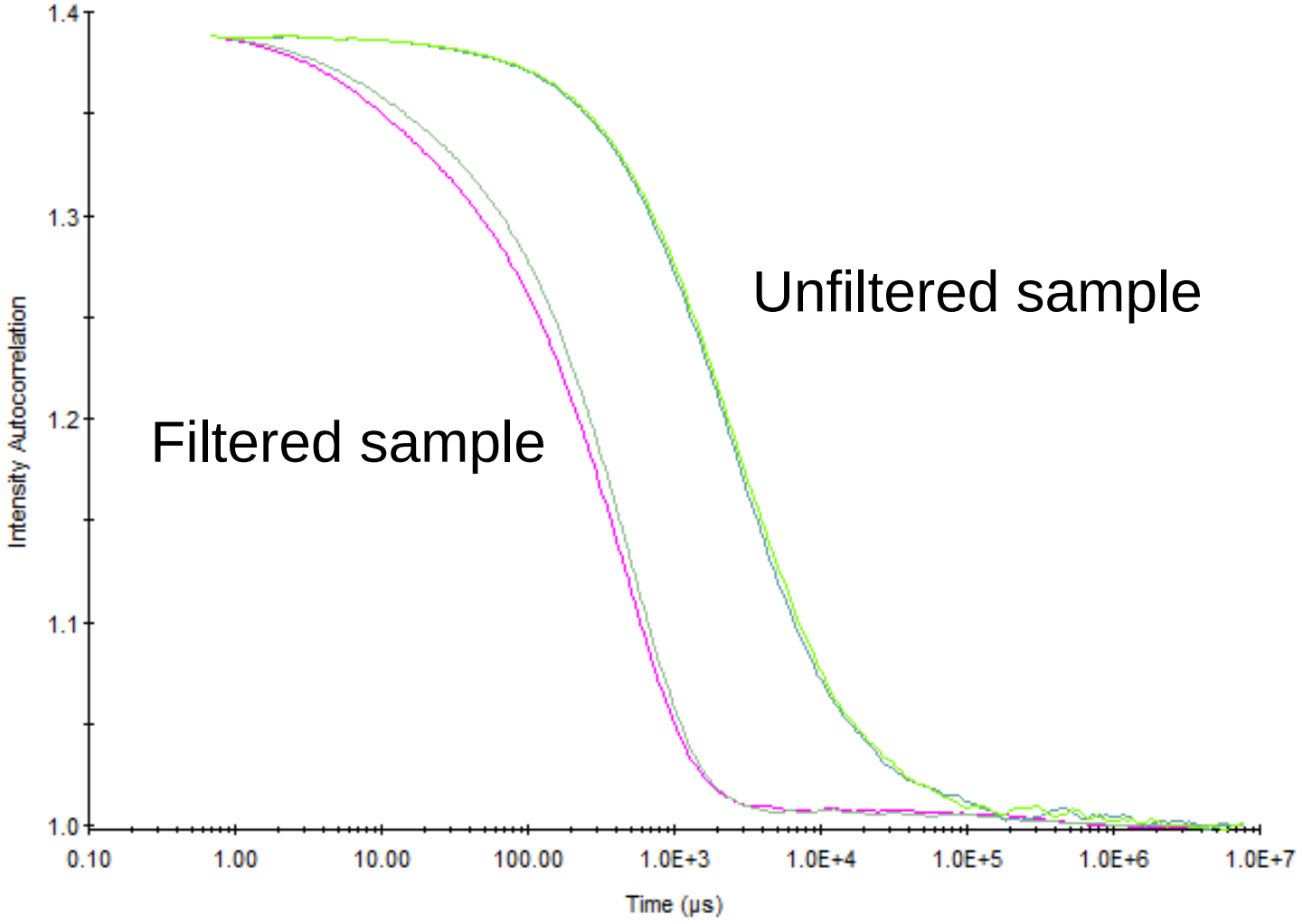
Autocorrelation quickly decays – half way down at 10 µs

