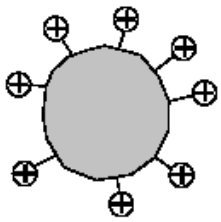
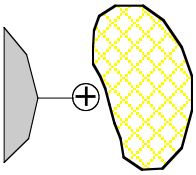
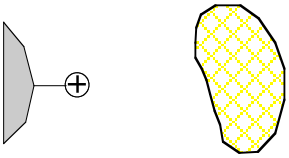
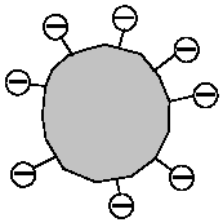
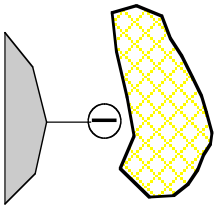
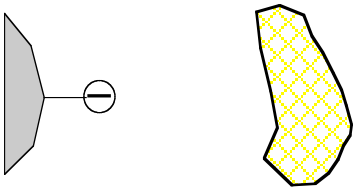
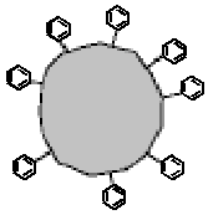
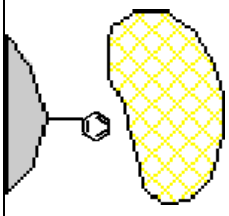
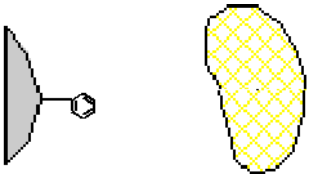
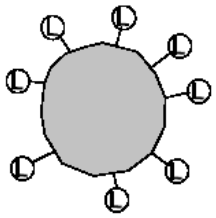
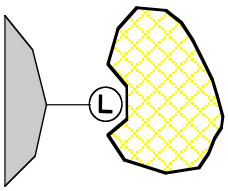
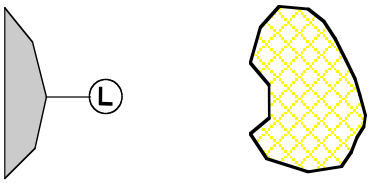
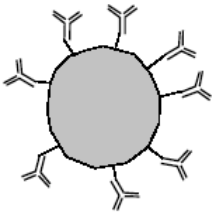
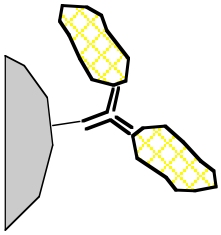
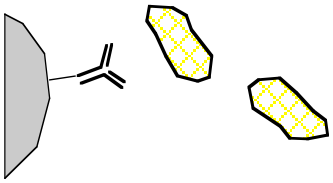
Lecture 21: Protein Purification and Quaternary and Tertiary Structure

Reading in Campbell: Chapter 5, 4.5

Key Terms:

- **Chromatographic Steps**
- **Molecular Weight Determination**
- **SDS Gel Electrophoresis**

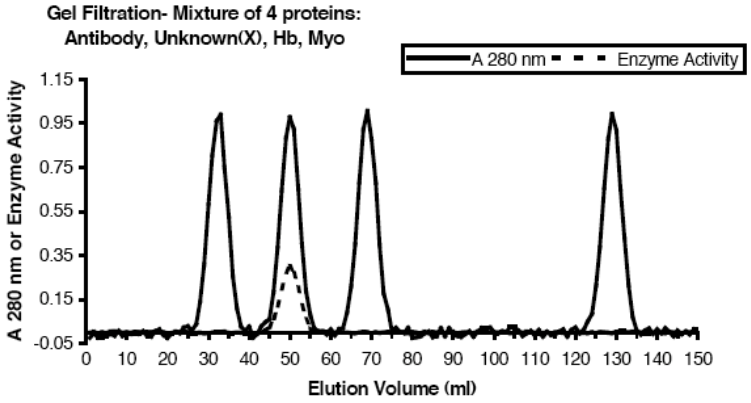
Type	Type of Resin	Principal of Separation	How to Elute the Protein
Anion Exchange *Separation by charge.*	• Beads with a **positive** charge 	Protein sticks to resin because of: • Overall **negative** charge (*anions*) • Proteins have patches of negative charge 	• Increase salt concentration to weaken electrostatic interaction. • Change of pH to pH < pI (protein becomes positively charged) 
Cation Exchange *Separation by charge.*	• Beads with a **negative** charge 	Protein stick to resin because of: • Overall **positive** charge (*cations*) • Proteins have patches of positive charge 	• Increase salt concentration to weaken electrostatic interactions. • Change of pH to pH > pI (protein becomes negatively charged) 
Hydro-phobic Chromato-graphy *Separation by hydro-phobicity*	• Beads with a **hydrophobic** group 	Protein sticks to resin in high salt because of: • **Hydrophobic** patches on surface of protein interact with hydrophobic groups on resin. (In high salt, less water is available to form a shell around these hydrophobic patches). 	• Decrease salt concentration to weaken hydrophobic interactions  (hydrophobic patches are once again surrounded by a shell of water)

