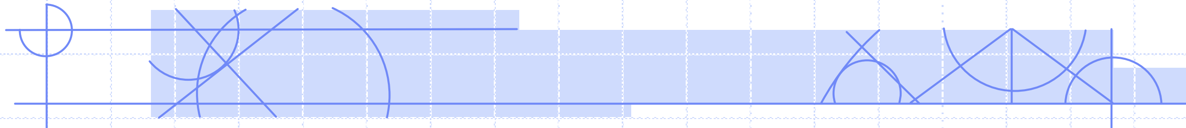
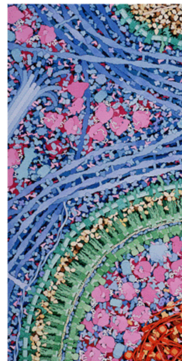
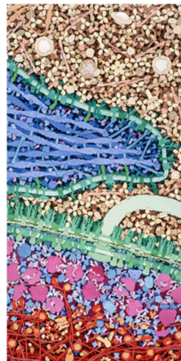
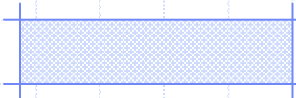
Macromolecules in solution



Nonideality is where biology occurs

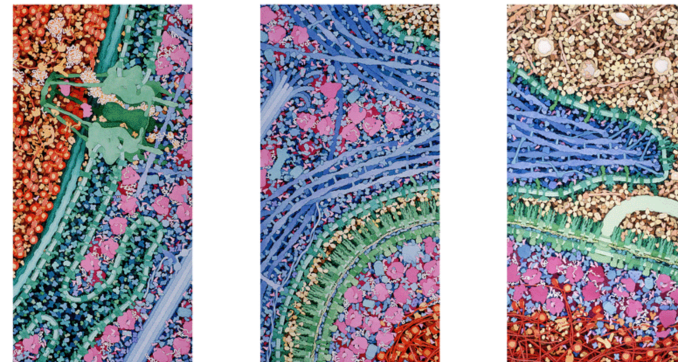



Watercolor of macrophage, blood and bacterium 2,000,000x magnification. David Goodsell



Macromolecules in solution

- Sedimentation equilibrium
- Osmotic pressure and solutions
- Nonideality and osmotic pressure
  - Entropic effects
  - Enthalpic effects

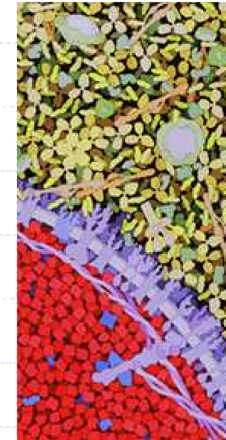


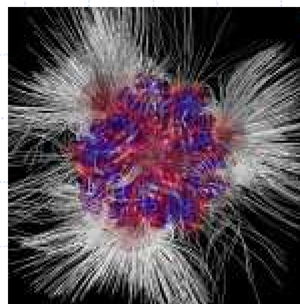
Watercolor of macrophage, blood and bacterium 2,000,000x magnification. David Goodsell

Solutions

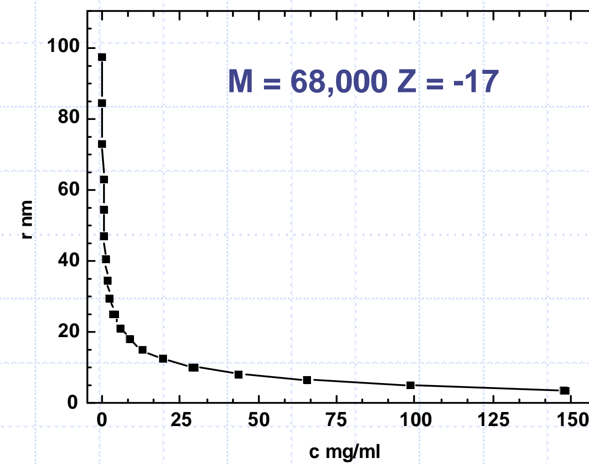
- Distance 100 – 1 nm
- Collisions $10^5 \rightarrow 10^{11}$ /s
- Rotation ~$10^7 - 10^9$ rad^2/s
- Proximity energies 1 nm

