CEITEC

Central European Institute of Technology

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Analytical ultracentrifugation

Introduction

Jan Komárek

What is it?

analytical ultracentrifugation (AUC)

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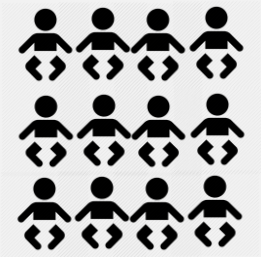
analytical ultracentrifugation

(AUC)

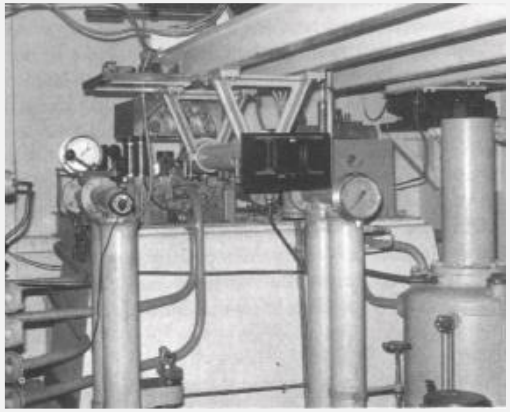
History

1924 – discovery of AUC



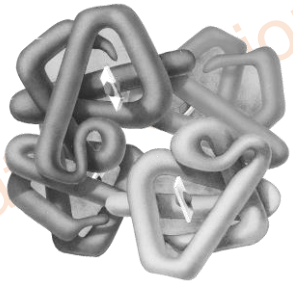
Theodor Svedberg
(1884-1971)

1926

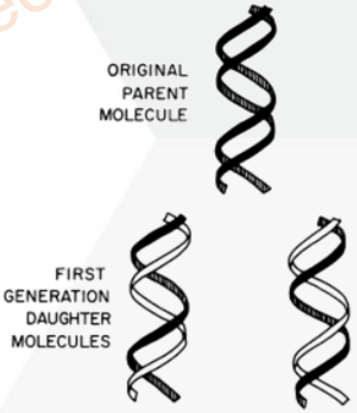


Svedberg´s ultracentrifuge

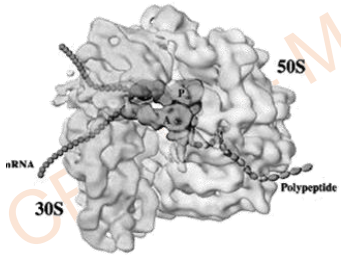
1926 (Svedberg)
Proteins as chemical entities with defined MW



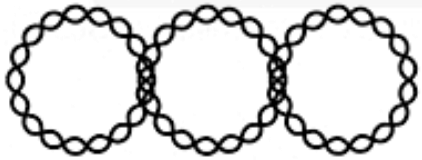
1958 (Meselson, Stahl)
Semiconservative DNA replication



1956 (Chao, Schachman)
1958 (Tissieres, Watson)
Structure of ribosomes



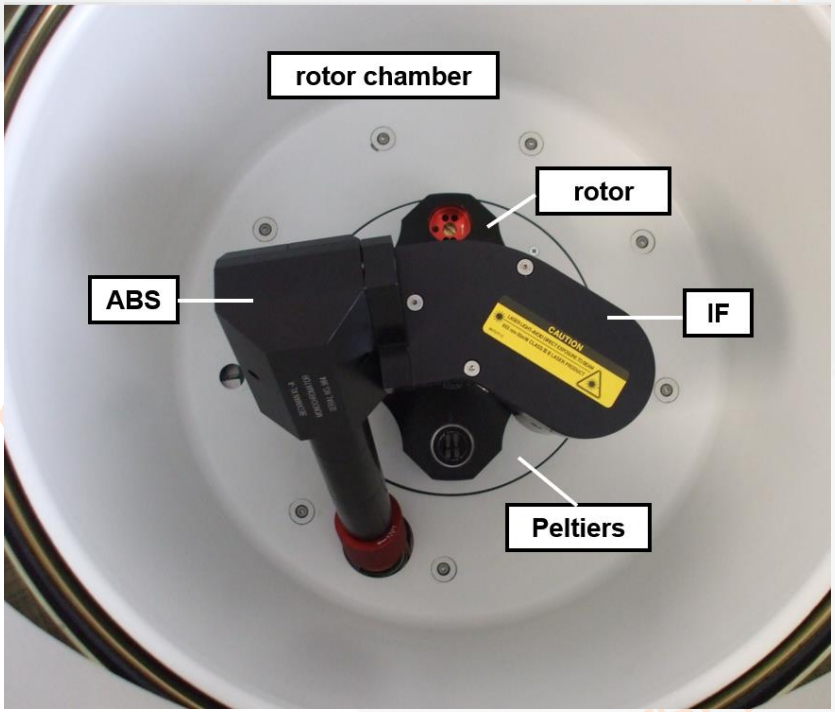
1963 (Vinograd)
DNA supercoiling



Instrumentation

Beckman Coulter ProteomeLab XL-I

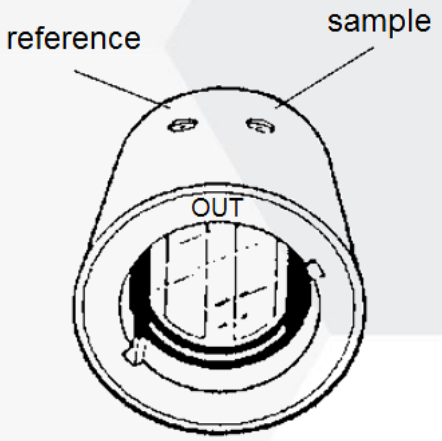
- speed up to 60 000 rpm
- temperature range 0° to 40° C
- **absorbance optics**
- wavelength 190 to 800 nm
- **Rayleigh interference optics**
- laser wavelength 660 nm



