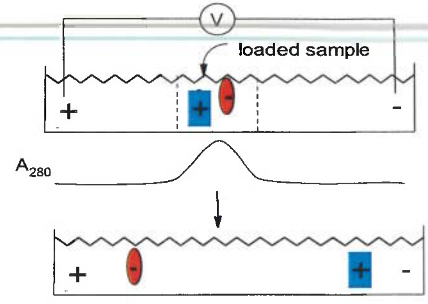
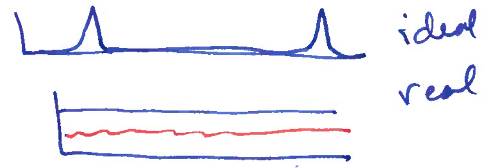
Lecture 22: SDS-PAGE, Quaternary Structure Determination

Evaluating Final Purity & Determining Molecular Weight.

After the protein is pure, its purity can be monitored by any of the following:

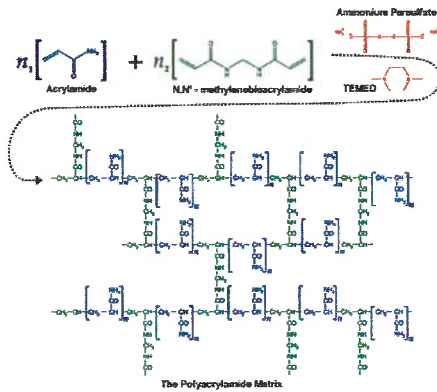
a) SDS-page gel electrophoresis.
b) Mass spectrometry.
c) Amino terminal sequencing.
d) Isoelectric focusing (separation by pI).

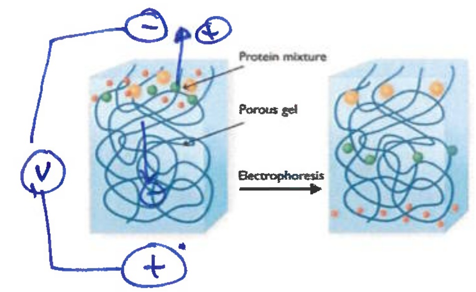


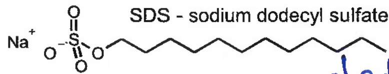


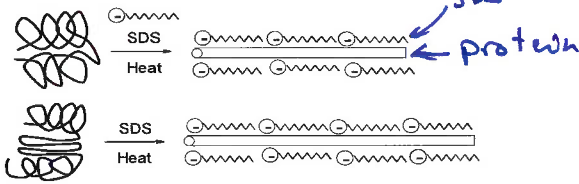
SDS-polyacrylamide gel electrophoresis (SDS-PAGE)
(separation of ***denatured*** proteins by size.)

1. **Electrophoresis:** Separation of molecules by migration due to electric field. v=q/M. Convection mixing destroys resolution, hence the need for a gel.
2. **Polyacryamide gels** – crosslinked polymer of acrylamide+crosslinker. Prevents convection mixing & causes separation by size.
3. **SDS** – denatures proteins, giving them a uniform charge to mass ratio, therefore separate only by size as they pass through the gel. Smaller proteins move faster

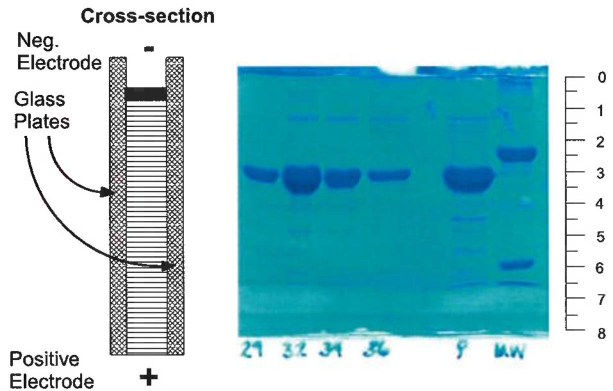








Gels are stained with a stain that is specific for protein. Most commonly used stain is Coomassie blue. Gel shows (left to right) four fractions obtained from chromatography, empty lane, pooled protein, molecular weight standards.



