Today’s class:

Centrifugation and electrophoresis

This lecture largely follows the parts of chapter 17 in the book ‘The Molecules of Life’ by Kuriyan et al.

What is centrifugation?

It is a method to measure velocity of molecules and separate/purify

Usage of centrifugation

- separating molecules having different densities by spinning them in solution around an axis (in a centrifuge rotor) at high speed.

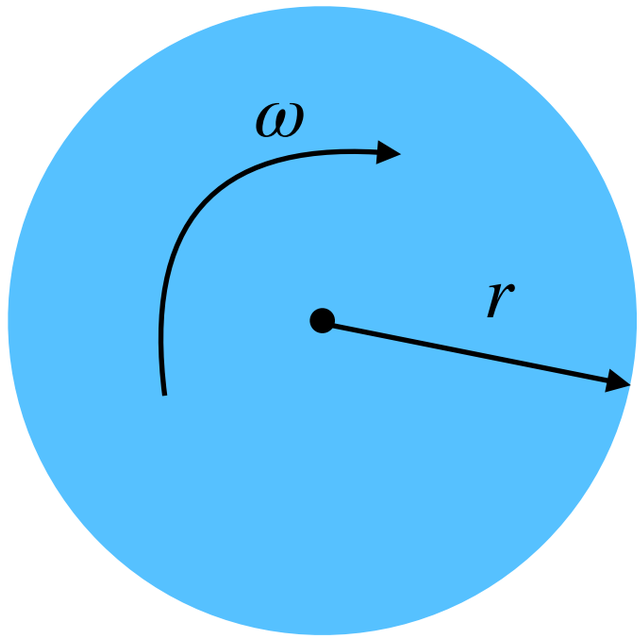
- To collect cells, to precipitate DNA, to purify virus particles, and to distinguish subtle differences in the conformation of molecules.

Centrifuge

A centrifuge is an instrument that spins a sample very rapidly, making the molecules in it move under the centrifugal force.

The principle of centrifugation

In a centrifuge, molecules in a solution are driven by centrifugal force which pushes the molecules away from the axis of rotation

