Light Scattering and its applications to Biological Molecules

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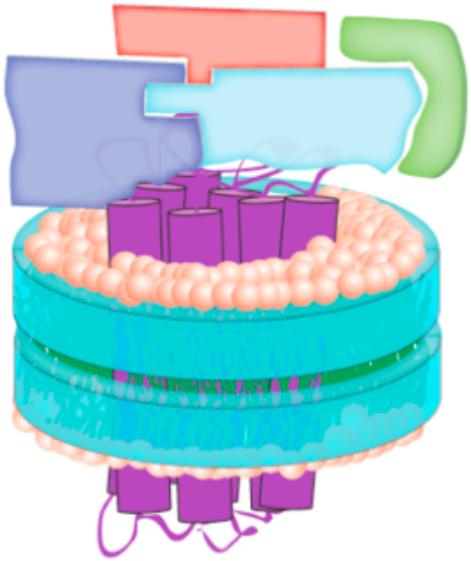
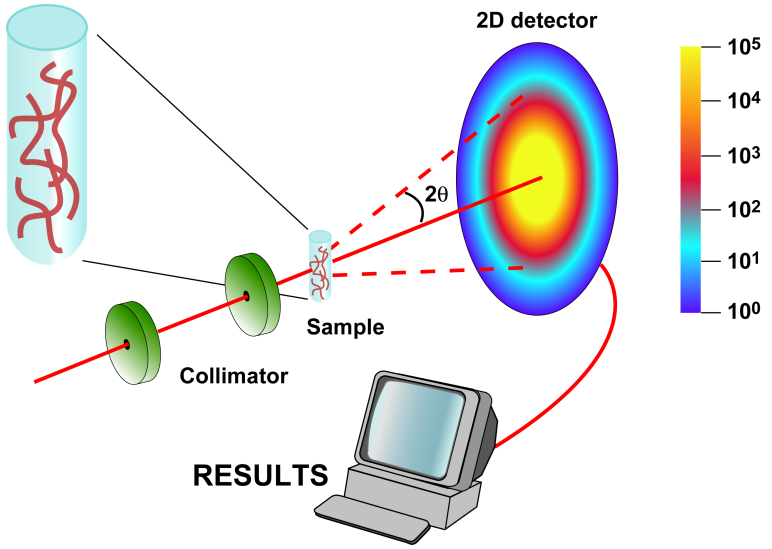
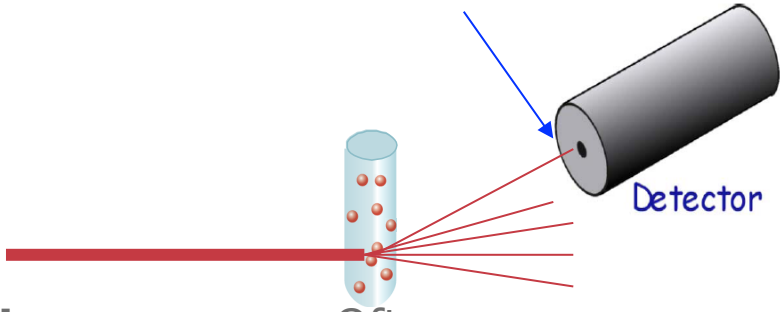
University of Copenhagen



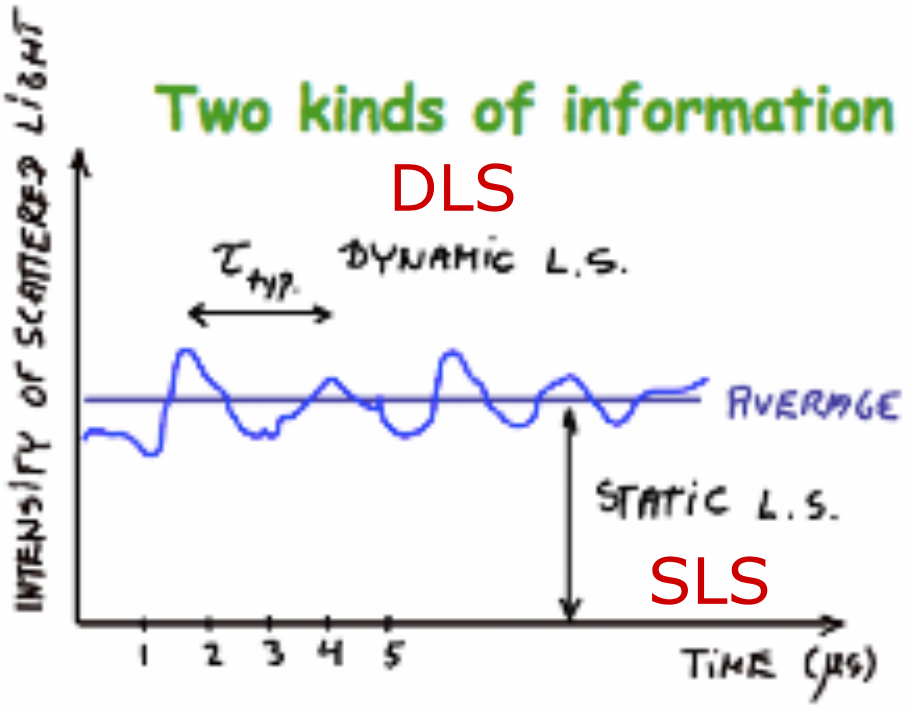


Light Scattering – The very basics:

Detector: Typically a point detector. Often placed on a goniometer-arm.



Laser source: Often Argon or HeNe lasers (polarized and monochromatic)



SLS: Static light scattering

DLS: Dynamic light scattering

SAXS, SANS, SLS and DLS

SAXS, SANS and SLS:

- Same theory
- Same experimental setup

- In theory...

Measure the **structure** of the particles in the sample

Dynamic light scattering (DLS):

- Another theory
- Another experimental setup

- In theory

Measures the **movements** of the particles in the sample

Abbreviations:

SAXS: Small-angle X-ray scattering

SANS: Small-angle neutron scattering

SLS: Static light scattering

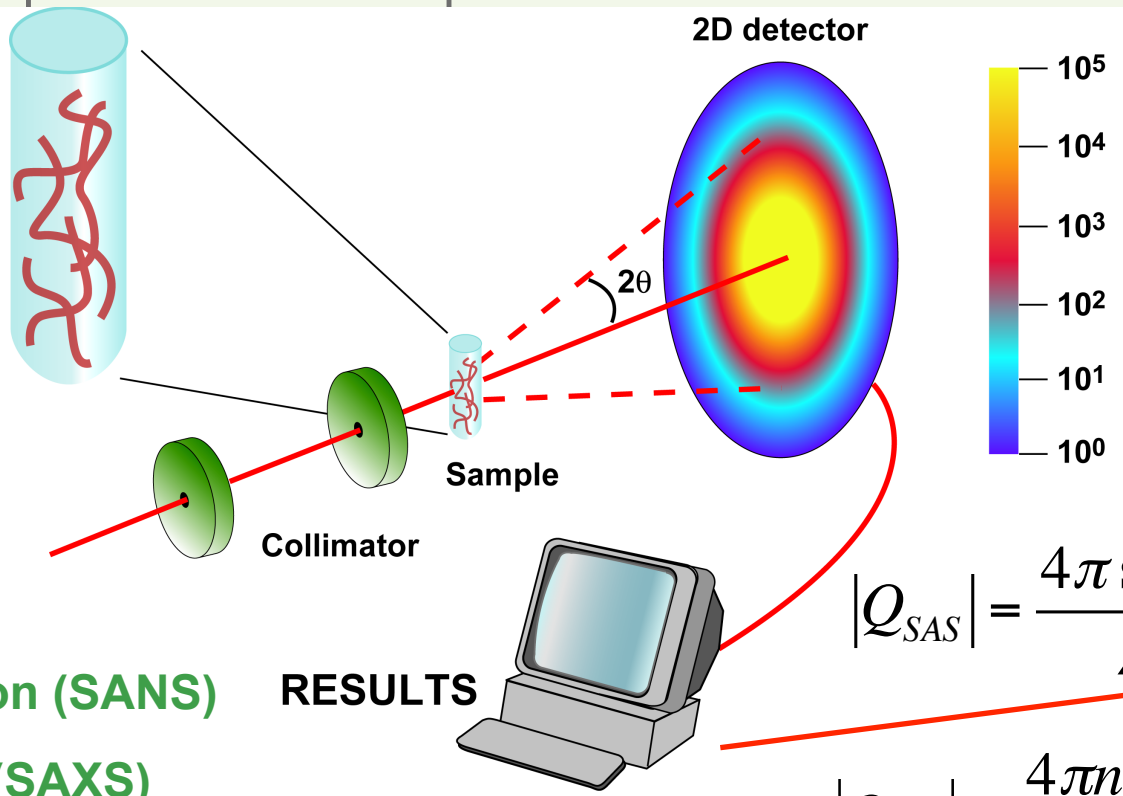
DLS: Dynamic light scattering

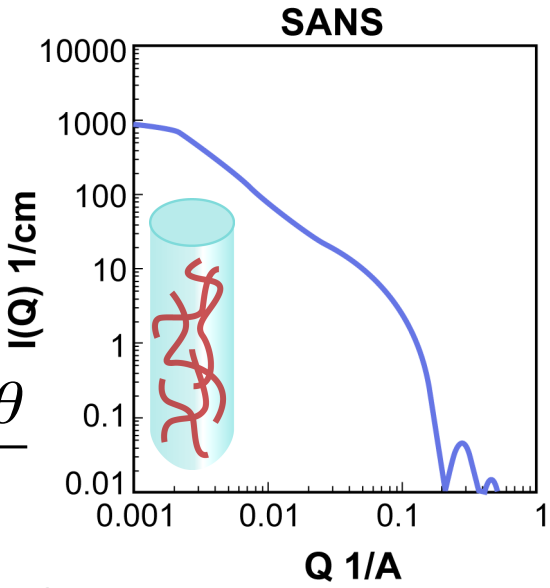
Plan for today:

1: Static light scattering, theory and instrumentation

2: Dynamic Light scattering, theory and instrumentation

Small Angle Scattering/Static light scattering – Principle of experimental setup





Beam:

Neutron (SANS)

X-ray (SAXS)

or light (SLS)

$$|Q_{SAS}| = \frac{4\pi \sin\theta}{\lambda}$$

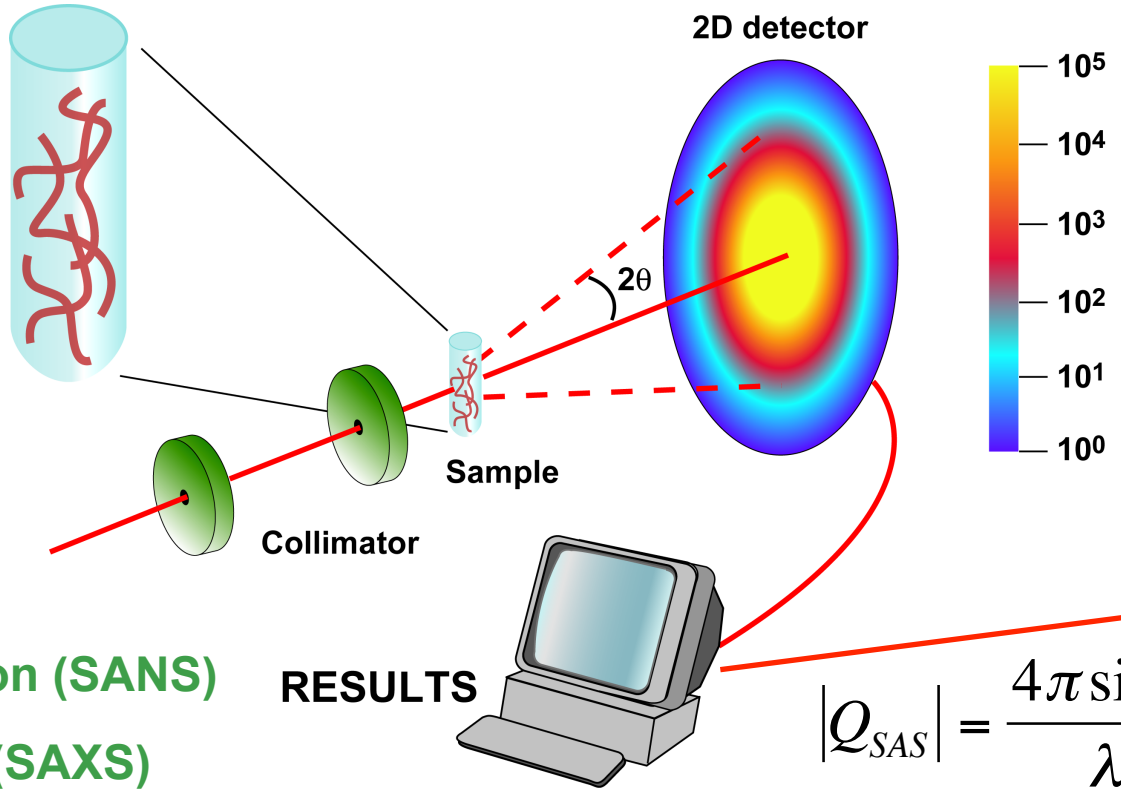
$$|Q_{SLS}| = \frac{4\pi n_0 \sin\theta}{\lambda}$$

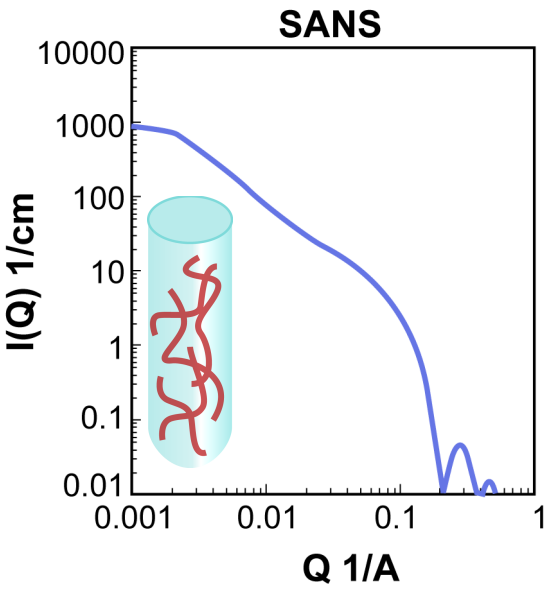
λ: Wave-length of X-ray, neutron or light

n_0: Refractive index of sample (=1.33 for water)

And where n_0="Speed of light in vacuum"/"Speed of light in medium"

Small Angle Scattering/Static light scattering – Principle of experimental setup





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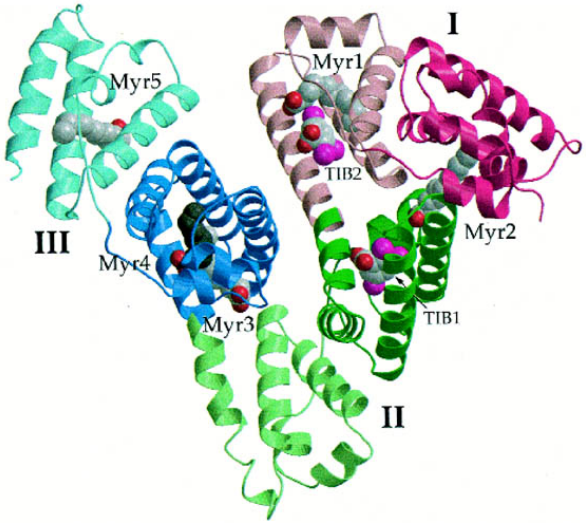
SAXS/SANS: $\theta_{min} \approx 0.03°$, $\theta_{max} \approx 3°$, Q=[0.001-0.5 1/Å], 1-200 nm