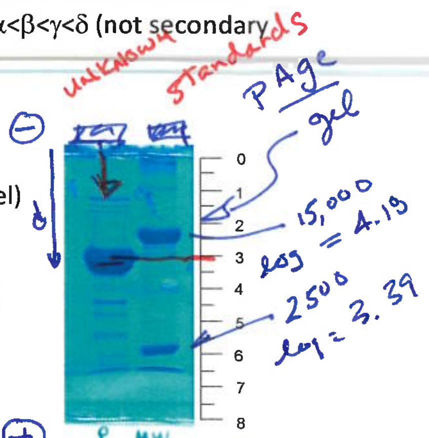
Quaternary Structure Determination:

i) Determine the molecular weight of each subunit by SDS-PAGE
ii) Obtain relative ratio of subunits from SDS-PAGE, based on relative band intensities.
iii) Determine the presence of disulfide bonds between subunits using SDS-PAGE.
iv) Obtain overall native molecular weight by size exclusion (gel filtration) Chromatography
v) Propose a quaternary structure (# of chains, location of S-S bonds) that is consistent with **all** of the data.

Nomenclature:

Homo__dimer__ = all chains equal Hetero__tetramer__= at least two different chains

Chain labels: Greek alphabet, usually in order of molecular weight: $\alpha<\beta<\gamma<\delta$ (not secondary structure) Examples: Hemoglobin: heterotetramer ($\alpha_2\beta_2$), HIV protease: homodimer (α_2).



Denatured Molecular Weight (SDS-PAGE).

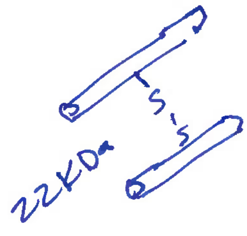
i) Load molecular weight standards on one lane (right lane in above gel)

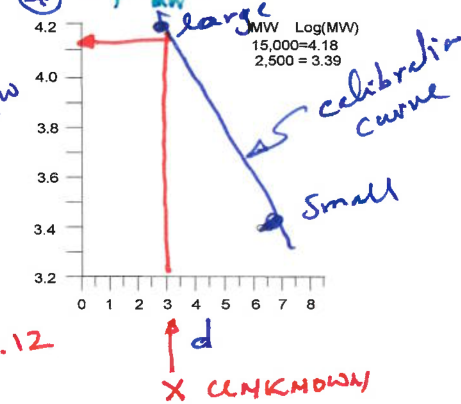
ii) load unknown(s) in other lane(s), apply voltage.

iii) Measure the distance migrated of each band.

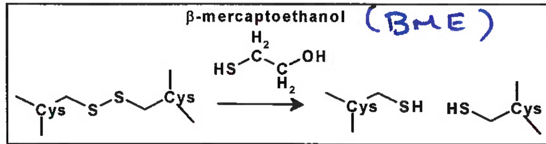
iv) Plot log MW versus *d* for standards, generating a calibration curve.

v) Use calibration curve to get logMW of unknown.