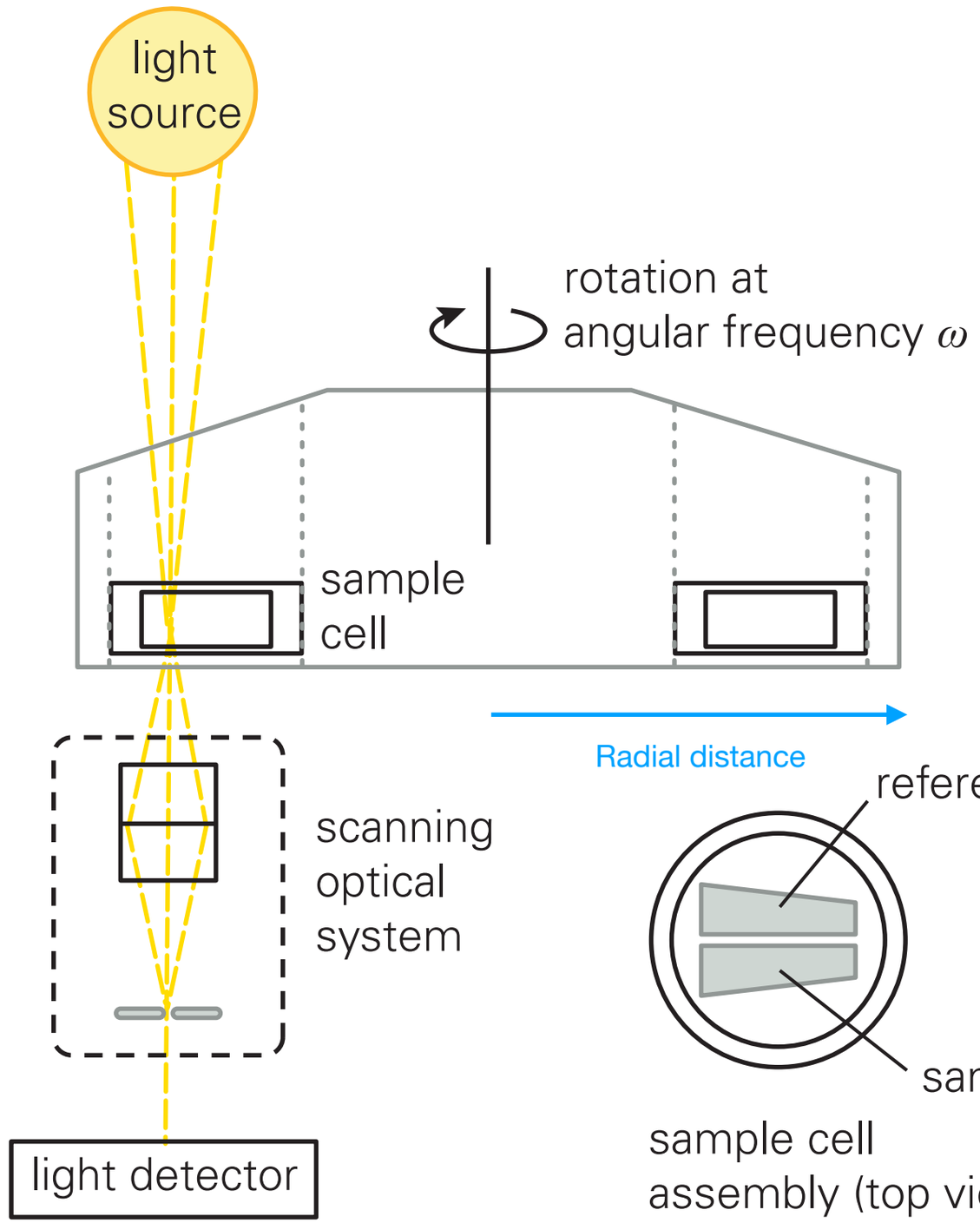


Centrifugal force, $f_{cen} \propto \omega^2 r$

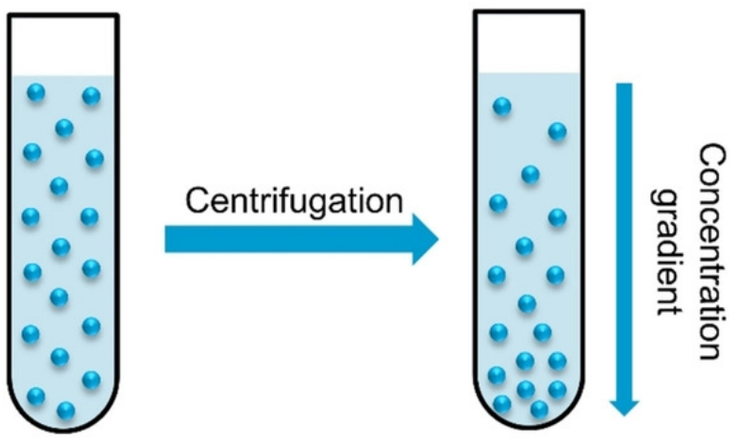
The molecules in the driven solution feel both centrifugal as well as Brownian force but the former is much stronger (typically $\sim 10^5$ times or more) than the latter

The set-up of a centrifuge

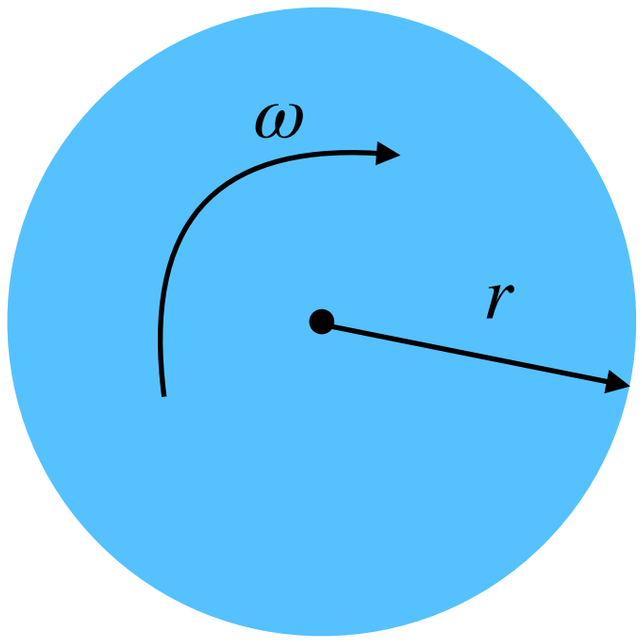
Figure 17.35 Diagram of a centrifuge. An analytical centrifuge spins samples inside a sample cell with windows so that the concentration can be measured as a function of position in the cell using absorption of light. The rotation rates in a laboratory centrifuge are as high as 40,000 rotations per minute.



Concentration gradient created by rotation can be tracked rapidly by following the absorbance of the solution



At what speed the molecules move in a centrifuge?



Centrifugal force, $f_{cen} \propto \omega^2 r$

For a molecule of mass m the centrifugal force becomes

$$f_{cen} = m\omega^2 r$$

But this is not the force the molecule experiences. Some of this force is cancelled by the centrifugal force on the solvent it displaces.

So the net centrifugal force on the molecule can be written as follows

$$f_{cen} = m_{eff}\omega^2 r$$

where $m_{eff} = m\left(1 - \frac{\rho_{solv}}{\rho_{mol}}\right)$

At what speed the molecules move in a centrifuge?

$$f_{cen} = m_{eff}\omega^2 r$$

The velocity of the molecule is set by a force balance of the centrifugal force and the friction force from the medium

$$f_{cen} = f v_{term}$$

where f = friction factor of the molecule

v_{term} = terminal velocity of the molecule

Thus, we get

$$v_{term} = \frac{m_{eff}\omega^2 r}{f}$$

Sedimentation coefficient

$$v_{term} = \frac{m_{eff}\omega^2 r}{f}$$

The terminal velocity of the molecule depends on the angular velocity, so it is usual to write it as

$$\frac{v_{term}}{\omega^2 r} = \frac{m_{eff}}{f}$$

The left hand side is called the sedimentation coefficient, S which depends on the nature of the molecule and medium

$$\Longrightarrow \quad S = \frac{m_{eff}}{6\pi\eta a}$$

Sedimentation coefficient is sensitive to density, size, shape, and even conformational changes of proteins

Sedimentation coefficient

The sedimentation coefficient, *S*, of a macromolecule is inversely related to the friction factor, *f*, of the macromolecule (see Equation 17.65). Because the friction factor depends on the shape, the value of *S* provides information on molecular shape.

Sedimentation coefficient and Svedberg constant

What is the dimension of sedimentation coefficient?

Dimension of sedimentation coefficient $= [T]$

What is the order of magnitude of typical sedimentation coefficients?

Let's assume a protein of mass 30 kDa and size 5 nm

$$\implies S \approx 5 \times 10^{-13} \text{ sec}$$

For convenience, it has been defined that: 10^{-13} sec $= 1$ Svedberg

This unit is named after Theodor Svedberg, who invented the high-speed ultracentrifuge for making such measurements.

