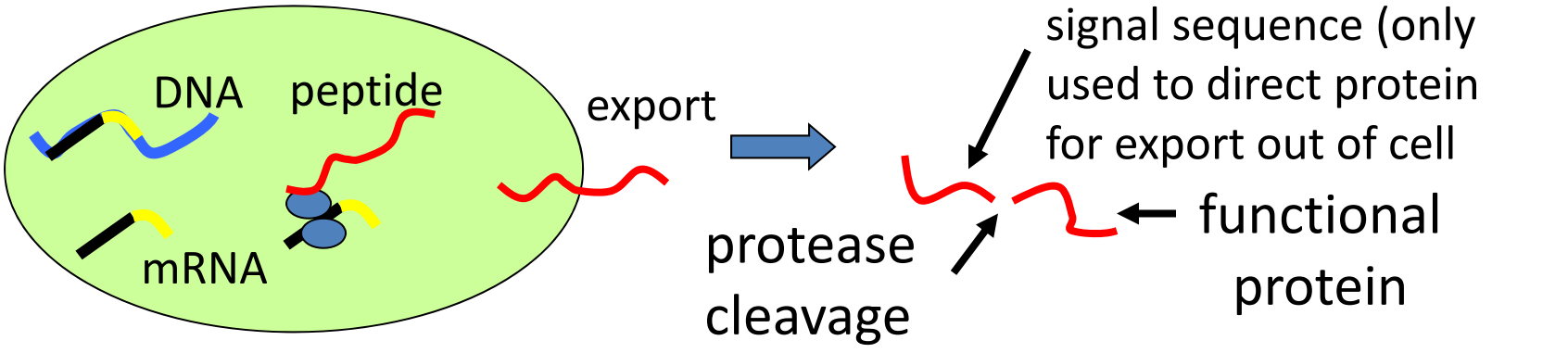
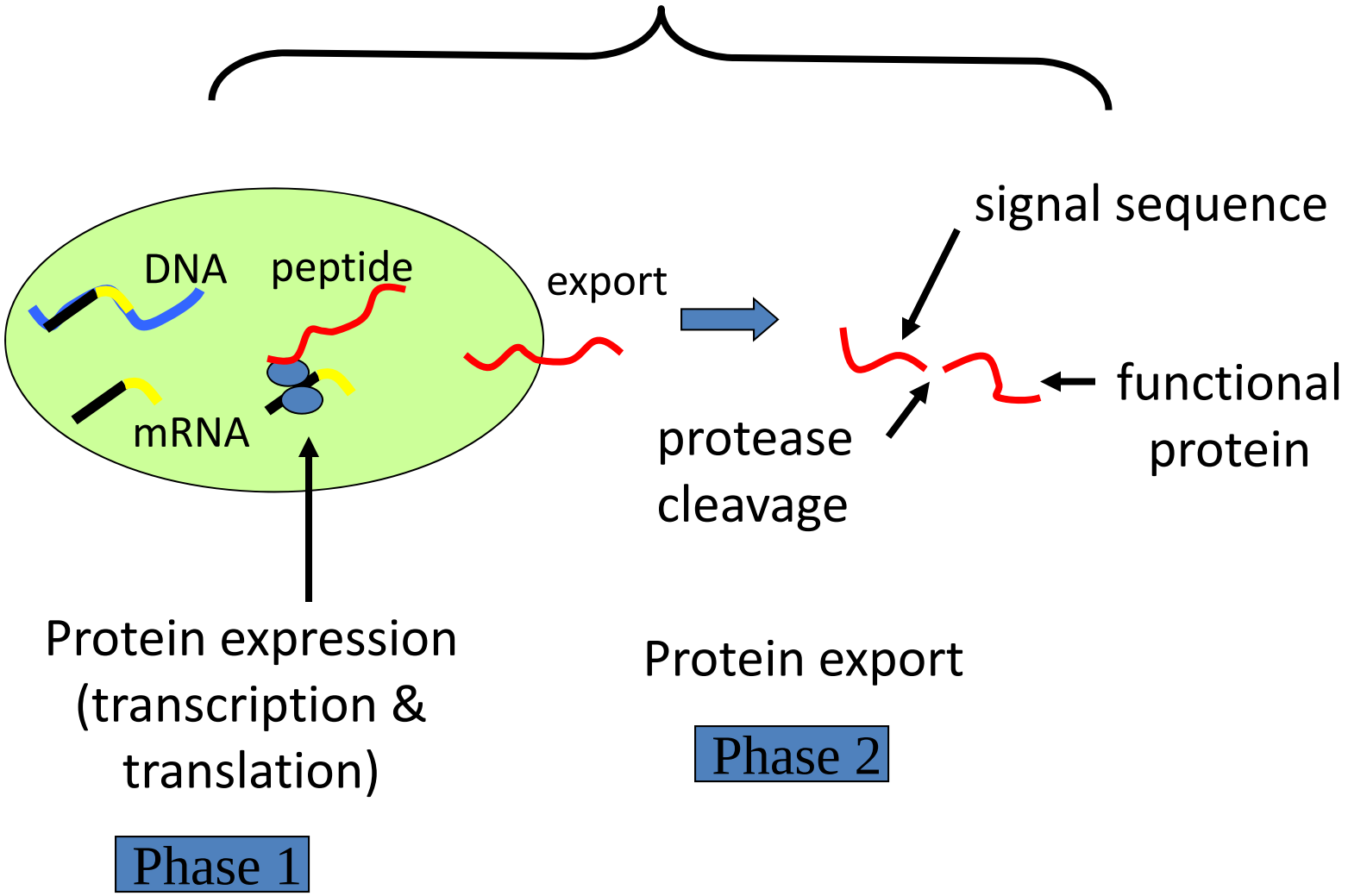
Protein Production

Protein engineering

- Hybrid proteins
  - a piece of one protein linked to a piece of another protein in the same amino acid chain.
    - This is often done to encourage *E. coli* cells to secrete protein into surrounding medium instead of holding it inside cell.
      - This makes it easier to recover and purify the protein.
      - An enzyme can be added after purification to cut off the part of the hybrid that signaled exportation.



Upstream processing



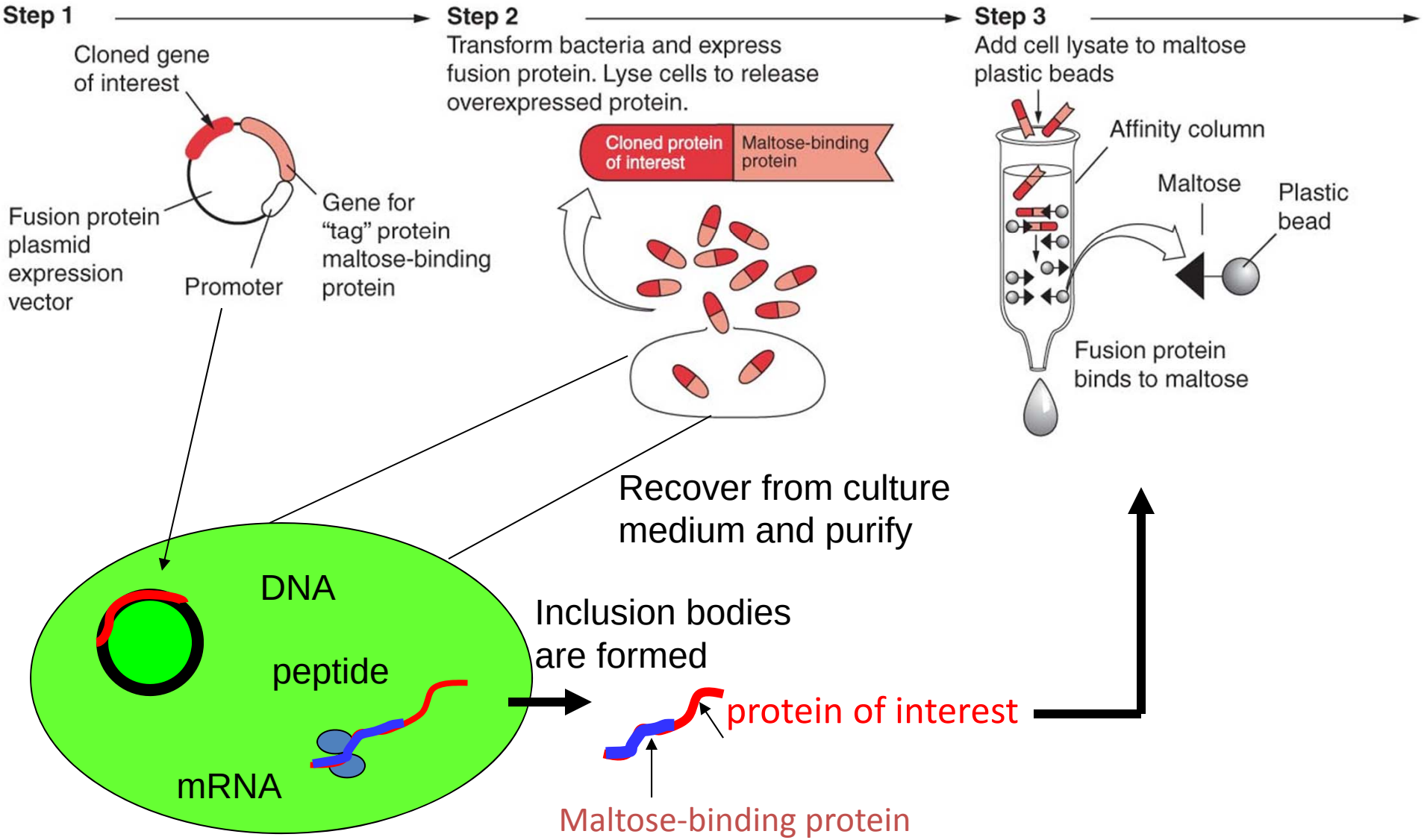
Cloning and Expression Techniques

- Bacterial fusion or hybrid proteins for synthesis and isolation of recombinant proteins
  - use recombinant DNA method to insert the gene for a protein of interest into a plasmid containing a gene for a well-known protein that serves as a "tag" for the protein of interest
  - the tag protein then allows for the isolation and purification of the recombinant protein as a fusion protein