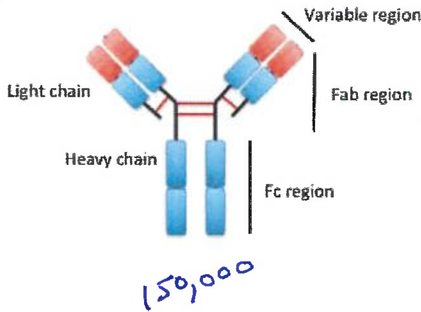


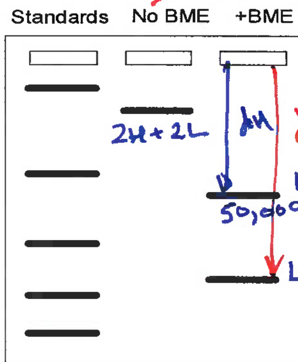


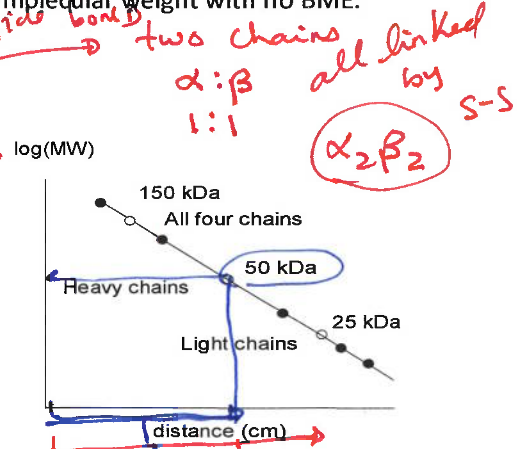
Disulfide Bonds: If proteins are crosslinked by disulfide bonds, and it is desirable to obtain the sizes of the subunits, then the S-S bonds have to be broken using β-mercaptoethanol (BME) or Dithiothreitol (DTT, not shown) before the electrophoretic separation. BME and DTT reduce the disulfide bond, generating free –SH groups on the Cys residues. Each chain will migrate separately in SDS-PAGE.



A change in the band pattern after treatment with BME suggests the presence of disulfide bonds. The molecular weight of the individual chains can be obtained from the +BME sample. The total number of disulfide-bonded chains can be estimated from the molecular weight with no BME.



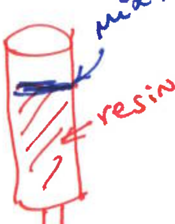


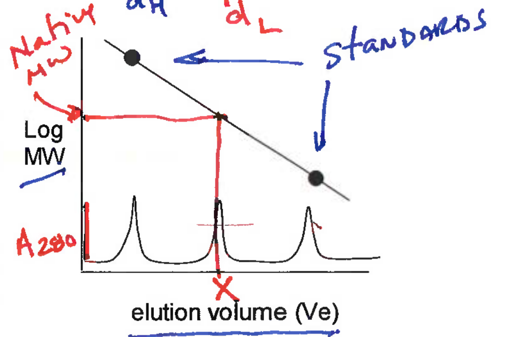


Native MW: Size exclusion Chromatography (gel filtration).

The beads in the gel-filtration column contains pores that allow smaller molecules to enter but exclude larger molecules. Gel filtration is usually performed under conditions where the quaternary structure of the protein is preserved, giving the **native molecular weight.**

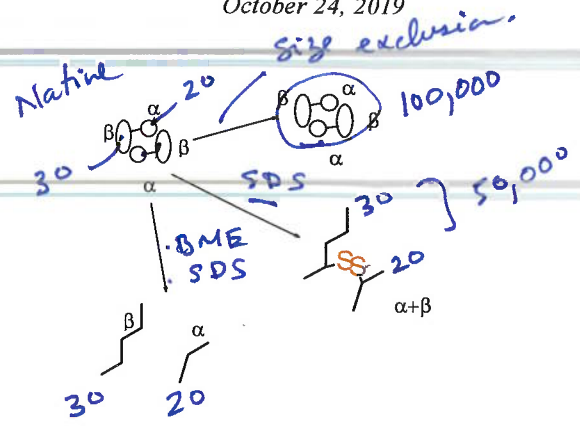
The volume that a particular protein elutes from a column is called the elution volume, V_e. For example, if a protein was contained in the 69th mL of liquid that dripped from the column then its elution volume would be 69 mL.





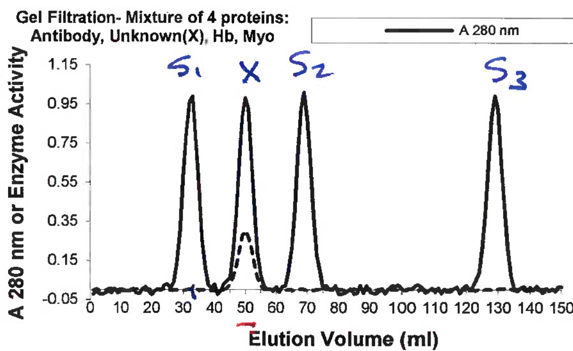
A plot of log MW versus elution volume is linear. Proteins with known molecular weights are used to define the calibration curve, from which the logMW of the unknown can be obtained.

