Question: You find that your protein sample loses activity during sample preparation/purification. What can you do to solve this?

a) Add an additional purification step

b) Use a protease inhibitor during purification steps

c) Perform each step as quickly as possible, in a cold-room

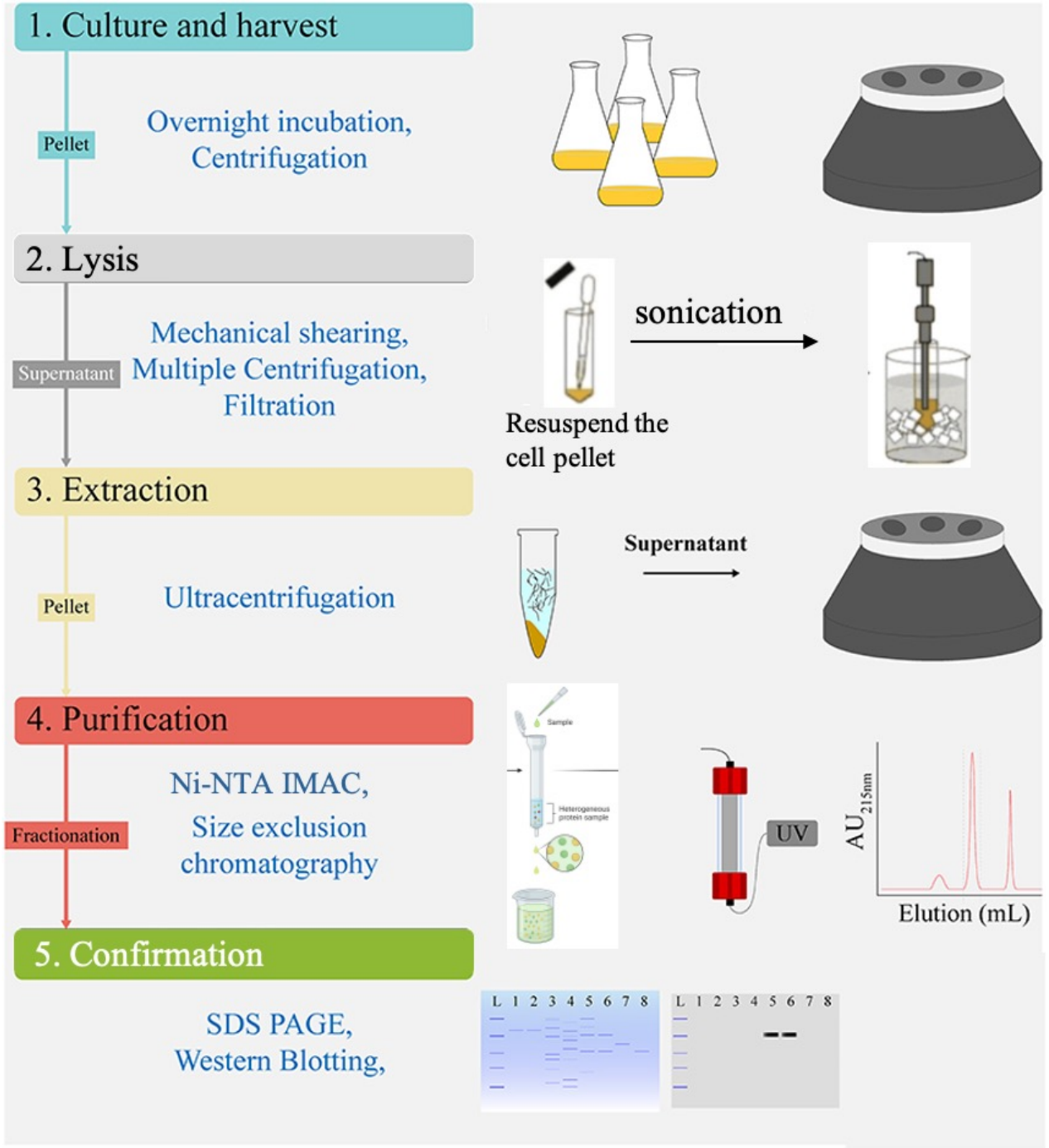
d) All of the above

Tutorial 1: Protein Separation

Bio312

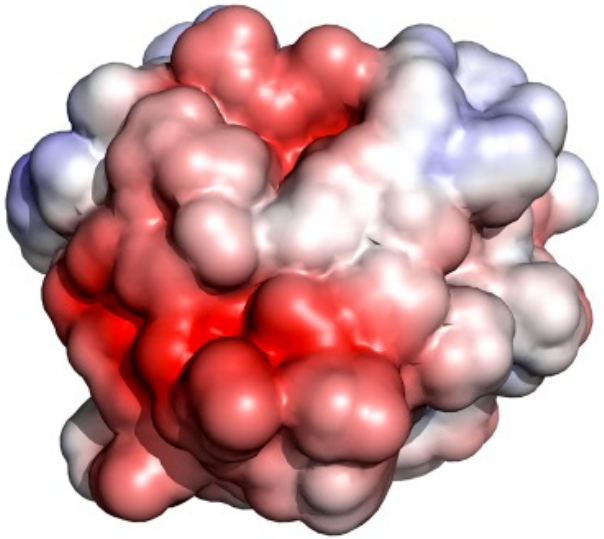
Dr. Han

Overview of Protein Purification Steps

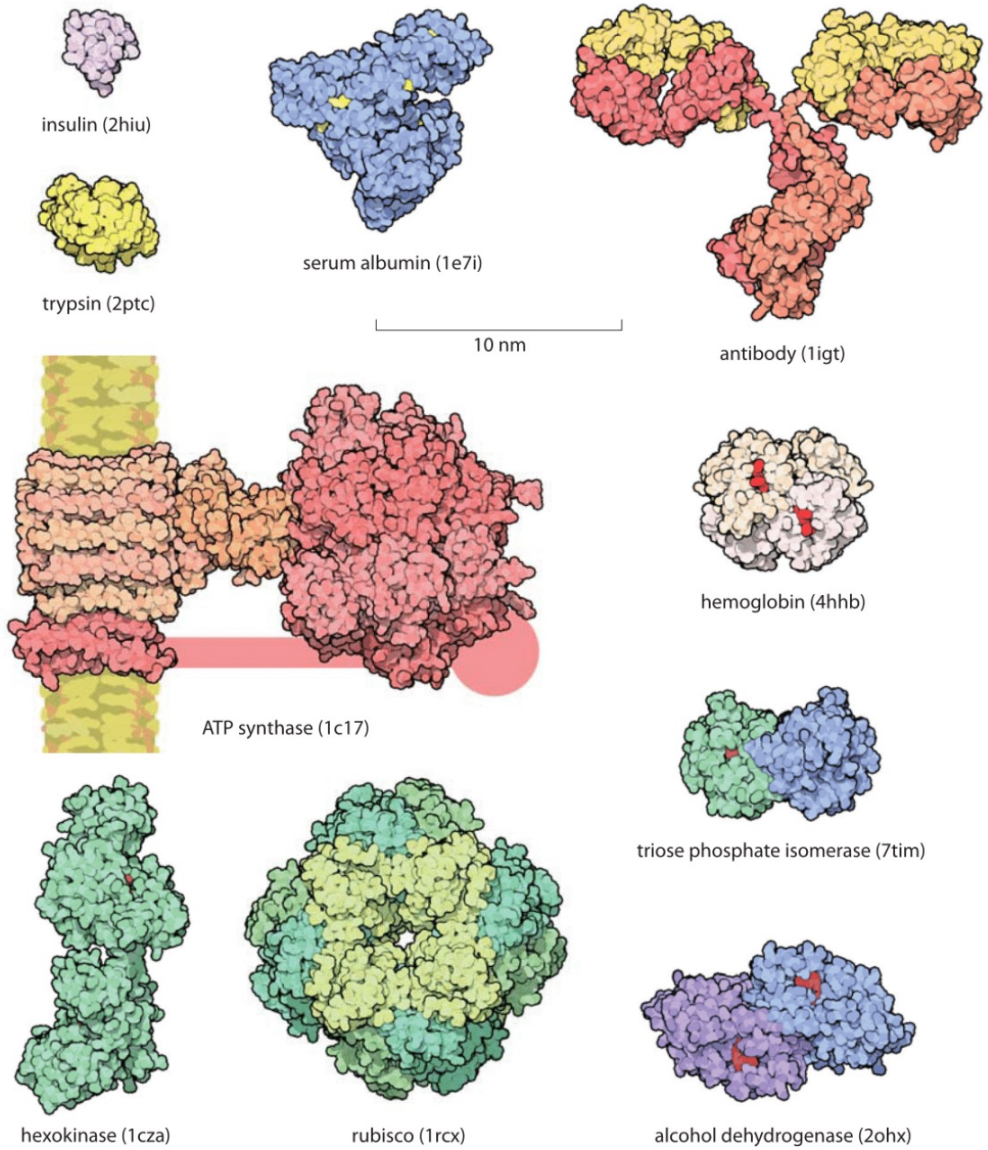


Modified from Acharya Dhruba et. al. *Frontiers in Cellular and Infection Microbiology*. 2019 (9):2235-2988

Proteins are Amphoteric Macromolecules with Different Sizes