Large particle

Intensity fluctuations correlate for a long time

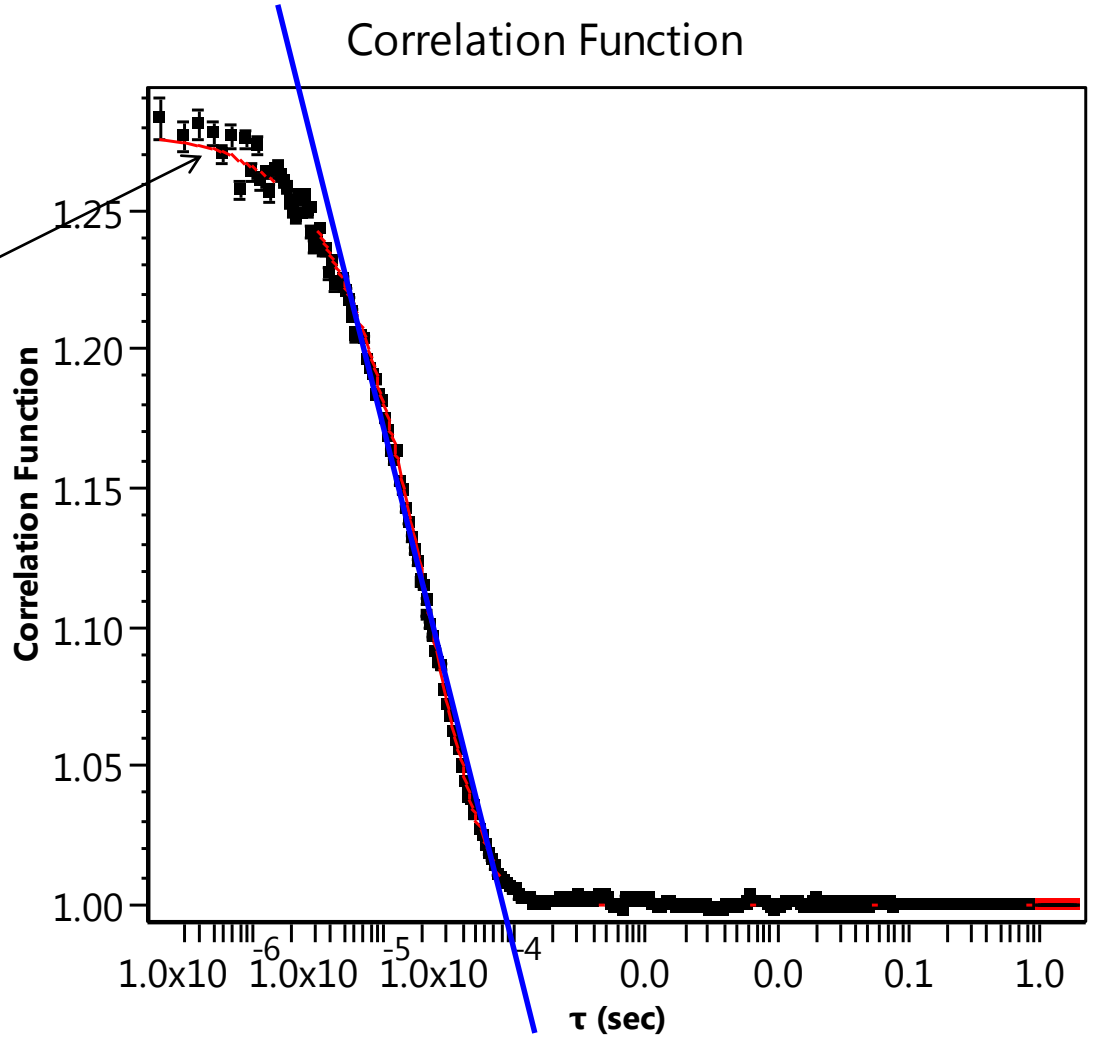


Autocorrelation takes a great deal of time to decay – half way down at around 100 µs.