An-60 Ti rotor

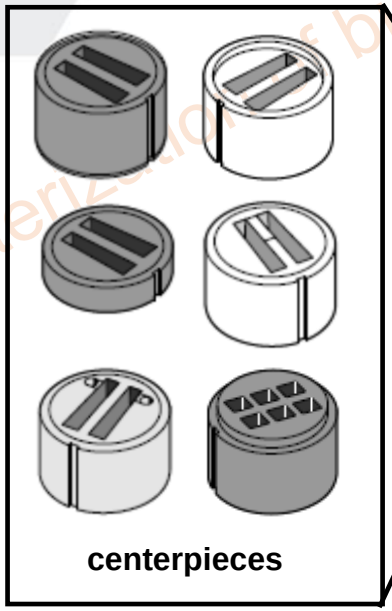
counterbalance

Instrumentation

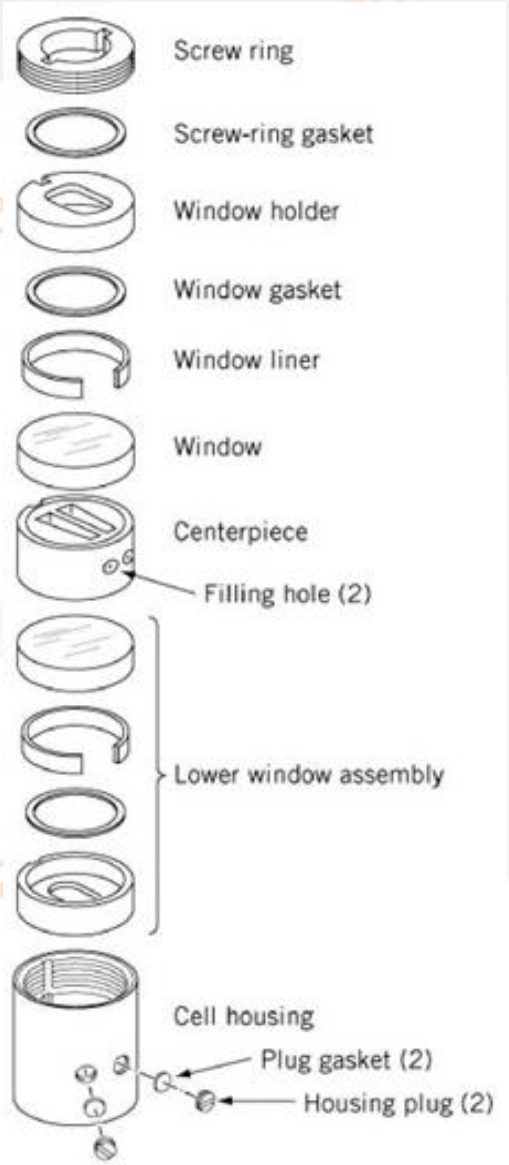


assembled double-sector centerpiece cell

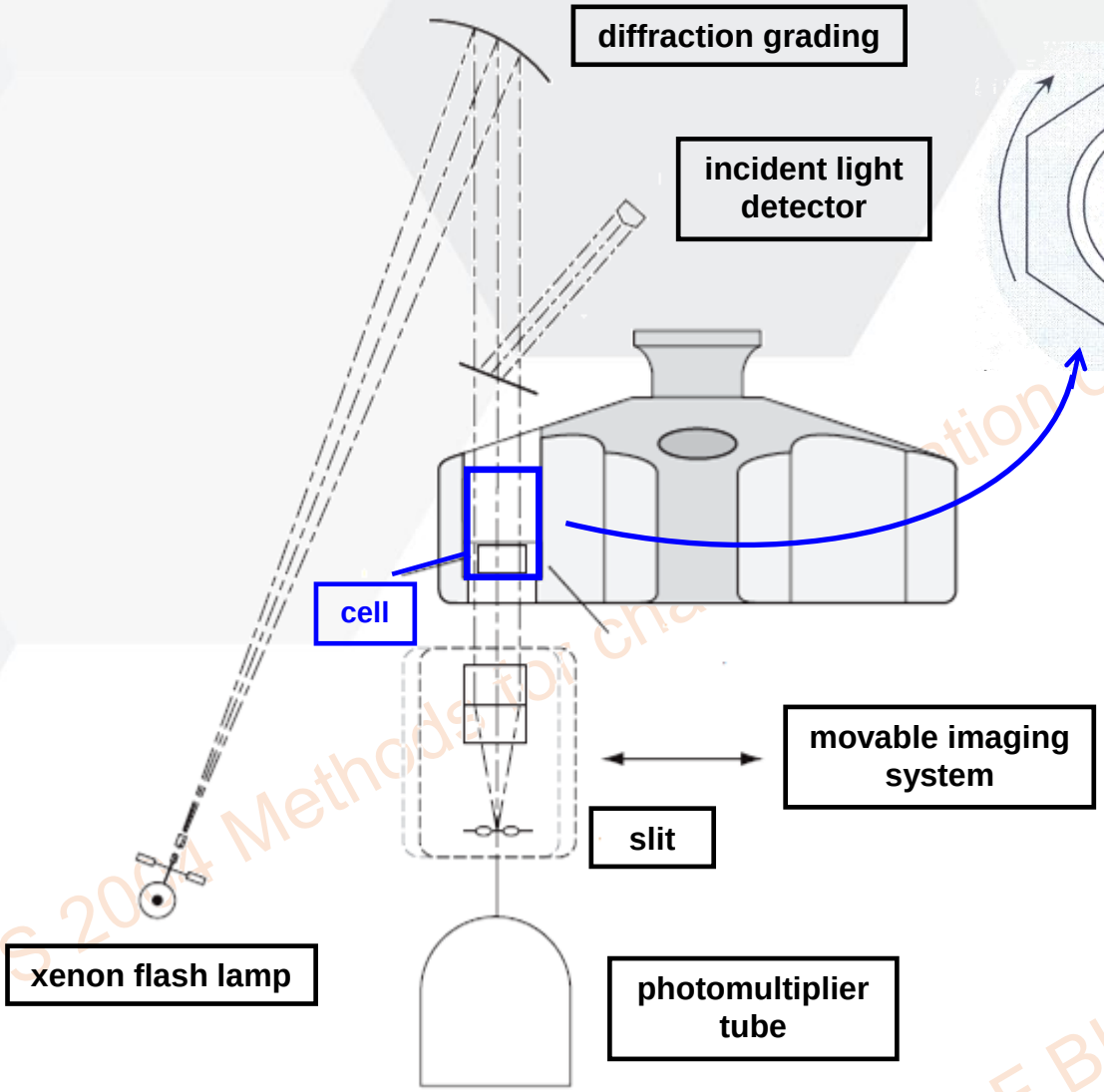
cell components

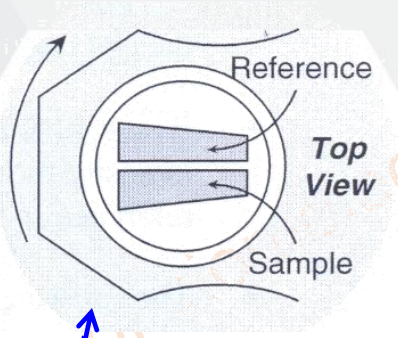


centerpieces



Absorbance optical system



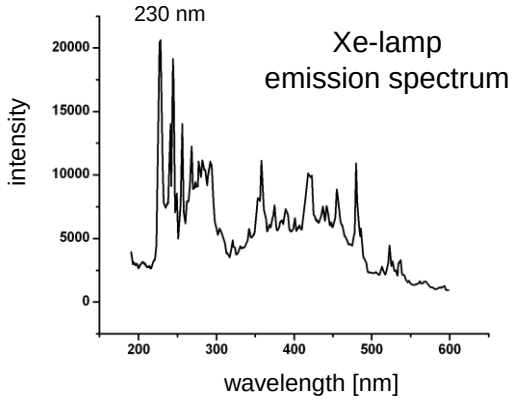


ABS optics - selective detection of absorbing compounds

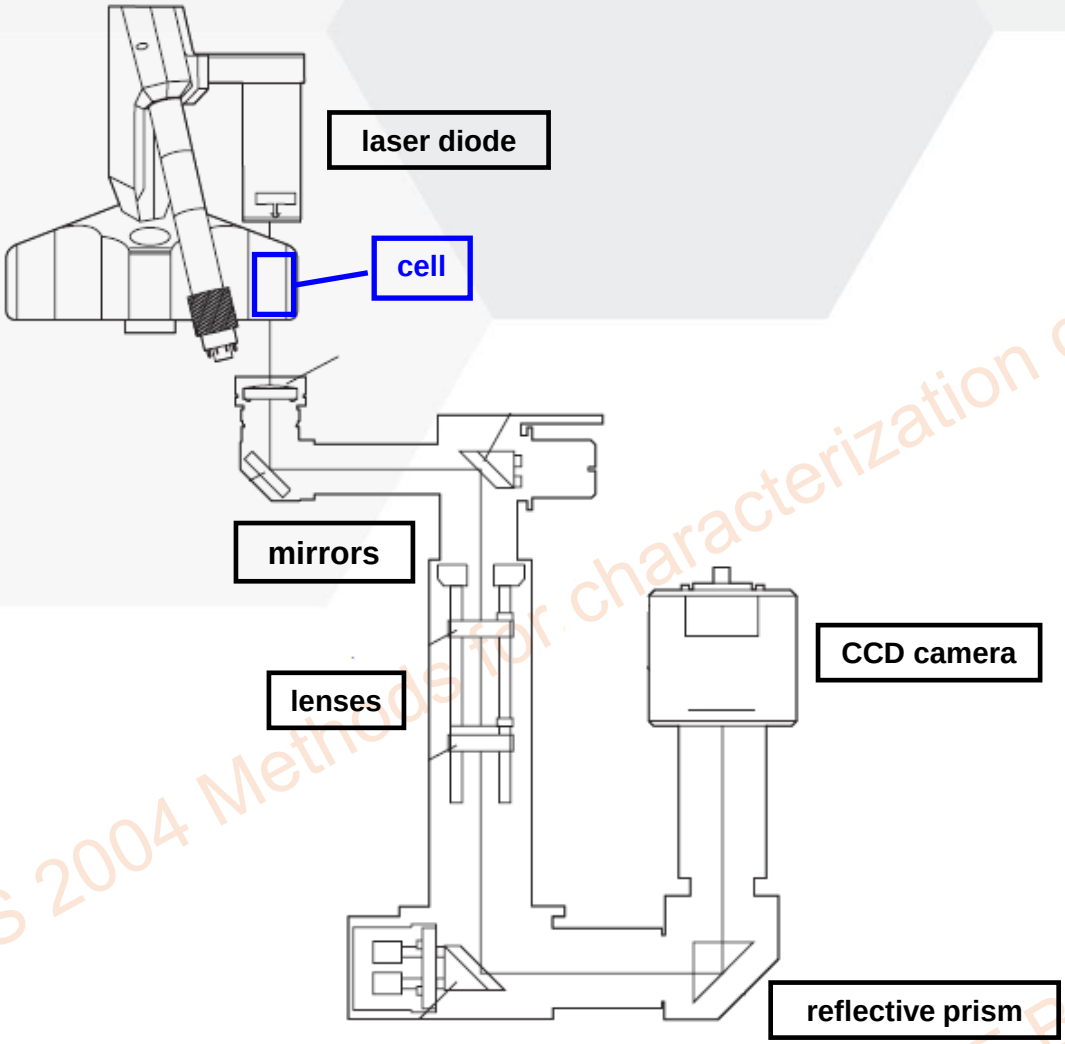
Lambert-Beer Law: $A = c\,\varepsilon\,l$

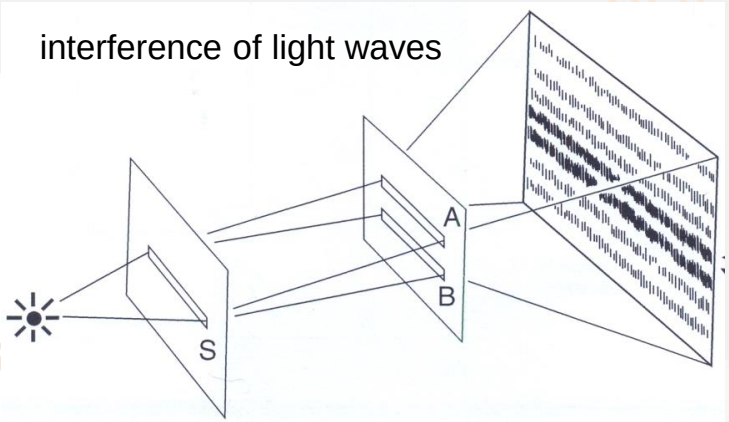
(limited linearity)

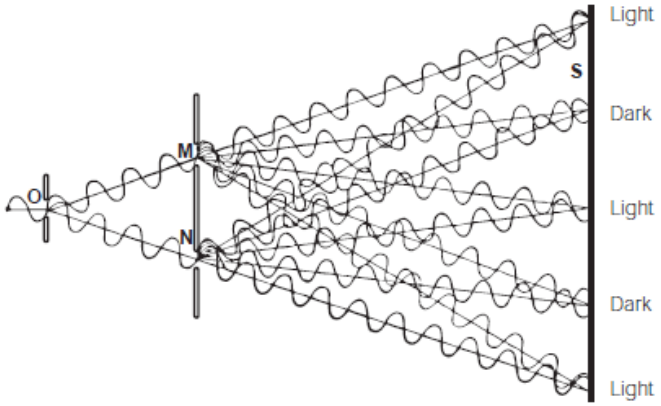
A – absorbance, *c* – concentration of solute [M], ε – extinction coefficient [$M^{-1}cm^{-1}$], , *l* – optical pathlength [cm]