David Goodsell

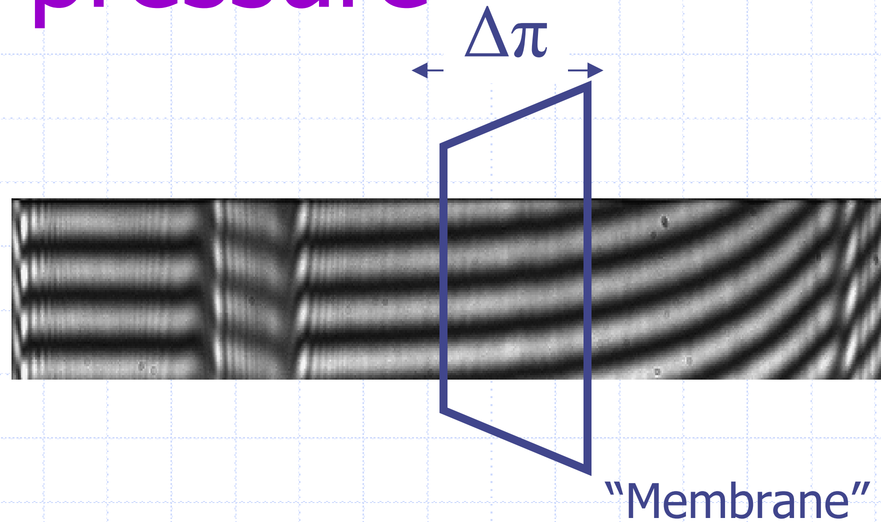




Eric Diluccio



Sedimentation equilibrium & Osmotic pressure



Osmotic pressure increment of component across the membrane

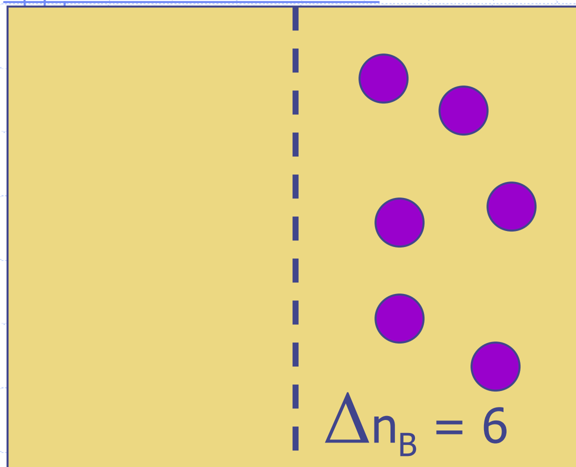
$$\Delta\pi V = \Delta n_B RT$$

$$\Delta n_B / V = \Delta C_B = \Delta c_B / M_B$$

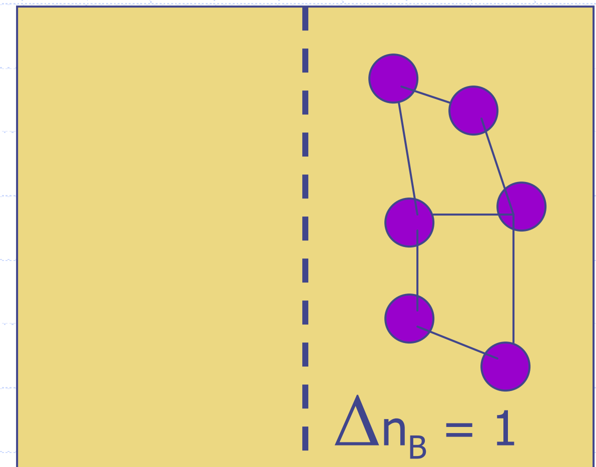
$$\Delta\pi / \Delta c_B = RT / M_B$$

Osmotic pressure

- Independence of motion
- Phases
- Components

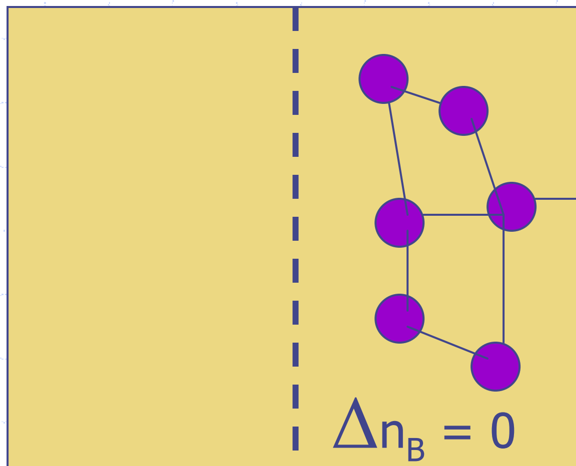


$$\Delta\pi V = \Delta n_B RT$$



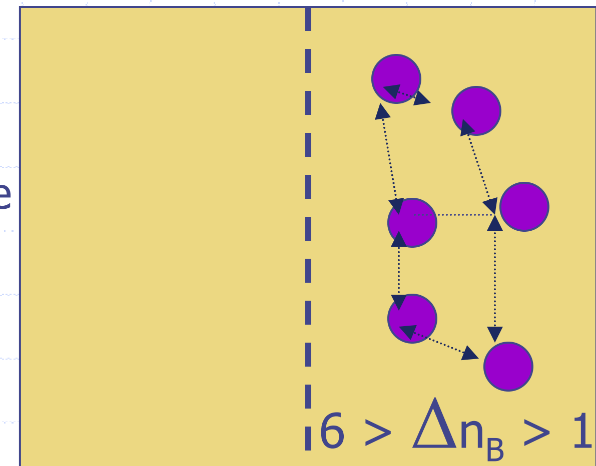
$$\Delta n_B/V = \Delta C_B = \Delta c_B/M_B$$

$$\Delta\pi/\Delta c_B = RT/M_B$$



$$1/M_B = C_B/c_B$$

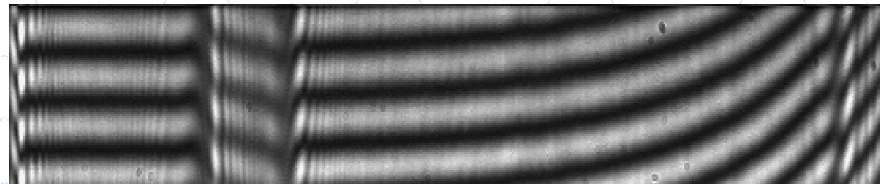
c_B g/l, C_B moles/l, M_B g/mole



Sedimentation Equilibrium
A balance of energies

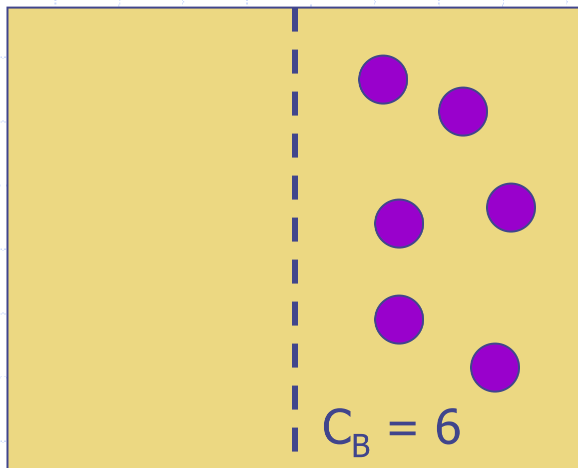
Gravitational potential gradient

$$= M_b g = M_b \omega^2 r \equiv -M_b \omega^2 d \frac{r^2}{2} \Longrightarrow$$



$$\Longleftarrow \frac{dG}{dC} \equiv \mu = -RT\, d \ln C \Longleftarrow$$

Chemical potential gradient

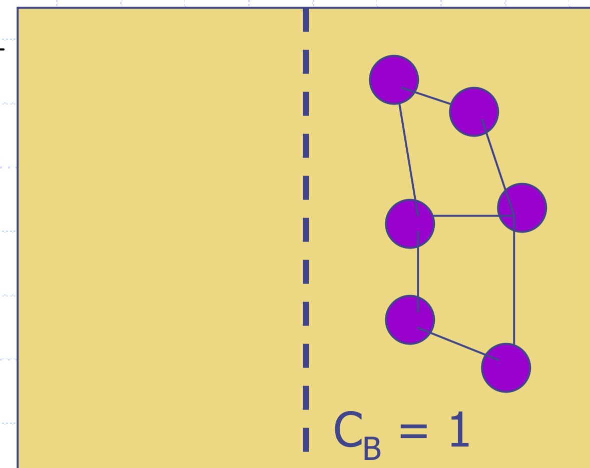


$$-\mu_B = RT \ln C_B = M_b \omega^2 d \frac{r^2}{2}$$

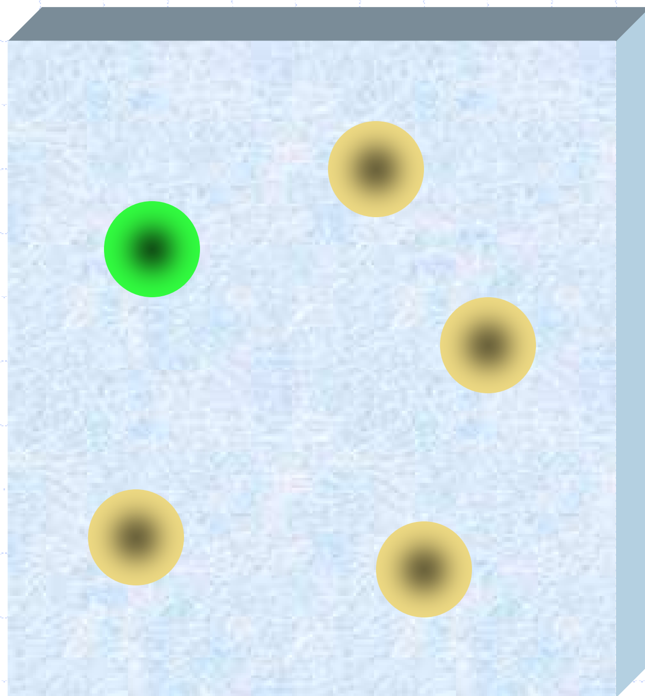
$\leftarrow \mu_B = -RT \ln 6$

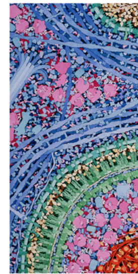
$\mu_B = -RT \ln 1 \rightarrow$

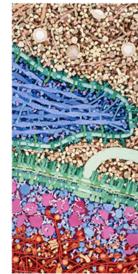
$c_B/M_B = C_B$



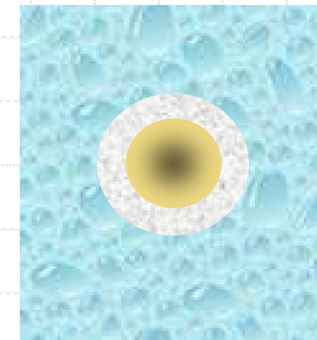
Nonideality: unequal interaction enthalpies