- pH < pI, positively charged
- pH > pI, negatively charged



The charged groups, hydrophobic region, size and solvation affect the biophysical properties of the protein and largely determine its purification behavior.

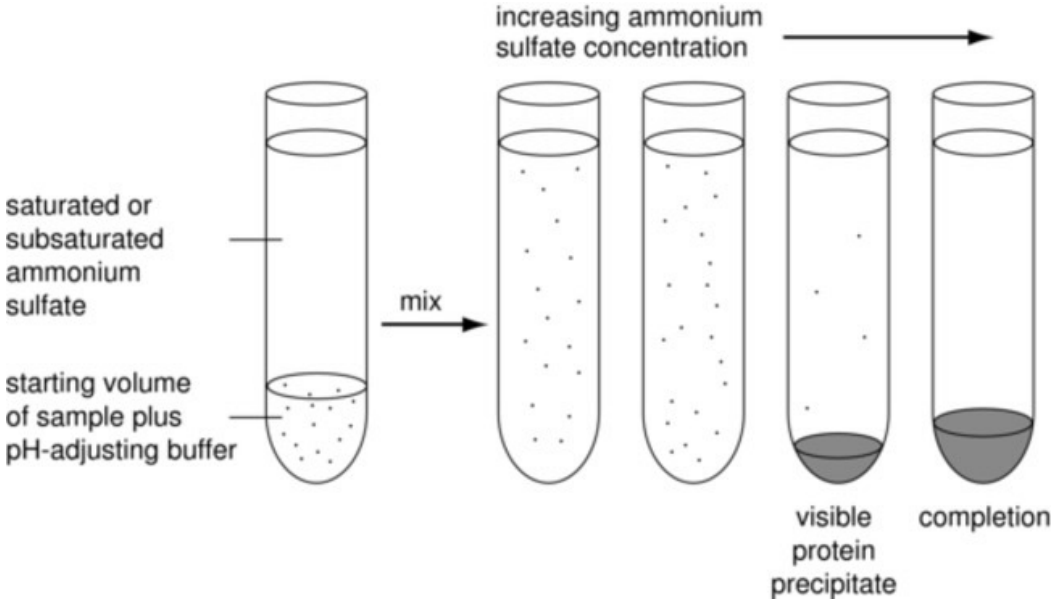
http://book.bionumbers.org/how-big-is-the-average-protein/

Methods for Protein Separation

Different sizes	Different solubility	Different charges	Different Ligand Binding
• Ultracentrifugation • Dialysis • Size exclusion chromatography • PAGE (SDS-PAGE or native PAGE)	Salting out e.g. Ammonium sulfate	• Ion exchange chromatography • Electrophoresis	Affinity chromatography