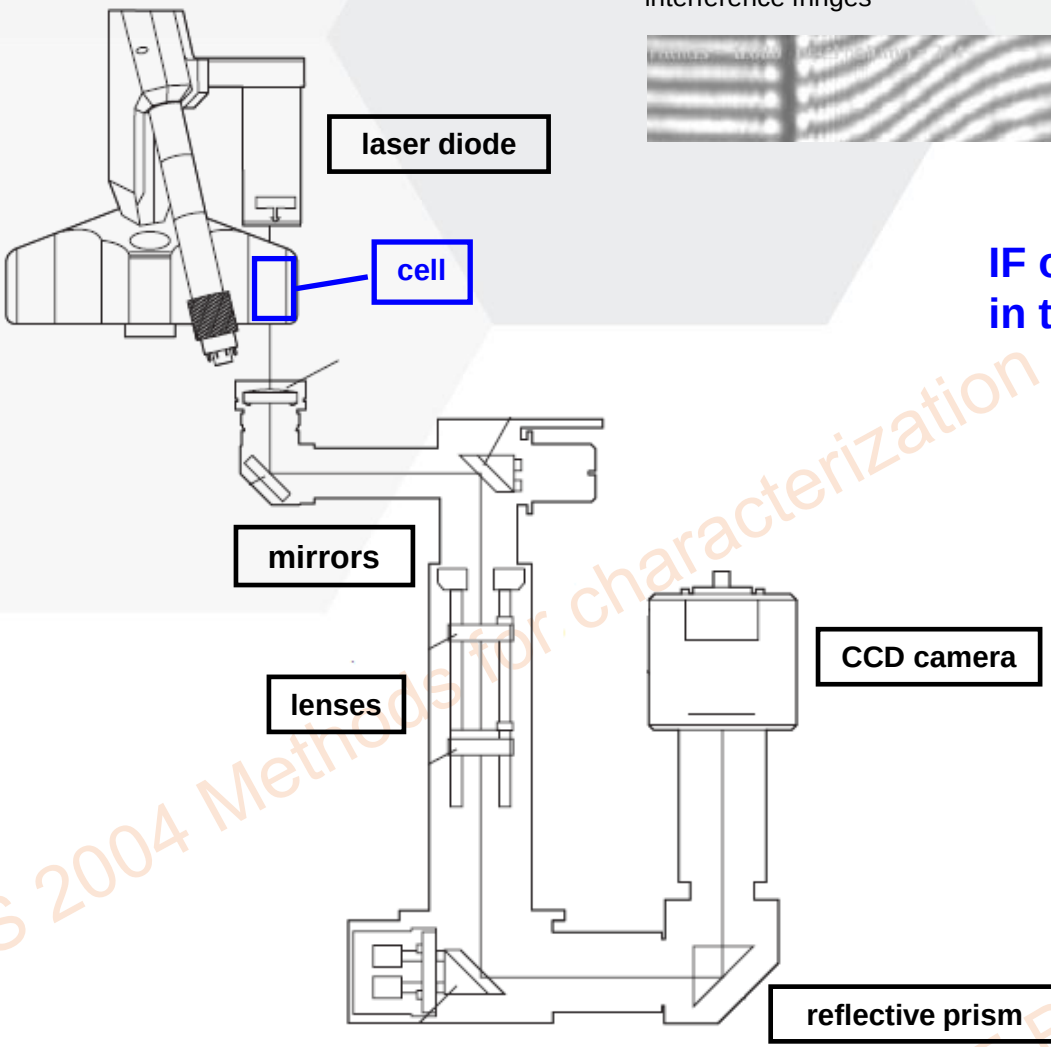
Interference optical system



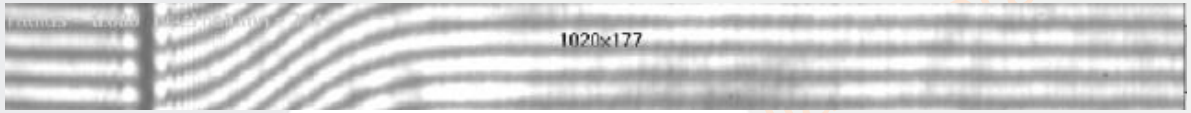




Interference optical system



interference fringes

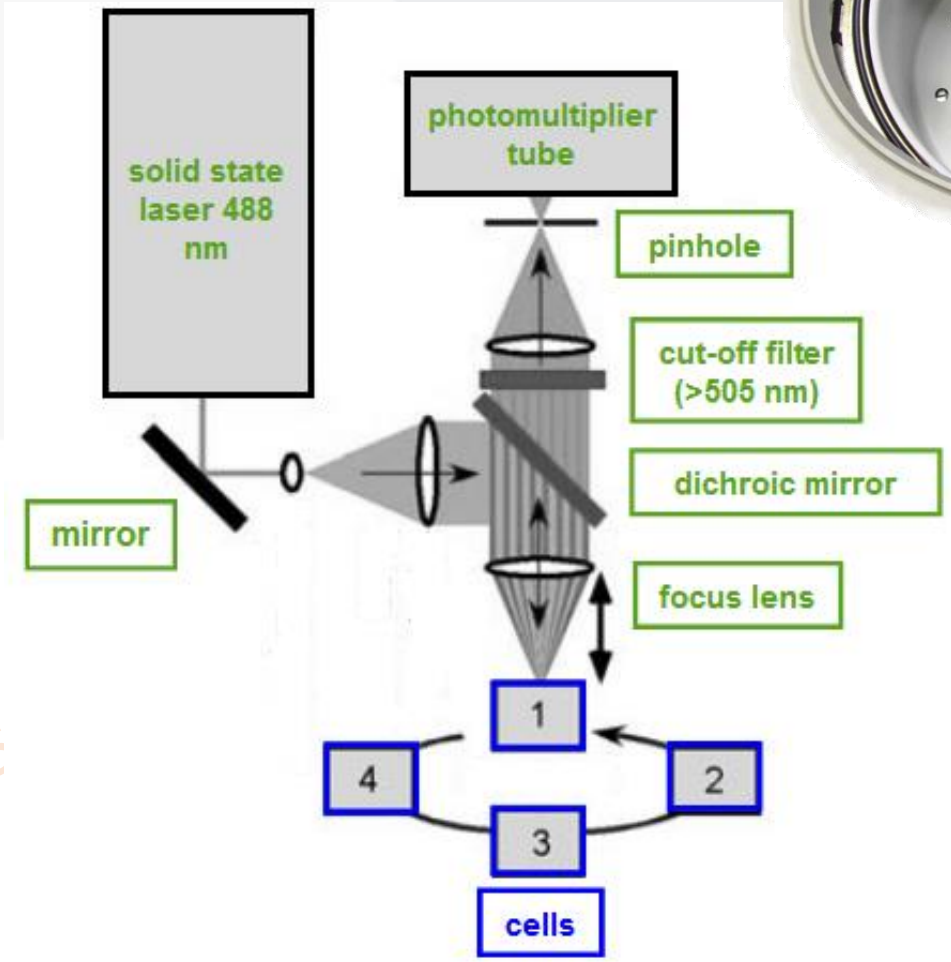


IF optics - sensitive to all components in the solution, including salts!

$$Y = c \frac{dn}{dc} \frac{l}{\lambda}$$

Y – measured fringe displacement [fringes], c – weight concentration [mg·ml^{-1}], dn/dc – refractive index increment, l – optical pathlength, λ – wavelength

Fluorescence detection system (FDS)



$$F = I_0 \cdot Q \cdot \varepsilon \cdot c$$

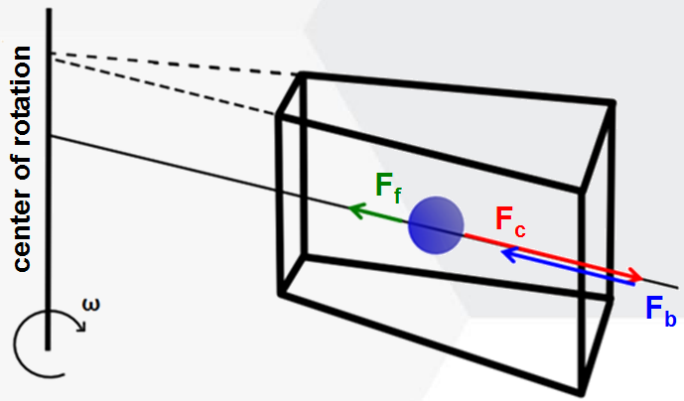
F – fluorescence intensity, I_0 – incident intensity of excitation beam, Q – quantum yield, ε – extinction coefficient, c –concentration

- higher sensitivity and selectivity

- analysis of high-affinity interactions (K_d in the pM range)

- analysis of labeled molecules present in complex media (e.g. blood serum, cell lysate)

Sedimentation of particles



centrifugal force: $F_c = m\,\omega^2\,r$

(m – mass of the particle, ω – angular velocity [rad.s^{-1}], r – radial distance)

frictional force: $F_f = -fu$

(f – frictional coefficient, u – velocity of particle)

buoyant force: $F_b = -m_0\,\omega^2\,r \qquad m_0 = m\bar{v}\rho = \frac{M}{N}\bar{v}\rho$

(m_0 - mass of solvent displaced by the particle, $\bar{v}$ – partial specific volume [cm^3·g^{-1}], ρ – density of solvent, M – molar mass, N – Avogadro´s number

forces balanced: $F_c + F_b + F_f = 0$

↓

Svedberg equation:

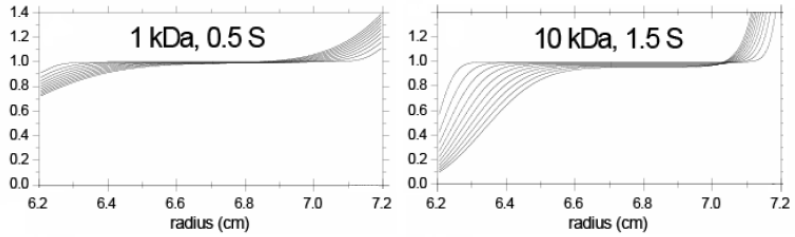
(definition of sedimentation coefficient)

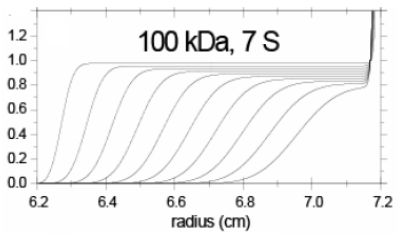
$$\frac{M(1-\bar{v}\rho)}{Nf} = \frac{u}{\omega^2 r} \equiv s$$

[s] = 1 S (Svedberg, 10^{-13} s)

sedimentation coefficient *s*:

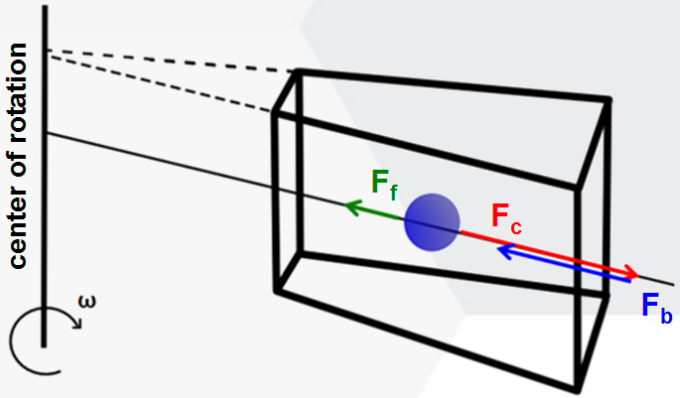
- depends on the **mass** and **shape** of particle