SLS: $\theta_{min} \approx 8°$, $\theta_{max} \approx 160°$, Q=[0.0004-0.001 1/Å], 200-2000 nm

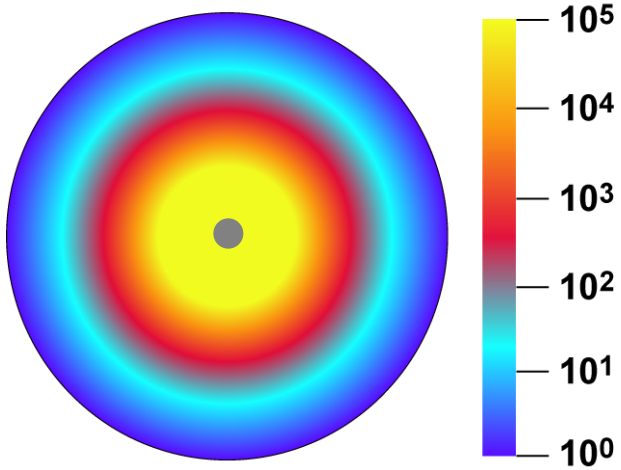
Sample structure versus scattering pattern

Direct space

Reciprocal space



Fourier Transform



Bovine Serum Albumin

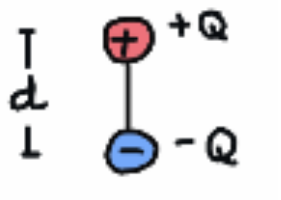
Scattering pattern

Small lengths ⟷ **Large angles**

Large lengths ⟷ **Small angles**

This is in principle still valid in light scattering

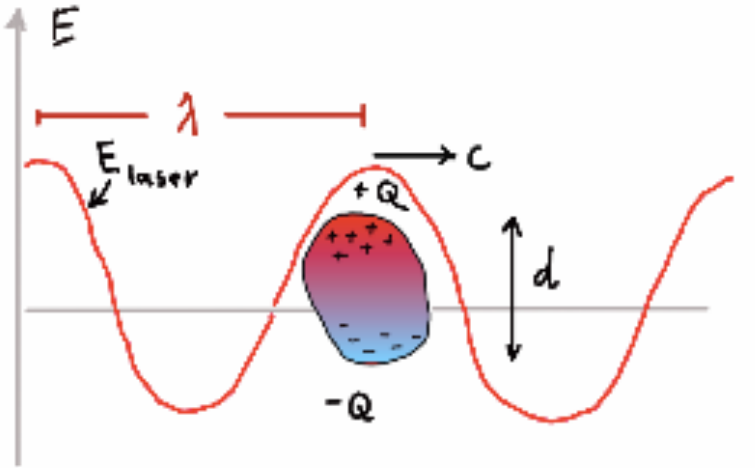
Why is light scattered? (Remember basic electrodynamics)



Dipole momentum

$$\mu = d \cdot Q$$

Dipole momentum is additive



Induced dipole momentum (oscillating)

$$\mu = d \cdot Q = \alpha \cdot m \cdot E_{laser}$$

$$\mu = \alpha \cdot m \cdot E_{0,laser} \cdot \cos(\omega \cdot t - \vec{k} \cdot \vec{r})$$

Polarizability — Mass of dipole

Underlying assumption:

Particle/dipole size << λ_{laser} (638 nm for HeNe laser)

Scattering from one small particle

The mathematics works just as it did for X-rays and Neutrons (SANS and SAXS) and will not be shown in detail.

$$I_{s,1} = I_{laser} \frac{\alpha^2 m^2 (4\pi)^2}{r^2 \lambda^4}$$

r is the sample detector distance

NB: $\alpha/\lambda^2 \sim \Delta\rho$, the excess scattering length density in SAS

Scattering from N small particles (assuming dilute non-interacting particles)

$$I_{s,N}(q) = N I_{s,1} = I_{laser} N \frac{\alpha^2 m^2 (4\pi)^2}{r^2 \lambda^4}$$

For dilute samples of small particles:

-The scattering intensity is proportional to the second power of the particle mass, m

-Proportional to the second power of the polarizability of the particle, α

-Inversely proportional to the fourth power of the wave-length, λ

-Proportional to the sample concentration, *N*

Scattering from larger particles?

The scattering becomes *q*-dependent (just like small-angle scattering):

$$I_{s,N}(q) = NI_{s,1}P(q) = I_{laser}N\frac{\alpha^2 m^2 (4\pi)^2}{r^2\lambda^4}P(q)$$

Where P(q) is the particle form factor

Note: Because light-scattering most often is a low-q technique, light scatterers make heavy use of low-q approximations and in particular the Guinier approximation.

A note on the contrast

$$I_{s,N} = I_{laser} N \frac{\alpha^2 m^2 (4\pi)^2}{r^2 \lambda^4}$$

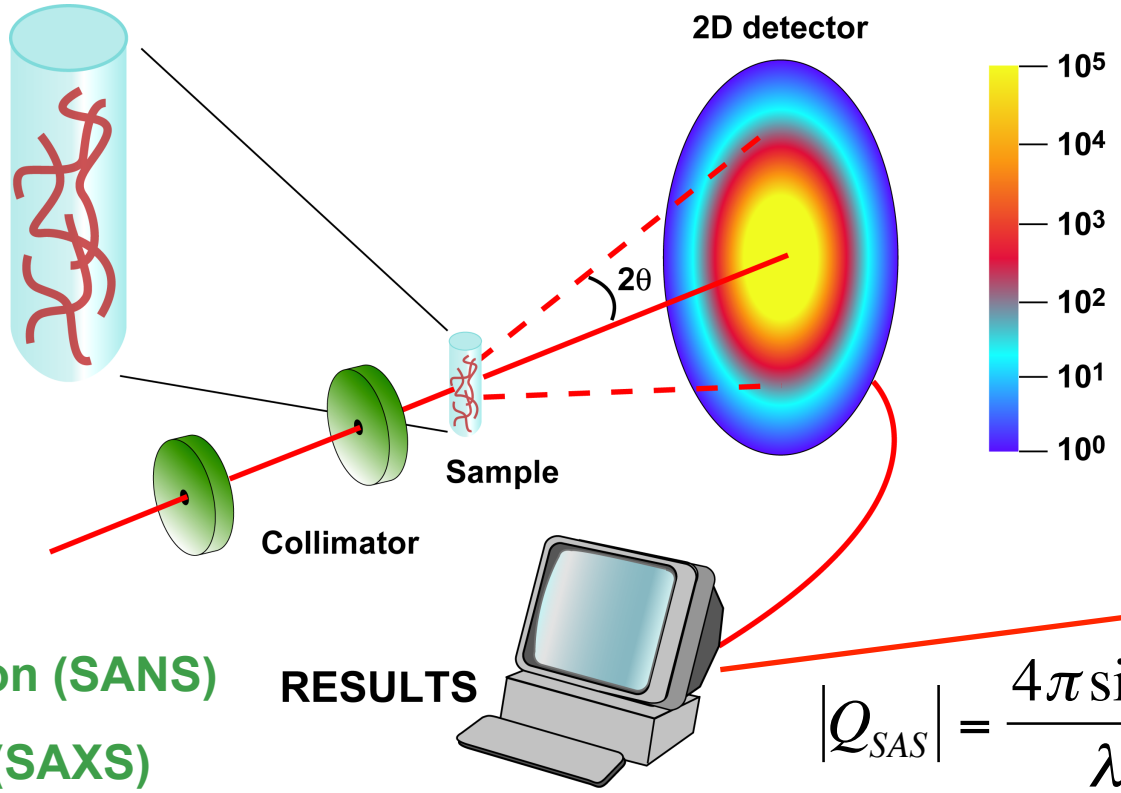
NB: $\alpha/\lambda^2 \sim \Delta\rho$, the excess scattering length density in SAS