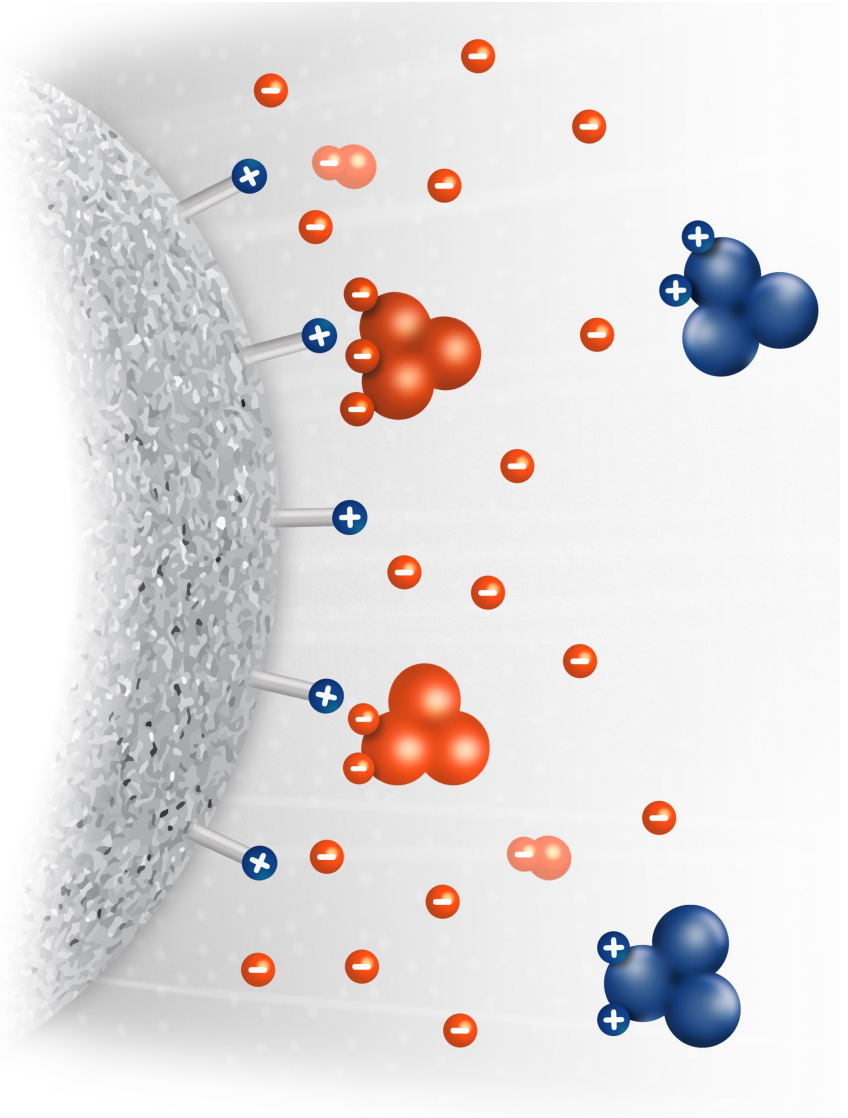
1. Salting out

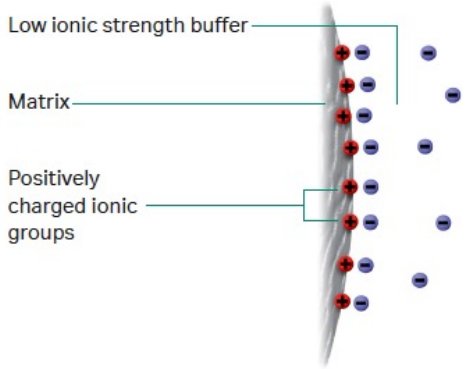
- A purification method that relies on the basis of protein solubility.
  - Most proteins are less soluble in solutions of high salt concentrations because the addition of salt ions shield proteins with multi-ion charges.
- Most common method is ammonium sulfate precipitation
  - Cheaper
  - Water soluble
  - No denaturation (change solubility)
  - But it requires prior knowledge of the protein's solubility.
- Useful in concentrating proteins



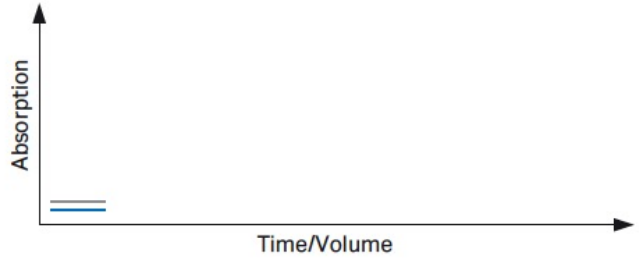
2. Ion Exchange Chromatography (IEC)

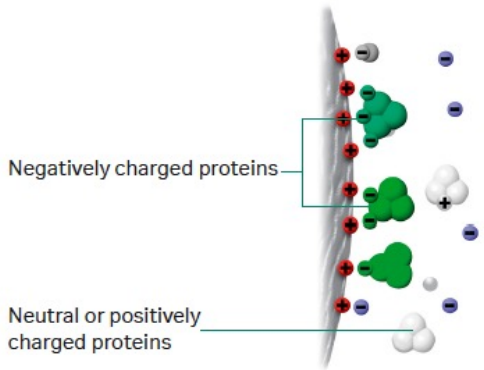
- Net surface charge is highly pH dependent
  - pH < pI, positively charged
  - pH > pI, negatively charged
- Cation exchange (separates based on positive charges of solutes/proteins, matrix is negatively charged)
- Anion exchange (separates based on negative charges of solutes/proteins, matrix is positively charged)