Expression vectors

- Plasmid vectors for making fusion proteins are called expression vectors because they enable bacterial cells to produce or express large amounts of protein
  - vectors have gene encoding
    - Maltose-binding protein

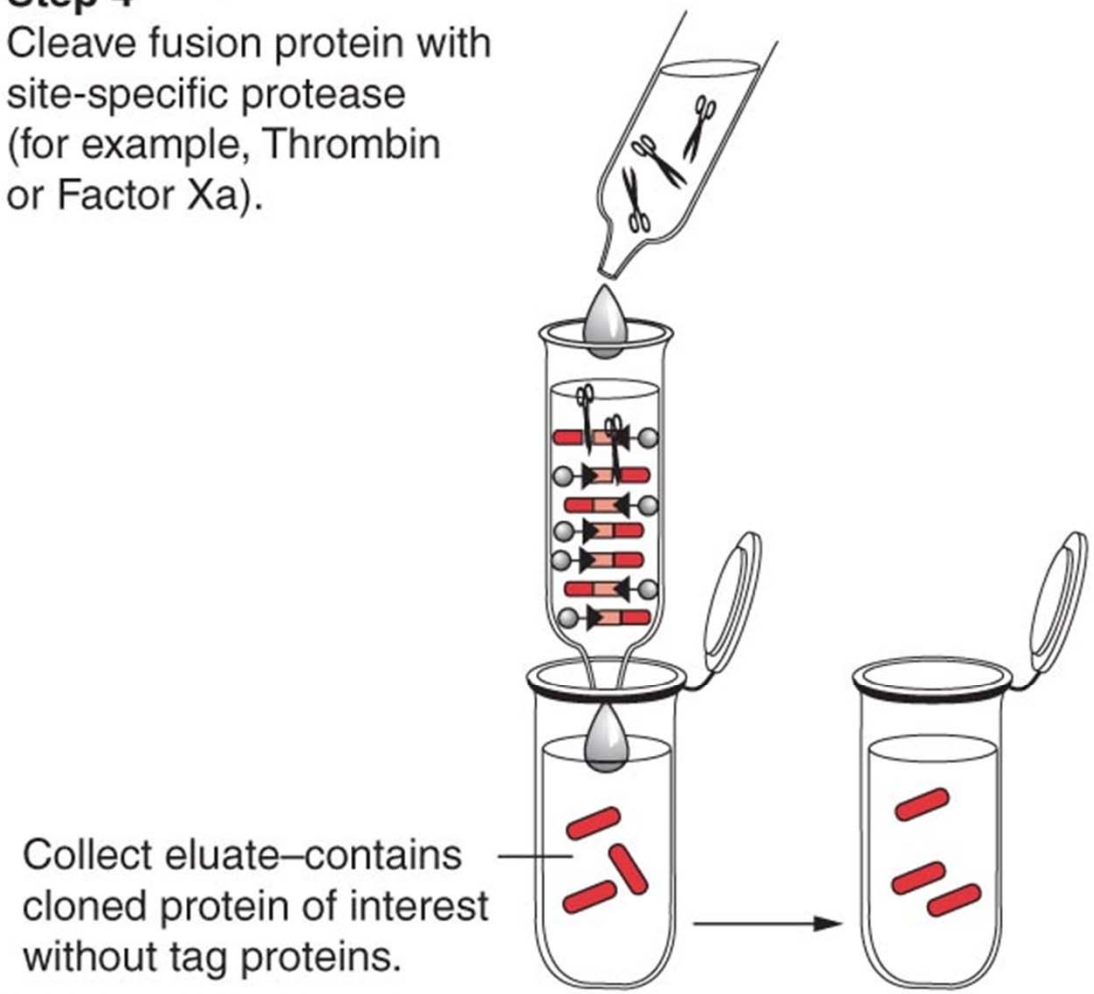
Fusion Proteins-how they are made and recovered



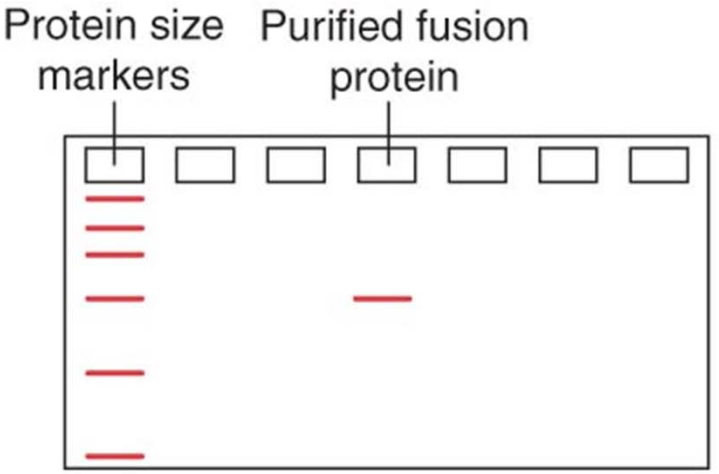
Fusion Proteins

Step 4
Cleave fusion protein with site-specific protease (for example, Thrombin or Factor Xa).

Step 5
Analyze by SDS-PAGE to check purity.



Collect eluate–contains cloned protein of interest without tag proteins.



Large-scale cultivation of microorganisms

- Fermentation vessels or bioreactors has impeller that stirs the culture so bacteria in suspension get good access to dissolved nutrients and oxygen for growth.

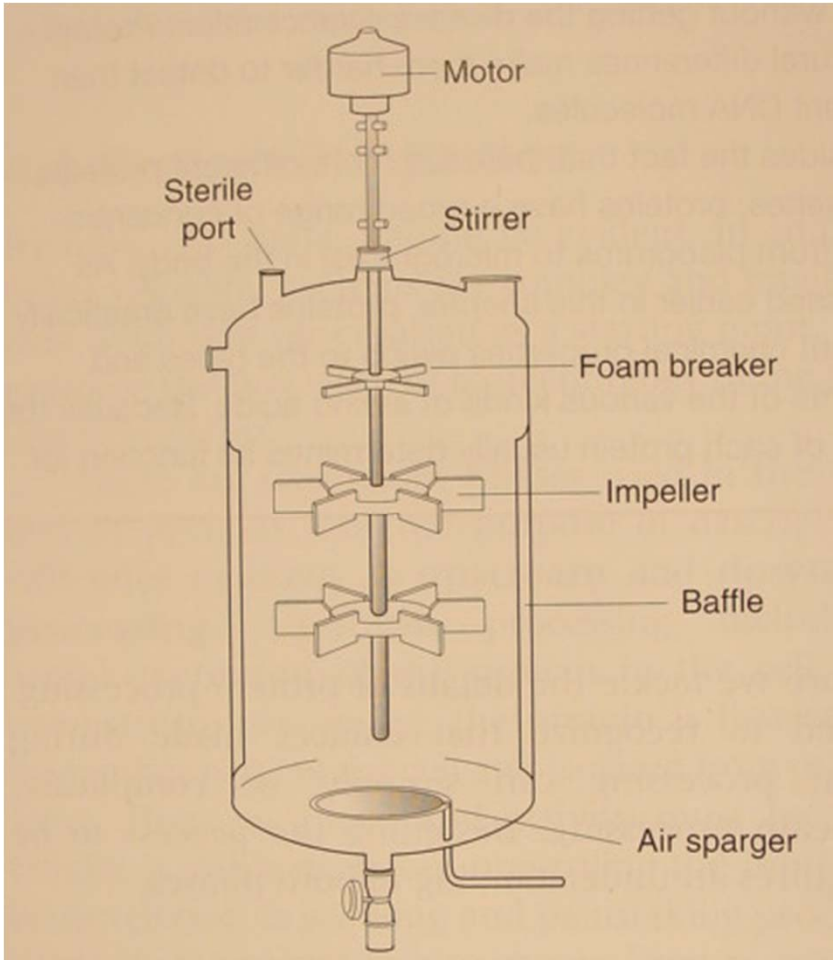


Figure 4.6 Microbial Batch Fermenter Fermentation

Recombinant protein production in *E.coli*

Advantages	Disadvantages
E. coli genetics are well understood	Foreign proteins produced as inclusion bodies must be folded
Almost unlimited quantities of proteins can be generated	Proteins can not be folded in ways needed for many proteins active in mammalian systems
Fermentation technology is well understood	Some proteins are inactive in humans