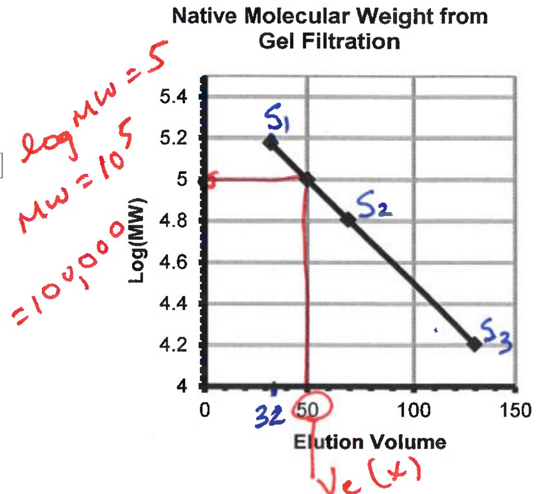
Example: An enzyme consists of four polypeptide chains. Two chains are 20 kDa in size (α-chain) and two are 30 kDa in size (β-chain). There is a single disulfide bond between the α and β subunits. The four chains associate as indicated in the diagram to form a hetero-tetramer, $(\alpha\beta)_2$.



1. **Size exclusion chromatography**, using three known standards (IgG, Hb, Myo).

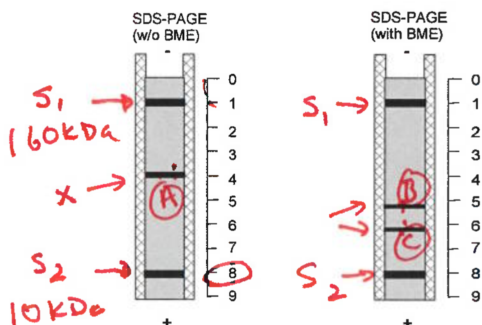
Protein	MW (gm/mole (Da))	Log MW
Antibody (IgG)	150,000	5.17
Unknown	100,000	5
Hemoglobin	64,000	4.8
Myoglobin	16,000	4.2

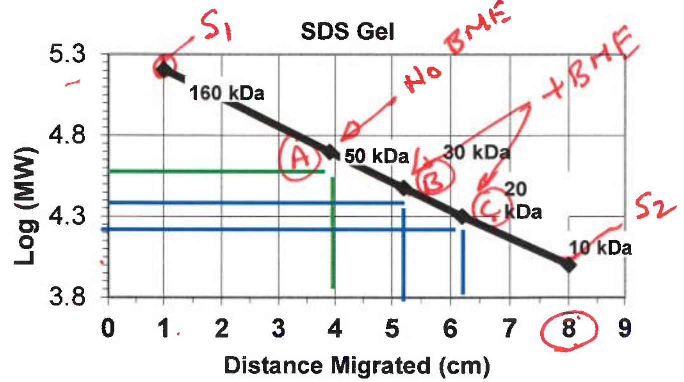




2. **SDS-PAGE** in the *absence* and *presence* of β-mercaptoethanol.

For calibration purposes, two proteins with known molecular weights, one with a molecular weight of 10 kDa and the other with a molecular weight of 160 kDa, were also included in this experiment. These two *standards* consist of a single polypeptide chain and will therefore give a single species in all experiments. Images of the two gels, as well as a plot of distance migrated versus log MW are shown.



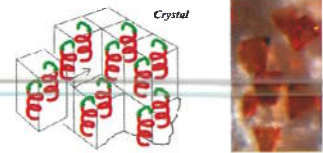


Analysis:

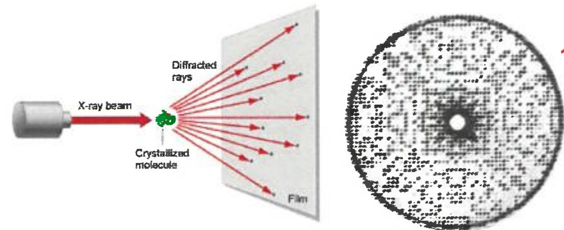
1. The gel with BME gives the size and relative stoichiometry of the chains. The MW of the α chain is 20 kDa, the β chain 30 kDa. The 1:1 intensity of staining indicates two chains, in a ratio of 1:1.
2. There is a difference in sizes between -BME and +BME, therefore some chains are joined by disulfides, the size to the crosslinked protein ~ MW α + MW β, therefore α-S-S-β.
3. Select chain combination that is consistent with the size exclusion data. All of the following agree with the SDS-PAGE data, but only one is consistent with the size exclusion MW.