Affinity Chromato-graphy *Separation by affinity.*	• Beads with a ligand: 	Protein stick to resin because of: • Binding site for ligand 	• Excess ligand • Change in pH, salt, solvent to weaken protein-ligand interaction. 
	• or Antibody 	Protein stick to resin because of: • Binding to antibody 	• Changes in solution conditions (pH, salt, solvent) to weaken protein-antibody interaction. 
Gel Filtration: *Separation by size*		• Proteins don't "stick". • Small proteins enter the interior of the beads, and therefore take longer to wash off of the column.	• Simply washing the column with buffer will eventually wash the proteins out of the column. • Smaller proteins elute last.

Size & Quaternary Structure Determination

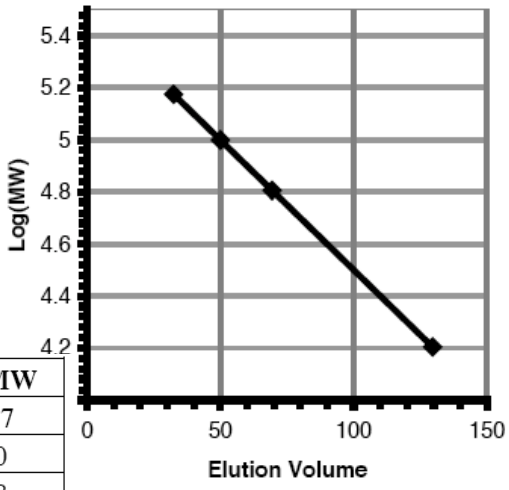
Native Molecular Weight: Gel filtration (size exclusion).
The matrix or beads in the gel filtration column contains pores that allow smaller molecules to enter but exclude larger molecules. The larger the molecule the less time it spends in the beads and the sooner it is washed (eluted) from the column.

- Large proteins elute first.
- Small proteins elute last.



The volume that it takes for a particular protein to elute from a column is called the elution volume, Ve. 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.

The relationship between the molecular weight of the protein and its elution volume is given by the following equation: $\log(MW) = \square V_E + \square$ where $\square$ and $\square$ are constants obtained by measuring the elution volume of proteins with known sizes. Gel filtration columns must be calibrated with known proteins.



Protein	MW (gm/mole (Da))	Log MW
Antibody (IgG)	150,000	5.17
Unknown	100,000	5.0
Hemoglobin	64,000	4.8
Myoglobin	16,000	4.2

Note: Gel filtration is usually performed under conditions where the quaternary structure of the protein is preserved, giving the *native* molecular weight.

Denatured (subunit) Molecular weight: SDS polyacrylamide gel electrophoresis (SDS-PAGE):

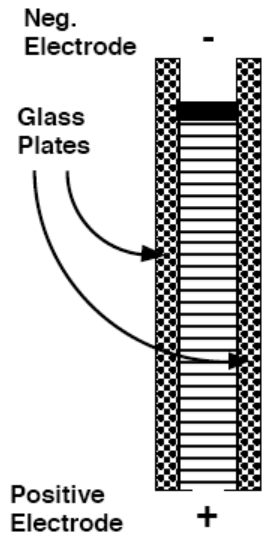
In SDS gel electrophoresis, proteins are denatured to form individual polypeptide chains and then they are forced through a gel by an electric field. For proteins, acrylamide gels (a chemically crosslinked polymer) are used. This material is very similar to Jello in its macroscopic properties, i.e. a tightly knit web of polymer fibers. After the experiment the gels are stained with a protein specific stain to visualize the separated protein bands.