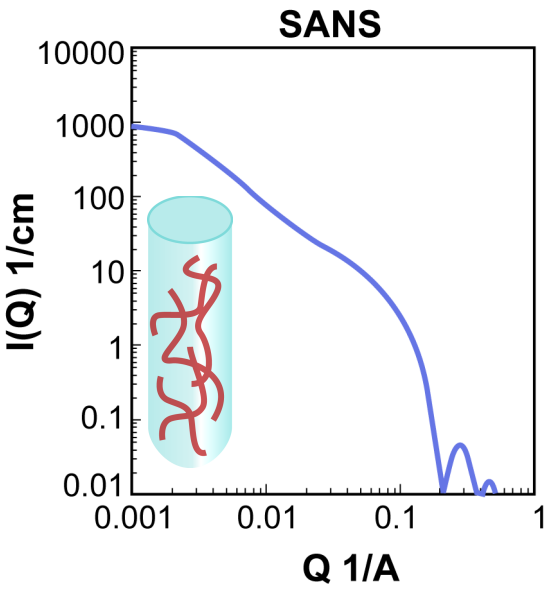
The polarizability, α, is related to the refractive index of the particle via (NB for obbjects smaller than λ):

$$\alpha = 3V \frac{m^2 - 1}{m^2 + 2} \qquad \text{where} \qquad m = \frac{n_{particle}}{n_{solvent}}$$

And V is the volume of the particle

Small Angle Scattering/Static light scattering – Principle of experimental setup





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SAXS, SANS, SLS and DLS

Small-angle X-ray scattering (SAXS), Small-angle neutron scattering (SANS), and Static Light scattering:

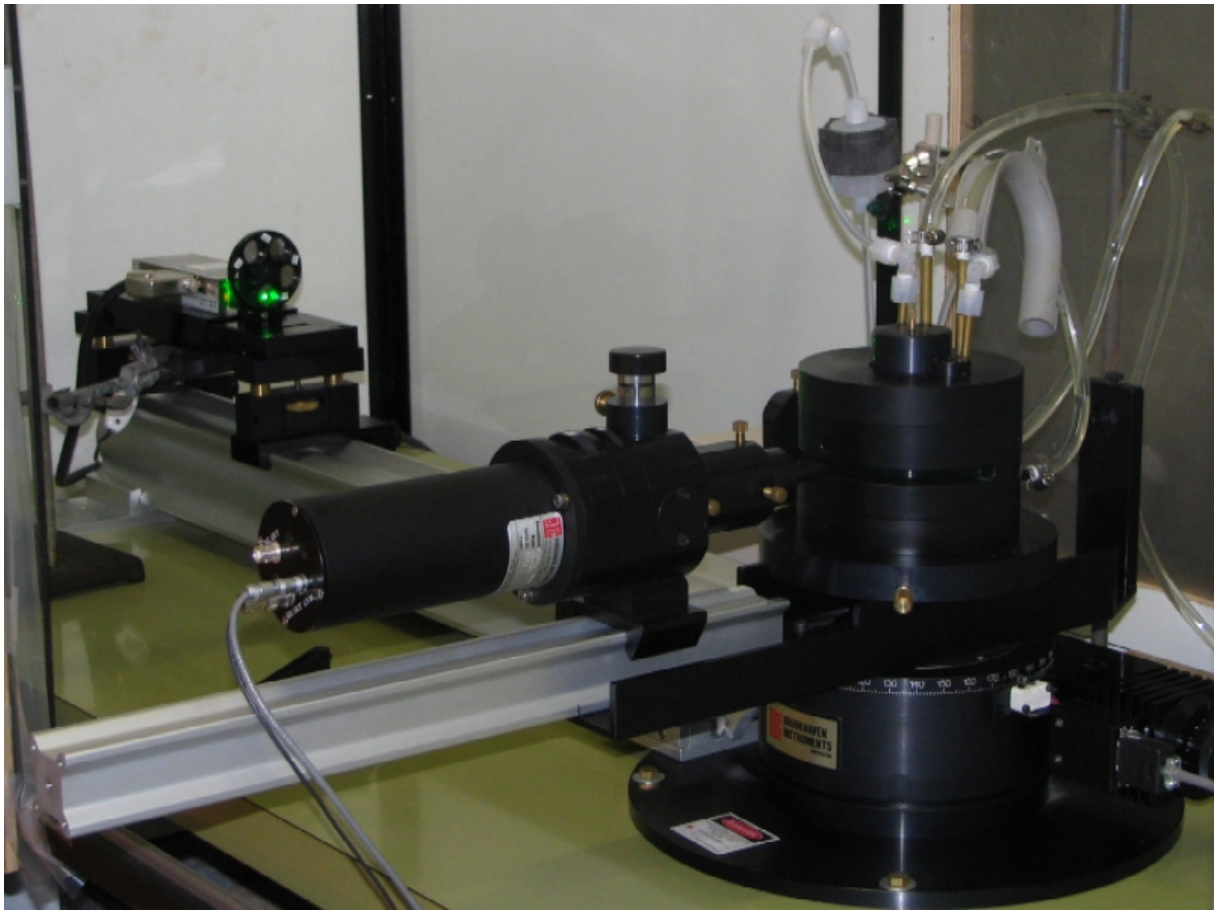
- Same theory
- Same experimental setup

With small variations

- In theory...

Measure the **structure** of the particles in the sample

Light scattering setup (Brookhaven Instruments).



-For both Static and Dynamic Light scattering (SLS and DLS).

SAXS, SANS, SLS and DLS

Small-angle X-ray scattering (SAXS), Small-angle neutron scattering (SANS), and Static Light scattering:

- Same theory
- Same experimental setup
  - *In theory...*

With small variations

SAXS and SANS instruments:
Big and expensive (from 0.5 M€)

SLS (and DLS) instruments:
Small and not very expensive (From 40 k€)

Measure the **structure** of the particles in the sample

How to determine the molar mass, M, from a static light scattering experiment:

Define "normalized" apparatus independent light scattering R the Rayleigh ratio.

$$R = \frac{I_{s\,total} \cdot r^2}{I_{laser} \cdot V_{scat.}}$$

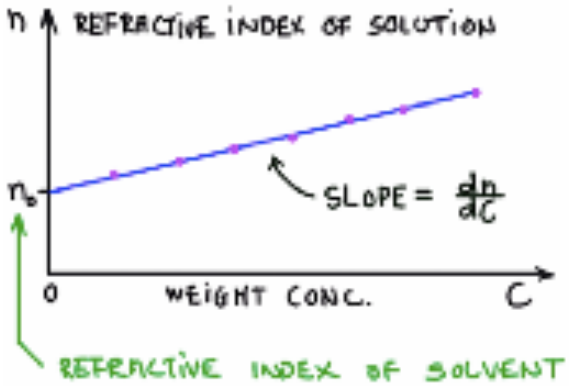
$$R = K \cdot C \cdot M$$

measured known known wt. conc.

Valid for small particles

The optical contrast constant K

$$K = \frac{4\pi^2 n_0^2 \cdot \left(\frac{dn}{dC}\right)}{N_A \cdot \lambda_0^4}$$



NB: For most proteins dn/dC = 0.18-0.20 mL/g

Why is the expression only valid for small particles?
I.e.: What is implicitly assumed?

How to handle large particles ?

$$R(q) = K \cdot C \cdot M \cdot P(q)$$

Measured known known (wt conc)

- Measure at many angles
- Calculate $M_{app.}$ at each angle (q)
- Extrapolate to zero angle (q = 0)

And what happens if the samples are concentrated?

$$\frac{K \cdot C}{R} = \frac{1}{M} + 2B_{22}C$$

Second virial coefficient, B_{22}

B_{22} is interesting because it tells whether the particles are attractive (B_{22} negative) or repulsive (B_{22} positive). And it quantifies the strength of interaction.