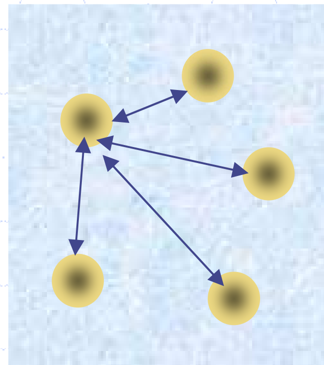




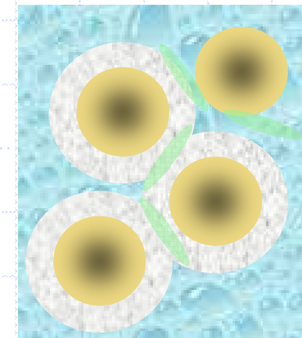
Solute-solvent



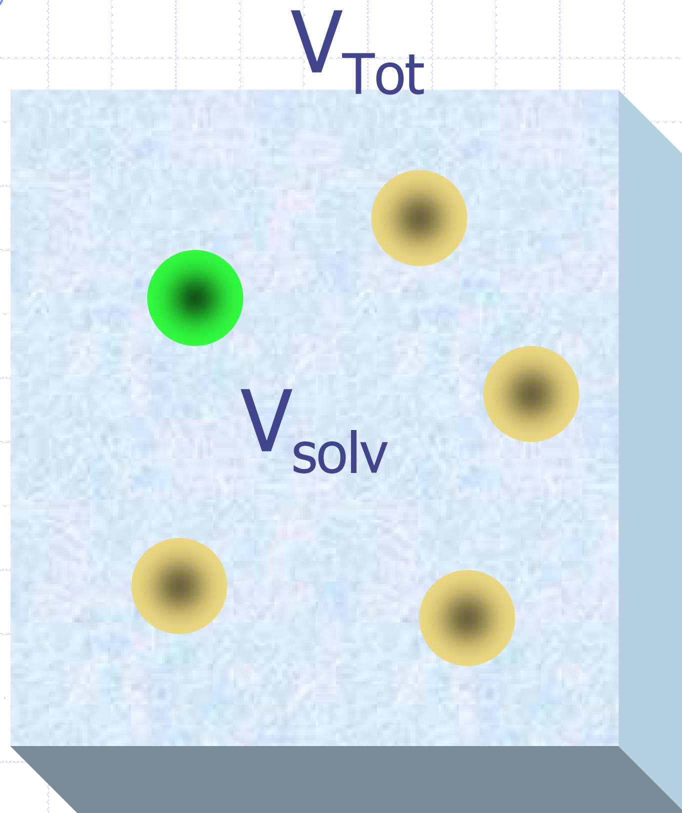
Solute-solute



Solvent-solvent



Entropic nonideality



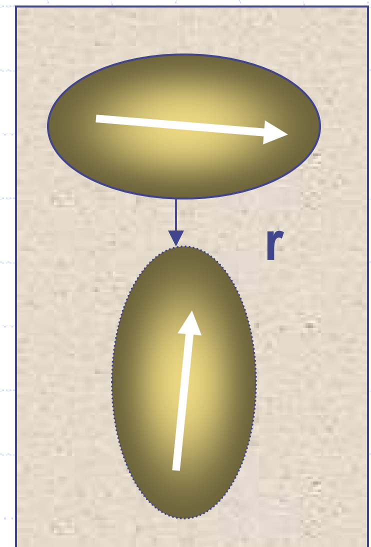
- $\Delta\pi\ V_{solv} = \Delta n_B RT$
- $\Delta\pi / \Delta c_B = RT\ (1/M_B + Bc_B\ ...)$
- $\Delta C_B = \Delta n_B / V_{solv} = c_B / M_B$
- $\Delta\pi$ gives the *apparent* Δ # particles/gram
- As $V_{solv}\downarrow$, $\Delta n_B/V\uparrow$, $\Delta\pi/\Delta c_B\uparrow$ $B > 0$

Enthalpic nonideality Proximity energies

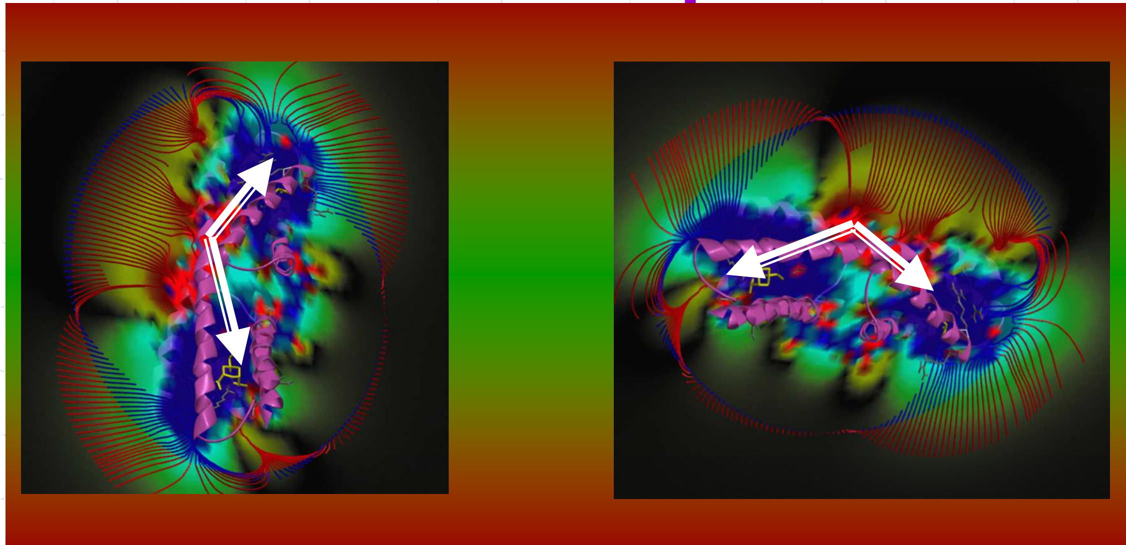
Type	X_1	X_2	n	Dependence[a]
Charge-charge	Q	Q	1	Γ
Charge-dipole	Q	μ	2	Γ, Θ
Dipole-dipole	μ	μ	3	Γ, Θ
Charge-induced dipole	Q	α	4	Θ
H-bond	E	E	4	Θ
Dipole-induced dipole	μ	α	5	Θ
Dispersion	α	α	6	Not D
VdW	-	-	12	Not D

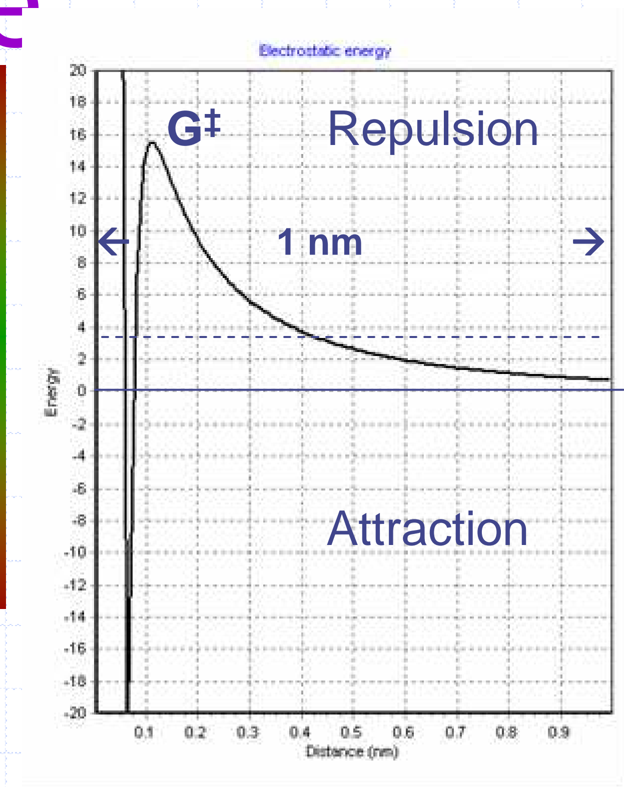
[a] Γ = ionic strength dependence, Θ = angular dependence