αβ - predicted	$\alpha_2\beta_2$ - predicted	$\alpha_3\beta_3$ - predicted
MW = 50 kDa	MW = 100 kDa	MW = 150 kDa

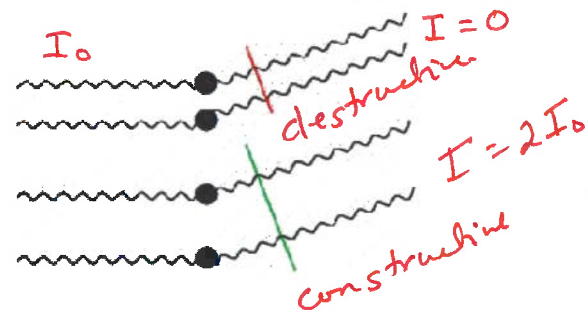
Atomic Resolution Structures: X-ray Diffraction



1. Proteins must be crystallized in a regular lattice, just like a normal salt. The **unit cell** is the basic unit of a crystal. It is repeated over-and-over again to form the macroscopic crystal lattice. A single unit cell can contain multiple protein molecules.
   (http://www.ruppweb.org/xray/tutorial/Crystal_sym.htm)
2. X-rays are scattered by electrons – the amount of scattering is proportional to the number of electrons (hydrogens scatter so weakly, that they usually cannot be detected, hence they are usually omitted from structures in the protein database).



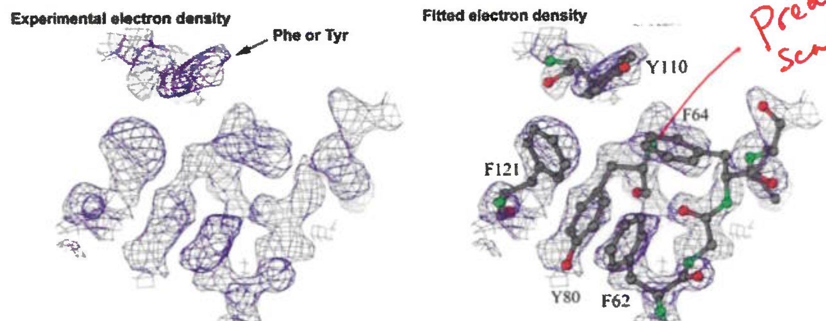
3. Interference between X-rays that are scattered from atoms in different locations changes the amplitude and the phase of the scattered X-rays. Therefore, scattered X-rays can be used to determine the position of atoms.



Top pair of atoms show ____________________ interference

Bottom pair of atoms show ____________________ interference

4. Intensities can be measured directly. The relative phases of the scattered X-rays have to be obtained indirectly. One common method of obtaining phases is called **molecular replacement**, where a homologous known structure is used to calculate the phases. Other methods include incorporating heavy atoms into the crystal to perturb the scattering.
5. A Fourier transform of the intensity and phases of the scattered X-rays produces an 'electron density map', or the number of electrons at each point in space in the crystal (ρ(x,y,z)). The crystallographer must figure out how to place, or "fit", the known primary structure of the protein into this map.



The overall process of obtaining crystal structures is:
Purify Protein→Grow Crystals→Collect Scattering →Determine Phase→Fourier Trans→Fit Map→Validate Model →Deposit structure in Protein Data Base (pdb).

Evaluation of structural quality (is the model correct?)

- Ramachandran outliers: most (99%) of residues should fall in low energy region of the Ramachandran plot.
- Agreement between observed data and predicted scattering, based on the model. The difference is the called the R-factor, and it should be ~20%.