Measuring sedimentation coefficient through terminal velocity

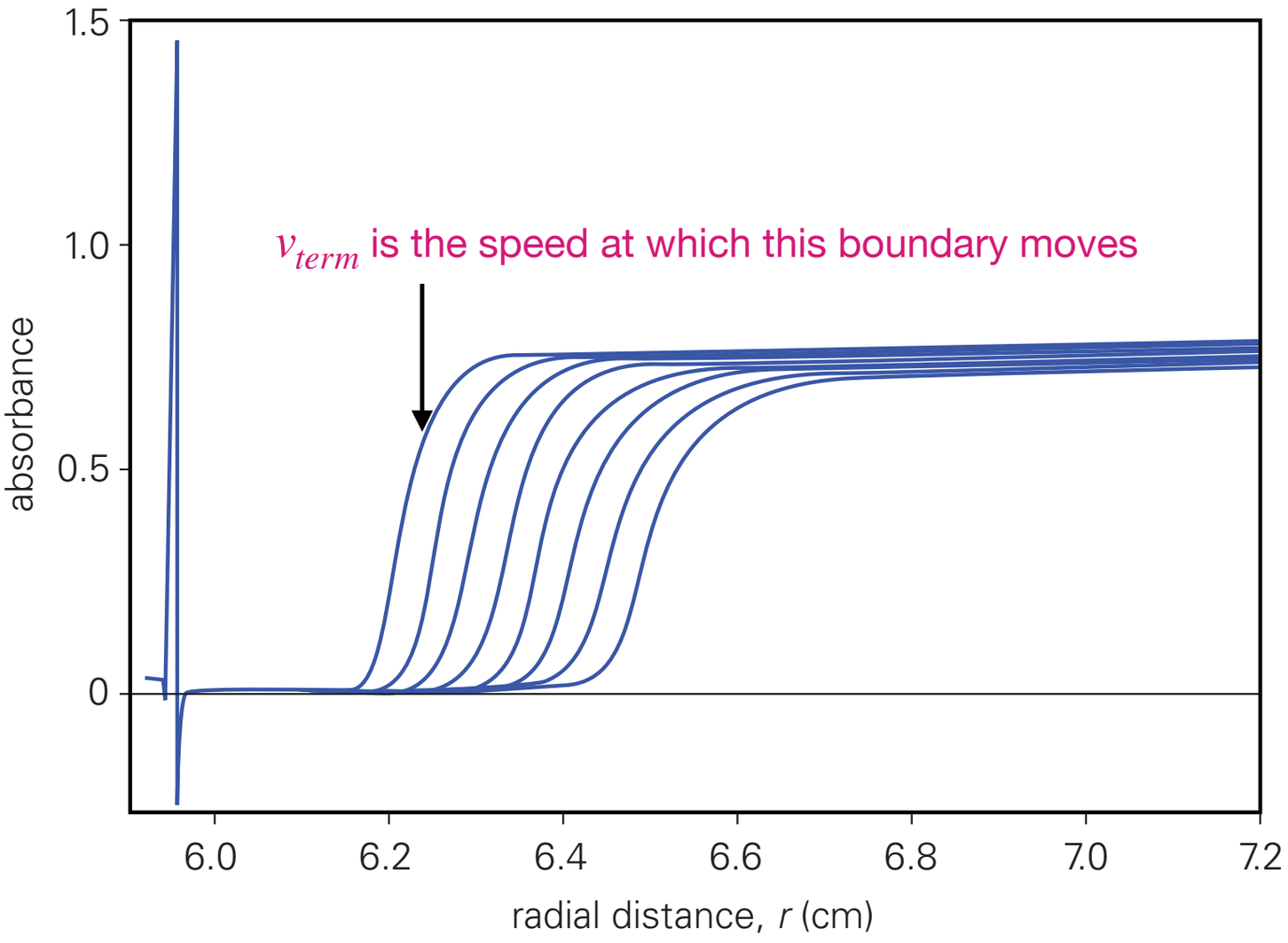
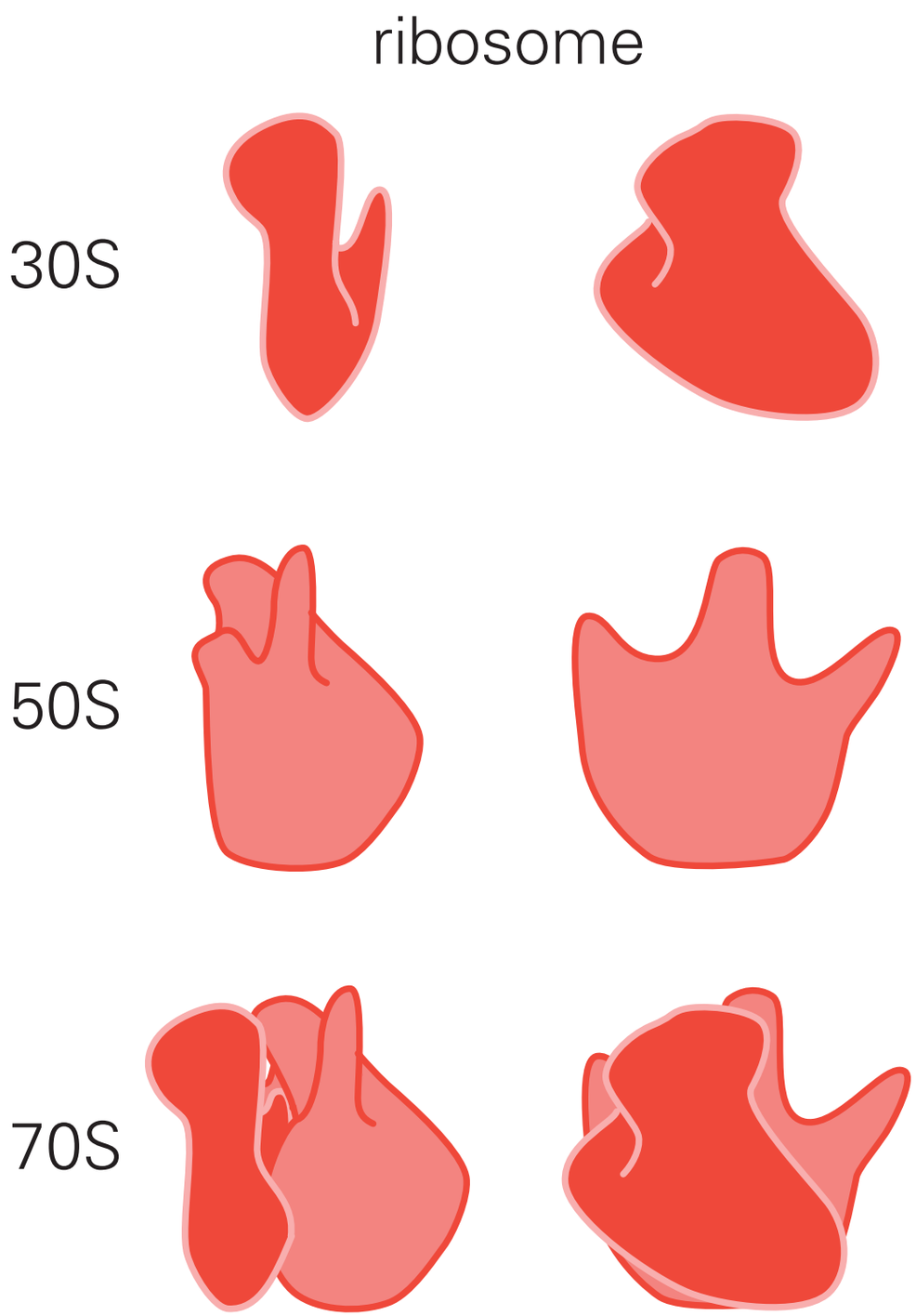