$$U = \frac{X_1 X_2}{D r^n}$$



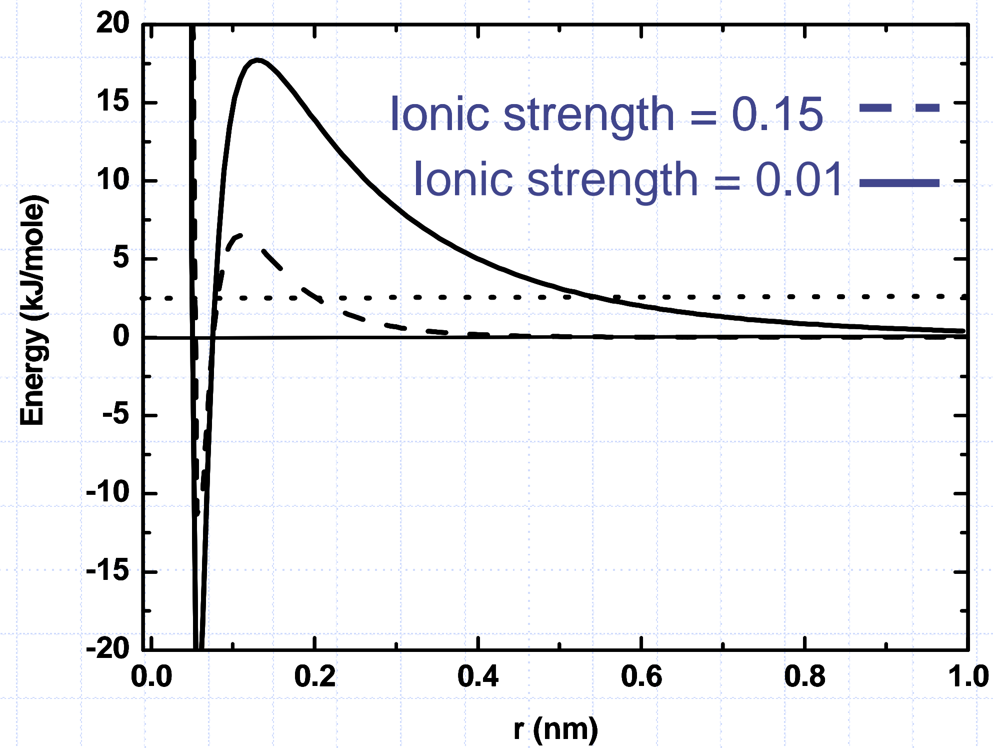
Distance dependence



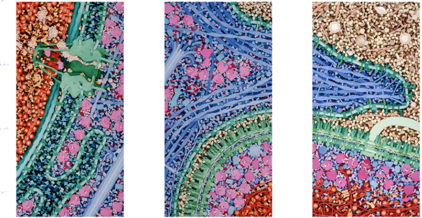


- May be positive or negative
- Orientation dependent
- Solvent dependent
- Kinetic control- activation energy

Ionic strength effect



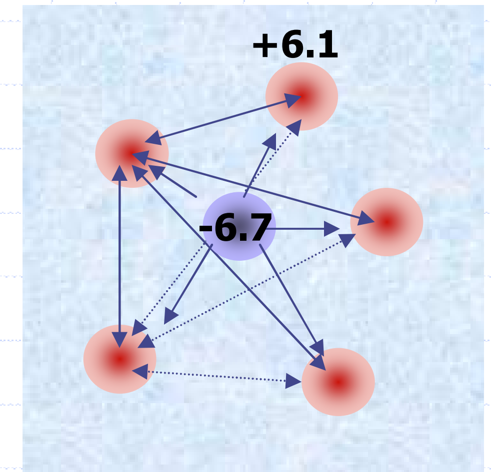
Effects of weak forces on non-ideality
$a_B/c_B \equiv \gamma = \pi/c_B = M/M_{app}$



	Lower M_{app}	
Repulsion		Total (dashed), Charge (dashed), Size, Size (dashed)
γ 1.0		Ideal
Attraction		Total, Charge
	Higher M_{app}	

2.0, 1.0, 0.0; 0 — 100

C_B (mg/ml)



$$\text{spheres}: \frac{16\pi N_A r^3}{3M_B^2} \qquad \text{for rods}: \frac{N_A \pi d L^2}{4M_B^2}$$

$$\text{Charge}: \frac{Z_1 Z_2 \bar{v}_A}{4m_3 M_B M_B}$$

Sedimentation

- General considerations
- Experiment design
- Data interpretation

An AUC experiment consists of…

- **The setup**
  - **Rotor**
  - **Cells**
    - **Centerpieces**
  - **Optical systems**
    - **Windows**
- **Method**
  - **Sample concentration range**
  - **Temperature**
  - **Rotor speeds**
  - **Number of scans**
  - **Delay before scans**
  - **Interval between scan**
- **Waiting**
  - **For pressure**
  - **For temperature**

Compatibility with sample and method

Optimizing information content

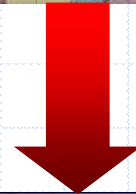
Always with sedimentation velocity

An AUC experiment consists of…

- **Analysis**
  - **Velocity: Size distribution** — Sedanal, Sedfit, Dcdt+
  - **Velocity: Discrete species** — Sedphat, Svedberg
  - **Equilibrium: Thermodynamics** — HeteroAnalysis, Nonlin, UltraScan
- **Interpretation**
  - **Solvent properties** — Measure or calculate; Sednterp, Sednterp2
    - **Density, viscosity**
    - **pH**
    - **Ionic strength**
  - **Solute properties**
    - **Buoyancy factor**
    - **Signal → concentration conversion**
    - **Size, asymmetry**

What do you want to know?

- Size distribution
- Stoichiometry- single component
- Reaction reversibility
- Stoichiometry and energetics of a self association
- Stoichiometry and energetics of a hetero association



What’s in the sample?

- What kind of macromolecules are we dealing with?
- What is in the solvent?
- How much sample do you have
  - Or get your hands on?
- What awful behavior does your molecule exhibit that you are reluctant to tell me about?
- How will you react if the sedimentation results don’t match your working hypothesis…
  - Or your delusional molecular fantasy?
- What are going to do to me if it gets sucked into the XLI vacuum system?

Proteins- general

- What is the amino acid composition?
  - Is it highly charged and small?
  - Globular of fibrous?
- Is it conjugated?
  - With what?
  - How much?
- Absorbance characteristics?
  - Fluorescence characteristics?
- Soluble? In what?
  - Be alert for the phrase "it loses activity if…"
- Is it alone, or did it bring its buddies with it?
  - How is the sample purified?
  - Is GPC part of the purification protocol?
  - What tests for purity are used?

v-bar
frictional coefficient

M, v-bar
frictional coefficient

Which detector to use

density, v-bar
aggregation

Expectations

Proteins- self association

- Is it known (expected) to self associate?
  - What is known about the association stoichiometry?
  - What is known about the strength of association?
- Is the self association ligand-linked?
  - What is the mass/association characteristics of the ligand?
  - Will the ligand interfere with any of the optical systems?
- What questions do you want answered by sedimentation?
  - E.g. reversibility of the reaction
    - Time scale of reversibility
  - Homogeneity of association
  - Effect of ligand on association
  - Strength and stoichiometry of association
  - Linkage energy between ligand and protein association

Molecular weight &
Concentration range
Optical system

Molecular weight
Number of components
Optical system

Proteins- hetero association

- All of the questions above must be asked about each component.
- Each component needs to be characterized individually
- Are they known (expected) to associate?
  - What is known about the association stoichiometry?
  - What is known about the strength of association?
  - Do the components self associate?
- Is the association ligand-linked?
  - What is the mass/association characteristics of the ligand?
  - Will the ligand interfere with any of the optical systems?

Polysaccharides

- What is the composition?
  - Is it charged or neutral?
  - Does it have any chromophores?
- Be prepared for severe hydrodynamic nonideality.
  - Characteristics are best determined by extrapolation to [C]→ 0
- If charged, be prepared for severe thermodynamic nonideality, too

Optical systems
Expectations

Expectations

Nucleic acids

- Be prepared for severe hydrodynamic and thermodynamic nonideality.
  - Characteristics are best determined by extrapolation to [C]→ 0
- The partial specific volume of highly charged molecules depends on the solvent composition
  - Best off determining vbar if possible

Expectations

M, vbar

Expectations

Others kinds of molecules

vbar
Expectations

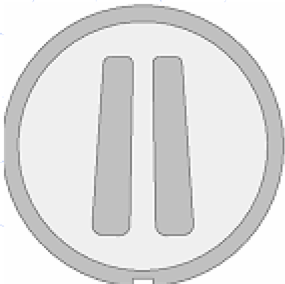
- Nearly any system will benefit from characterization by sedimentation
- Hetero-associations (e.g. protein-DNA)
- Small molecules: drugs, ligands, gasses
  - Is it monomeric?
  - Can approximate vbar from composition/density
- Large aggregates: viruses, organelles

- Be fearless!!

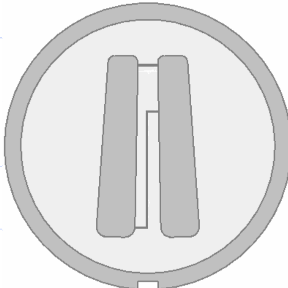
What is in the solvent?

- Compatibility with centerpiece
- Does it absorb UV?
  - BME, DTT, unreduced Triton X100
  - Nucleotides, flavones
- What is the solvent viscosity and density?
  - Salts and neutral molecules will affect density
  - PEG, glycerol affect viscosity strongly
- Will any of the solvent components sediment significantly?
  - Will the gradients matter biochemically?