Brown, 2008

Sedimentation of particles



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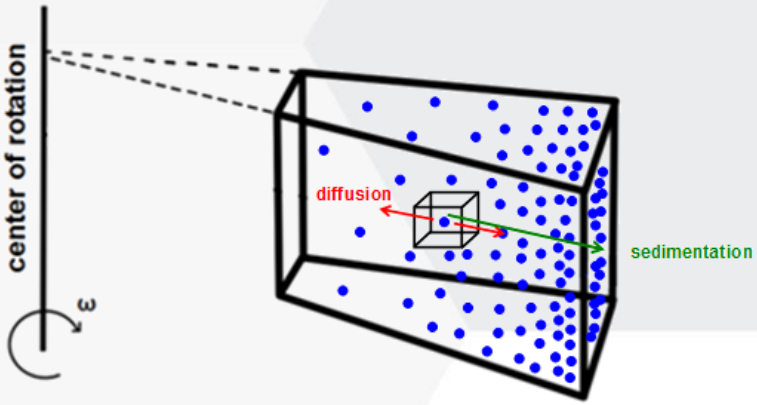
sedimentation coefficient s:

- depends on the **mass** and **shape** of particle

typical values of s:	
peptides:	< 1 S
proteins:	1 – 10 S
bacterial ribosome:	70 S
viruses:	100 – 600 S

Sedimentation of particles



diffusion as a result of random thermal motion of particles

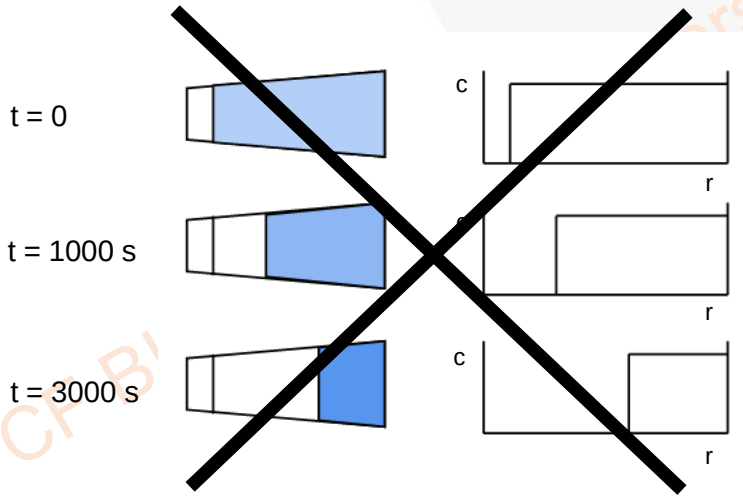
First Fick´s law:
(description of diffusion)

$$J_D = -D\frac{dc}{dr}$$

(J_D – diffusional flux, D – translational diffusional coefficient, dc/dr – concentrational gradient)

Stokes-Einstein equation: $$D = \frac{RT}{N_A f}$$

limiting case of no diffusion:



Applications

What can be studied?

- **peptides**
- **proteins, glycoproteins, membrane proteins**
- **nucleic acids (DNA, RNA)**
- **lipids, lipoproteins, liposomes**
- **polysaccharides**
- **viruses, viral vectors**
- **nanoparticles**
- **organic/inorganic polymers**

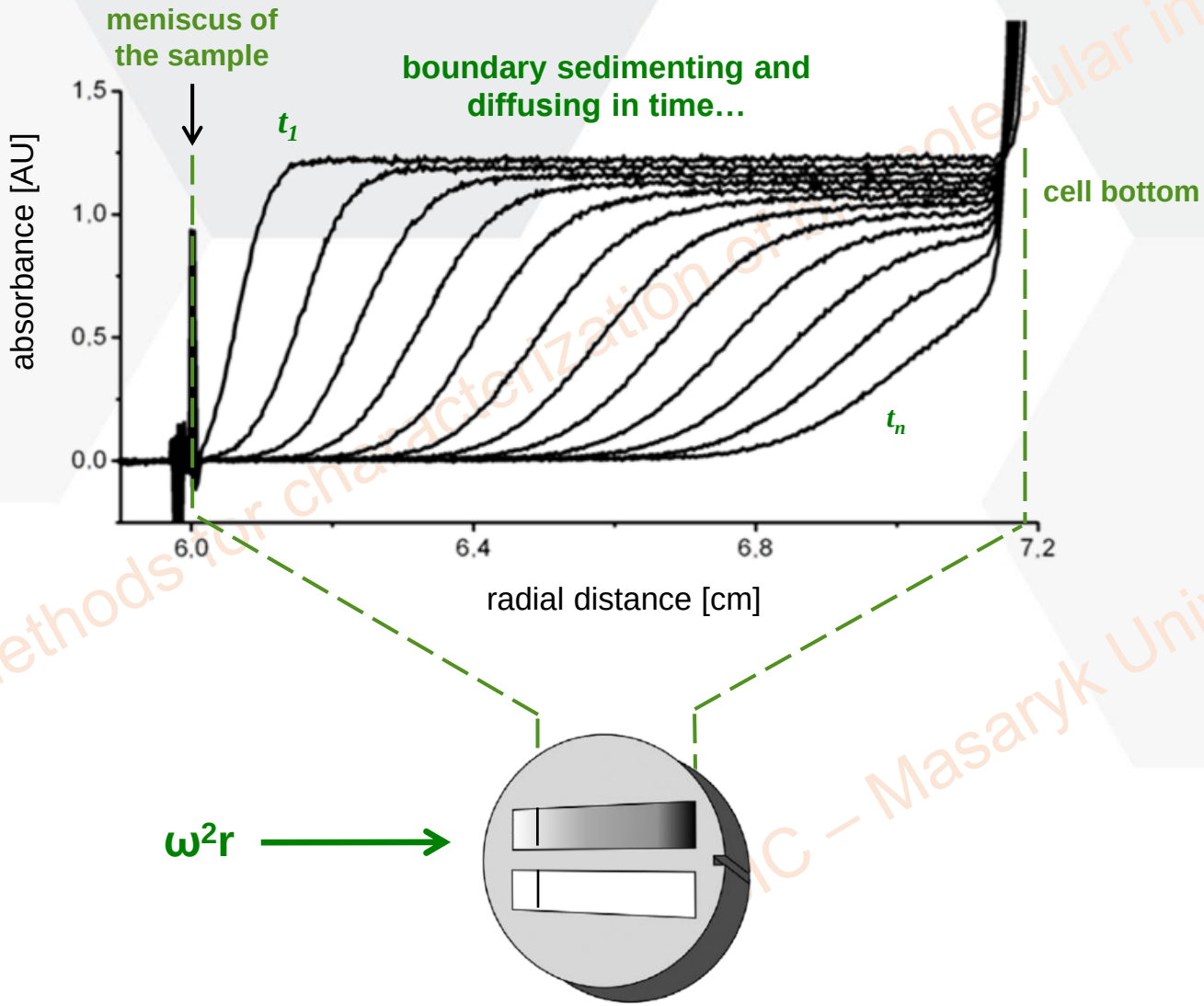
What can we learn?

- **purity/homogeneity**
- **molar mass**
- **size and shape**
- **aggregation**
- **biomolecular interactions**

Advantages of AUC:

- in-solution technique, no interaction with matrix (unlike SEC)
- variability in solvent (ionic strength, pH, co-factors) → closer to physiological conditions
- calibration-free, first-principle method, no imobilization or labeling
- non-destructive method
- broad dynamic range (small peptides – viruses)
- low sample consumption (~hundreds of µg)