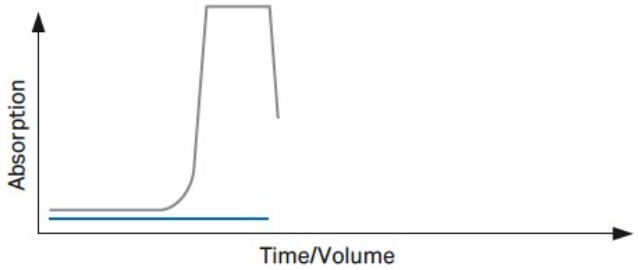


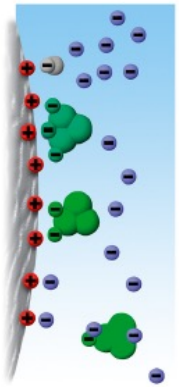
Equilibration



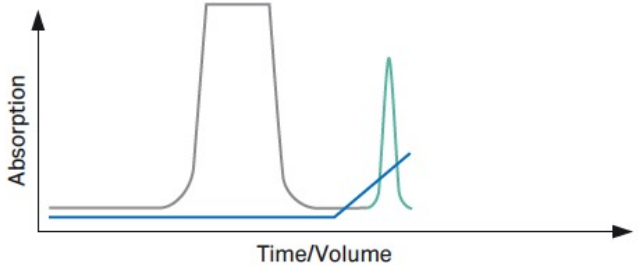


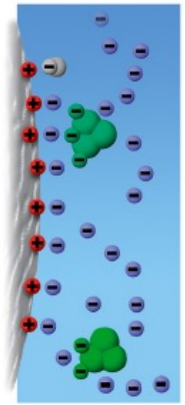
Sample application and wash



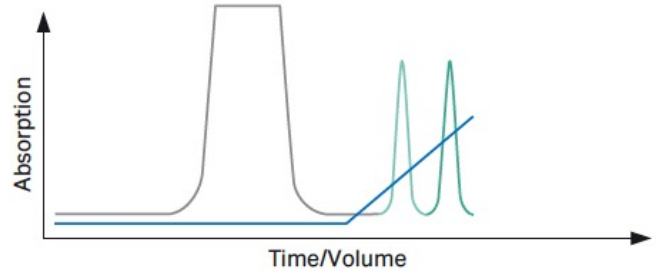


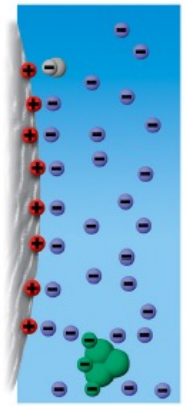
Elution 1



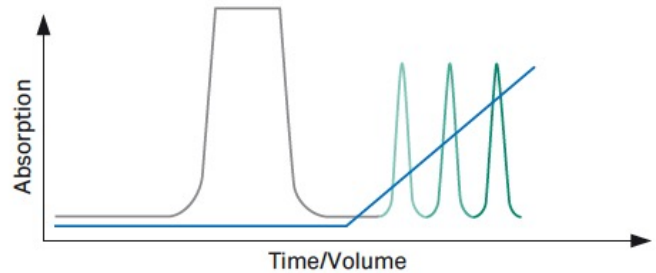


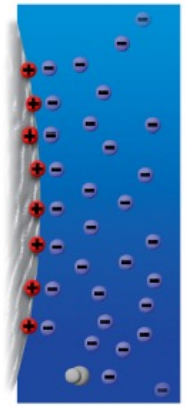
Elution 2



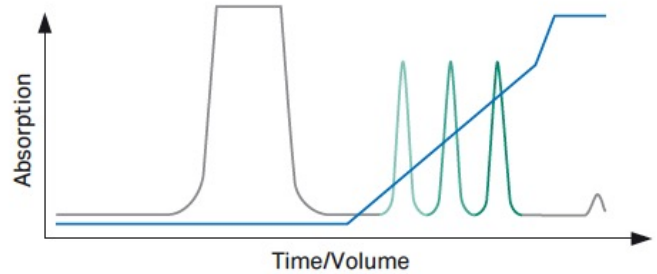


Elution 3





Regeneration



Think: What is the starting point for selection of a suitable IEX matrix for purification of a recombinant protein?

a) Prediction of isoelectric point (pI) from the amino acid sequence

b) Test protein binding to an IEX matrix at a range of pHs and salt concentrations

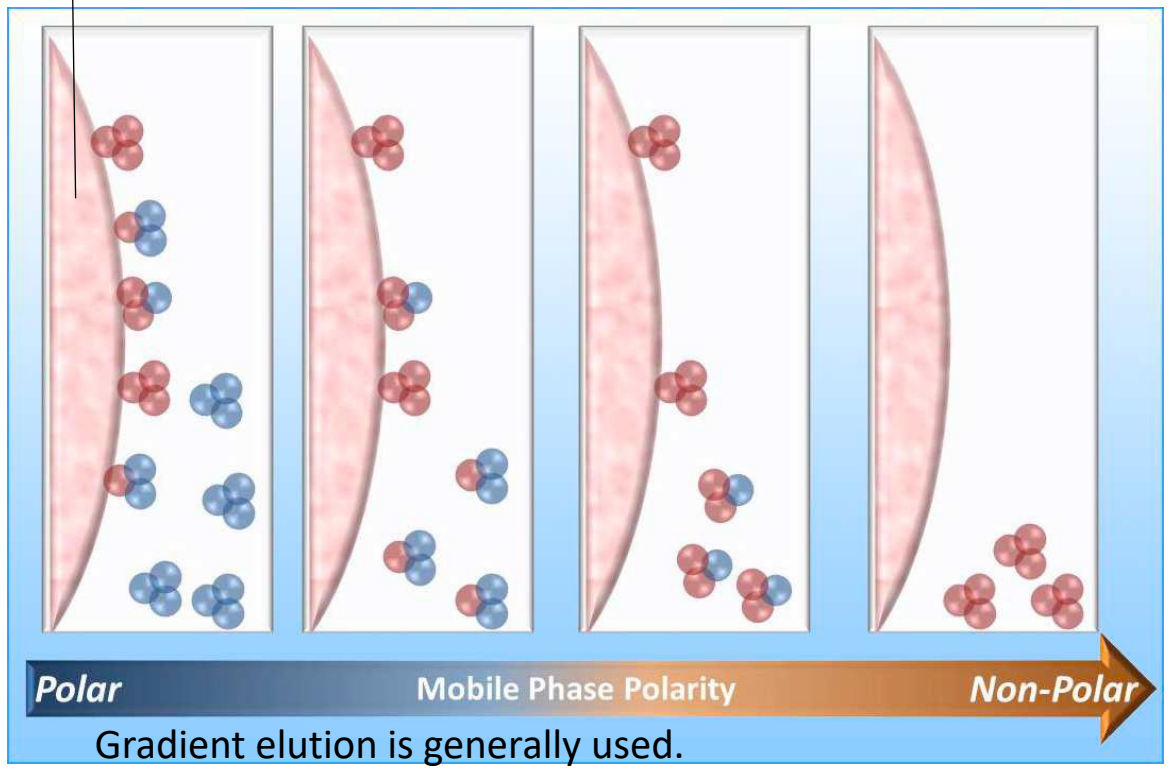
c) Test protein binding to a selection of anion and cation exchange matrices

d) Pass your sample through a preparative column and elute with a salt gradient

3. Reverse-Phase Liquid Chromatography (RPLC) Hydrophobic Interaction Chromatography (HIC)

- Based on the surface hydrophobicity of molecules.
- Shorter alkyl chains (C4 and C8) are typically preferred for intact protein separation because they are less retentive.