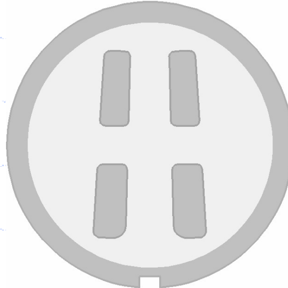
Centerpieces



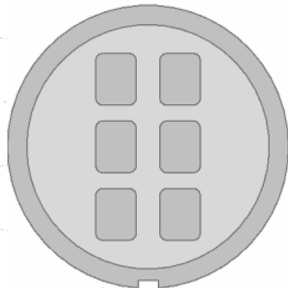
SedVel60K
SedVel50K



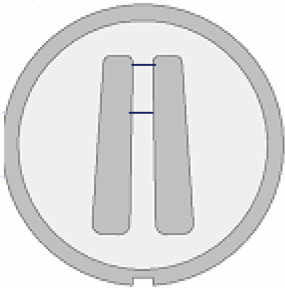
Meniscus matching



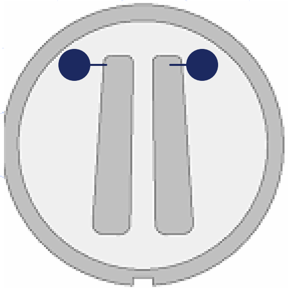
4-channel Velocity/Equilibrium



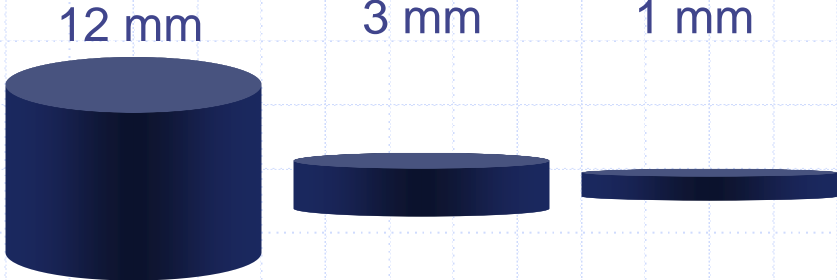
6-Channel Equilibrium



Synthetic boundary



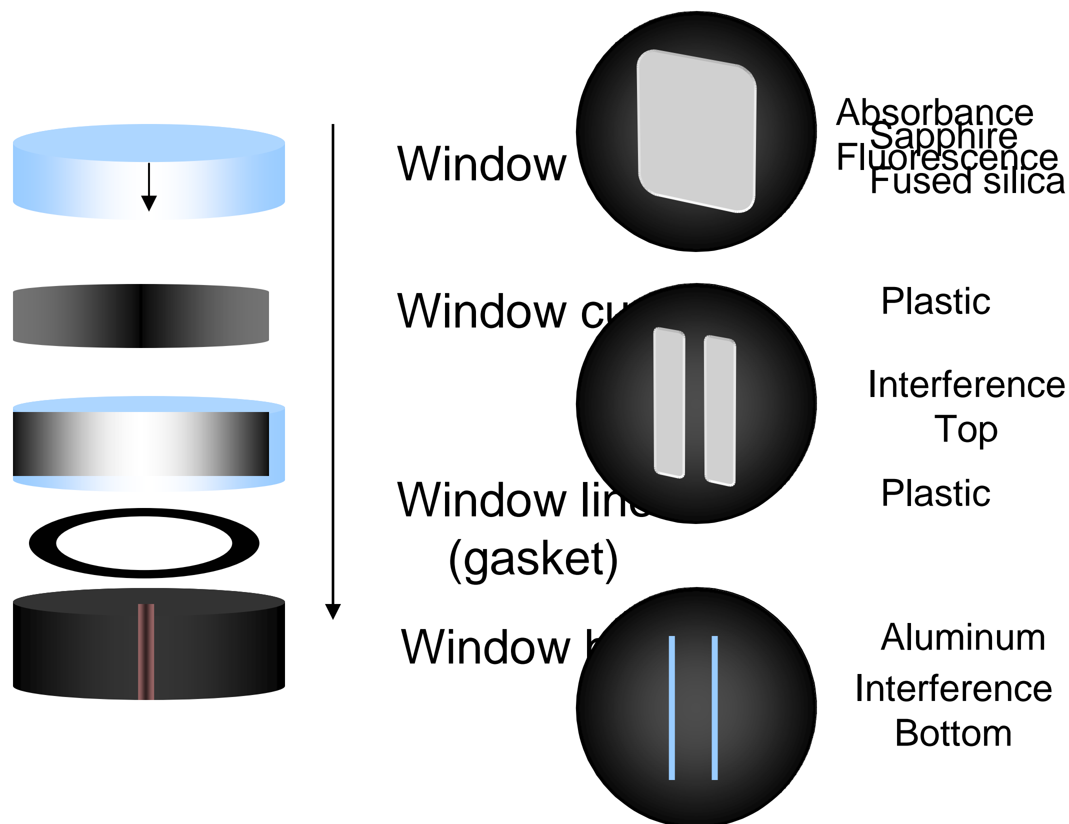
Band forming



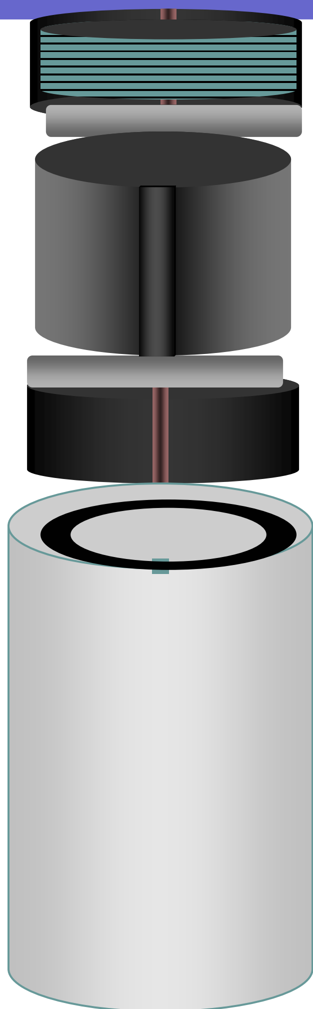
- Inspection and polishing

- Charcoal-filled Epon
- Aluminum-filled Epon
- Aluminum
- Titanium

Windows and holders



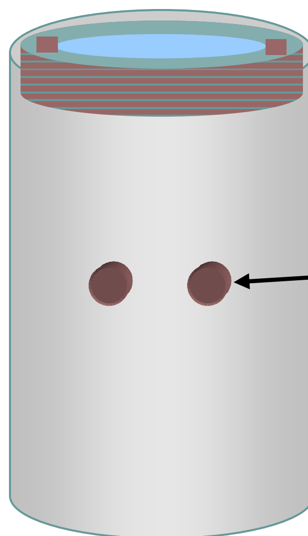
Cell assembly



Lube
- Screw ring
- Housing thread
- Rotor hole