Example: Using static light scattering to investigate the interplay between protein aggregation dynamics and glycosylation of an enzyme

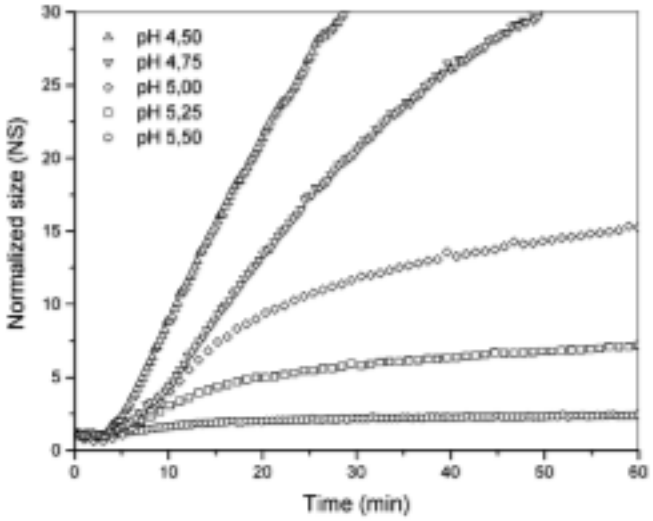


FIGURE 2: Time course of aggregation of Phy at T_d (see Table 1) as a function of pH.

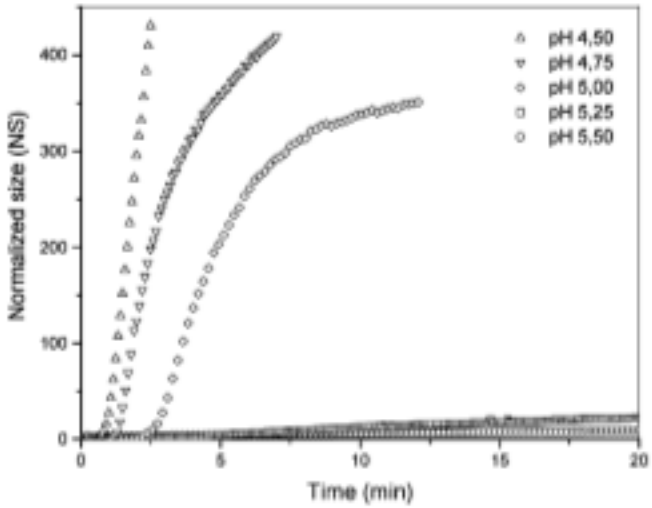


FIGURE 3: Time course of aggregation of dgPhy at T_d as a function of pH. The figure highlights the pronounced increase in the aggregation rate when the pH is lowered, particularly between pH 5.25 and 5.00.

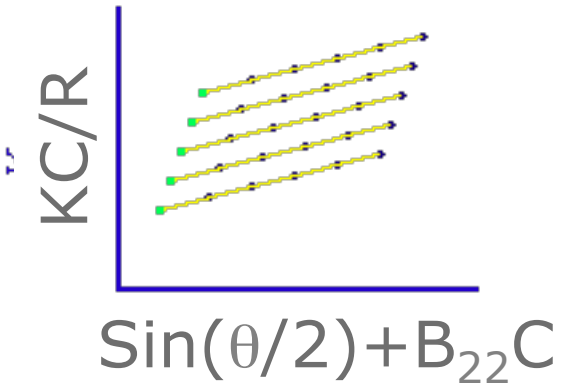
Protein samples were placed in the SLS instrument and left to aggregate while being monitored with SLS (w. time resolution of a few seconds)

Observation: Deglycosylated protein aggregates much faster than glycosylated protein *(Høiberg-Nielsen et al, 2006)*

Measurements of second virial coefficients

The Zimm plot: Measure scattering as a function of angle and concentration

Extrapolate to zero angle and zero concentration



$$\frac{K \cdot C}{R} = \frac{1}{M} + 2B_{22}C$$

Example:

Table 3: Second Virial Coefficients, B_{22} (±standard error of the mean) of Phy and dgPhy Measured at 20 °C

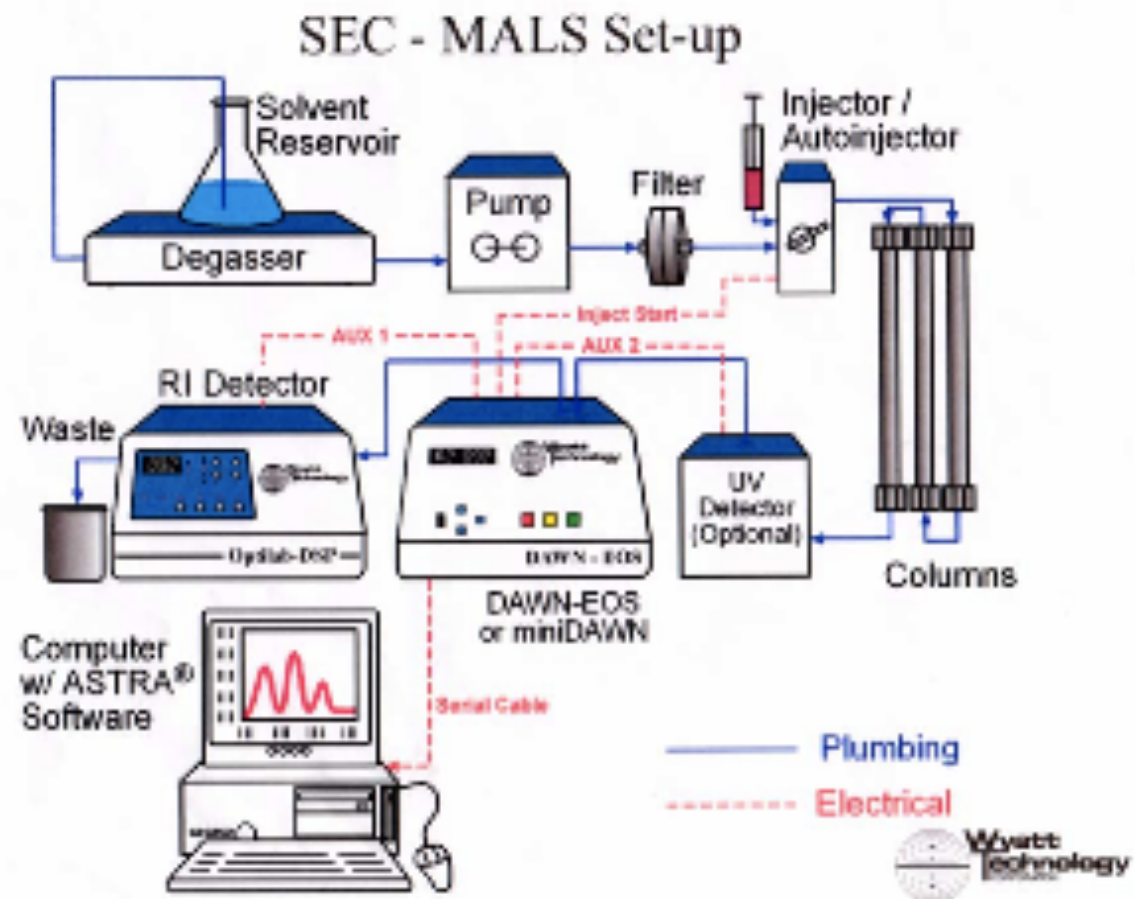
	B_{22} ($\times 10^4$ mL mol g^{-2})	
pH	Phy	dgPhy
6.50	7.10 ± 1.40	7.90 ± 1.80
5.50	4.71 ± 0.16	5.25 ± 0.24
5.25	3.10 ± 2.40	4.90 ± 2.80

From Høiberg-Nielsen et al, 2006

Observation: Both Glycosylated and deglycosylated protein have repulsive interactions. The interactions are of equal size.