Figure 17.36 Changes in protein concentration during centrifugation. Concentration profiles are shown at different times for a sample undergoing centrifugation. The concentration at any position, *r*, within the sample cell is determined from the absorbance at 280 nm, which is linearly proportional to protein concentration. The boundary moves toward the outer edge of the rotor (right) with time. The rate of movement of the boundary allows the sedimentation coefficient to be determined.

$v_{term}/\omega^2 r$ gives the sedimentation coefficient

A possible source of error to this calculation of sedimentation coefficient can come from a spread in the boundary layer introduced by the random Brownian forces on individual molecules.

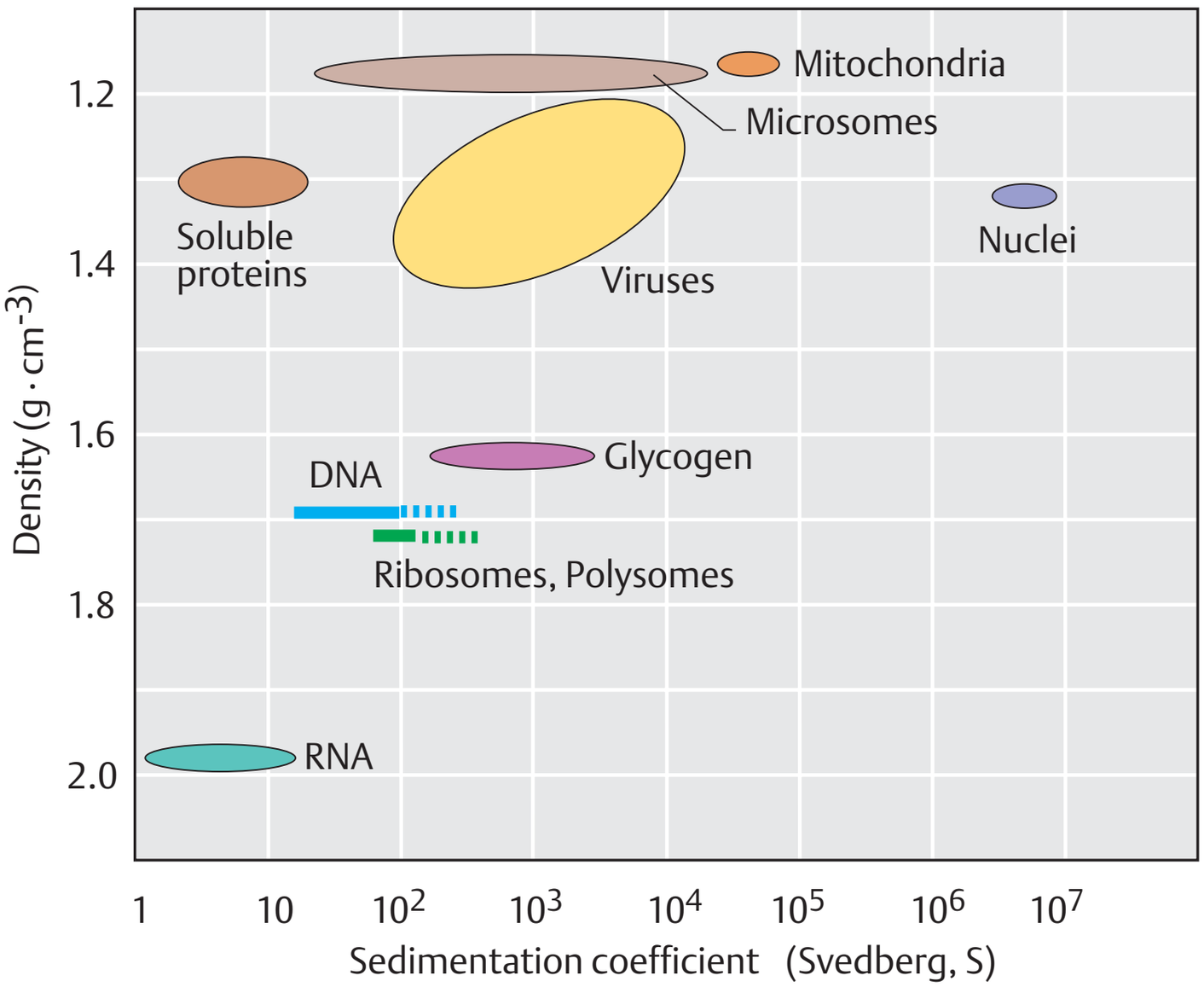
Sedimentation coefficient is not additive