- Torque to 130
- Torque slowly
- Torque 3 x
- If “chattering,” re-lube

- Re-torque after ΔT

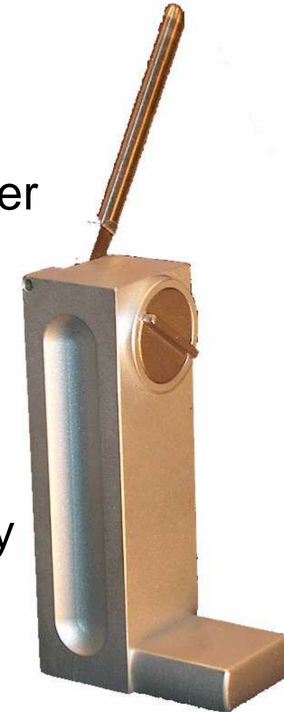


- Use softer gasket
- Teflon, neoprene
- Hex-head screws
- Torque screwdriver

Cell alignment in rotor

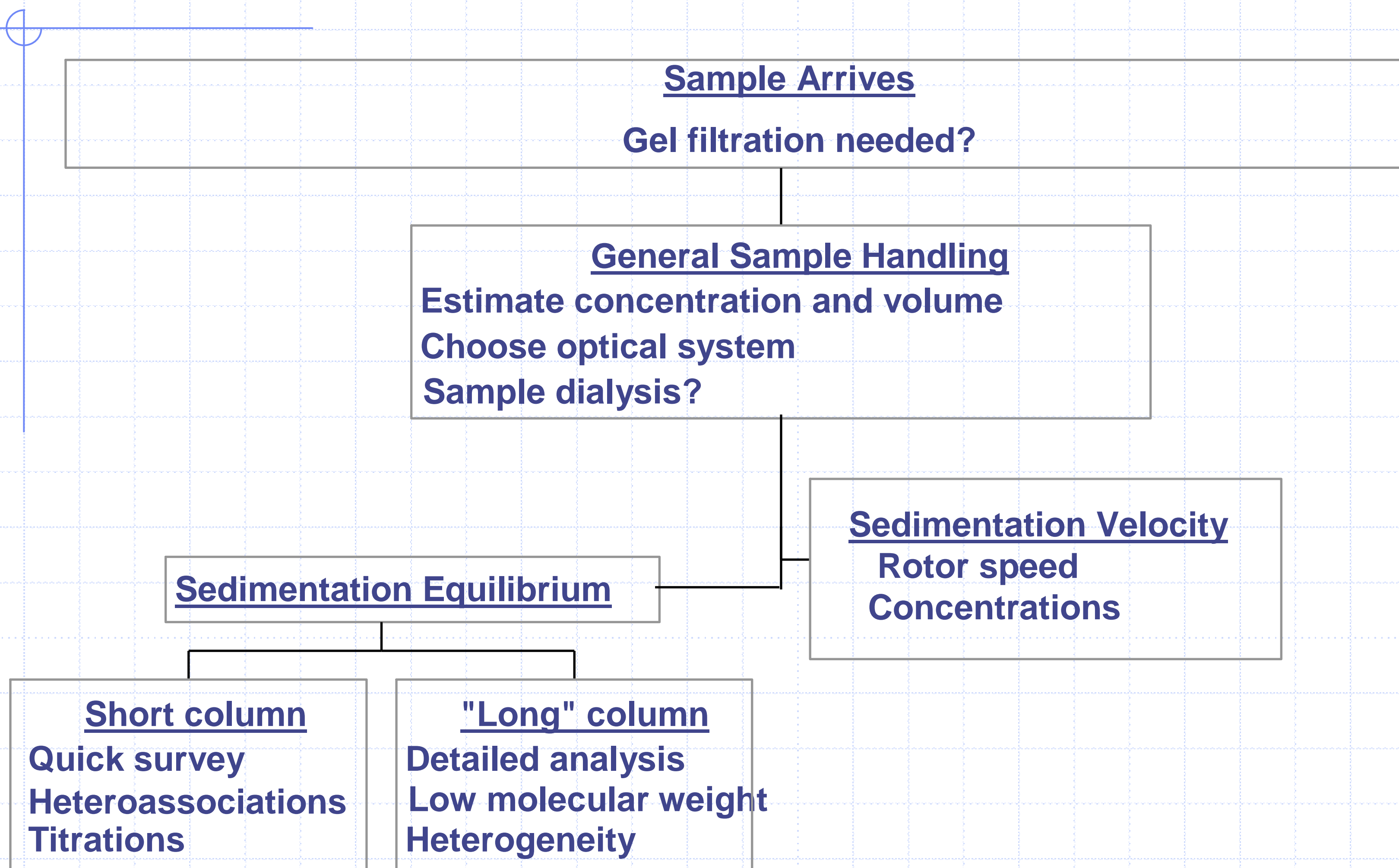
Gabrielson J, Randolph TW, Kendrick BS and Stoner MR (2007) "Sedimentation velocity analytical ultracentrifugation and SEDFIT/c(s): Limits of quantitation for a monoclonal antibody system" Anal. Biochem. 361:24-30.

- < ±0.2° to prevent false peaks
- Limits of visual detection
- Rely on accuracy of centerpiece
- Scribe lines mark cell housing center
- Want cell walls radially directed