Static (or dynamic) light scattering combined with size separation:

Separate the molecular species according to size on a HPLC column
Measure light scattering and molar mass on individual fractions
Measure wt. conc. of individual fractions via the refractive index



Static Light Scattering

Pro's:

SLS is a good and fast method for obtaining information about the molar mass of biomolecules in solutions

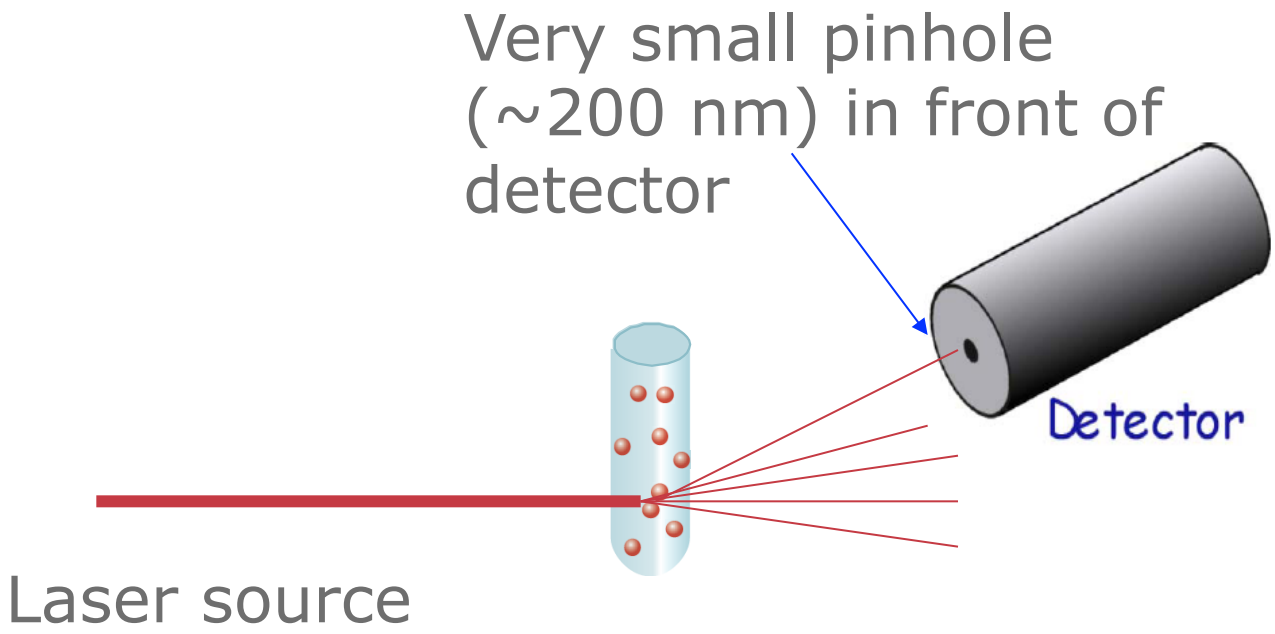
SLS is really useful for characterization of fast dynamic processes. Or test the stability of a sample over time

Con:

Everything in the sample scatters!! A few dust particles in the sample will create a lot of noise in the signal.

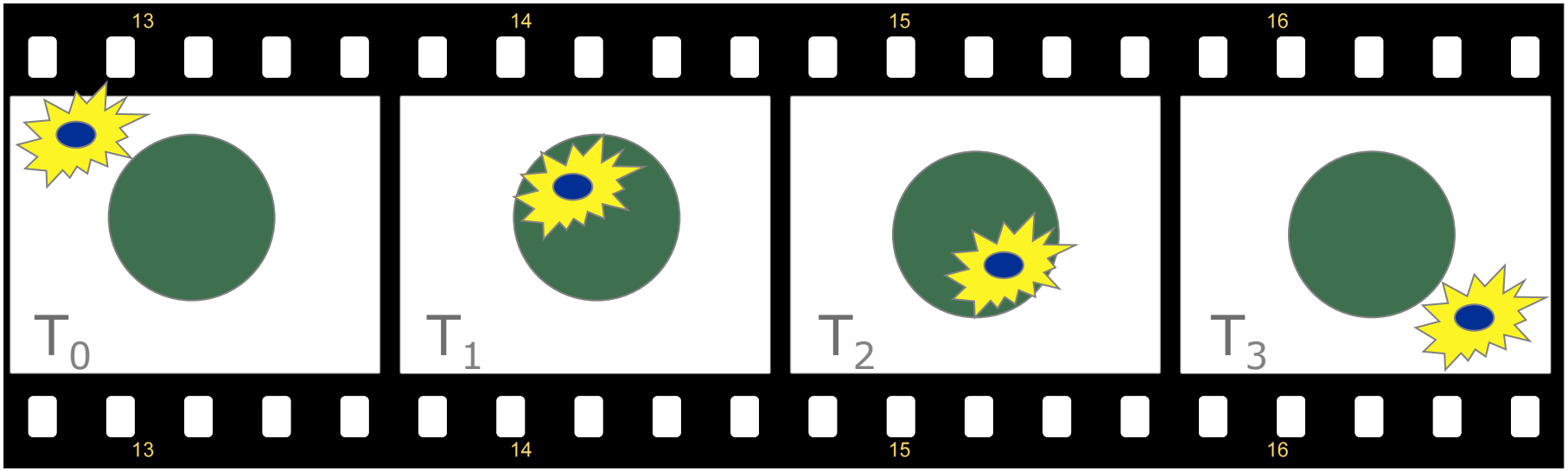
=>Filter your samples!

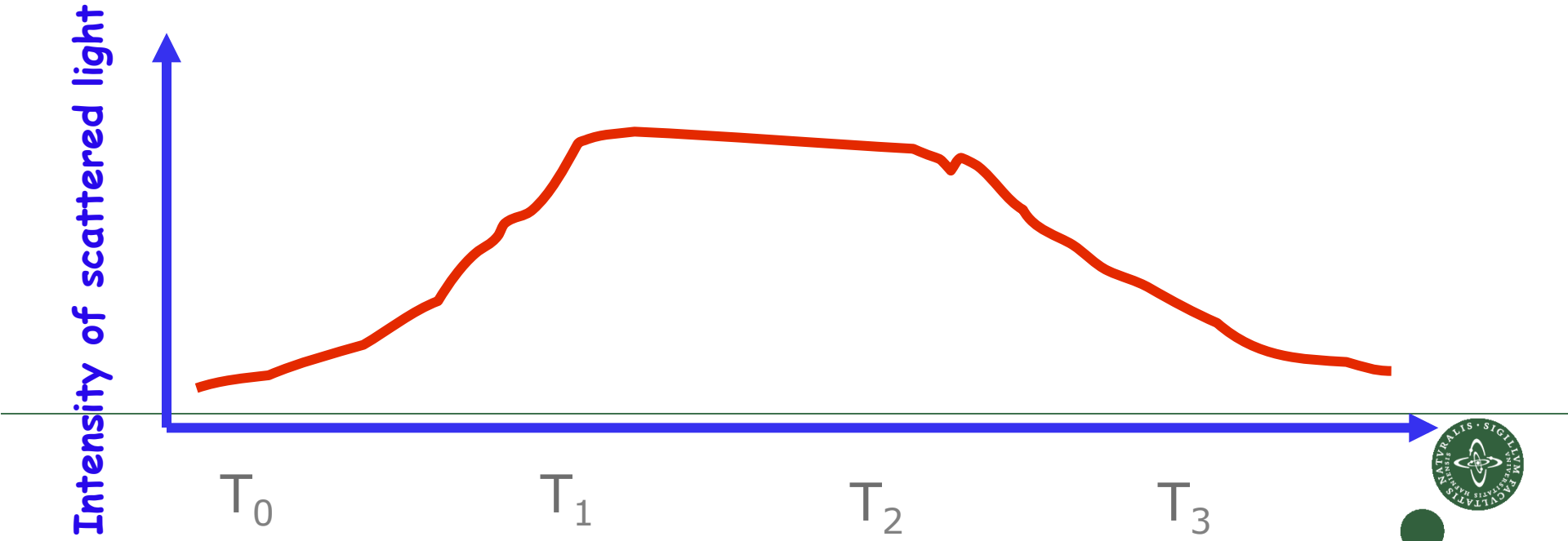
Dynamic light scattering – Principle of experimental setup