$$30\ S + 50\ S \neq 70\ S$$

Sedimentation can be used for separation of biomolecules

Koolman, Color Atlas of Biochemistry, 2nd Ed, 2005



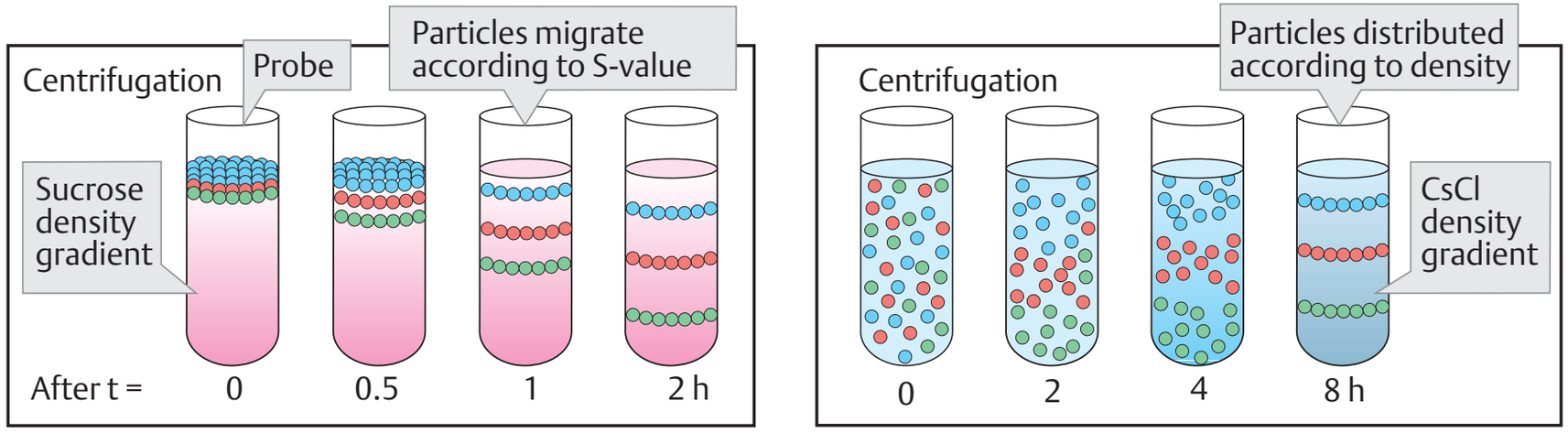
Such differences in sedimentation coefficient is leveraged to separate molecules through density gradient centrifugation

Sedimentation can be used for separation of biomolecules…*contd*

Two types of centrifugation-based separation assays are used

Zonal centrifugation
Separation of molecules based on a differences in sedimentation coefficients

Isopyknic centrifugation
Separation of molecules based on a density gradient set up by centrifugation



Equilibrium centrifugation can be used to determine molecular weights and existence of oligomers

During centrifugation an equilibrium is established between the flux of centrifugation and diffusive flux

Flux balance: $[c(r)](S)(\omega^2 r)(Adt) = (D)\left(\frac{dc(r)}{dr}\right)(Adt) \quad \Longrightarrow \quad [c(r)]\left(\frac{m_{\text{eff}}}{f}\right)(\omega^2 r) = \left(\frac{k_B T}{f}\right)\left[\frac{dc(r)}{dr}\right]$

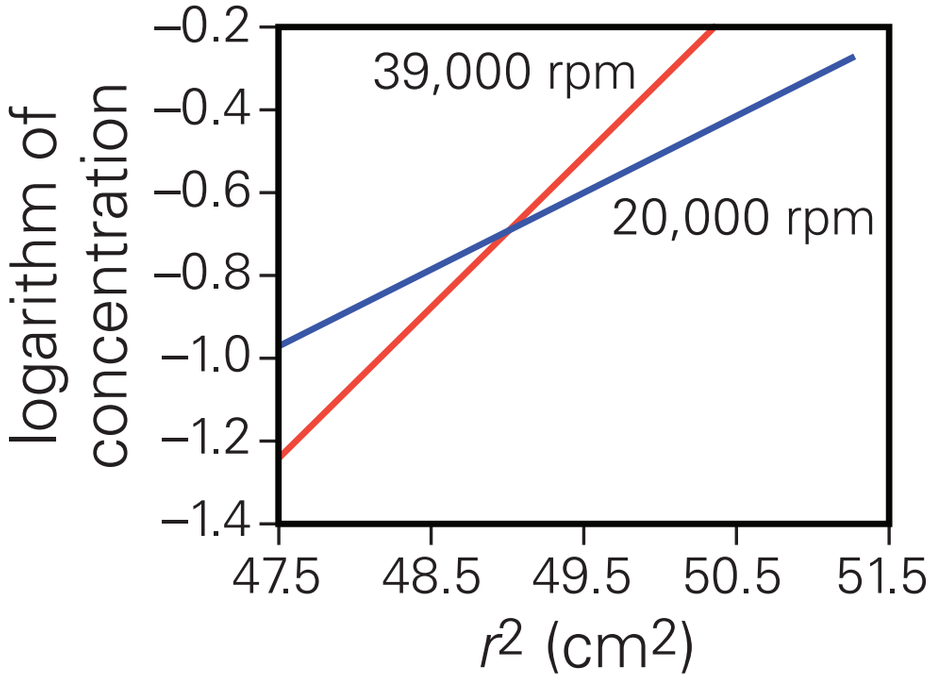
Using $M_{eff} = N_0 m_{eff}$ and $R = k_B N_0 \quad \Longrightarrow \quad M_{\text{eff}} = M\left(1 - \frac{\rho_{\text{solv}}}{\rho_{\text{macromol}}}\right) = \left(\frac{RT}{\omega^2}\right)\left(\frac{dc}{c}\right)\left(\frac{1}{r\,dr}\right)$

$$\Longrightarrow \quad M = \frac{2RT}{\omega^2\left(1 - \frac{\rho_{\text{solv}}}{\rho_{\text{macomol}}}\right)} \frac{d(\ln c)}{d\,(r^2)}$$