Recombinant proteins produced in eucaryotic fungi

TABLE 4.4 SOME RECOMBINANT PROTEINS FROM FUNGI

Protein	Fungi
Human interferon	*Aspergillus niger, A. nidulans*
Human lactoferrin	*A. oryzae, A. niger*
Bovine chymosin	*A. niger, A. nidulans*
Aspartic proteinase	*A. oryzae*
Triglyceride lipase	*A. oryzae*

Fungi are good hosts for producing **glycosylated proteins** since, as eucaryotic cells, they have the molecular machinery to add sugars molecules to peptide chains, whereas, bacteria do not have this machinery

Plant cells for protein expression

- 85% of all current drugs originated in plants
- Eg papain – proteolytic enzyme, produced on industrial scale and used as an meat-tenderizing agent.
- Plants can be genetically modified to produce proteins.
- Eg, tobacco plant is genetically engineered to produce million seeds from a single plant.
- Genetic material is integrated into it to produce million copies of the proteins of interest

Disadvantages

- Not all proteins can be expressed in plants
- Tough cell walls of plants make it difficult to extract the expressed proteins
- glycosylation in plant cells is different than the animal cells

Mammalian cell culture system

Problems

nutritional requirements of mammalian cells is complex

slower cell growth

easily contaminated

Animal bioreactor production systems

- Living animals are also protein producers
- eg monoclonal antibodies – they react against only one target.
- Antibodies are proteins produced in reaction to antigens (invading virus or bacteria), are part of immune response
- Mice are injected with an antigen. It produces the desired antibody, which is extracted by different methods.
- Another example – using milk or eggs containing recombinant genes from transgenic animals

Insect systems

- Glycosylation is slightly different than mammals
- Used to produce only when small quantities of proteins are needed

Protein Production

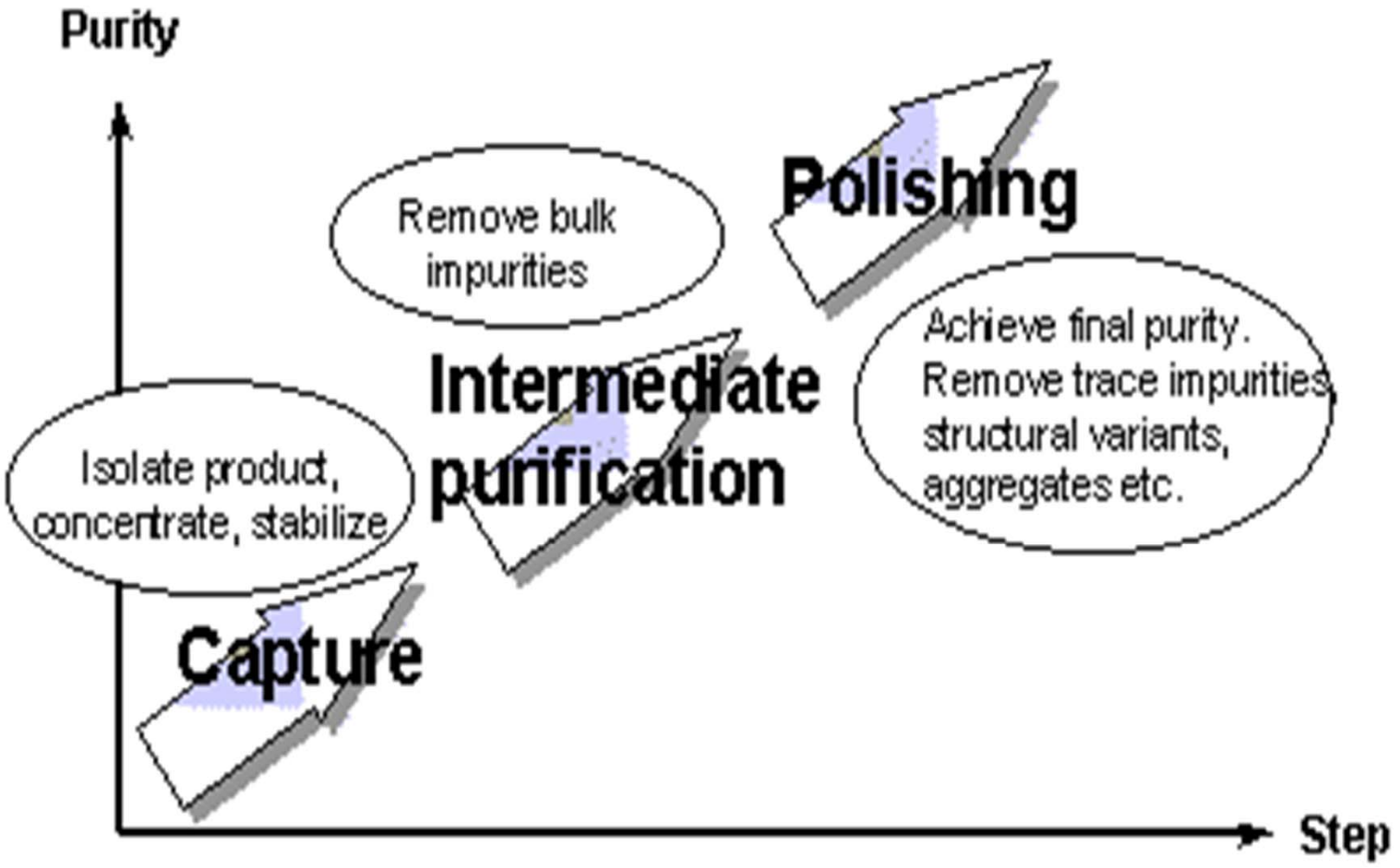
- Proteins are valuable
- Proteins are complex and fragile products
- Production of proteins is a long and painstaking process
  - *Upstream processing* includes the actual expression of the protein in the cell
  - *Downstream processing* involves purification of the protein and verification of the function; a stable means of preserving the protein is also required

Protein Production

- Protein Expression: The First Phase in Protein Processing
  - Selecting the cell to be used as a protein source
    - Microorganisms
    - Fungi
    - Plants
    - Mammalian cell systems
    - Whole-animal production systems
    - Insect systems

Protein Purification

Three Phase Strategy



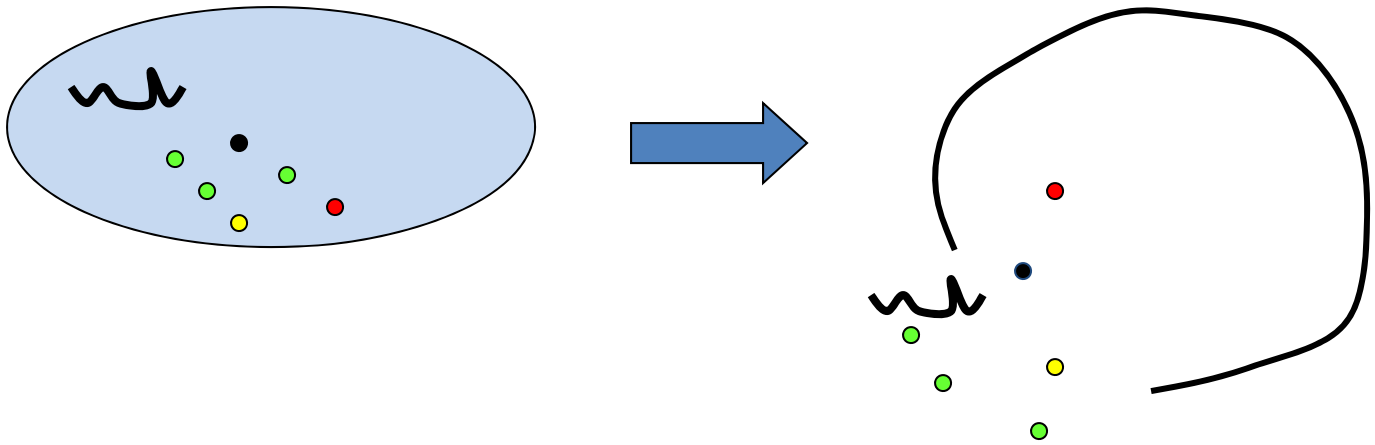
Protein Purification Methods

- Protein Must Be Harvested
  - Target protein is usually < 1% of total culture medium.
  - Entire cell is harvested if protein is intracellular
    - Requires cell lysis to release the protein
    - Releases the entire contents of the cell
  - Culture medium is collected if the protein is extracellular

Cell lysis
(when protein is still inside cell)

Disrupting the cell wall to release the protein

- Freezing and thawing
- Detergents (to dissolve cell walls)
- High-energy sound waves,
- High salt concentration



Proteins are extremely fragile!