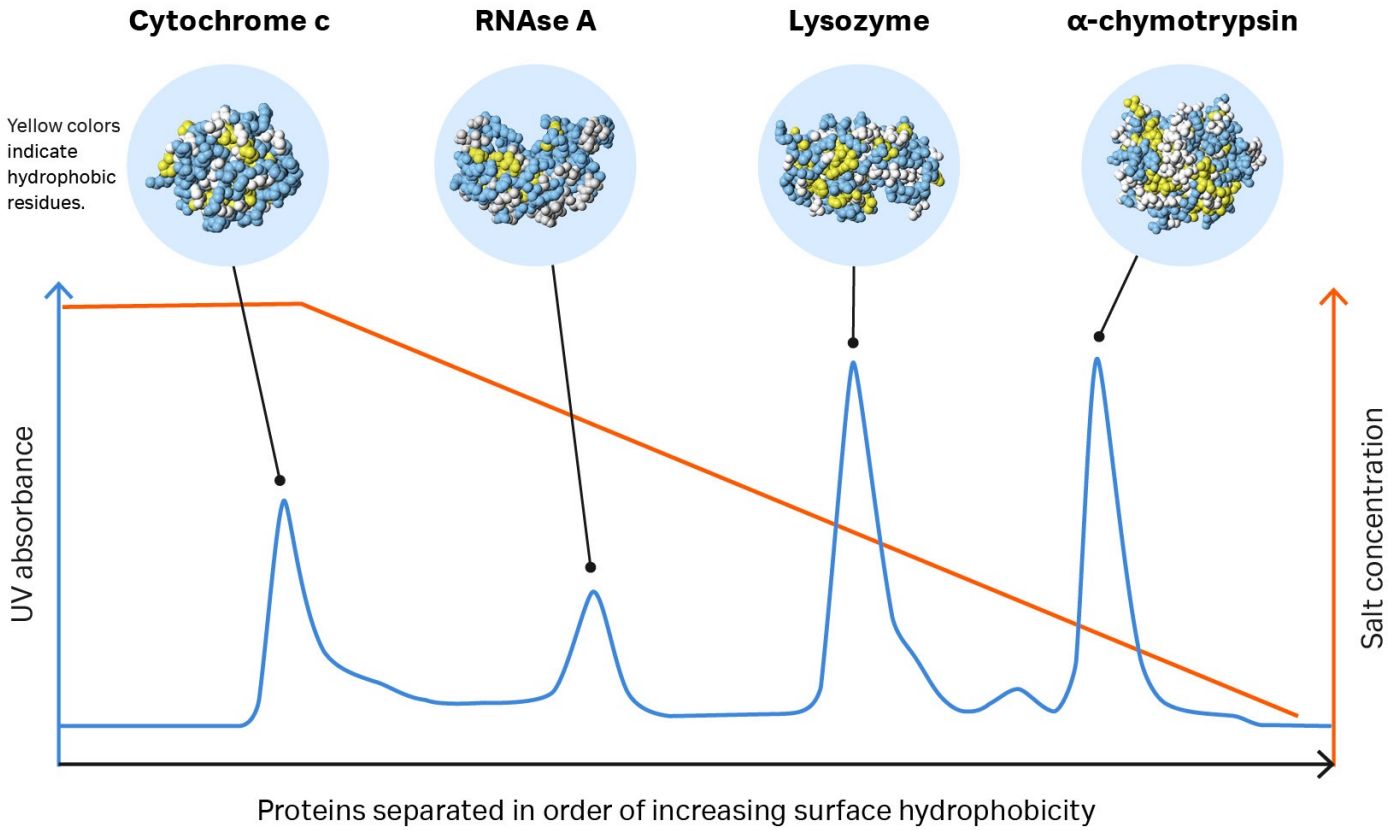
- RPLC is most commonly applied as the final dimension of separation in proteomic study. This is due to the solvent used in RPLC is compatible with MS.



0.1% formic acid in water, acetonitrile with 0.1% formic a

Hydrophobic Interaction Chromatography (HIC)

- HIC uses hydrophobic amino acids on the surface of the protein to interact with a matrix carrying other hydrophobic groups, such as butyl or phenyl.
- When the ionic strength of the buffer is reduced, the interaction is reversed.
  - the protein with the lowest degree of hydrophobicity is eluted first;
  - The most hydrophobic protein elutes last



https://www.cytivalifesciences.com/en/us/solutions/protein-research/knowledge-center/protein-purification-methods/hydrophobic-interaction-chromatography

Question: What properties of a protein does hydrophobic interaction chromatography exploit for purification?