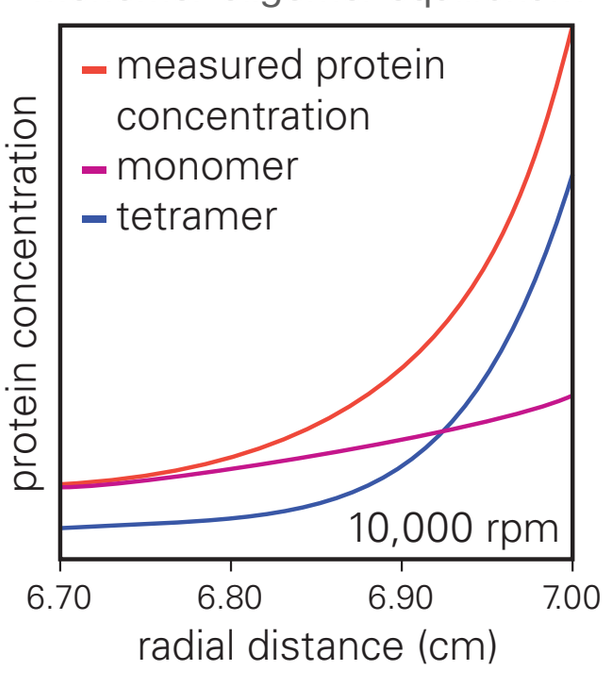
Using the slope of $ln(c) - r^2$ we can estimate the MW

Any non-linearity indicates the presence of one species or a multi-species equilibrium in the solution

One type of protein species



Monomer-oligomer equilibrium



Electrophoresis: driving molecules by electrical force

Electrophoresis

Electrophoresis is the driven movement of charged molecules in an electric field, analogous to movement in a centrifugal field.

Basic principle:

- An electric field is applied to a sample by placing electrodes at each end and connecting the electrodes to an electrical source.
- An ion in the sample will be attracted to the oppositely charged electrode.

This creates a mobility of the molecules under the electrical force called the electrophoretic mobility

Electrophoretic mobility

The electrical force on an ion of charge Z moving in an electric field E is given by

$$f_{elec} = ZeE$$

This is balanced by the friction force to set the The terminal velocity

$$v_{term} = \frac{f_{elec}}{f} = \frac{ZeE}{f}$$

Similar, to sedimentation coefficient, we define the electrophoretic mobility

$$\frac{v_{term}}{E} = \frac{Ze}{f}$$

The electrophoretic mobility depends on the molecular size and shape through the friction factor and the charge of the molecule