These gels act as size exclusion media, impeding the motion of larger protein. As the proteins are forced through the gel, they separate according to size.

- Smaller proteins have a much easier moving and therefore migrate a larger distance.
- Larger proteins have a more difficult time and therefore migrate a smaller distance.

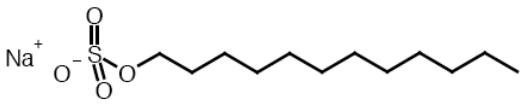
Remarkably, the relationship between the distance migrated, d, and the molecular weight is:

$\log(MW) = \square d + \square$ where $\square$ and $\square$ are constants determined by calibration with known standards.



Denaturation by SDS:

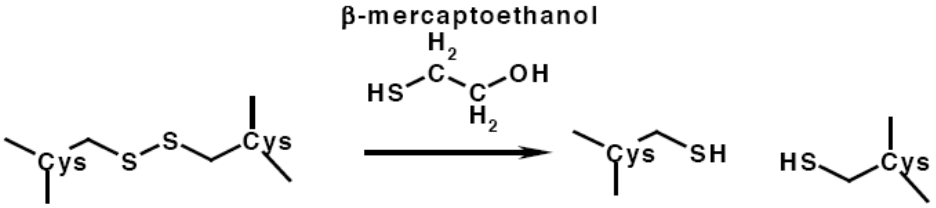
The proteins are denatured by the use of a detergent called SDS (sodium dodecyl sulfate). This negatively charged detergent binds to the protein, giving each protein a constant charge-to-mass ratio. It is estimated that each residue in the protein binds approximately 3 SDS molecules.



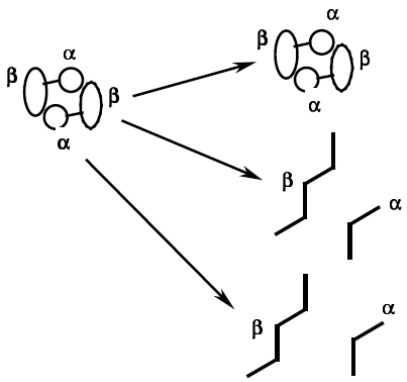
The formation of a uniform charge to mass ratio is important because the actual velocity of a charged particle in an electric field depends on the ratio of the charge to the mass of the particle. $v \quad \frac{q}{M}$ Since all proteins have the same charge to mass

ratio after treatment with SDS, they will migrate in the same direction, toward the positive electrode, or anode. (Anions, or negatively charged particles, migrate towards the anode). In free solution (e.g. without the gel) they would all migrate with the same velocity because their intrinsic charge has been overwhelmed by the charge from the SDS. However, they separate according to molecular weight because of the sieving properties of the gel.

Disulfide Bonds:
If proteins are crosslinked by disulfide bonds (e.g. antibodies), and it is desirable to obtain the sizes of the individual subunits, then the S-S bonds have to be broken using β-mercaptoethanol (βME) before the electrophoretic separation. β-mercaptoethanol reduces a disulfide bond to free Cysteines:



Example Determination of Quaternary Structure:
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, $(\alpha\beta)_2$.



The following three experiments were performed:

1. Gel filtration chromatography
2. SDS-PAGE in the absence of β-mercaptoethanol
3. SDS-PAGE in the presence of β-mercaptoethanol

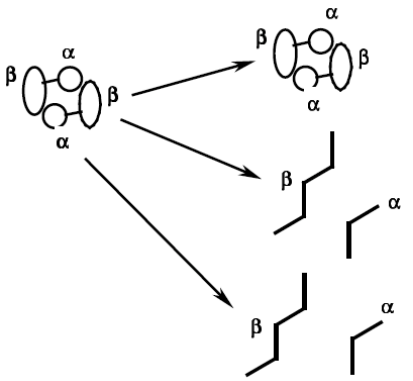
Two proteins with known molecular weights, one with a molecular weight of 10 kDa and the other with a molecular weight of 160 kDa, were also included in these experiments. These two standards consist of a single polypeptide chain and therefore give a single species in all experiments.