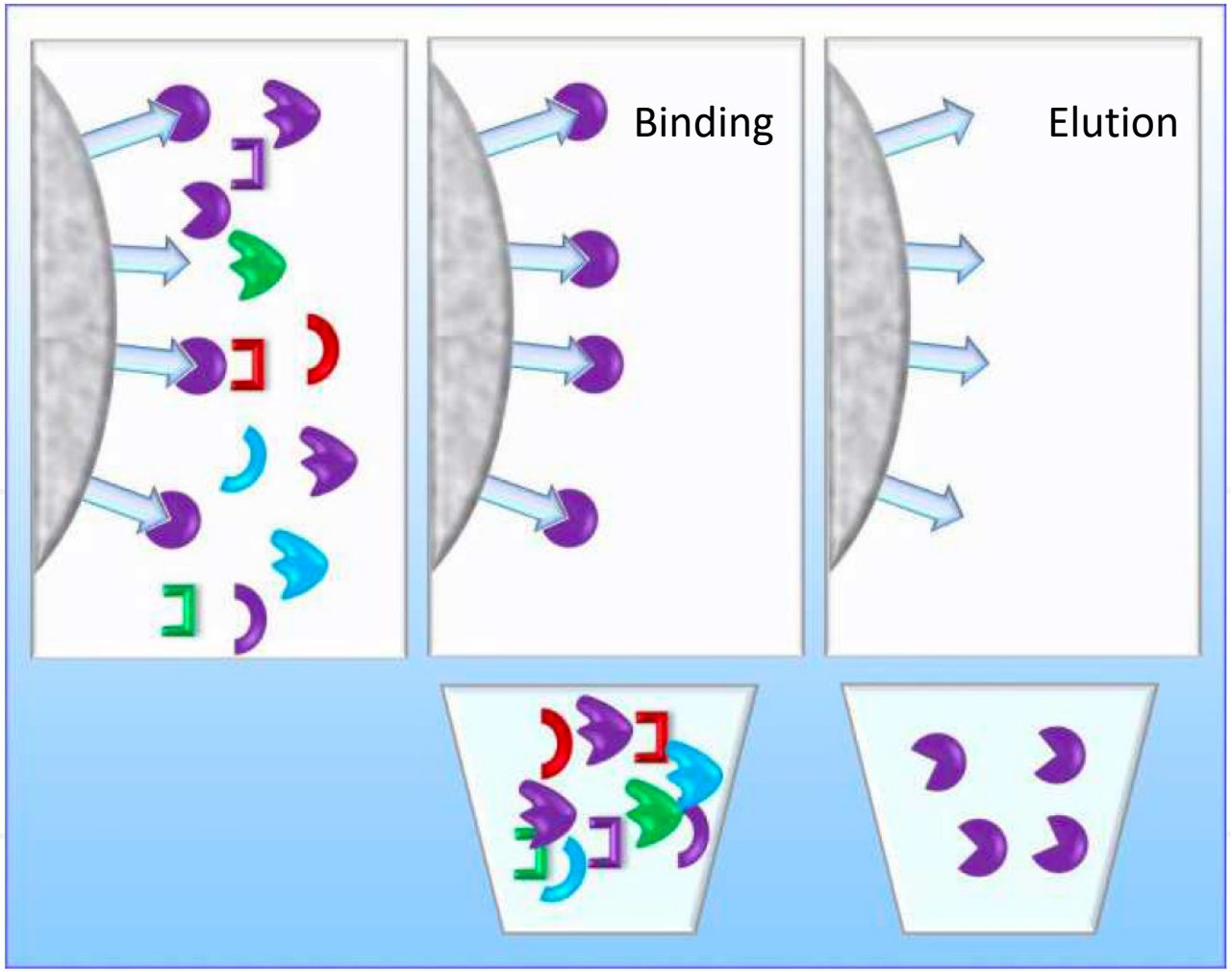
a) Charged amino acids

b) Hydrophobic amino acids on the protein surface

c) Molecular weight

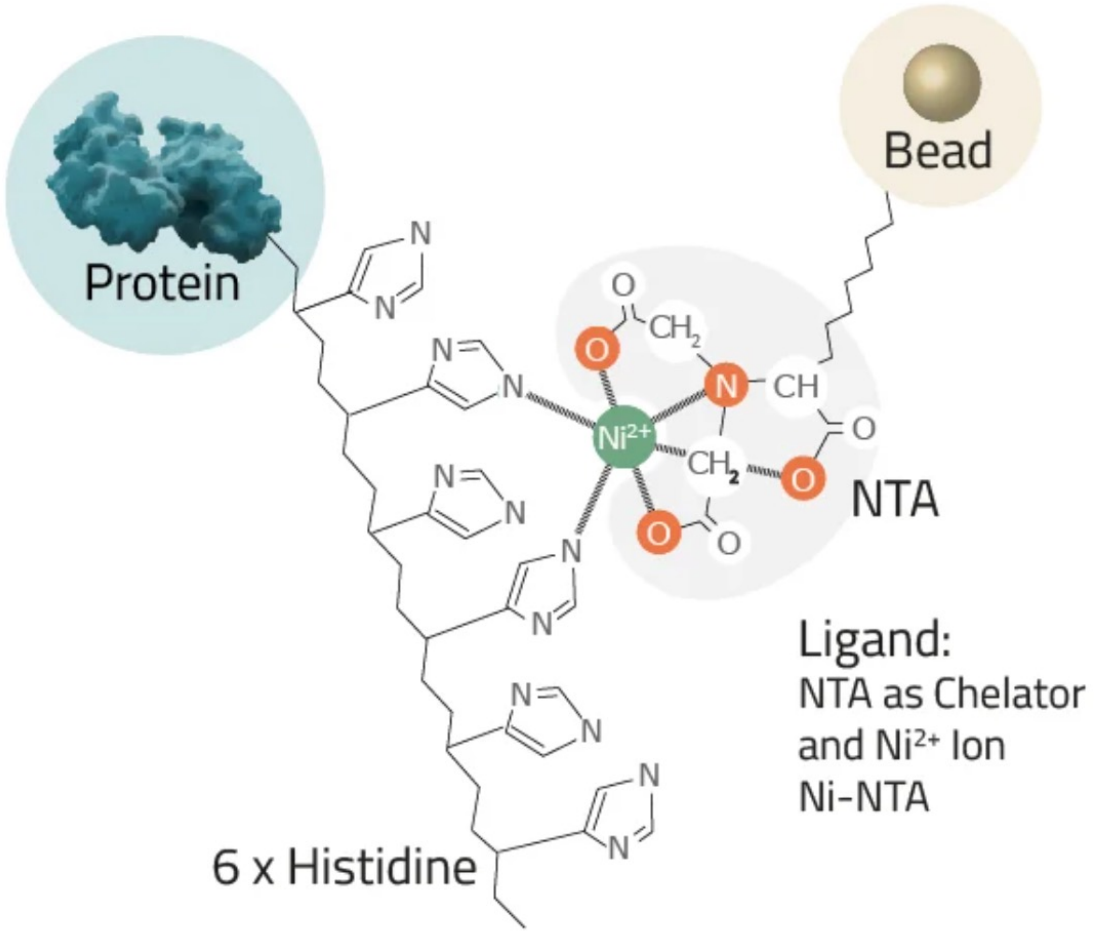
d) Enzyme activity

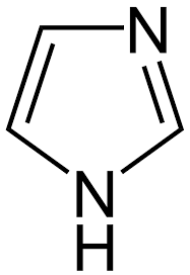
4. Immobilized Metal Affinity Chromatography