Stabilizing proteins in solution

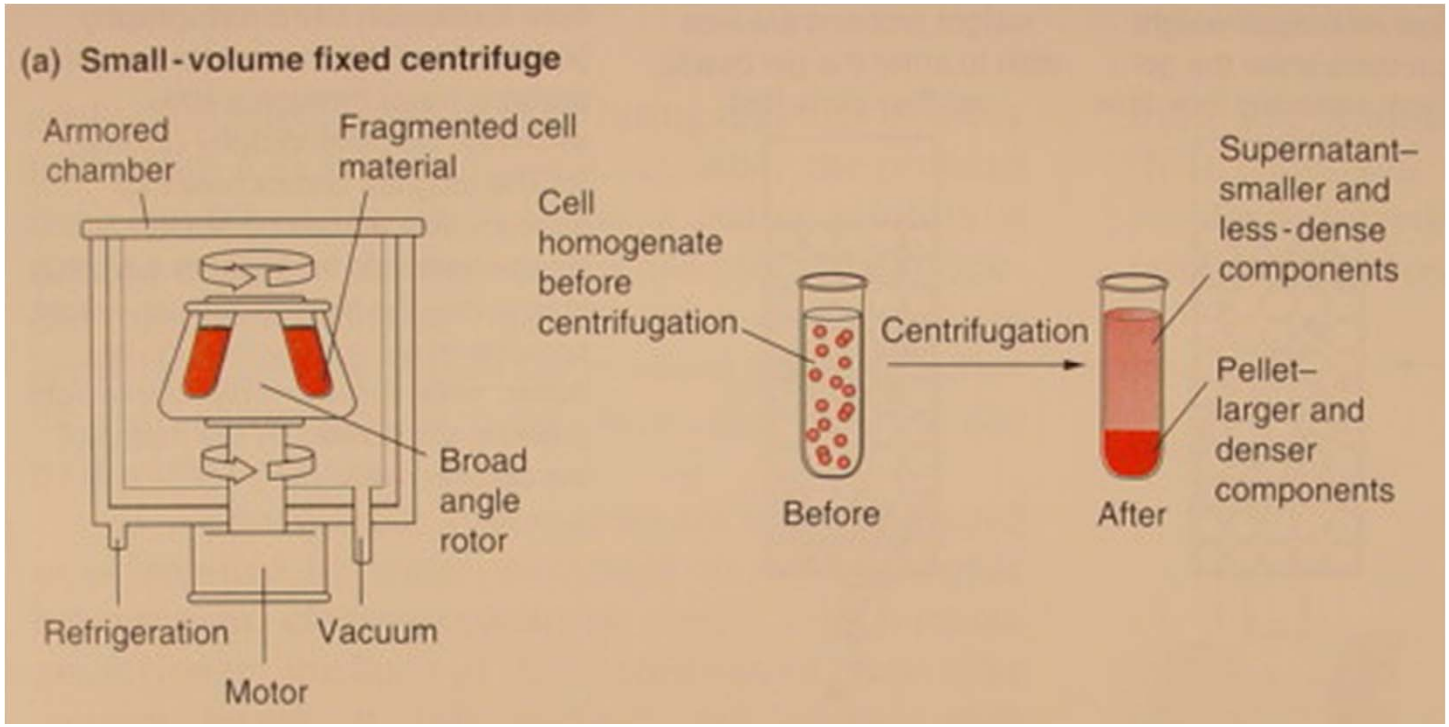
- Important to maintain the bioactivity of the protein
- Proteins are relatively fragile – prevent their degradation
- Low temperature
- Proper pH
- Protection against natural proteases – protease inhibitors and antimicrobials
- Prevention of mechanical destruction by foaming and shearing.

Protein Purification Methods

- Separating the Components in the Extract
  - Similarities between proteins allow the separation of proteins from non-protein material
    - Hydrophilic residues on protein surfaces are utilized for protein precipitation – salts (ammonium sulphate) cause proteins to settle out of solution
    - Ammonium sulphate reacts heavily with stainless steel used in industrial facilities
    - Other solvents are used – ethanol, isopropanol, acetone, and diethyl ether.

Protein Purification Methods

- Separating the Components in the Extract
  - Filtration (size-based) separation methods
    - *Centrifugation:* separate samples by spinning them at high speed

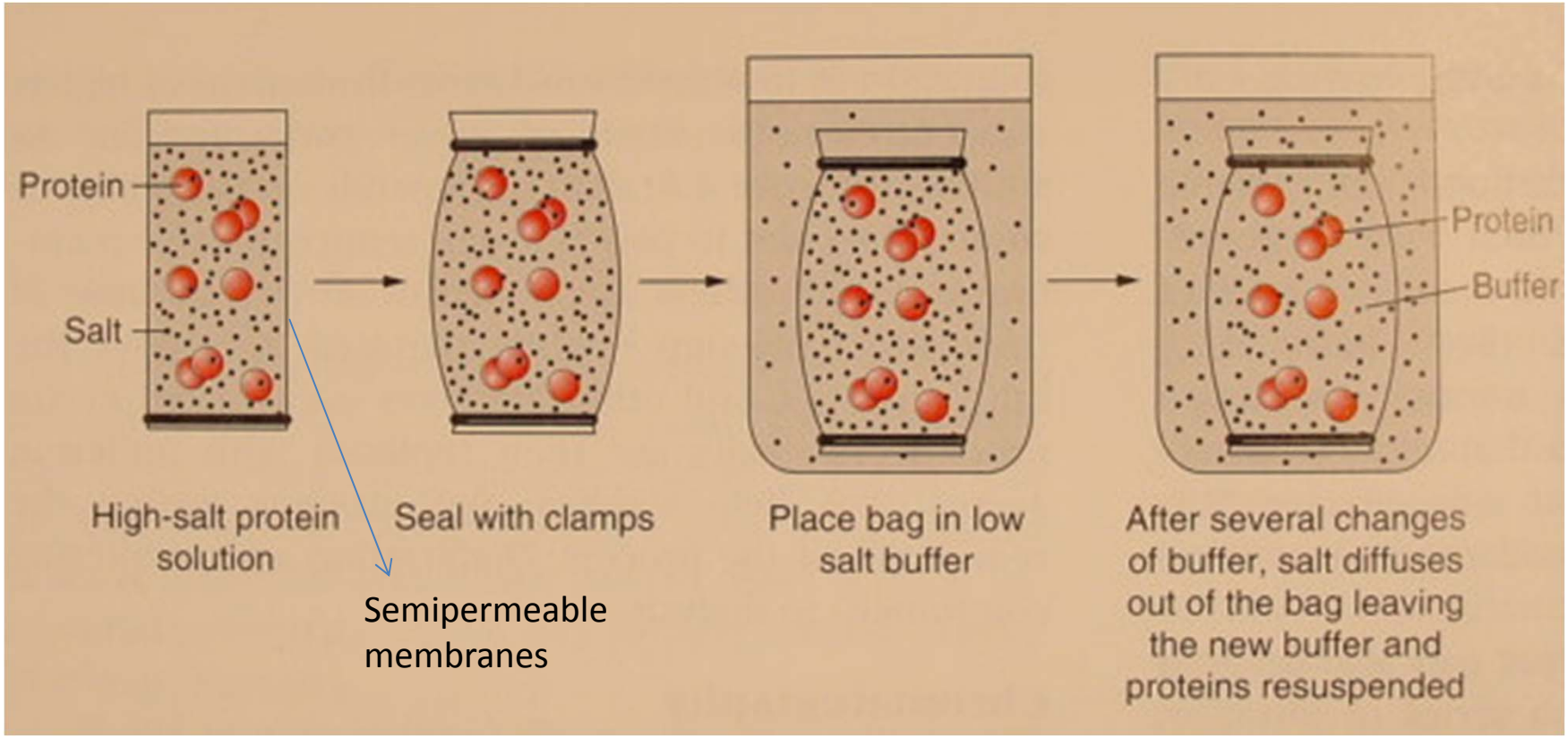


Protein Purification Methods

- Separating the Components in the Extract
  - Filtration (size-based) separation methods
    - *Membrane filtration* (membranes of nylon with varying pore sizes)
    - *Microfiltration*: removes precipitates and bacteria
    - *Ultrafiltration*: removes proteins and nucleic acids
    - Clog easily but faster than centrifugation

Protein Purification Methods

- Separating the Components in the Extract
  - **Diafiltration and dialysis** rely on the chemical concept of equilibrium – migration of dissolved substances from areas of higher concentration to areas of lower concentration



Dialysis

- **Dialysis**-a process that separates molecules according to size through the use of semipermeable membranes containing pores of less than macromolecular dimensions**.**
- **Pores** in the membrane allow solvents, salts and small metabolites to diffuse across but block larger molecules.
- Cellophane (cellulose acetate) most commonly used dialysis material.
- Usually used to change the solvent in which the protein is dissolved in.

Protein Purification Methods

- Separating the Components in the Extract
  - **Chromatography** – allows the sorting of proteins based on size or by how they cling to or dissolve in various substances
  - Long glass tubes are filled with microscopic resin beads (filtration system) and a buffered solution
  - Protein extract flows through the resin beads
  - Depending upon the resin, protein either sticks to the beads or passes through the column

Protein Purification Methods

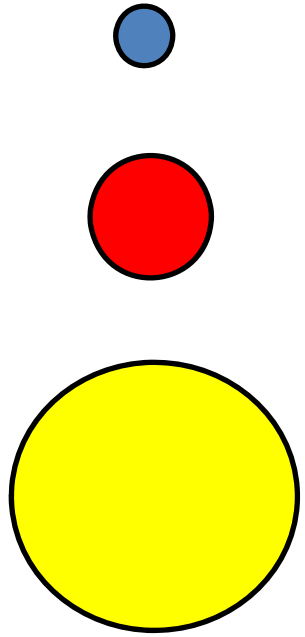
- Separating the Components in the Extract
  - Chromatography
    - **Size exclusion chromatography (SEC)** – uses gel beads with pores
      - Larger proteins move quickly around the beads and smaller proteins slip through the pores and therefore move more slowly through the beads

A gel filtration column has beads with channels running through them.