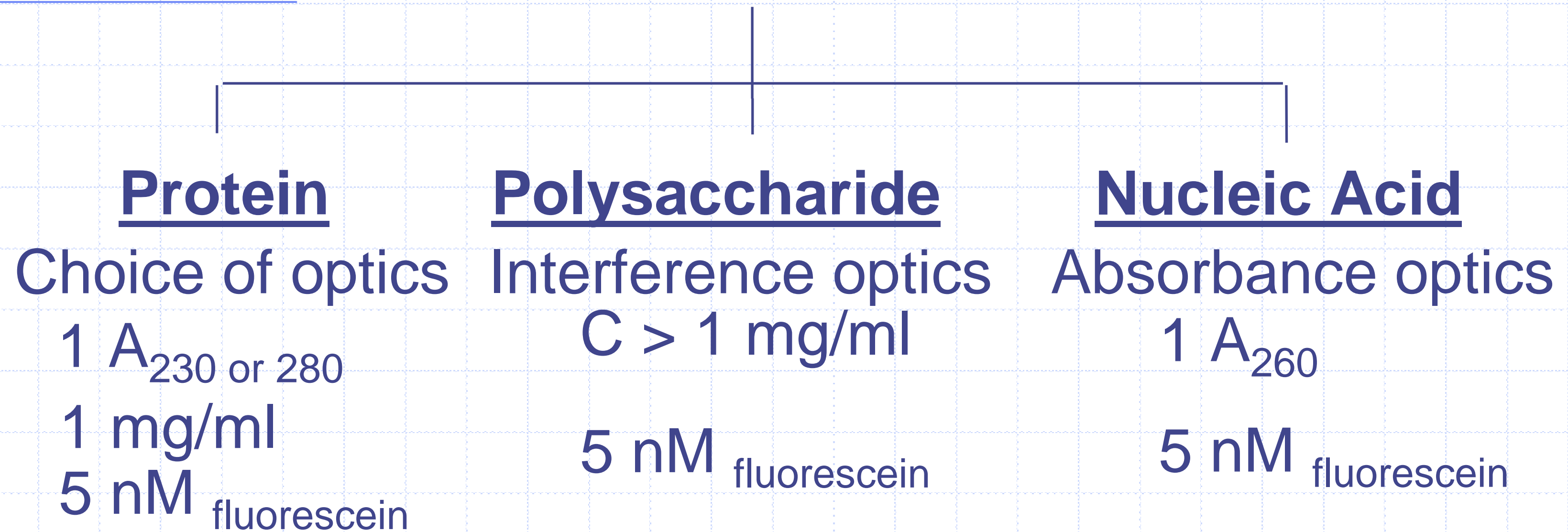


- Tool provides reproducibility
- Require accuracy
- Tool to test alignment

Sample Handling

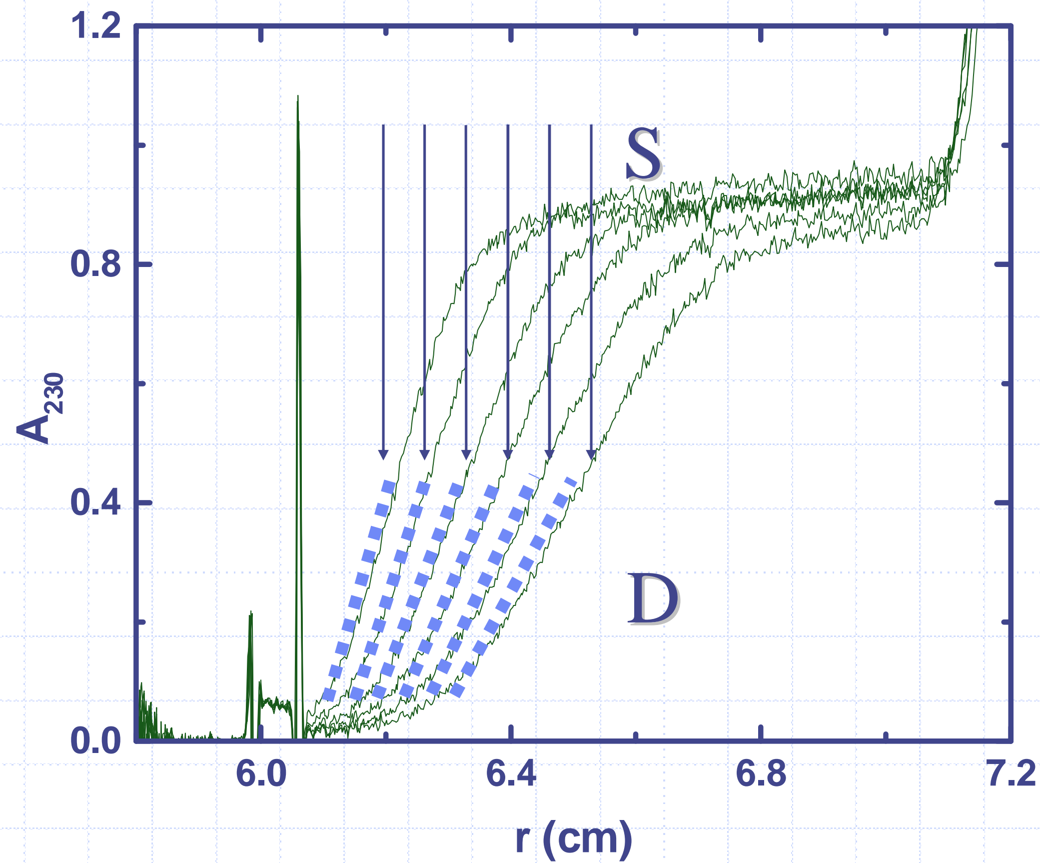


Optical system choices by sample type and desired initial concentrations



	Absorbance	Interference	Fluorescence
Sensitivity	0.1 OD	0.05 mg/ml	100 pM fluorescein
Range	2-3 logs	3-4 logs	6-8 logs
Precision	Good	Excellent	Good

Sedimentation velocity



Distance moved by s & D

For $s = 5 \times 10^{-13}$ s = v/a

At 60,000 rpm, $\omega^2 = 3.959 \times 10^7/s^2$

at 6.5 cm $\omega^2 r = 2.57 \times 10^8$ cm/s²

$v = 5 \times 10^{-13} * 2.57 \times 10^8$ cm/s²

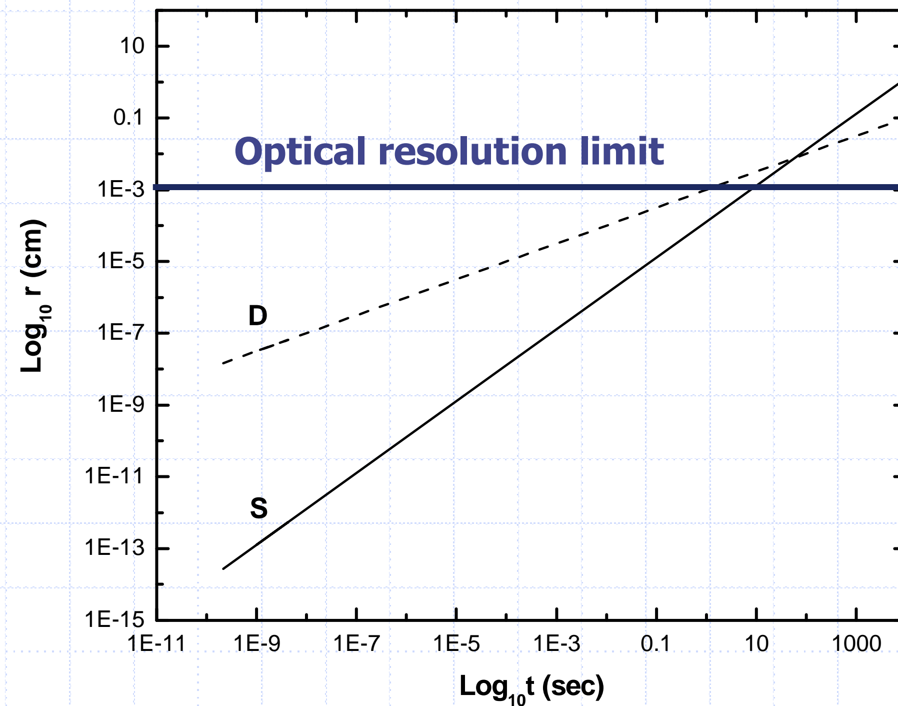
$v = 1.29 \times 10^{-4}$ cm/s or 1.29 μm/s

In 1 second, the molecule sediments ~1.29 μm

For $D = 5 \times 10^{-7}$ cm²/s

$\langle x \rangle = (2Dt)^{1/2}$

in 1 second $\langle x \rangle = 1 \times 10^{-3}$ cm diffuses 10 μm

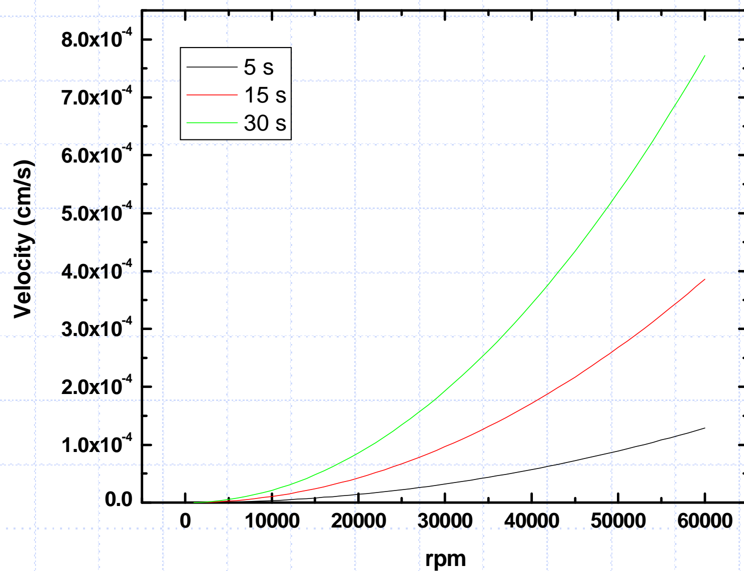


Choosing a rotor speed

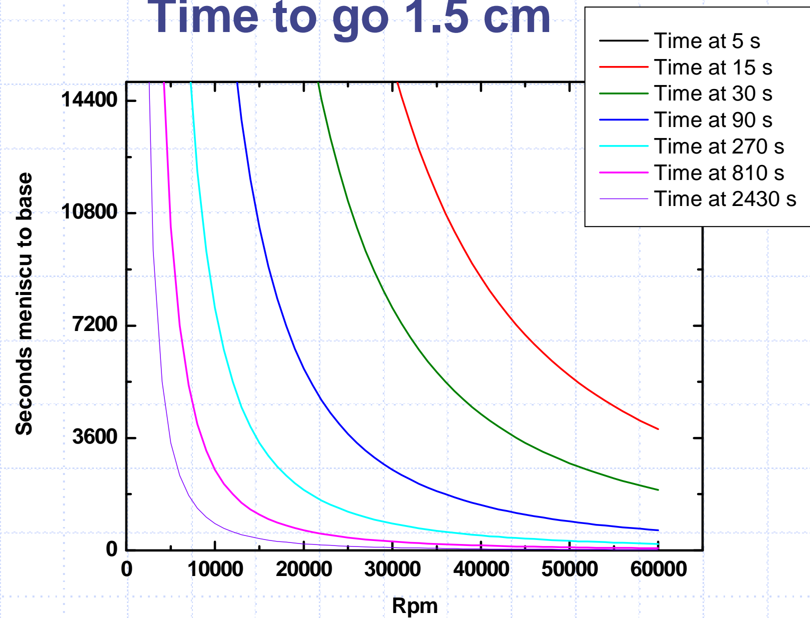
- Component resolution improves as ω^2
- Need sufficient scans for analysis
- Do not want boundaries to shift position significantly during a scan