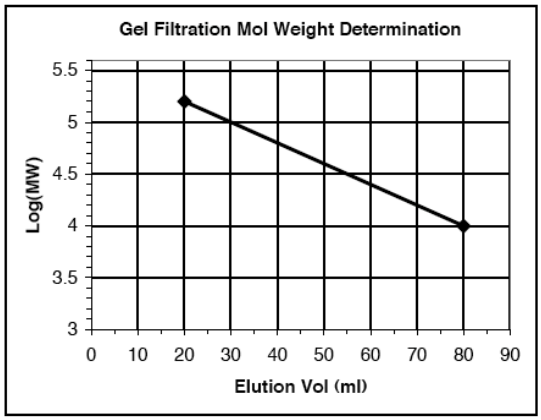
The elution volumes and the distance migrated for these two standards are given on the plots below. The table of logarithms will be useful for this problem.

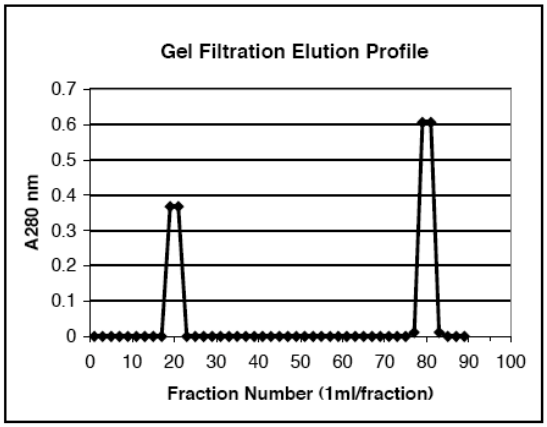
	Molecular Weight	Log (MW)
standard 1	**10,000**	**4.00**
α	20,000	4.30
β	30,000	4.48
αβ	50,000	4.70
$(\alpha\beta)_2$	100,000	5.00
standard 2	**160,000**	**5.20**

1. Determine the expected elution volume of the tetramer on the gel filtration column. Draw the contribution of tetramer to the elution profile. Assuming that one mL fractions were collected, which fraction would contain the pure tetramer?

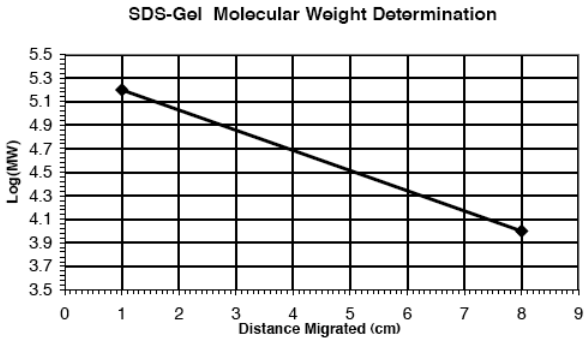
	Molecular Weight	Log (MW)
standard 1	**10,000**	**4.00**
α	20,000	4.30
β	30,000	4.48
αβ	50,000	4.70
$(\alpha\beta)_2$	100,000	5.00
standard 2	**160,000**	**5.20**

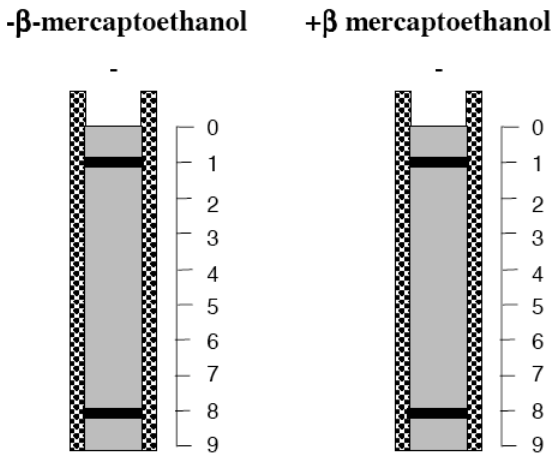






2. What distance(s) would the protein migrate on each SDS-PAGE gel? Sketch the location of the protein(s) on each SDS-PAGE gel.





Atomic Resolution Structures: X-ray Diffraction

Proteins must be crystallized in a regular lattice, just like NaCl.

1. No real limitations as to the size of structures.
2. X-rays are scattered by electrons – the amount of scattering is proportional to the number of electrons.
3. Interference between X-rays that are scattered from atoms in different locations change the amplitude and the phase of the scattered X-rays. Therefore, scattered X-rays can be used to determine the position of atoms.
4. 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 ($\rho(x,y,z)$). The crystallographer must figure out how to place, or "fit", the known primary structure of the protein onto this map (see diagram).