Gel filtration chromatography

- Also called **size exclusion chromatography** or **molecular sieve chromatography**.

How does it work? If we assume proteins are spherical…



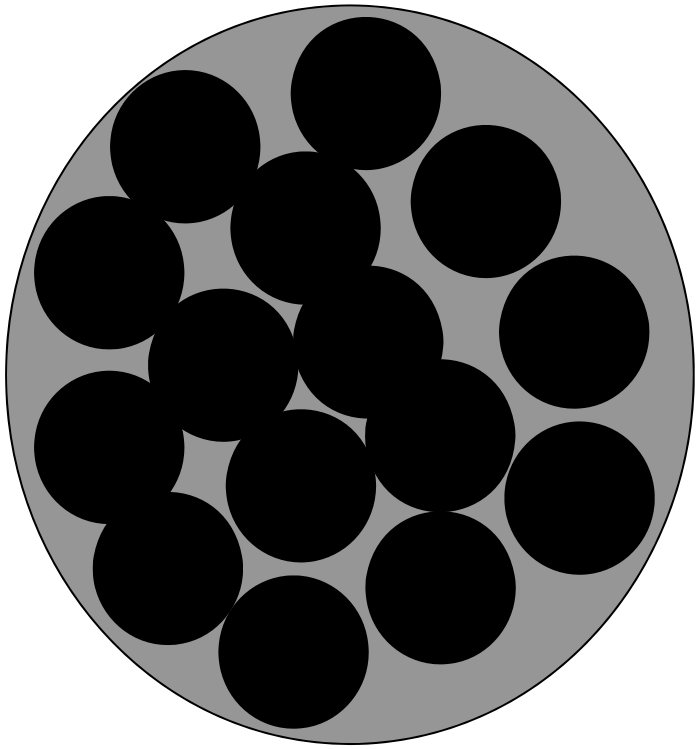
size

Molecular mass (daltons)