-Dynamic Light Scattering is sometimes referred to as quasi-elastic light scattering (QELS)
-And basically the same theory works for photon correlation spectroscopy PCS (Fluorescence based method)

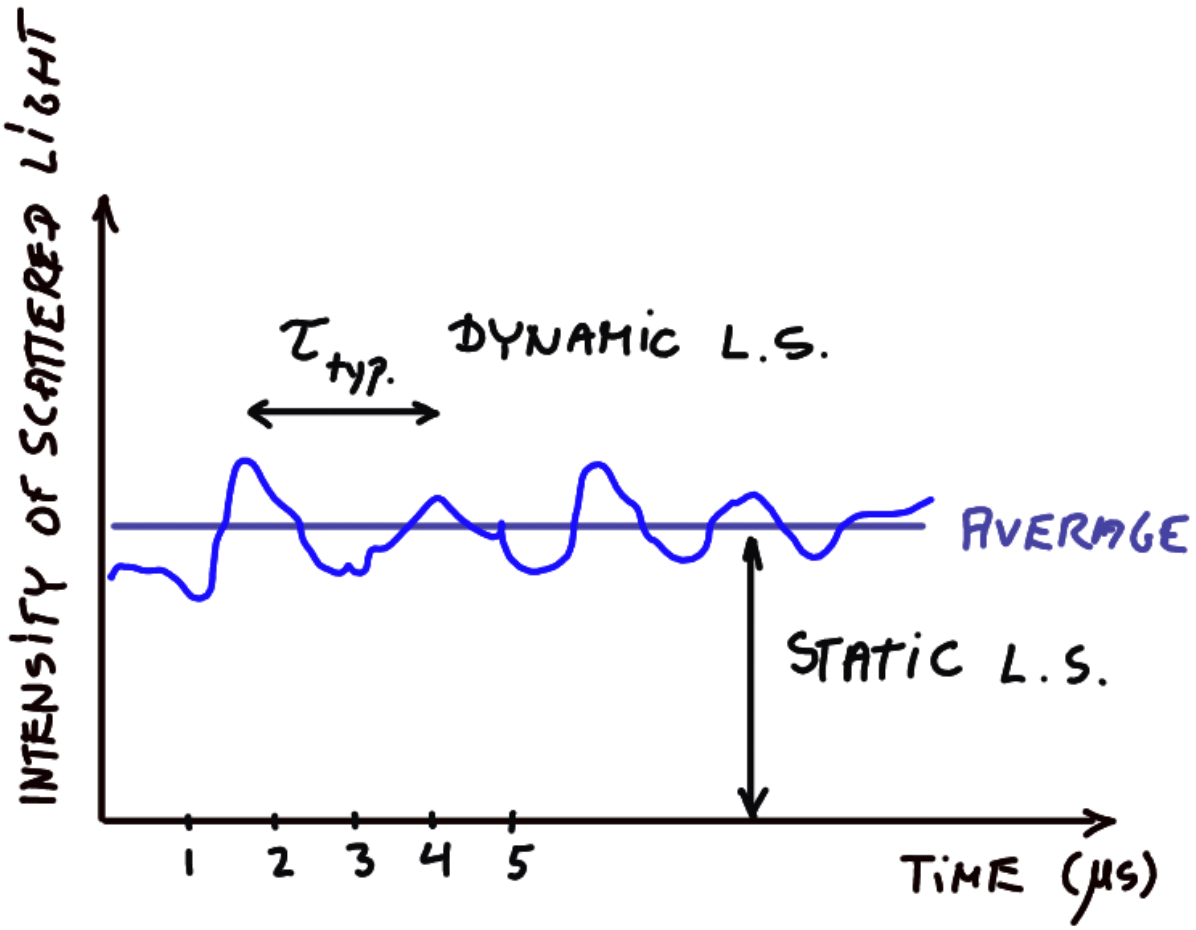
Dynamic light scattering –Principle of measurement (1):





Dynamic light scattering –Principle of measurement (2):

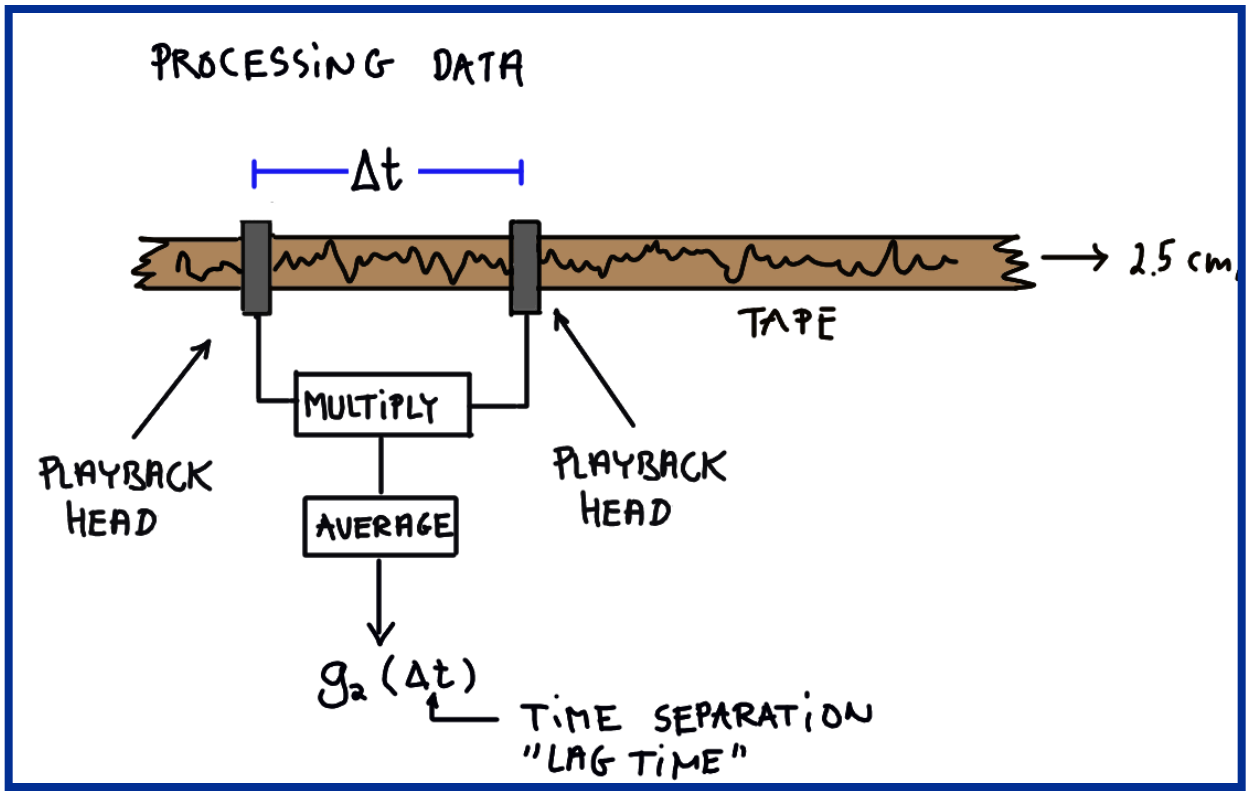
Measure for longer time....