- Separate proteins based on their specific, ligand binding affinity.
  - Metals (Ni^{2+}): 6x His tag
  - Phosphoprotein/peptide
  - Proteins binds to specific drug or substrate
  - Isolate proteins that interact to form a complex

Ni-Affinity Chromatography: commonly used

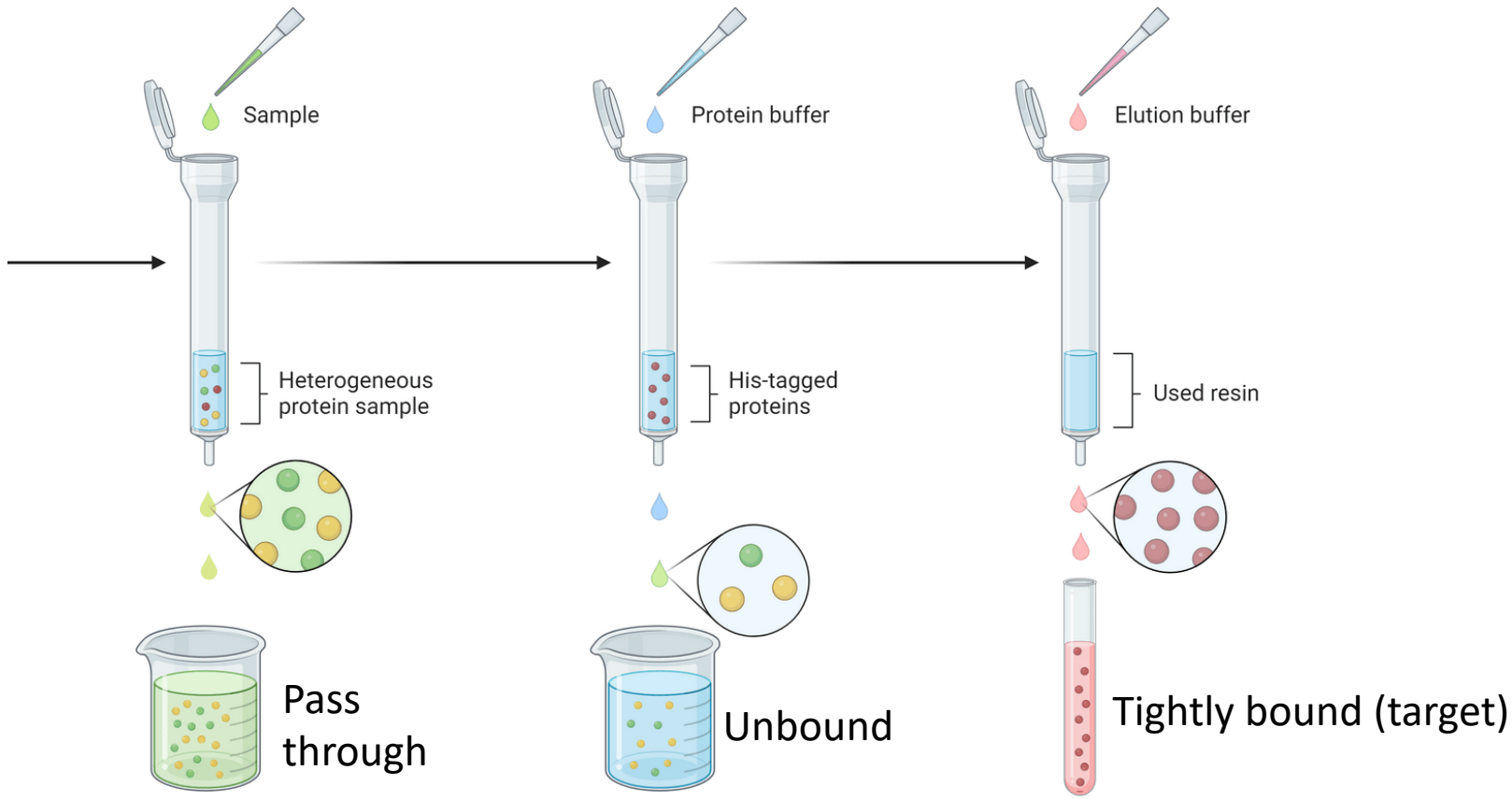




Imidazole
(the competitor)

300 mM Imidazole

pre-mix with the Profinty™ IMAC Ni-Charged Resin for 2 hours at 4°C



Question: To elute target proteins from an affinity chromatography matrix, which of the following conditions would be the most appropriate?