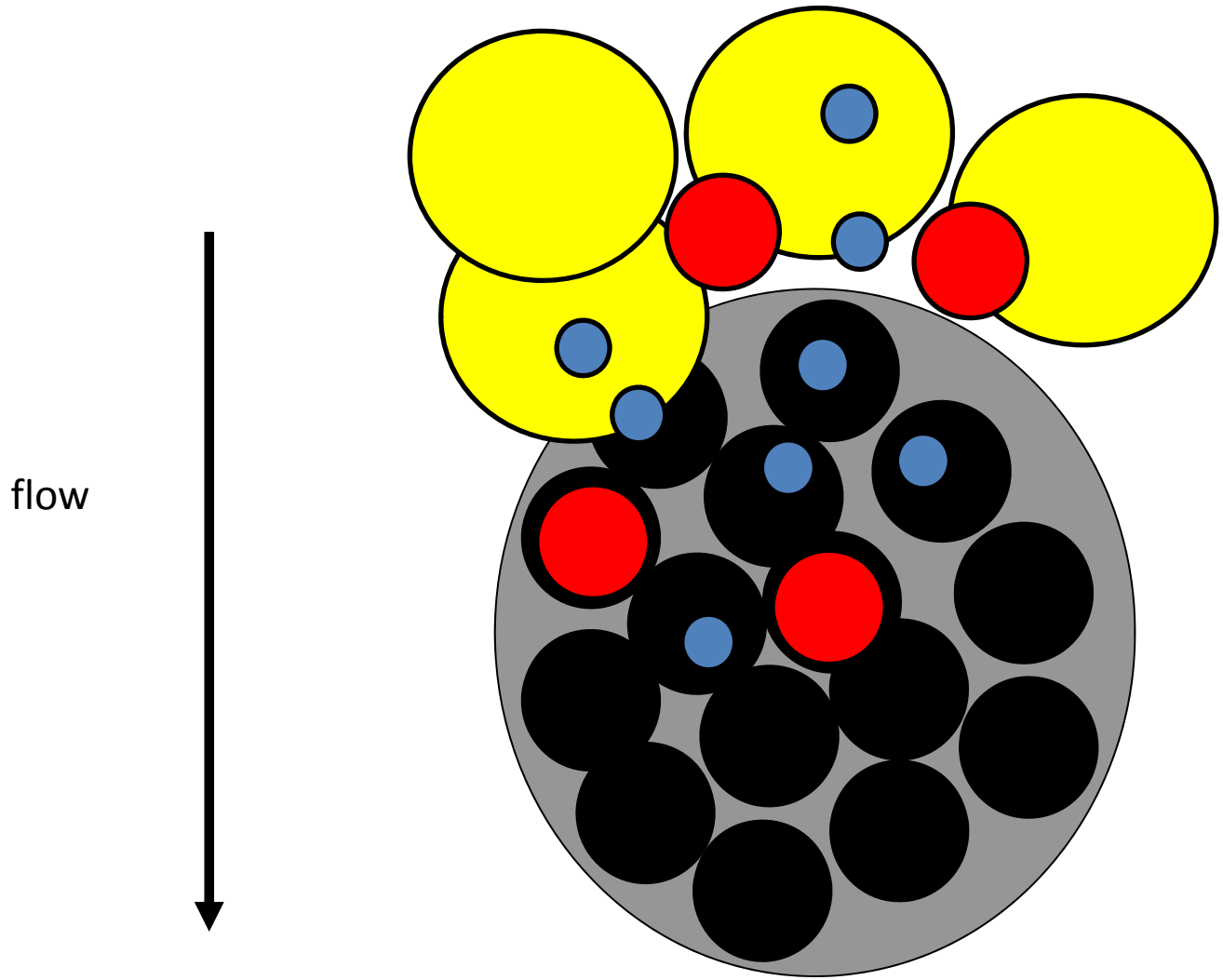
10,000

30,000

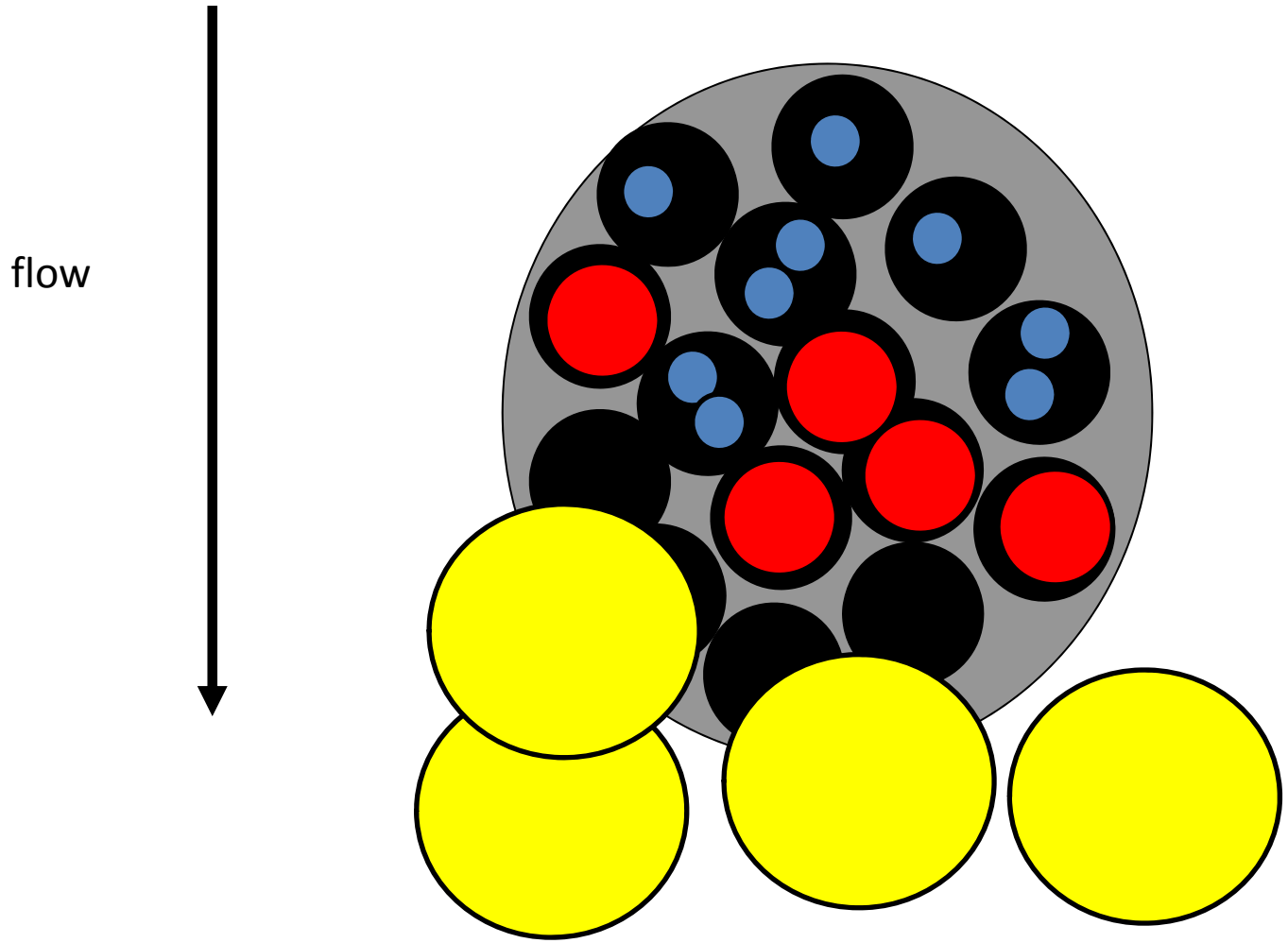
100,000



Gel filtration chromatography

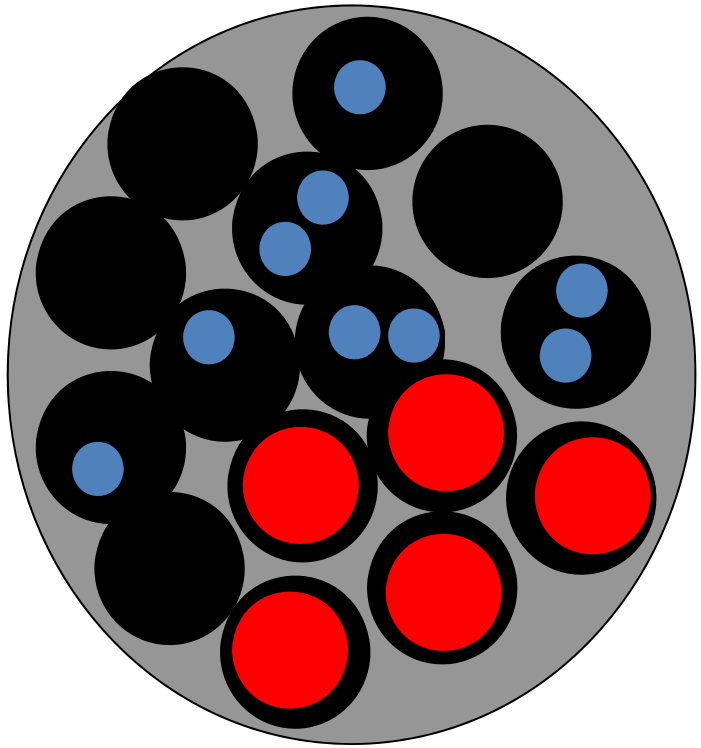


Gel filtration chromatography

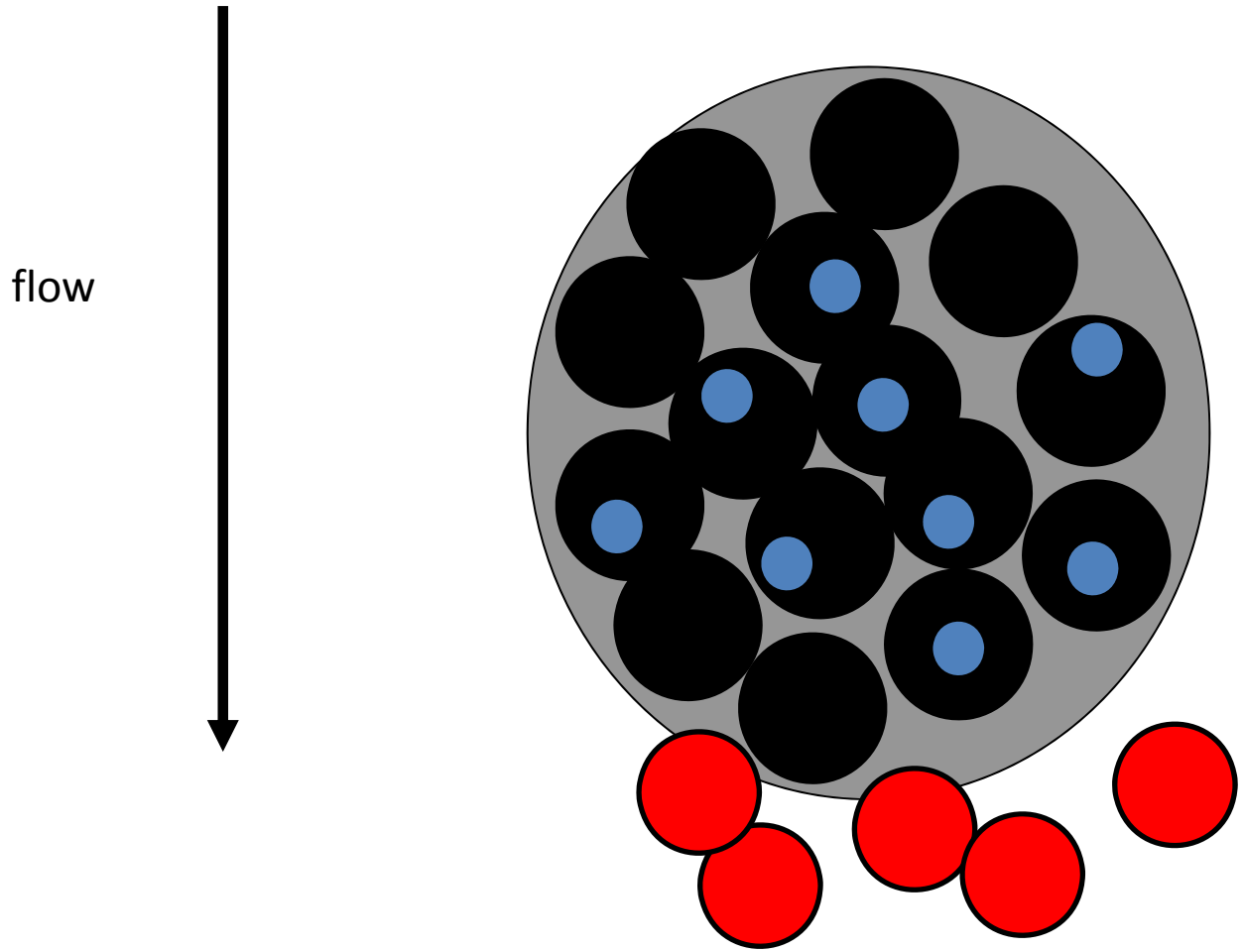


Gel filtration chromatography

flow



Gel filtration chromatography



Gel filtration chromatography

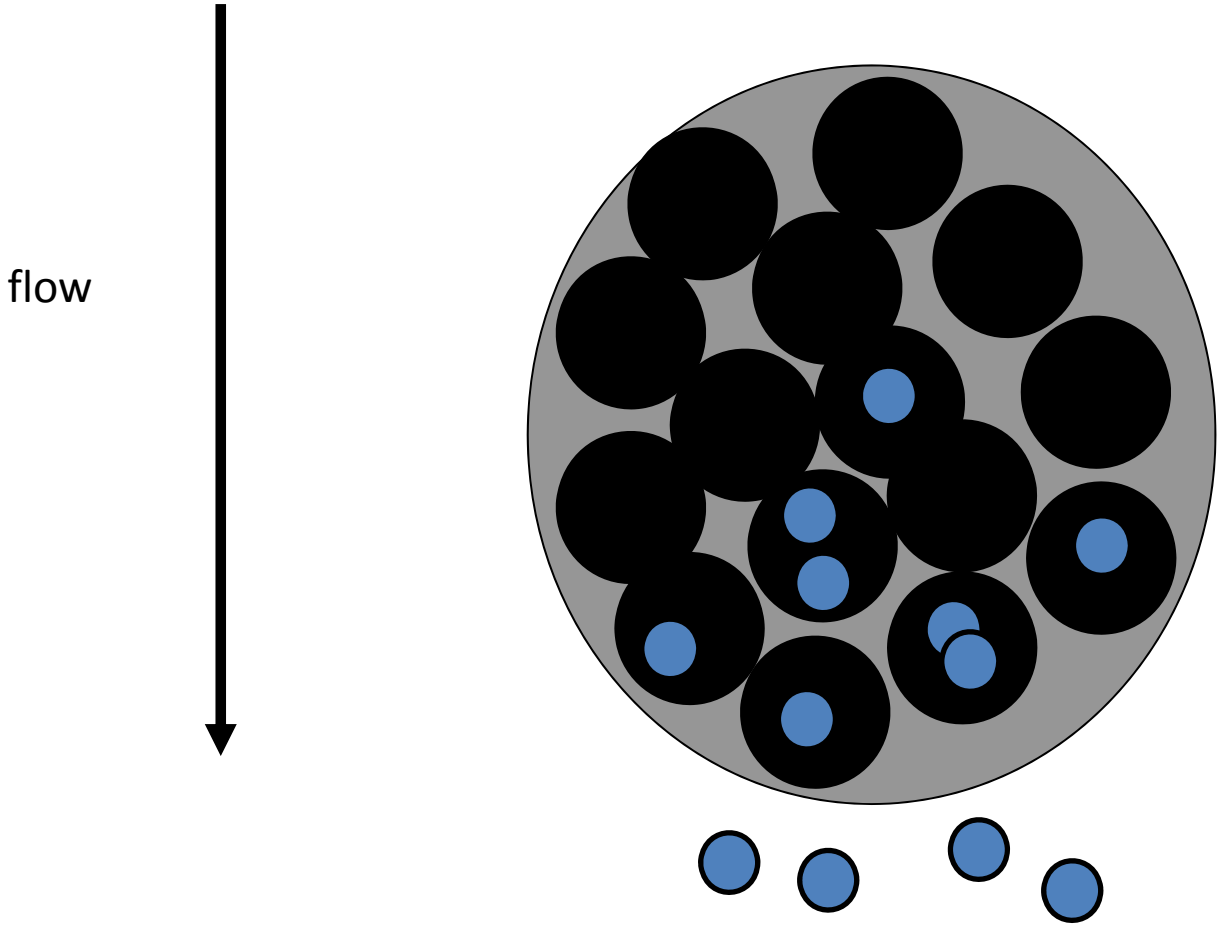
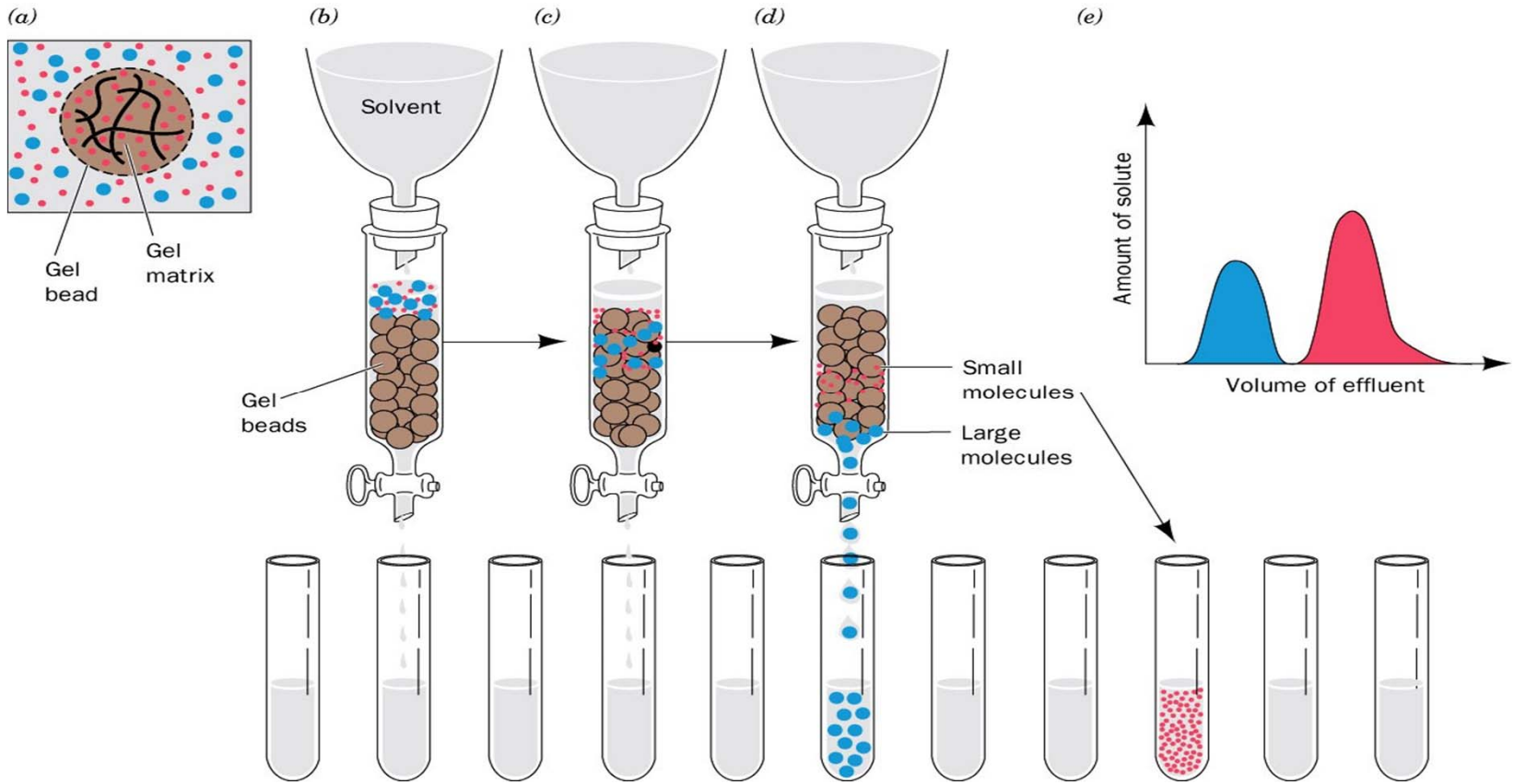
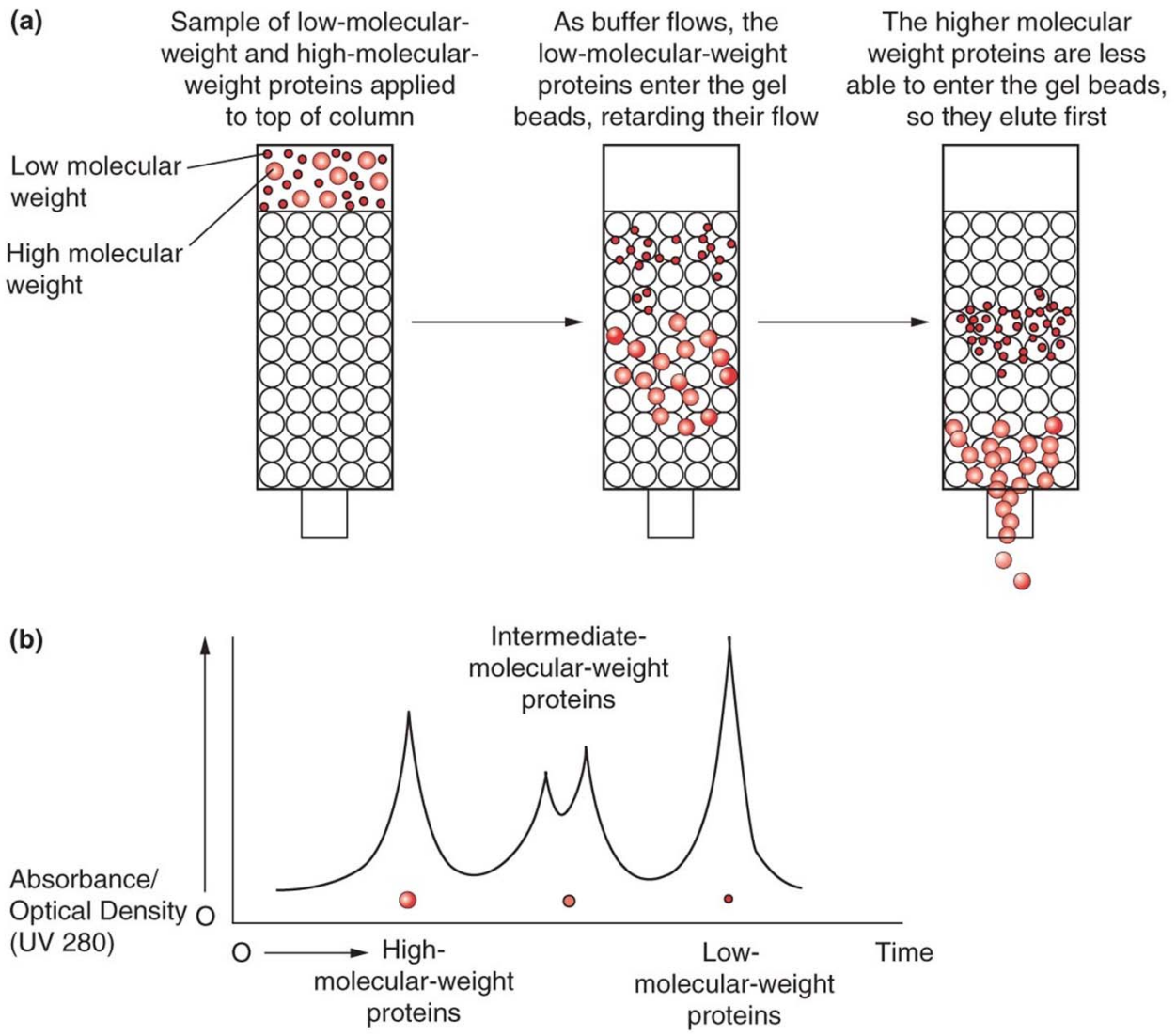


Figure 6-9 Gel filtration chromatography.



Protein Purification Methods

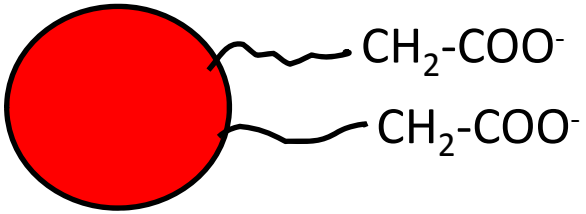


Protein Purification Methods

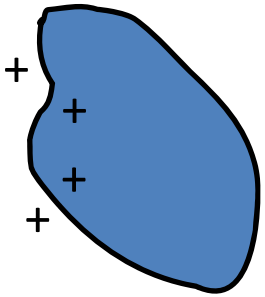
- Separating the Components in the Extract
  - Chromatography
    - **Ion exchange chromatography** – relies on the charge of the protein
      - Resin is charged
      - Opposite charged proteins will stick to resin beads
      - Can be eluted by changing the charge with salts of increasing concentration

Ion exchange chromatography

- Ion exchange resins contain **charged groups**.
- If these groups are *acidic* in nature they interact with positively charged proteins and are called **cation** exchangers.