Unfiltered sample
Filtered sample
Intensity Autocorrelation
1.4
1.3
1.2
1.1
1.0
0.10
1.00
10.00
100.00
1.0E+3
1.0E+4
1.0E+5
1.0E+6
1.0E+7
Time (µs)

The slope of the exponential decay corresponds to polydispersity: The steeper the slope = the sample is less polydisperse (and vice-versa)

From the fit to the data, e.g., using the CONTIN algorithm, **D** can be extracted from which $\mathbf{R_h}$ can be estimated.

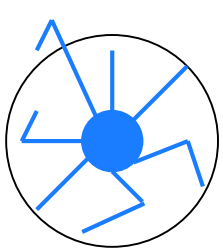


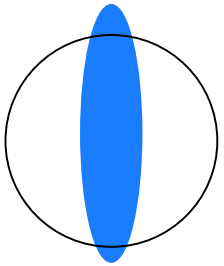
$$D = \frac{k_b T}{6\pi\eta R_h}$$

*Provencher, S (1982). "CONTIN: A general purpose constrained regularization program for inverting noisy linear algebraic and integral equations" (PDF). Computer Physics Communications. 27 (3): 229.

Notes on the hydrodynamic radius.

- Hydrodynamic radius:
  - Is what it implies: relates to the hydrodynamic behavior, i.e., the diffusion of a particle in a particular solution (taking into account temperature and viscosity).
  - We talk about ‘hard-sphere equivalents’: the R_h of a sample particle rotating in all directions plus the hydration layer is equivalent to the radius of a hard-sphere that diffuses in the ***same fashion*** as the sample particle under the same conditions.
  - The R_h is proportional to the inverse of the time decay in the autocorrelation function.





The shape factor.

The shape factor is the ratio:

$$R_g/R_h,$$

The shape factor offers an additional structural parameter for evaluating the mass distribution of a particle