Selecting rpm

Velocity versus rpm

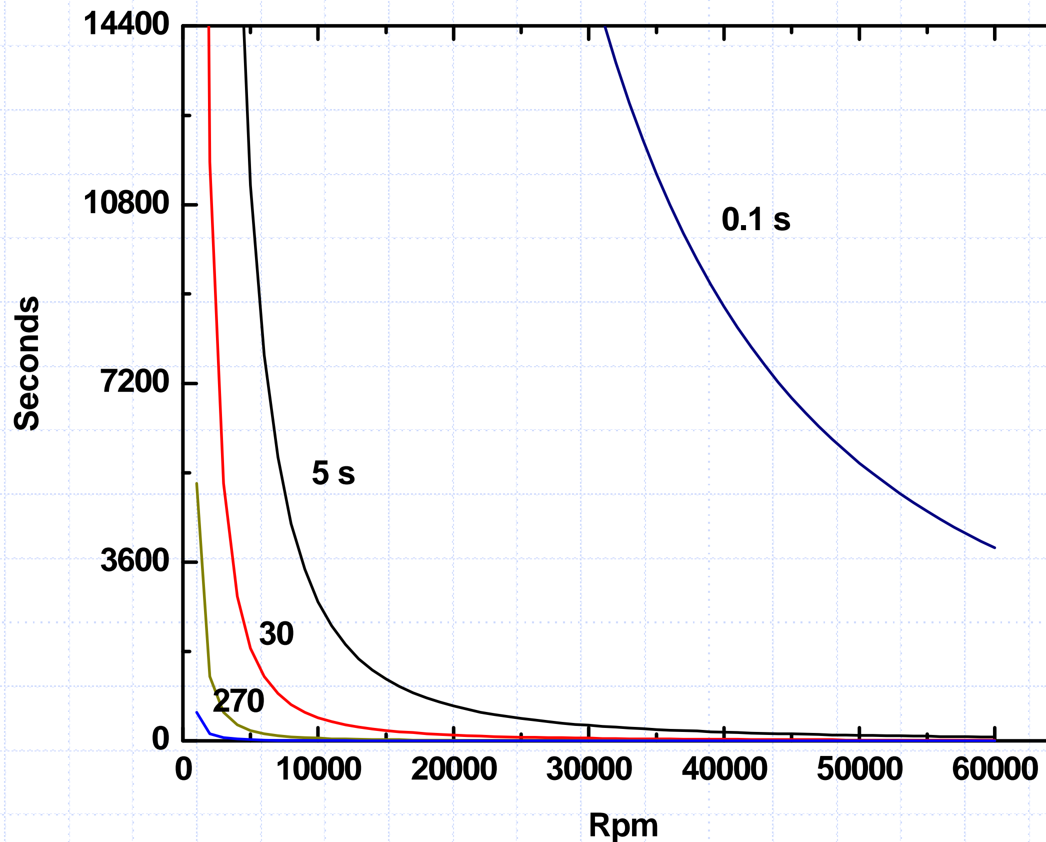


Time to go 1.5 cm



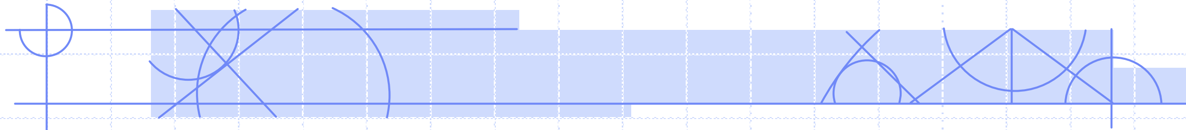
Keep optical resolution in mind

Time needed to move 100 μm

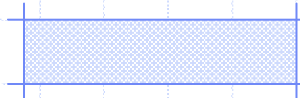


Sets the maximum resolution in s.

AUC Fundamentals



Data interpretation



Correcting for Buoyancy

- MB = M (1 - vr)
  - M is the anhydrous molecular weight
  - v is the partial specific volume
  - r is the solvent density
  - Approximate $M (1- \Sigma\delta_i v_i \rho)$
- Using neutral buoyancy
  - Set $1-v_i\rho = 0$ for a component
  - Useful with detergents

Determining ρ

- Depends on solvent component concentrations
- Depends on T
- Estimation from buffer concentration
  - Adjust to T using H_2O ρ(T)
  - Best if only one component in high concentration
- Measurement
  - Pycnometry, density meter, etc.

Partial Specific Volume

- Measure, but more frequently calculated
  - Depends on composition
  - Depends weakly on T
    - $v_T = v_{25} + 4.25 \times 10^{-4} (T - 25)$
  - Highly charged proteins need adjusting
    - v smaller than calculation
- Depends on solvent composition
  - Special care needed for high C components
  - Worked out for 6 M Gdn and 8 M Urea, some others

The buoyancy factor is $(d\rho/dc_2)_\mu$

- $(1-v\rho)$ is an approximation, only valid for a 2-component system
  - I.e. mass of solvent displaced is $M_2 v\rho$, leading to the buoyant force
- Gravitational field really acting on volume elements of the solution
  - correct term in place of $(1-v\rho)$ is $d\rho/dc_2$
- For dialysis equilibrium, $(d\rho/dc_2)_\mu$

When to worry about using (1-vρ)

- High concentration of co-solvent
  - e.g. 8 M Urea, 6 M GdHCl, 2 M NaCl
- Significant binding of a solvent component to the solute
  - e.g. Detergent with a protein
  - Cations with DNA or RNA
- The solvent used for determining v differs from the solvent used in the experiment
  - E.g. the v from Sednterp is for the anhydrous molecule, so M is the anhydrous molecular weight

Detergent-solubilized proteins

- Make the solvent density match the v of the detergent
  - Then M is the anhydrous molecular weight
- Tables of detergent partial specific volumes are available
- If possible, use D_2O to match density
  - Use of other solvent components (e.g. salt, sugar) to match density may be problematic
  - Due to preferential solvation effects
- Be careful if K is to be measured in detergents

So what does M refer to in a multi-component solution?

- Dissolve NaDNA in a solution of CsCl
  - What does M_2 refer to?
    - NaDNA or CsDNA or some in-between mixture?
- Depends c_2 when you measure $d\rho/dc_2$
  - If c_2 is measured as the g/ml of NaDNA added to a solution of CsCl, then M refers to NaDNA.

Correcting Viscosity

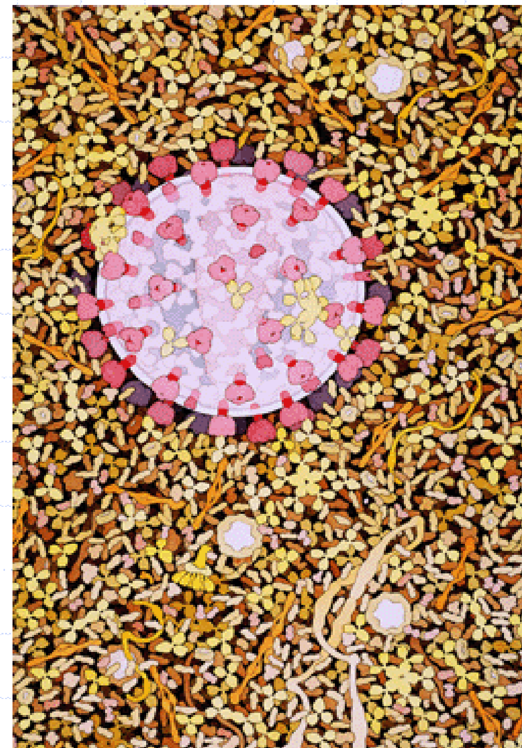
- η affects velocity directly
  - Affects time to reach equilibrium
- η depends on T and composition
  - η decreases ~4% per ^{o}C increase
  - Composition effect is small for salts
    - Organics (e.g. glycerol) can have large effect

Viscosities in complex solutions

- Arrhenius:

$$\log(\eta_{mix}) = \sum x_i \log(\eta_i)$$

  - x_i weight %
- Valid for ternary mixtures of mAbs
- Also valid for mAb:HSA mixtures
- Valid so long as preferential solvation not strong



David Goodsell

Based on work presented by William Galush at AAPS May 2011

Summary
Adjusting s for solvent effects

- Adjust to standard conditions
- Standard conditions are water at 20 ^{o}C
- s = M(1-vρ)/Naf and f = 6πηRS
  - v = v(T), weak function
  - ρ = ρ(ci,T), ci stronger than T
  - η = η(ci,T), both ci, T strong
- Use Sednterp

$$s_{20,w} = s\left[\frac{(1 - v_{20}\rho_{20,w})}{(1 - v_T\rho_{T,c_i})}\right]\left[\frac{\eta_{T,c_i}}{\eta_{20,w}}\right]$$

Ad hoc