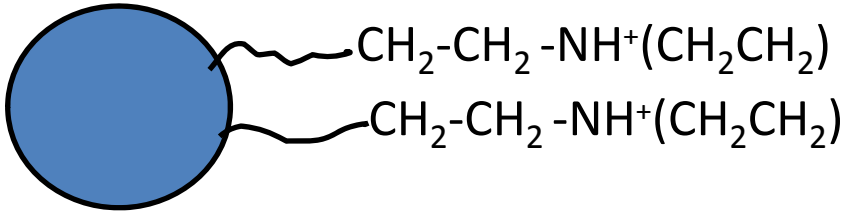


CM cellulose
cation exchanger

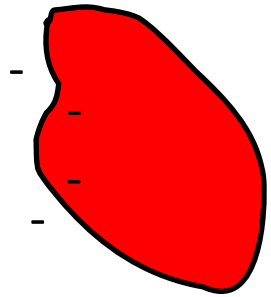


Positively charged (basic) protein or enzyme

- If these groups are *basic* in nature, they interact with negatively charged molecules and are called **anion** exchangers.



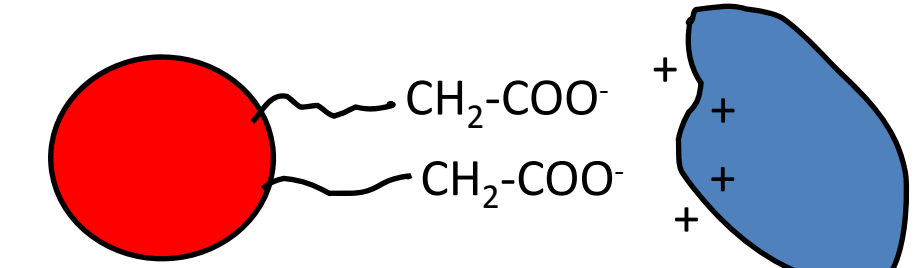
DEAE cellulose
anion exchanger



Negatively charged (acidic) protein or enzyme

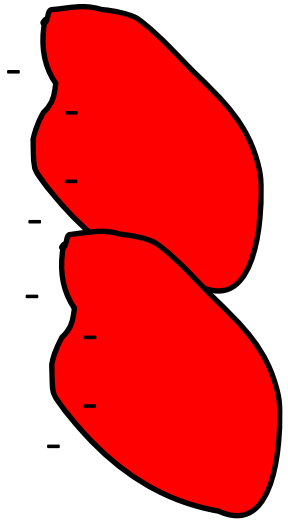
Ion exchange chromatography

For protein binding, the pH is fixed (usually near neutral) under low salt conditions.
Example cation exchange column…



CM cellulose
cation exchanger

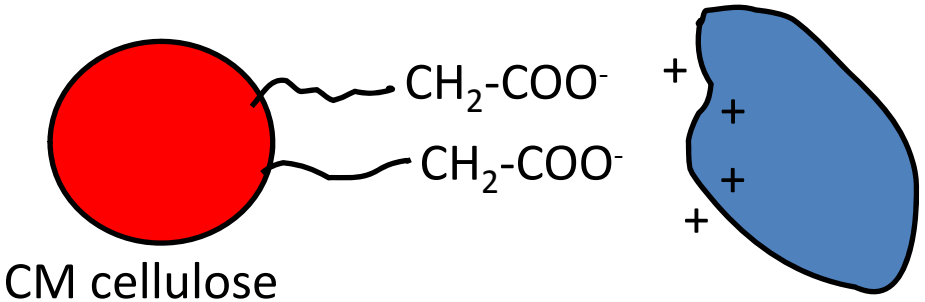
Positively charged protein or enzyme bind to the column



Negatively charged proteins pass through the column

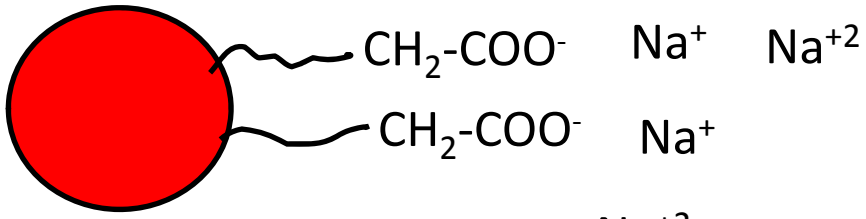
Ion exchange chromatography

To elute our protein of interest, add increasingly higher amount of salt (increase the ionic strength). Na^+ will interact with the cation resin and Cl^- will interact with our positively charged protein to elute off the column.



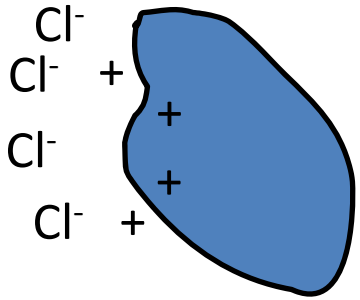
CM cellulose
cation exchanger

+ Increasing [NaCl] of the elution buffer



Na^+ Na^{+2} Na^+ Na^{+2}

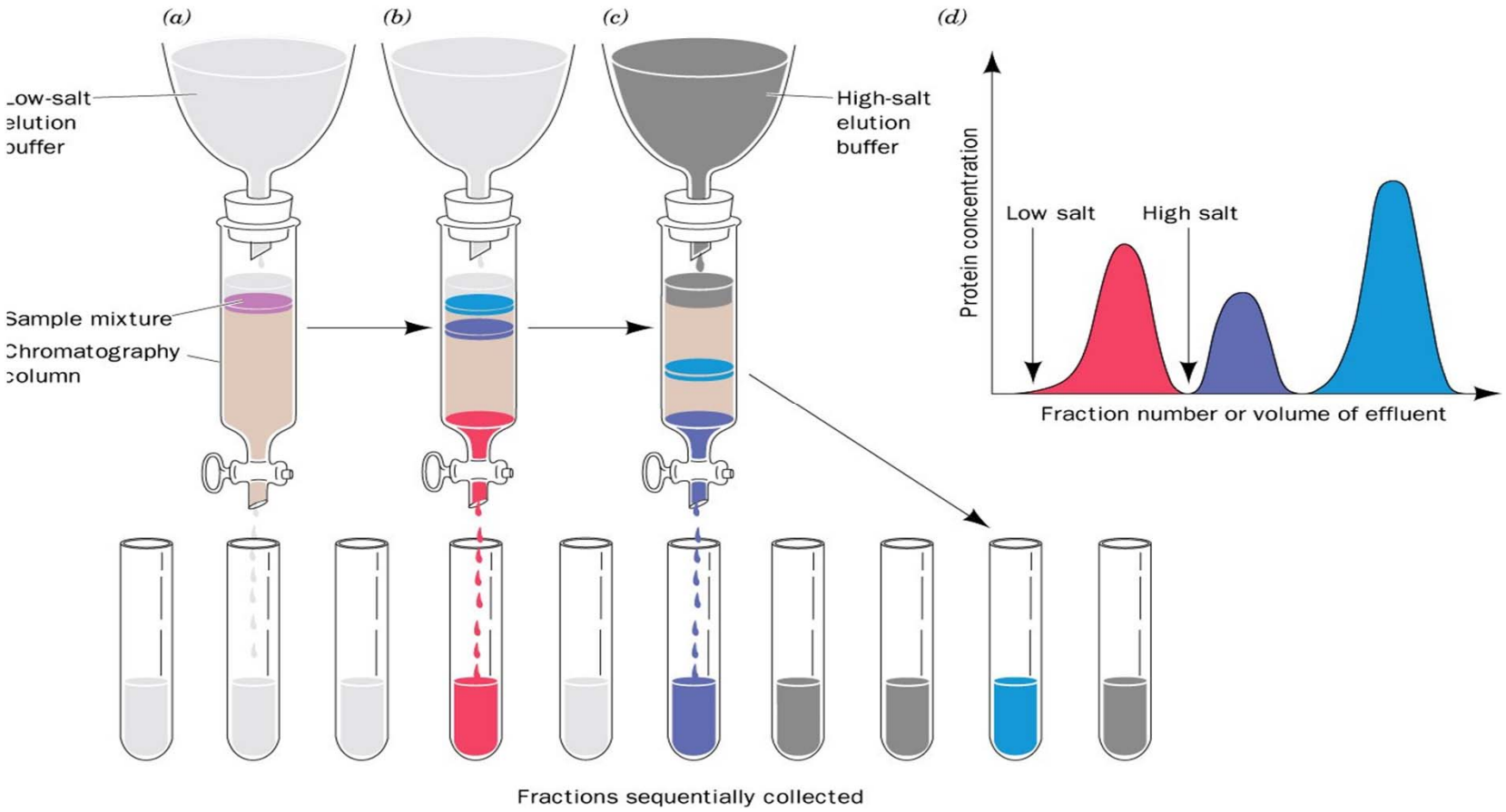
CM cellulose
cation exchanger



Ion exchange chromatography

- Proteins will bind to an ion exchanger with different affinities.
- As the column is washed with buffer, those proteins relatively low affinities for the ion exchange resin will move through the column faster than the proteins that bind to the column.
- The greater the binding affinity of a protein for the ion exchange column, the more it will be slowed in eluting off the column.
- Proteins can be eluted by changing the elution buffer to one with a higher salt concentration and/or a different pH (stepwise elution or gradient elution).
- Cation exchangers bind to proteins with positive charges.
- Anion exchangers bind to proteins with negative charges.

Figure 6-6 Ion exchange chromatography using stepwise elution.



Protein Purification Methods