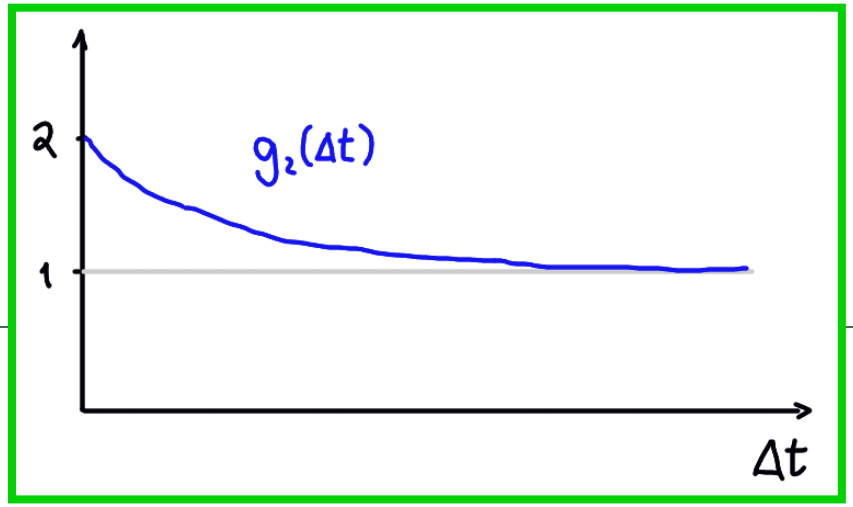
- Frequency of fluctuations depends on how fast the particles move (Large particles move slowly – small particles move fast ...)
- Intensity of fluctuations depends on particle size, contrast, and concentration (for a given fixed wave-length)

Dynamic light scattering –Principle of measurement (3):

-Quantify the light intensity fluctuations:



-And obtain the auto-correlation function



The characteristic decay time

Diffusion in one dimension:

$$\langle x^2 \rangle = 2D \cdot t$$

D: Diffusion coefficient
t: time
x: displacement

Characteristic diffusion distance for change in interference:

$$x = \frac{1}{q}$$

$$\left(\frac{1}{q}\right)^2 = 2D\tau_0 \Rightarrow \tau_0 = \frac{1}{2Dq^2}$$

Guess: $$g_2(t,q) = A \cdot e^{-t/\tau_0} + B = A \cdot e^{-2Dq^2 \cdot t} + B$$

Characteristic decay time

Models for the autocorrelation function

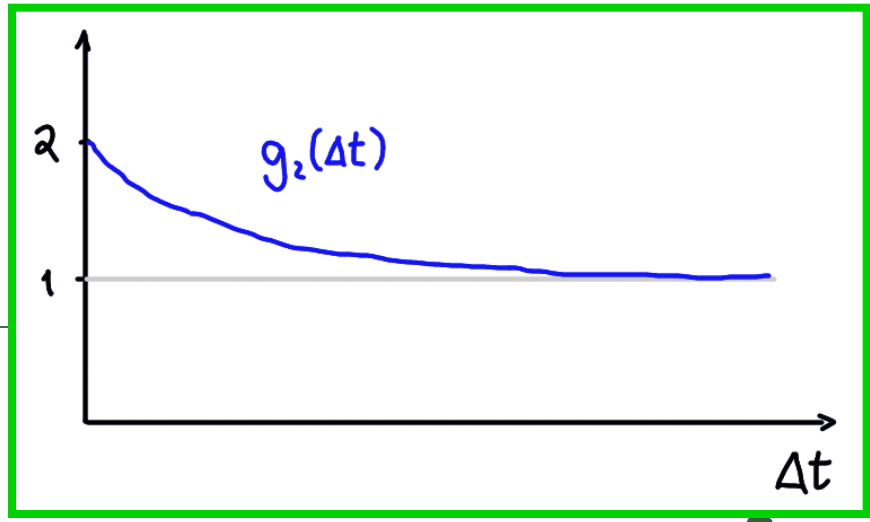
Assume mixture of *n* different species:
Theory:

$$g_2(\tau) = A_1 \cdot e^{-\Gamma_1 \tau} + A_2 \cdot e^{-\Gamma_2 \tau} + \ldots + A_n \cdot e^{-\Gamma_n \tau} + 1$$

$$\Gamma_i = D_i \cdot q^2 \quad \left[\mathrm{s}^{-1}\right]$$

Determine A_1, Γ_1, A_2, Γ_2, ... using a fitting procedure.

Problem: There is not always that much information in the auto-correlation function ....



Particle sizing of spherical particles with DLS

The Stokes-Einstein relation for spherical particles:

$$D = \frac{k_B T}{6\pi\eta r}$$

$$r_h = \frac{k_B T}{6\pi\eta D_{meas}}$$

The hydrodynamic radius

Measure the diffusion coefficient *D*
Then calculate equivalent hydrodynamic radius:

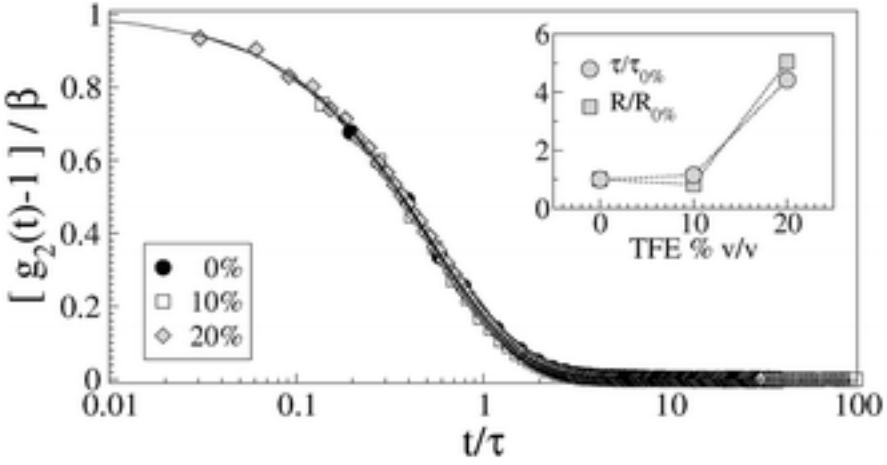
$$g_2(t) = A \cdot e^{-t/\tau_0} + B = A \cdot e^{-2Dq^2 \cdot t} + B$$

Range: Down to $r_h \sim 1$ nm
Up to $r_h \sim 1000$ nm

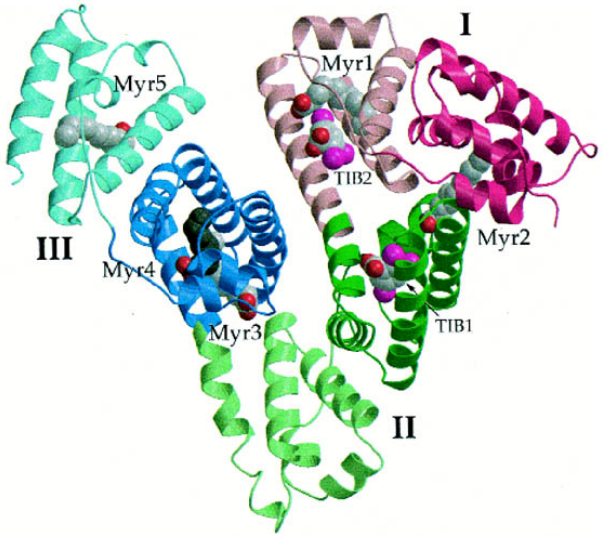
Other models for the autocorrelation function:

-Cumulant analysis (1st or 2nd order): -For monodispersed samples
-Single exponential, Double exponential, mulitiple exponential: For multi-dispersed samples
-Methods based on Indirect La Place transforms: For broad and unpredictable sample distributions:

Problem: There is not always that much information in the auto-correlation function ....
⇒Go for the simplest model that still gives a good fit to the auto-corelation function
⇒Remember to critically evaluate the fit to the auto-corelation function, by visual inspection (at least)



Example: Human Serum Albumin (HSA):



Non-trivial structure/shape!

SLS: HSA is too small to allow for extracting other information but that the Mw is ~66000 Da

DLS: Tells us that BSA has a hydrodynamic diameter of 6.4 nm

+Useful to check that the protein formulation is OK
+Information extremely quickly obtained,
- Information is not very detailed (As compared to SAS)

Closing remarks

Pro's:

DLS and SLS are good, fast and easily accessible methods for obtaining crude information about biomolecules in solutions

The methods are extremely useful for testing and optimizing a protein formulation in a home-lab

The methods are really useful for obtaining a characterization of fast dynamic processes

Con:

The methods most often require a larger sample volume than a SAXS experiment does – And gives less information

NB: *The commercial software for DLS analysis is often very optimistic with respect to the information contents in DLS data. Be careful!*

Thank you!