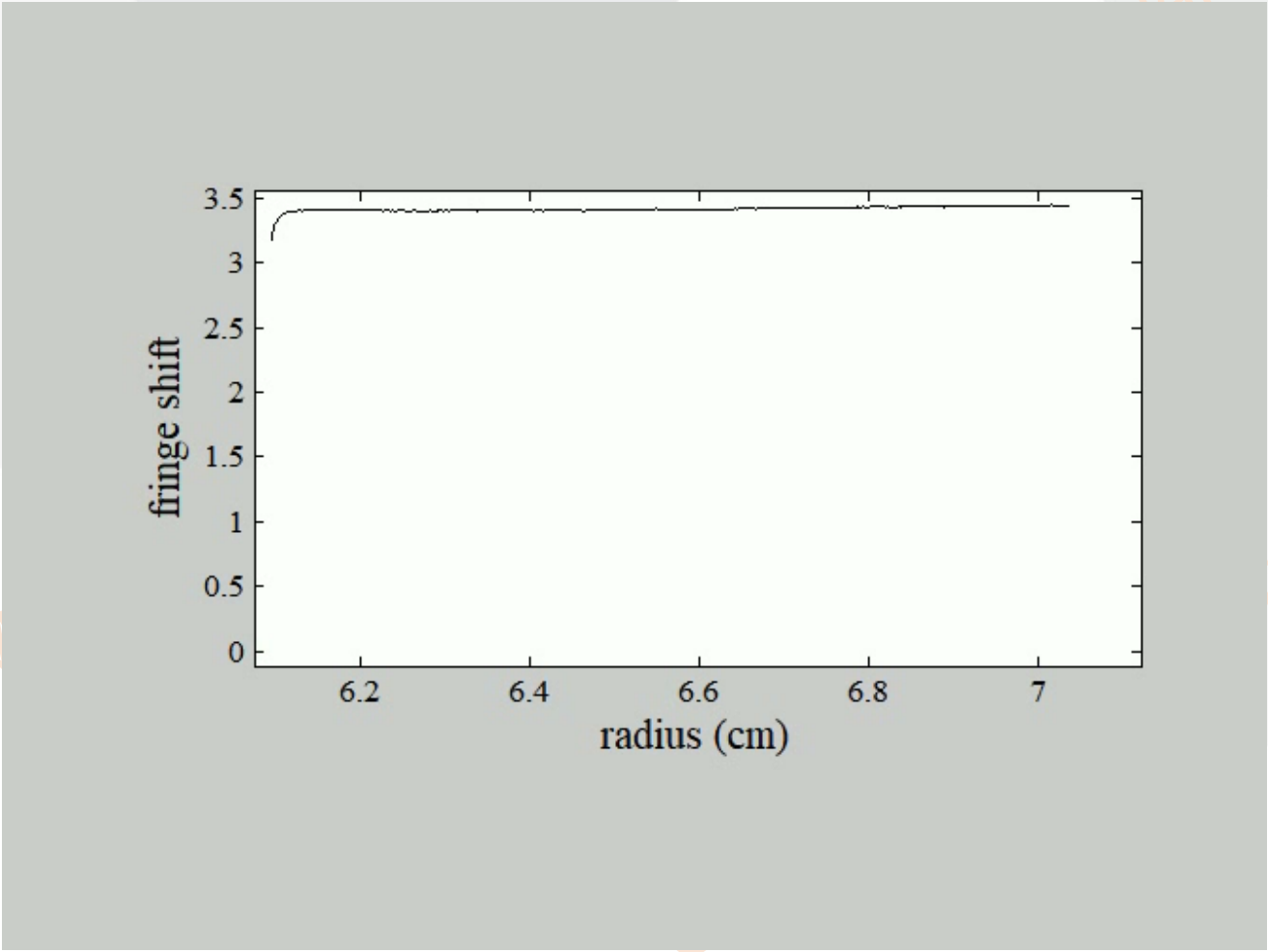
Sedimentation velocity



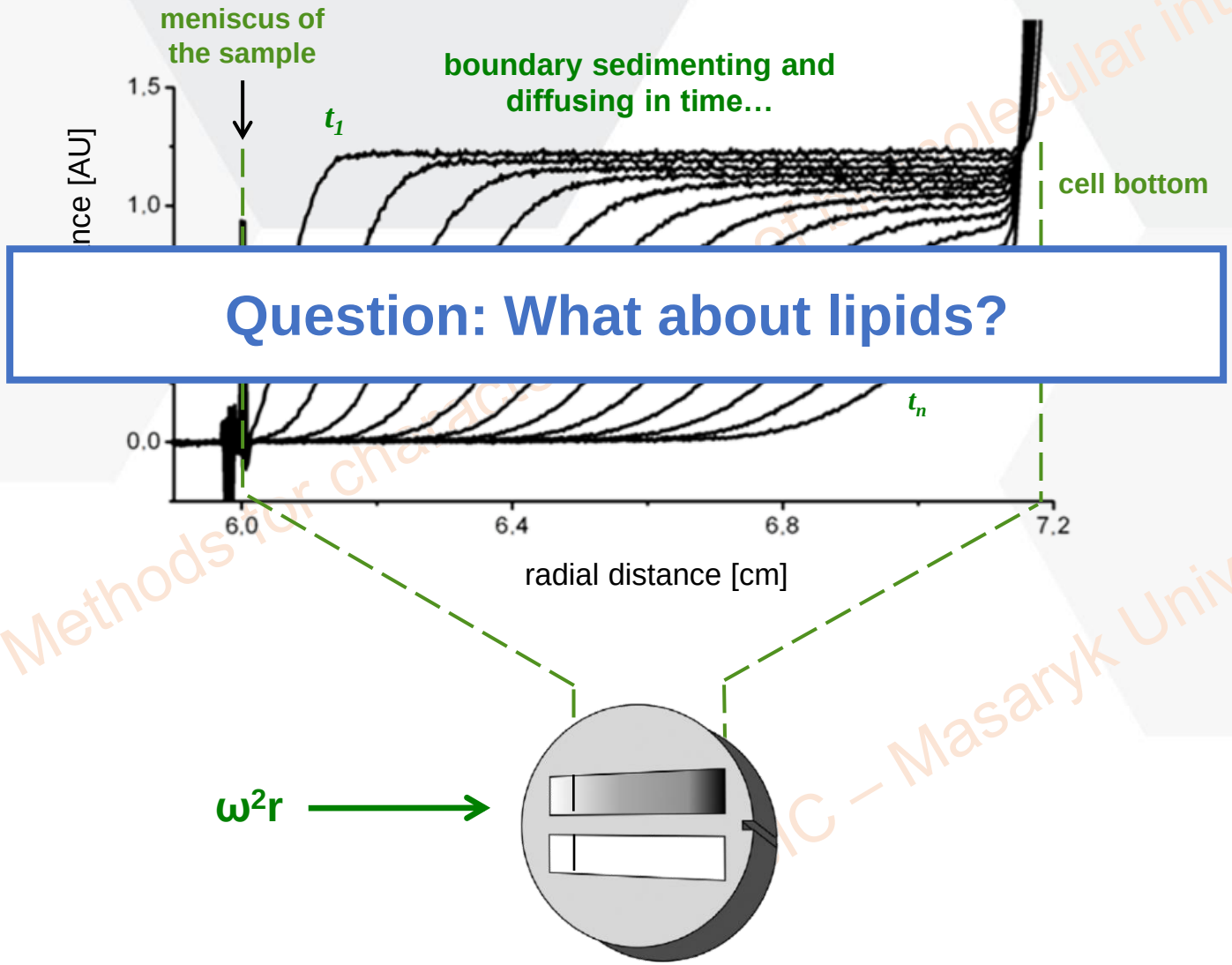
Sedimentation velocity

Sedimentation of a 48 kDa protein (3.4 S)

(50,000 rpm, 20 °C, IF detection, 4 hours)