Gel electrophoresis is a method for the measure electrophoretic mobility

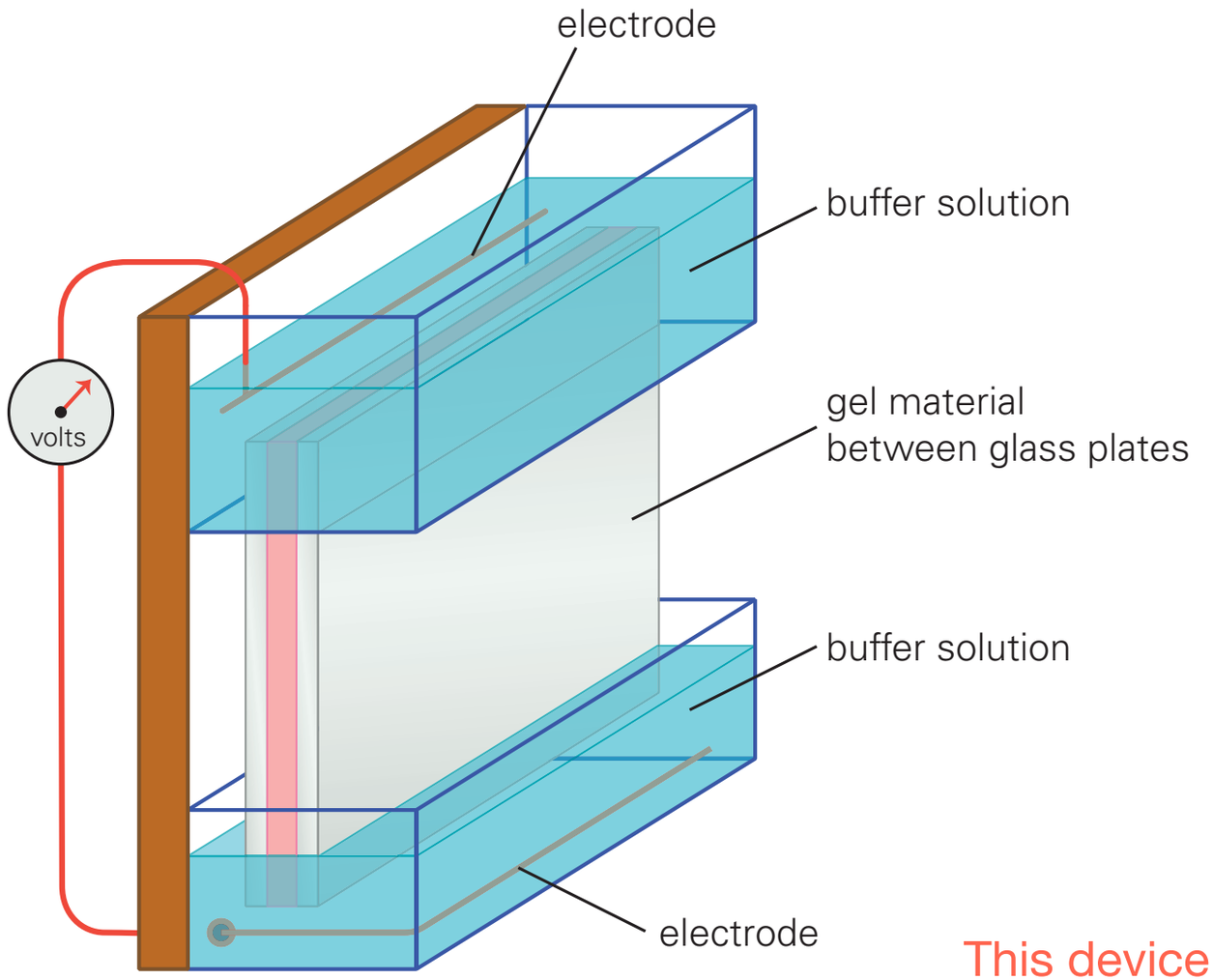


Figure 17.40 A gel electrophoresis apparatus. Electrophoresis experiments are commonly run in laboratories with the gel sandwiched between two vertical glass plates, each end immersed in a buffer solution. Wire electrodes are placed in the upper and lower buffer trays with a voltage source to provide the driving force to move the molecules from the top to the bottom. Samples are loaded into slots at the top of the gel so that many samples can be run in parallel (see Figures 17.42 and 17.43 for examples of such gels).

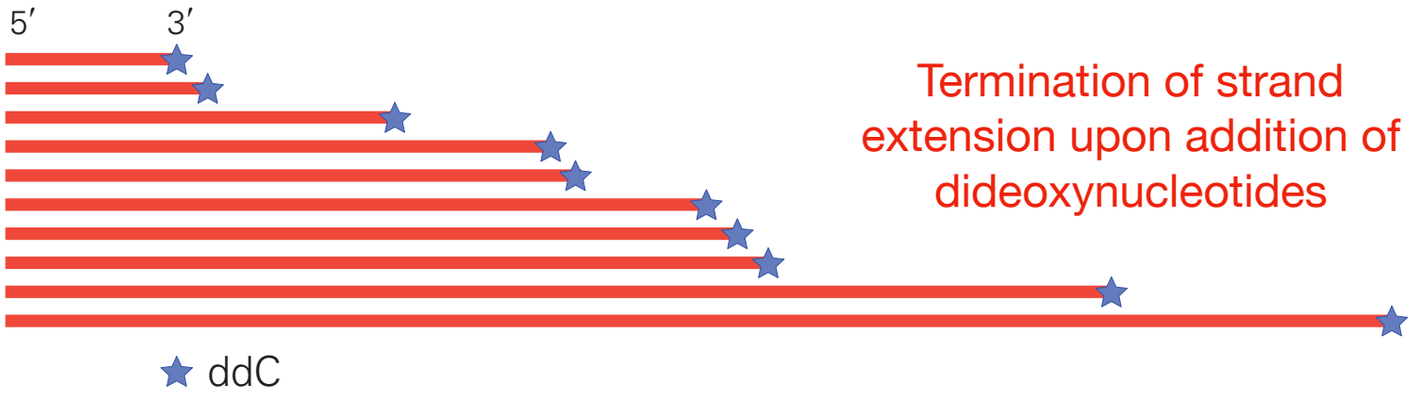
This device

- Removes the effects of convective flows in the medium, allowing measurement of just mobility
- Utilizes gel-forming polymers, like polyacrylamide or agarose (found in red algae or seaweed)
- Alters the mobility based on gel polymer concentration - so the mobility here is measured w.r.t a reference standard

Electrophoretic mobility of nucleic acids decreases with size

DNA strand to be sequenced used as template

3′ ATTTCGGCATTAGCATCGGAAAGGGTTAAAACTTCGGGAATTCCG 5′

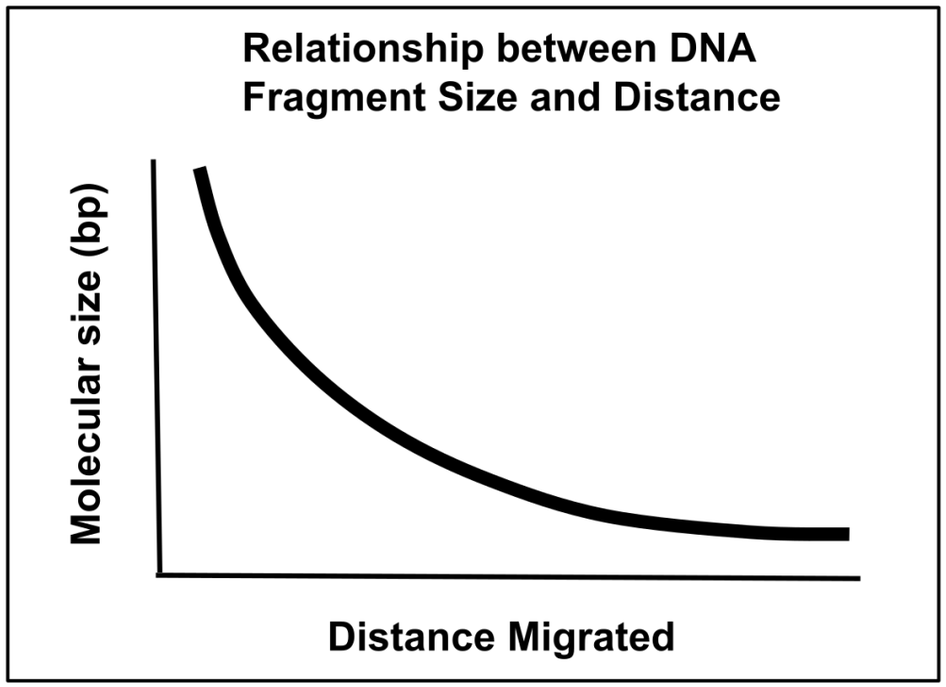


Termination of strand extension upon addition of dideoxynucleotides

The sequence of the parent DNA strand can be determined by noting the order in which strands with different terminal dideoxynucleotides migrate down the gel.