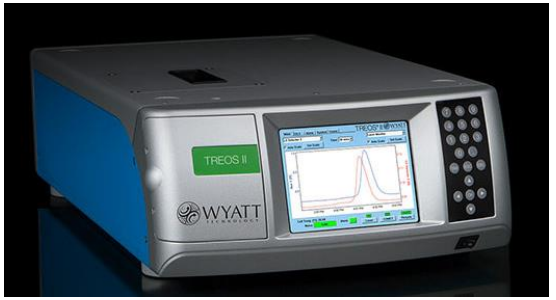
R_g/R_h of a sphere = 0.78

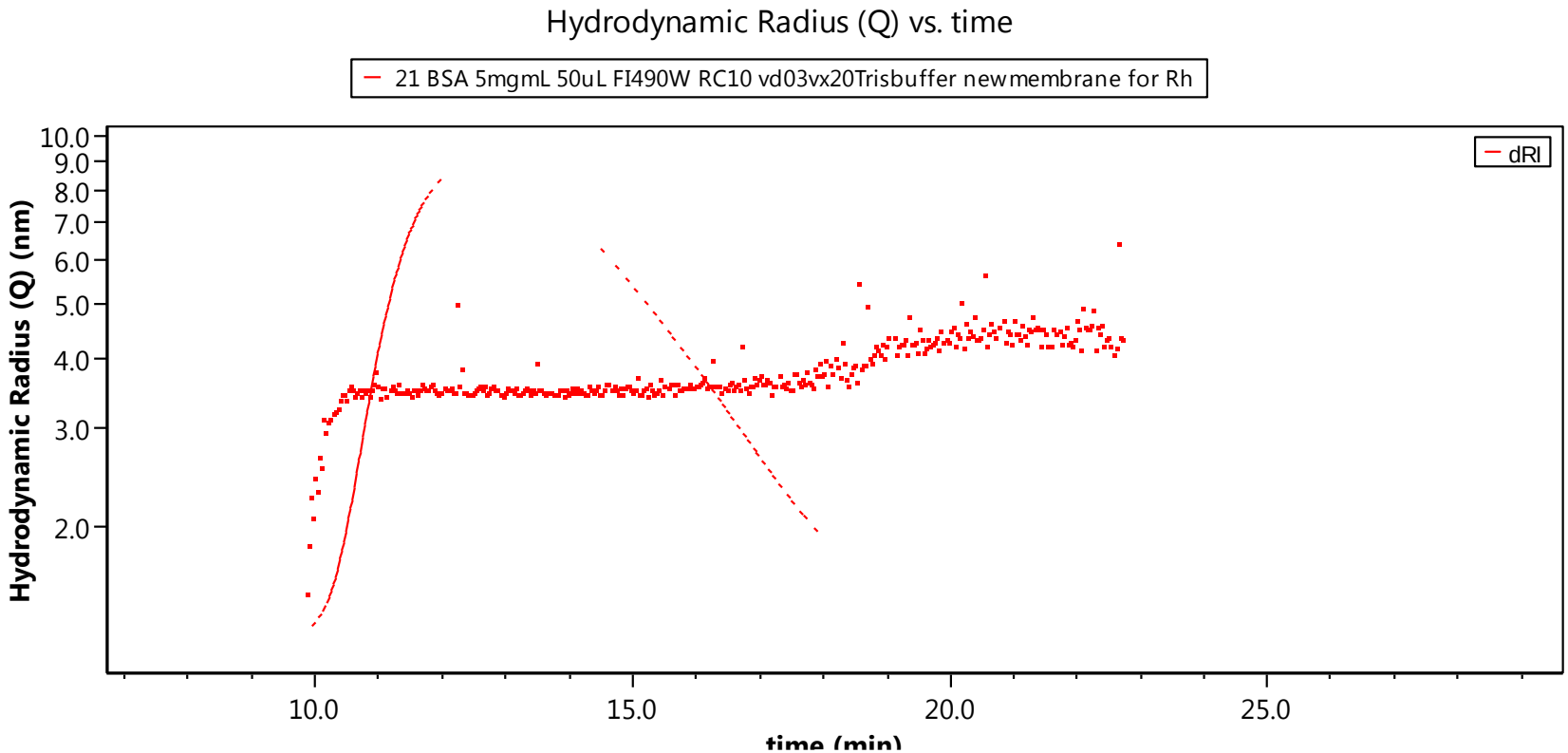
Flexible random coils (or self-avoiding walks) = 1.44–1.63 (depending on solvent and excluded volume effects)

Oblate spheroids = 0.88–0.99
Prolate ellipsoids = 1.36–2.24 (depending on the axial ratio61)

Long cylinders or stiff rods = 1.8 to >2

Continuous-flow AFFFF DLS (‘QELS’) measurements separation of BSA. MALLS and DLS are measured in the same cell (Wyatt TREOS)





Plot of R_h vs elution time. The DRI Signal is shown as a overlay.
Experiment was performed with Vd=0.3ml/min Vx=2.0 Loading: 1mg BSA

A reasonable read…

BIOCHEMISTRY AND MOLECULAR BIOLOGY EDUCATION
Vol. 40, No. 6, pp. 372–382, 2012

Laboratory Exercise

Protein Analysis by Dynamic Light Scattering: Methods and Techniques for Students‡

Received for publication, May 23, 2012; accepted 23 July 2012

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Summary

- Light scattering techniques can be used to:

  Obtain the molecular mass, mean-squared R_g, R_h, translation diffusion coefficient and second virial coefficient of a particles in solution.

  Useful over a wide molecular weight range.

  Can be used in conjunction with continuous-flow separation methods.

- Light scattering disadvantages:

  Requires a solvent with a different refractive index compared to the solute (usually this is fine for most biomacromolecules in aqueous buffers).

  Extremely sensitive to high-molecular weight species/dust/aggregates. ***HOWEVER! If you see aggregates it in DLS, you will probably see them in SAS!***

  DLS *cannot* resolve monomer-dimer equilibrium.

Acknowledgements

- Melissa Graewert
- BioSAXS Group at EMBL-HH