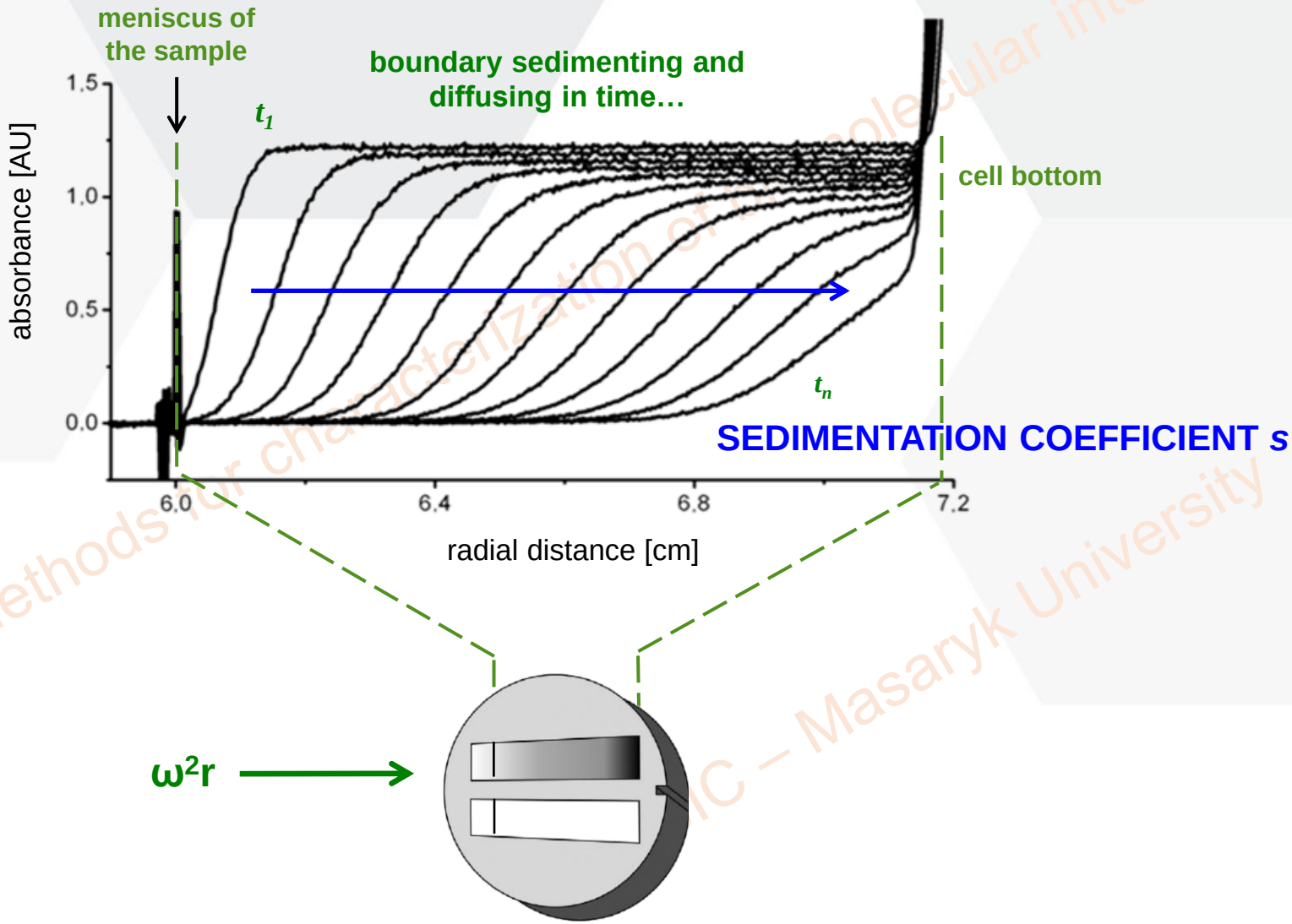


Sedimentation velocity

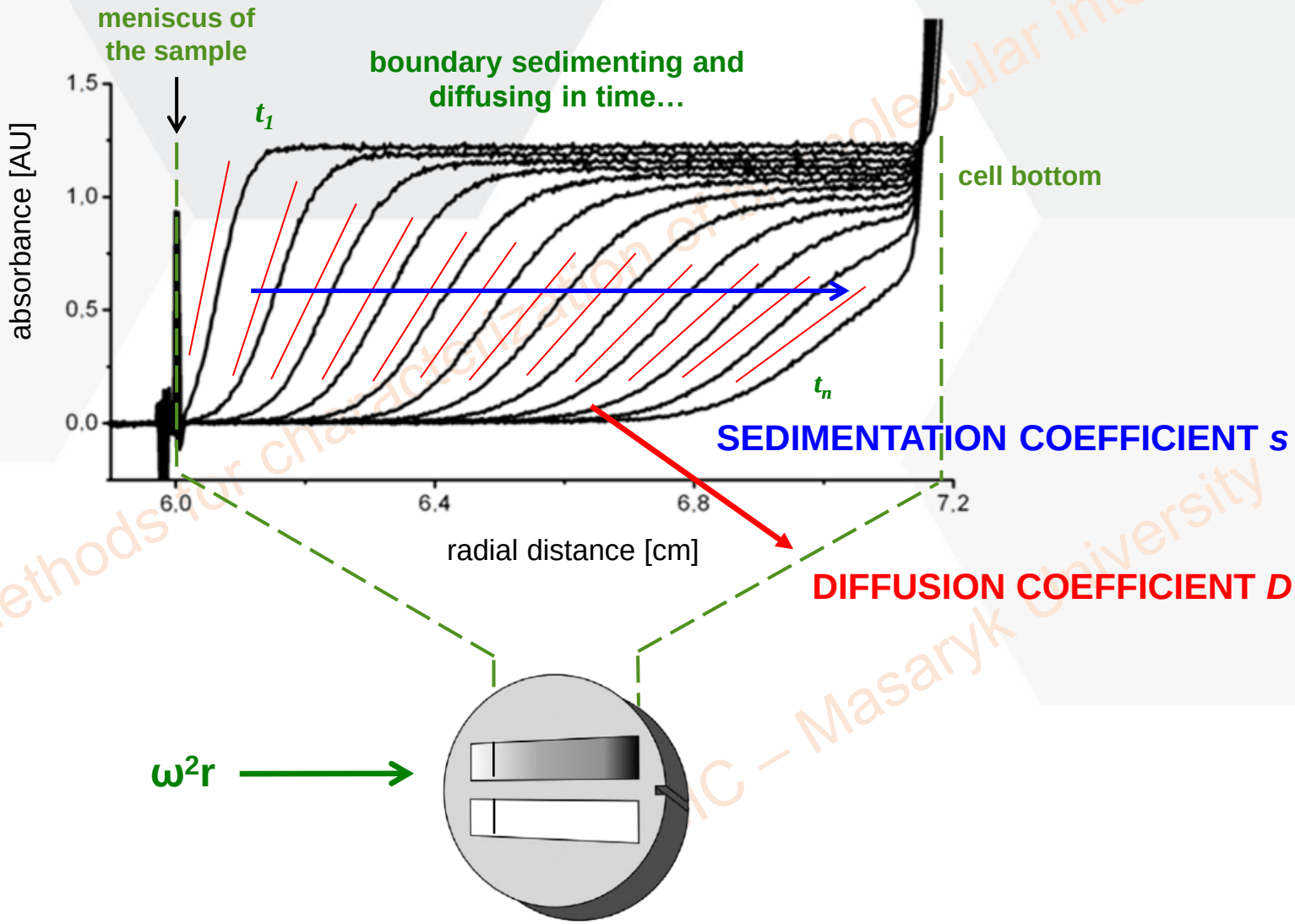


Question: What about lipids?

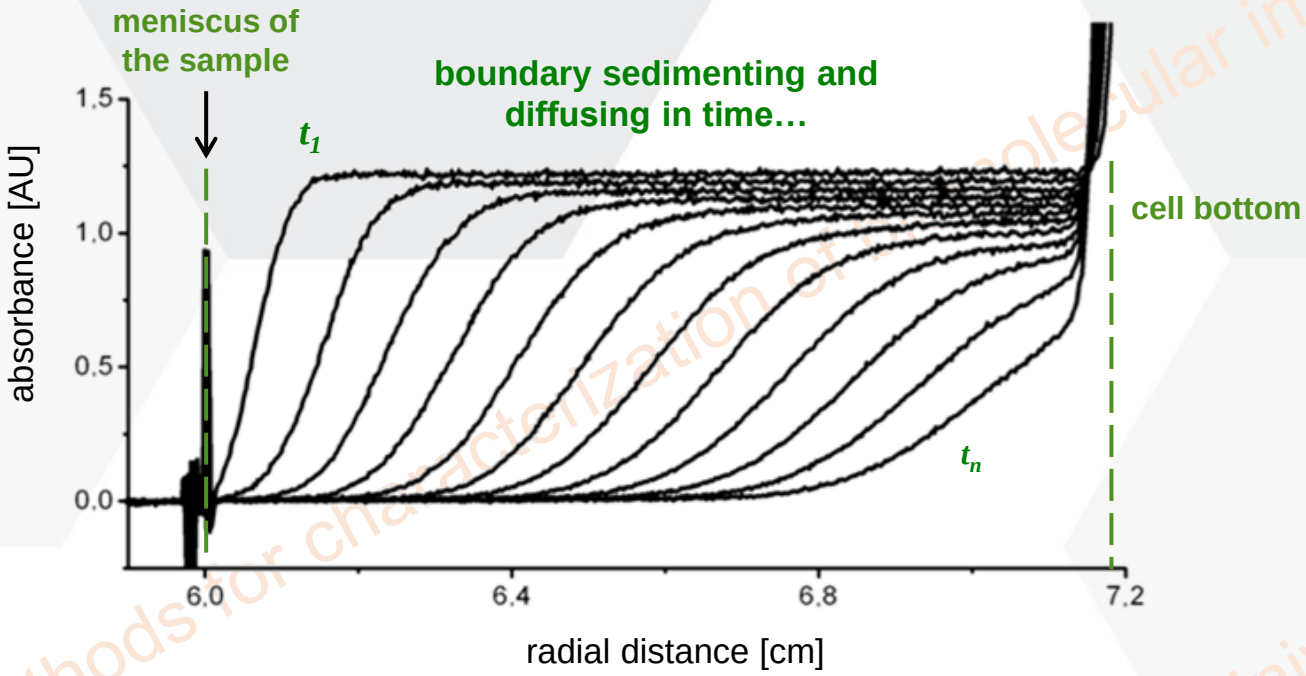
Sedimentation velocity



Sedimentation velocity



Sedimentation velocity



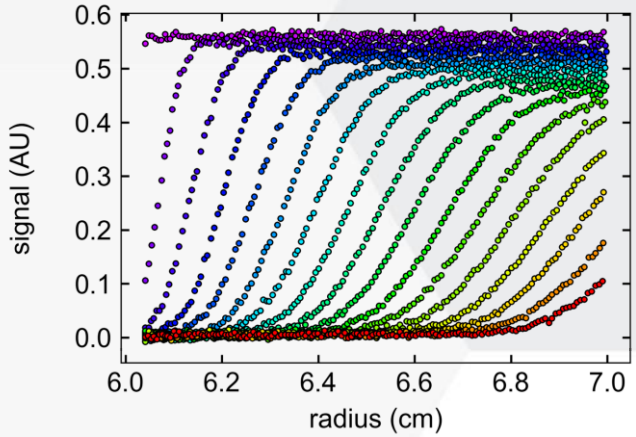
Lamm equation:

(describes the movement of sedimentation boundary in time)

$$\frac{\partial c}{\partial t} = D\left[\frac{\partial^2 c}{\partial r^2} + \frac{1}{r}\frac{\partial c}{\partial r}\right] + s\omega^2\left[r\frac{\partial c}{\partial r} + 2c\right]$$

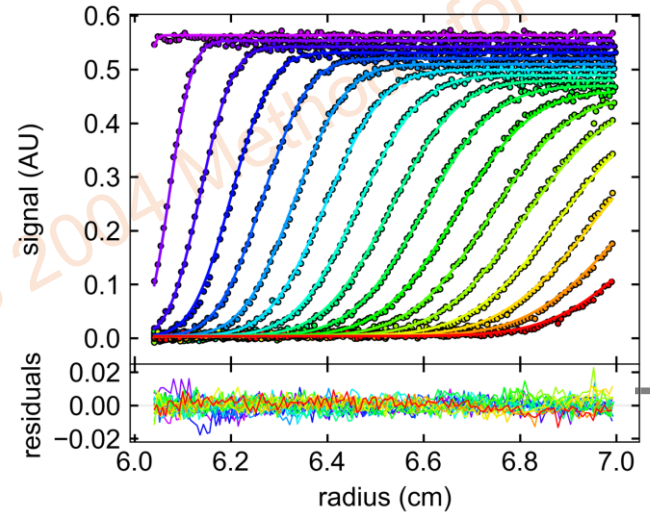
Sedimentation velocity

raw SV data:



$$\frac{\partial c}{\partial t} = D\left[\frac{\partial^2 c}{\partial r^2} + \frac{1}{r}\frac{\partial c}{\partial r}\right] - s\omega^2\left[r\frac{\partial c}{\partial r} + 2c\right]$$

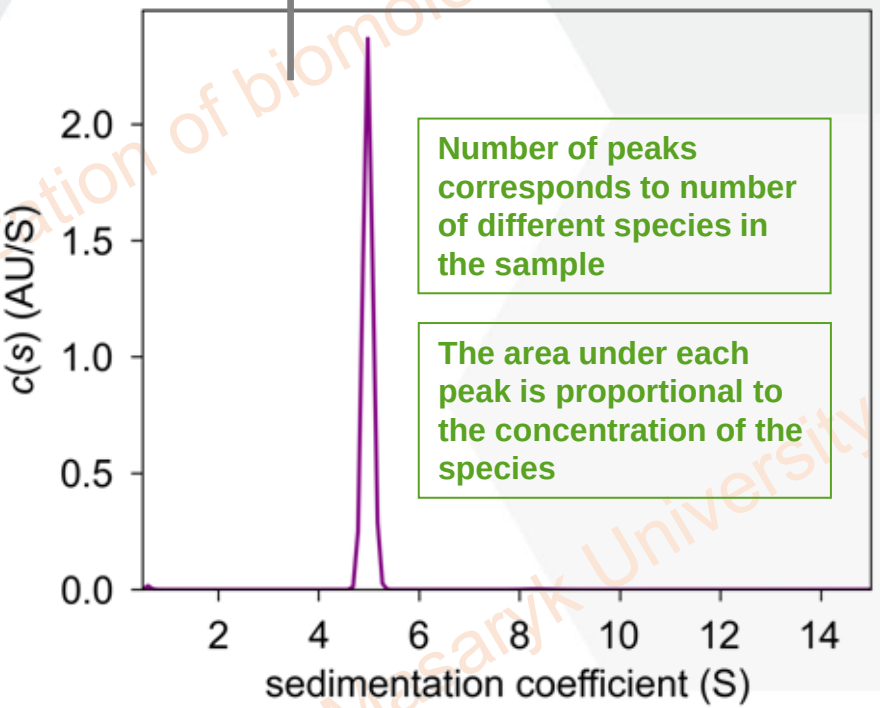
fitted data:



Residual graph showing goodness of the fit

result:

Distribution of sedimentation coefficients in the sample



Number of peaks corresponds to number of different species in the sample

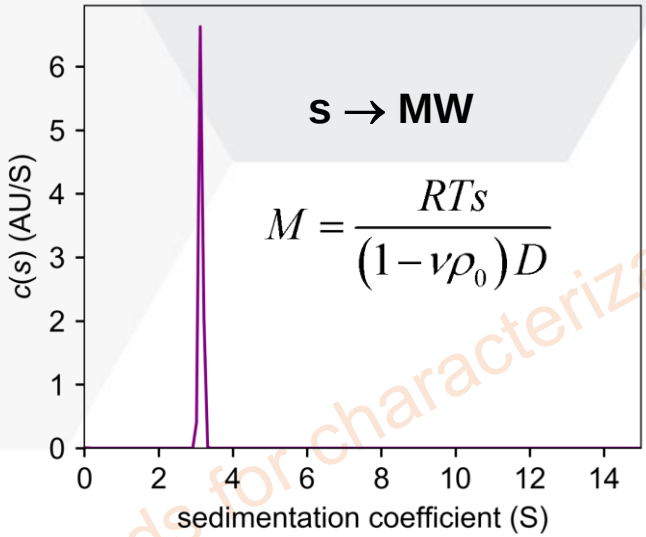
The area under each peak is proportional to the concentration of the species

→ Homogeneity

Molar mass determination

Sedimentation velocity (SV)

s → MW conversion using Svedberg equation:



Shape

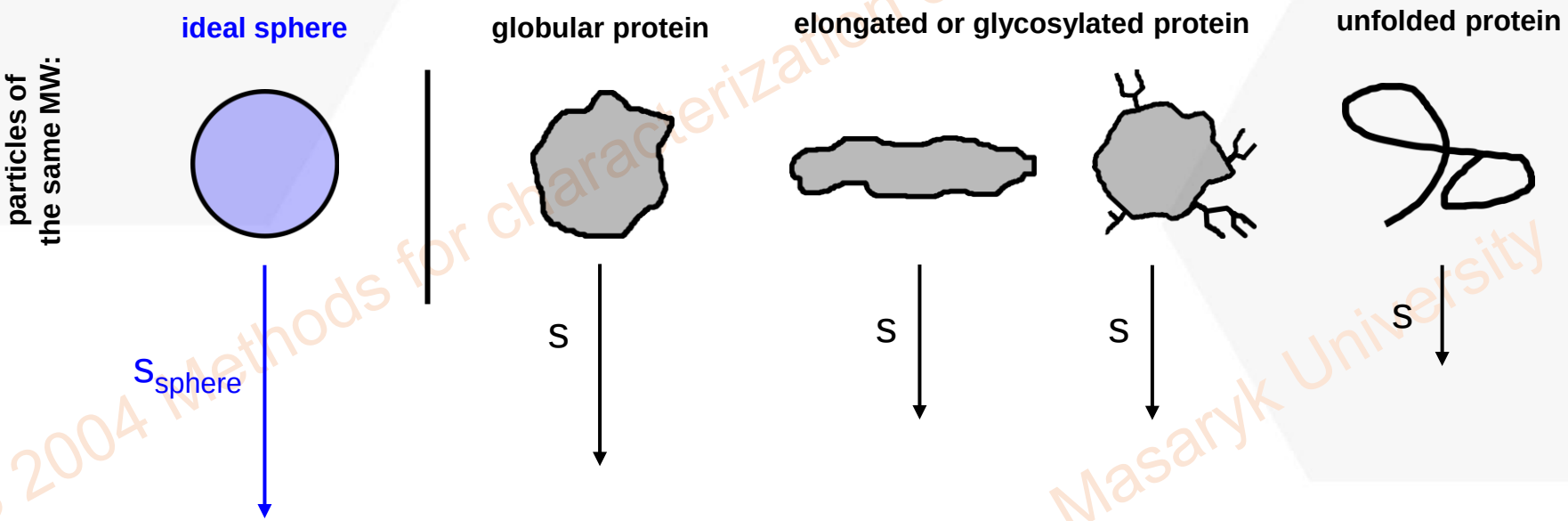
frictional ratio f/f_0:

(describes how much the particle differs in its shape from an ideal sphere)

$$f/f_0 = \frac{s_{sphere,20,w}}{s_{20,w}}$$

$$s_{sphere,20w} = 0.012 M^{2/3} \frac{(1-\bar{v}\rho)}{\bar{v}^{1/3}}$$

($\bar{v}$ – partial specific volume, ρ – density of solvent, M – molar mass)



f/f_0 = 1.0 | 1.2 - 1.3 | 1.5 - 1.8 | > 2

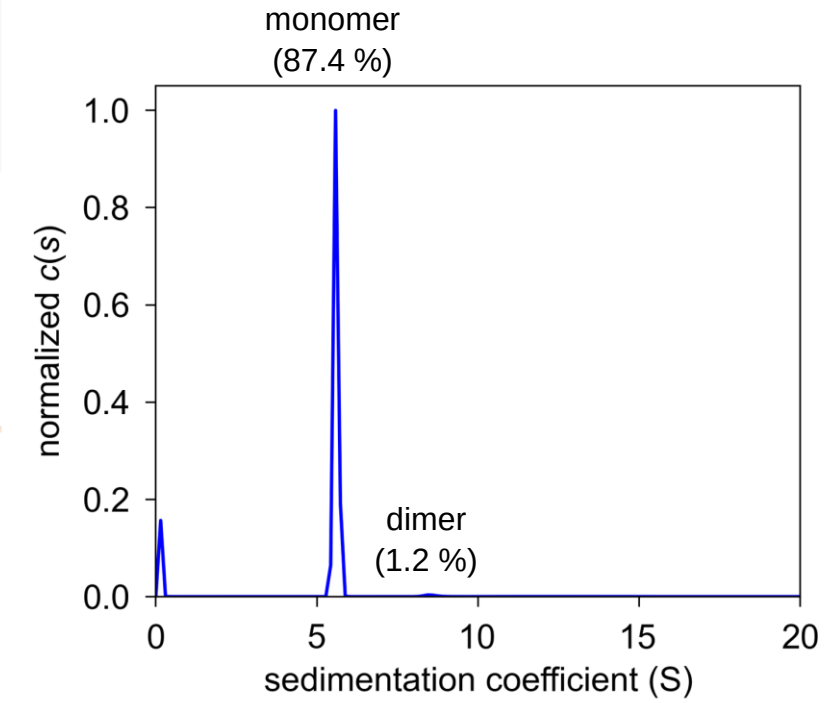
Folded or unfolded? Spherical or elongated/flexible?

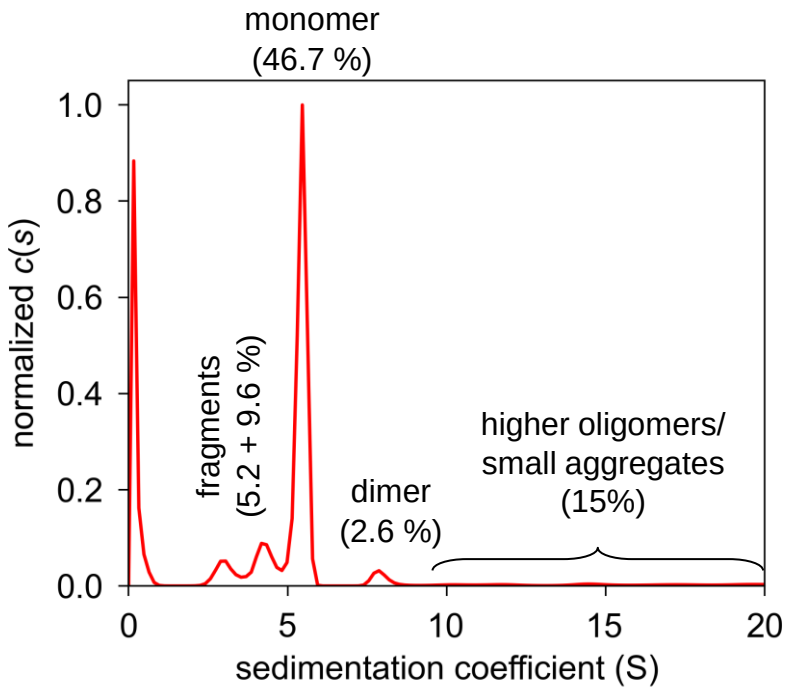
Detection and quantification of aggregates