a) Low salt concentrations

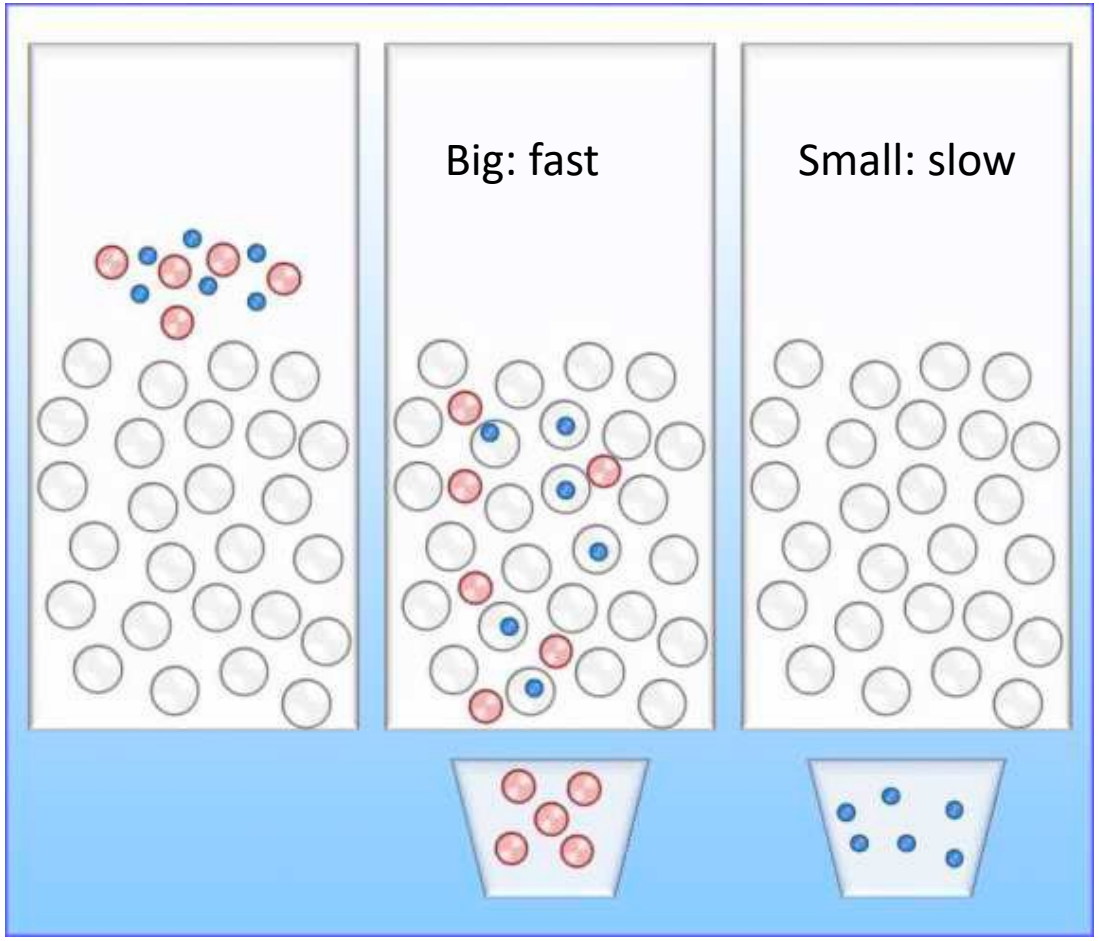
b) High salt concentrations

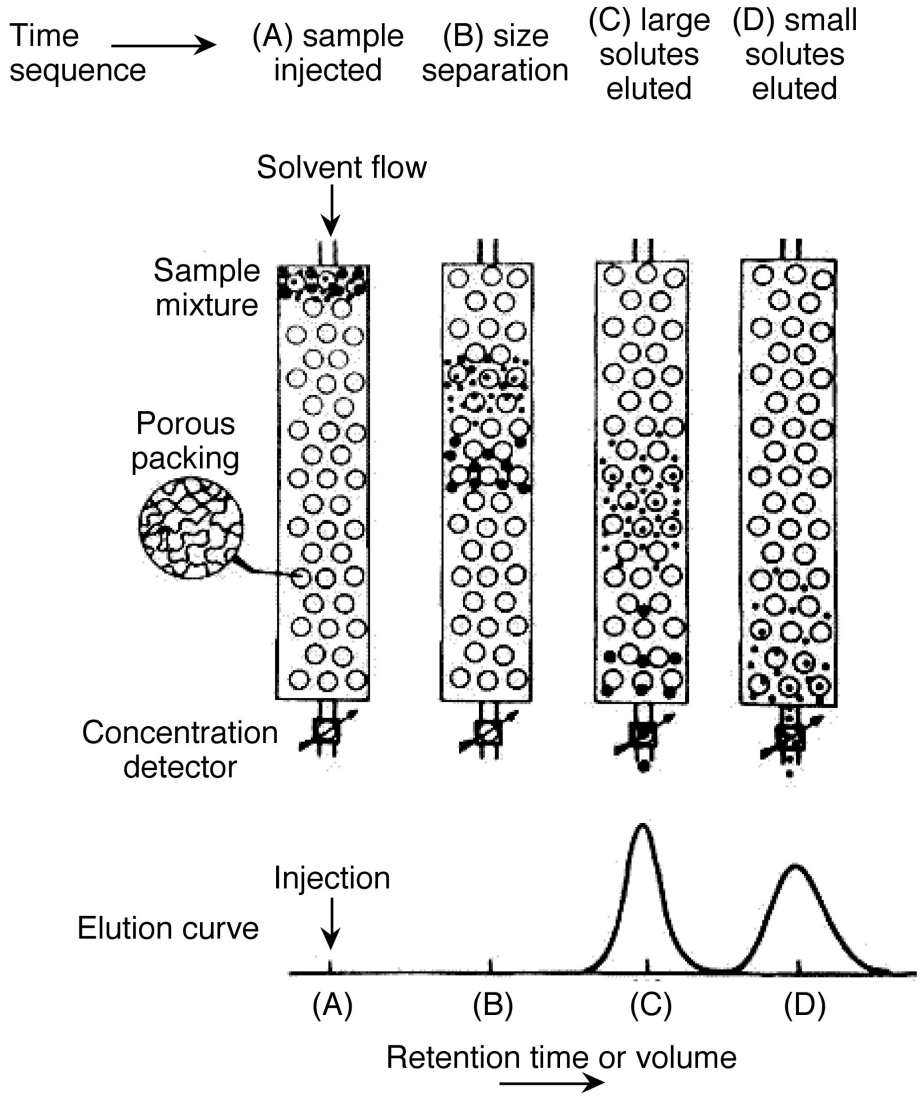
c) Adding a soluble ligand which competes with the affinity tagged protein for binding to the column

d) Just keep washing buffer through the column, isocratic elution

5. Gel Filtration/Size Exclusion Chromatography

- It separates proteins based on their sizes.





Different Types of Chromatography: Summary

(If don't know, first) 2. **Ion exchange** (cation exchange and anion exchange) - separates by surface charge on proteins

- **Cation exchange**: separates based on positive charges of solutes/proteins, matrix is negatively charged

- **Anion exchange**: separates based on negative charges of solutes/proteins, matrix is positively charged

3. **Hydrophobic interaction** - separates by hydrophobicity of Proteins

(If know the protein, first) 4. **Affinity** - separates by some unique binding characteristic of protein of interest for affinity matrix in column

5. **Gel filtration/size exclusion** - separates by size (molecular weight) of proteins

Question: Which of the following methods could be used to check the molecular weight of your purified protein?

a) SDS-PAGE only

b) Mass spectrometry only

c) Analytical SEC only

d) All of the above.

Question: Which of these chromatography types are suitable as a "capture" step in the purification of non-tagged proteins?

a) SEC

b) Dialysis

c) IEX and HIC

d) Ammonium sulphate precipitation