Distance migrated

Length of DNA fragment

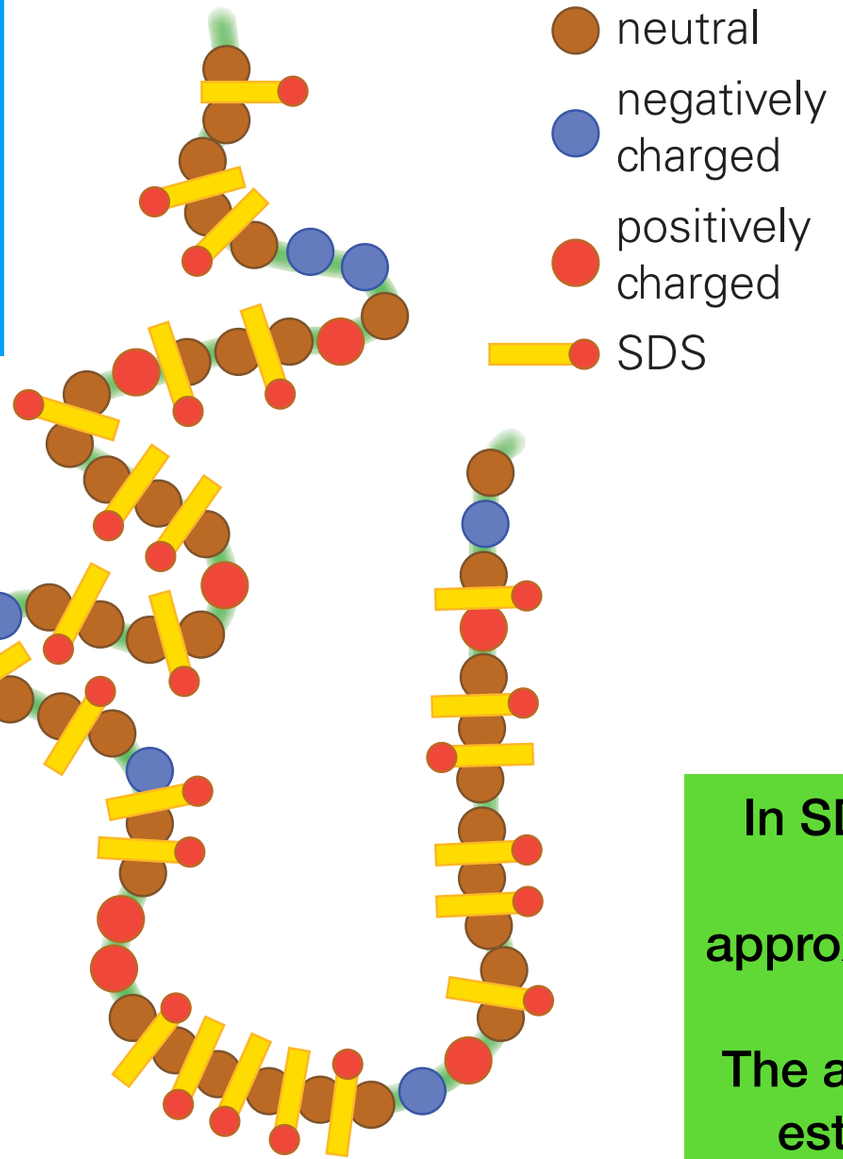


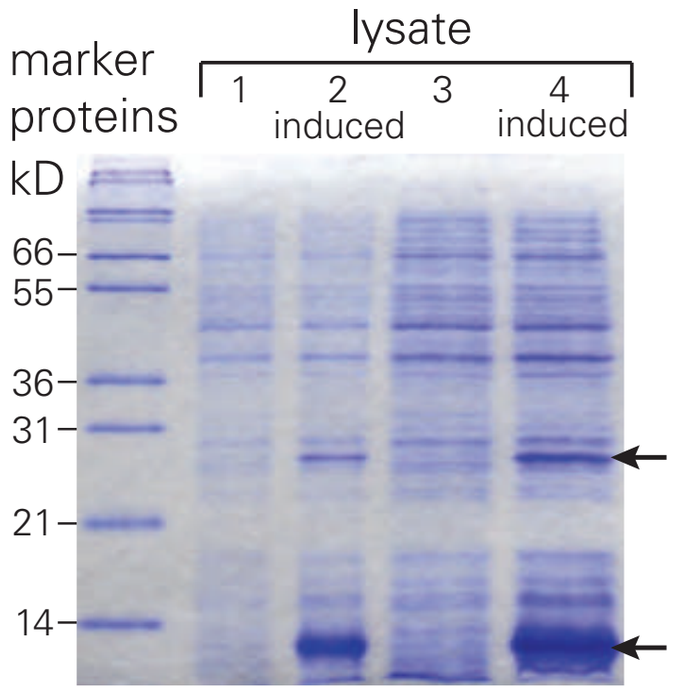
Success of this procedure hinges on the fact that total -ve charge on DNA is nearly proportional to the number of nucleotide

Gel electrophoresis analysis for proteins in useful for size determination

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The charge-length proportionality does not hold for proteins. So, SDS is used to unfold the protein and create a situation where this proportionality can be achieved.





One SDS molecule binds to on average two amino acids

In SDS-PAGE, the proteins separate according to MW, because approximately the -ve charge becomes proportional to MW.
The approximate MW of the protein is estimated by comparisons to the mobility of known MW reference proteins in the same gel