Biopharma industry - mAb as therapeutical drugs

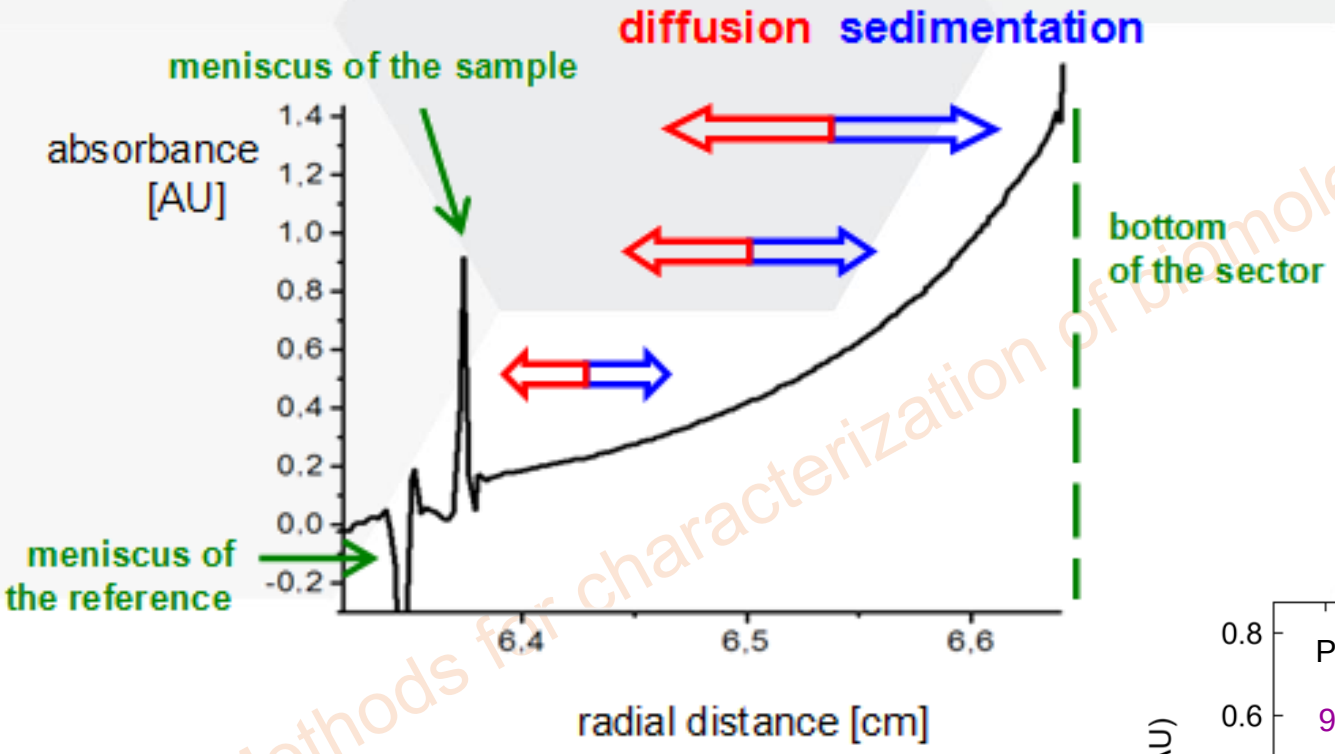
Aggregation ~ drug activity
immunogenicity
pharmacokinetics, pharmacodynamics

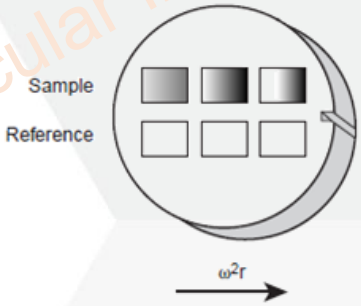
→ important to detect aggregates during mAb production, formulation and storage





Sedimentation equilibrium





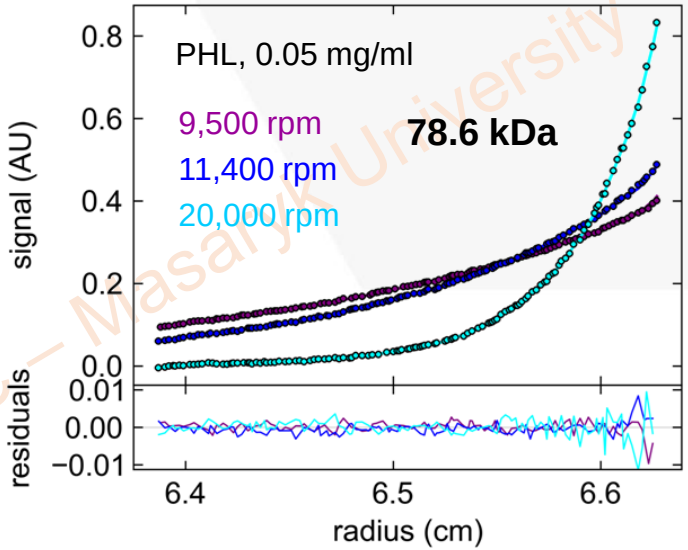
time to equilibrium
~ column height^2

exponential shape of curve:

one-component system:

$$c(r) = c(r_0)\exp\left(M(1-\bar{v}\rho)\omega^2(r^2 - r_0^{\,2})/2RT\right)$$

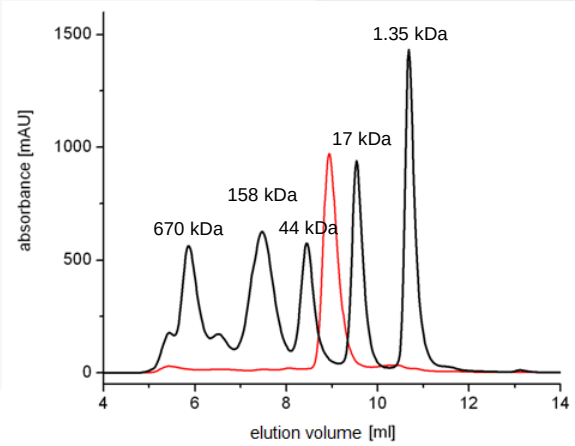
c(r) – concentration of molecule at radial distance *r*, $c(r_0)$ – concentration of molecule at reference position r_0



Molar mass determination

Protein AFL – comparison of SEC and AUC results

SIZE-EXCLUSION CHROMATOGRAPHY



by SEC: **30.4 kDa**

monomer (from amino acid sequence): **34.6 kDa**

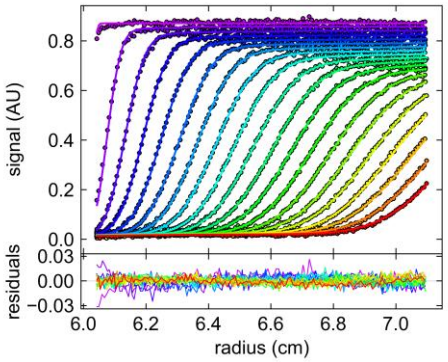
MONOMER!

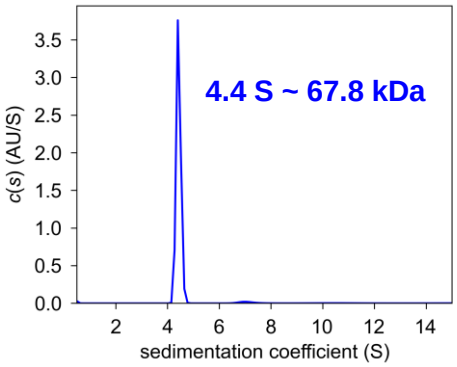
BAD RESULTS!

Interaction with matrix

ANALYTICAL ULTRACENTRIFUGATION

Sedimentation velocity:

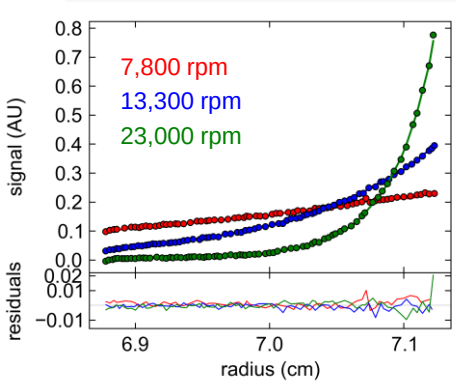




Sedimentation equilibrium:

by SE-AUC: **68.0 kDa**

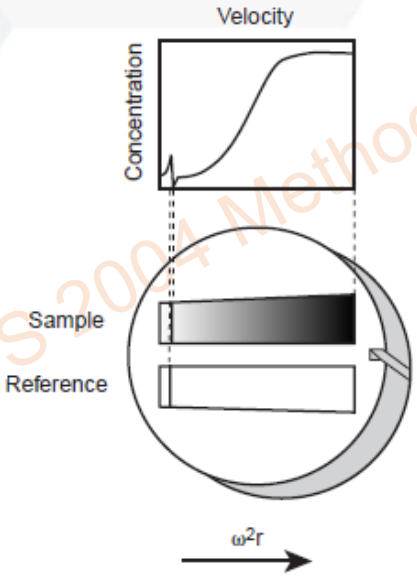
DIMER!



Overview of AUC techniques

SEDIMENTATION VELOCITY (SV)

- hydrodynamic technique
- sensitive to the **mass** and **shape** of the particle
- performed at high rotor speeds
- time: few hours
- determination of **sediment. coefficient** (then s → MW)

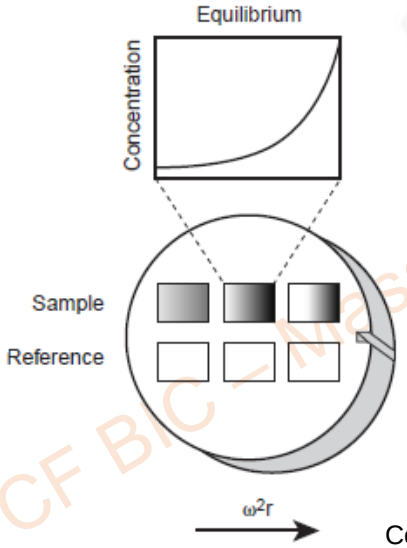


Applications:

- Homogeneity
- Size and shape
- Molar mass
- Biomolecular interactions

SEDIMENTATION EQUILIBRIUM (SE)

- thermodynamic technique
- sensitive to the **mass** (but not the shape)
- performed at lower rotor speeds (several speeds)
- time: few days
- determination of **molar mass** directly



Applications:

- Molar mass
- Biomolecular interactions

Cole, 1999

Thank you for your attention

Sample requirements

- **the requirements dependent on the nature of experiments and a particular sample of interest (some requirements „negotiable“)**
- **purity:** as pure as possible (>95 % for SE experiment)
- both sample and solvent necessary - sample should be equilibrated into experimental buffer by dialysis, SEC or spin columns (buffer matching most critical for IF optics)
- **buffers** (usually 10-20 mM): should not absorb at wavelength where the sample is measured (e.g. phosphate buffers work well for ABS optics, Tris and Hepes are tolerable at low concentrations for 280 nm)
- **ionic strength** (ideally 100-200 mM NaCl): needed to prevent electrostatic interactions (that would affect sedimenation rate and underestimate determined sedimentation coefficient)
- if possible, substances generating density gradients (glycerol, sucrose, CsCl) should be avoided
- reductants (DTT, βME) should be used at lowest possible concentrations
- **sample concentrations:** dependent on absorbtivity and type of the sample, but usually not higher than 1-2 mg/ml
- **recommended volumes and concentrations for SV/SE experiments** (recommended to measure at least 3 different concentrations (to see eventual reversible interactions or sample non-ideality)

SV experiment:	450 ul of both sample and reference buffer
	OD 0.1-1.0 (ABS), loading concentration > 0.1 mg/ml (IF)
SE experiment:	150 ul of both sample and reference buffer
	optimal loading OD 0.1-0.4 (ABS)