Ultrafiltration

- Buffer exchange can be done via ultrafiltration
- Process: passing protein solution through a membrane filter under centrifugal force
- Molecules < MWCO → pass through filter
- Molecules > MWCO → retained within filter
- Allows high protein concentration in a low salt buffer

Protein Purification – Differential Solubilities

Function of: pH, salt conc (ionic strength) and temp

- pH:
  - Least soluble at pI
  - Proteins have unique pI → can be precipitated at different pH values
  - Can also use pI in purification and analysis
- Salt concentration:
  - Solubility decreases as [salt] increases → salting out w/ammonium sulfate
  - Can also concentrate dilute proteins
  - Allows for selective extraction
- Remove proteins from leftover solution by precipitation and low speed centrifugation
- High [salt] removes hydration shell from protein → exposes hydrophobic patches → clumping

Hydration Shell

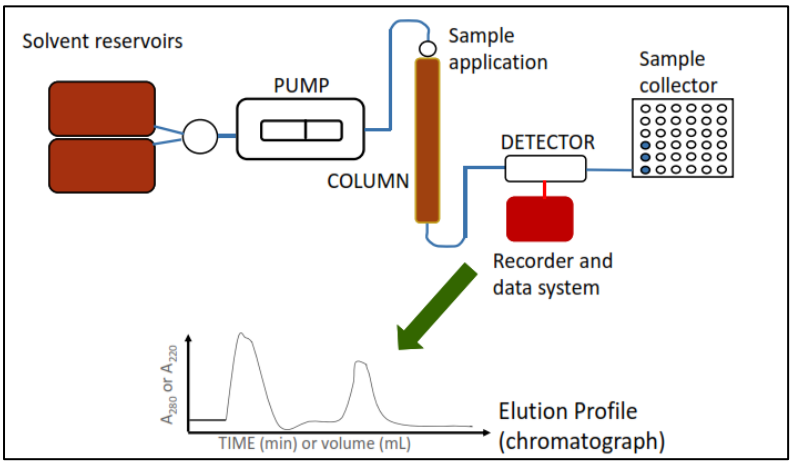
- Water solvates proteins → polar residues interact with water at dipoles
- Very few hydrophobic residues outside protein → water forms 'shell' around them
- Addition of salt draws the water away → exposes the residues → clumping
  - This does not deactivate the protein → just resuspend it

Column Chromatography

- Reservoir → supplies constant flow of buffer solution (contains mobile)
- Stationary Phase → porous solid matrix with chemical properties inside a column
- Mobile Phase → buffered solution that flows through matrix of stationary phase
- Eluent → Solution that passes out the bottom of the column → eluted volume V_e of each sample

- Protein sample is layered on top of column
- Buffer added from reservoir @ constant flow
- Proteins flow down column at different speeds due to different interactions with the stationary phase

Components



Two Types

Low Pressure Liquid Chromatography (LPLC)

- Simple and easy column setup
- Beads of size so low pressures are enough to push mobile phase through
- Slow flow rate → 0.1 mL/min

High Pressure Liquid Chromatography (HPLC)

- Very small rigid bead column setup
- High pressures required in order to push mobile phase through
- Very fast flow rate 1-10 mL/min

Ion-Exchange Chromatography

- Separates based on net charge
- Stationary Phase → polymer resin containing bound charged groups (+ or –)
- Proteins move through based on their fractional charge at the mobile phase buffer pH

Cation Exchangers → resin bound anionic (–) groups to attract cations: carboxyl-methylcellulose
Anion Exchangers → resin bound cationic (+) groups to attract anions: DEAE-cellulose

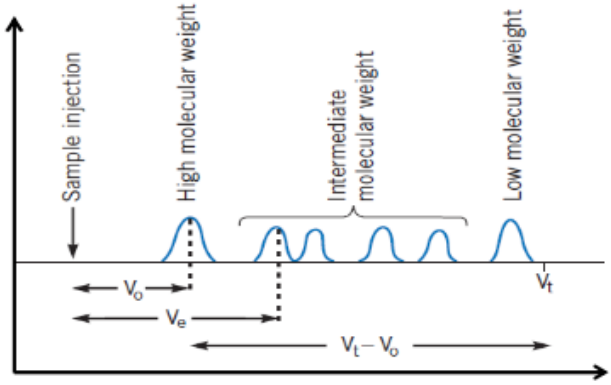
The net charge of each protein allows for separation using the matrix → proteins can then be eluted from the matrix using a linear NaCl gradient using 0 M and 0.5 M solutions to go from 0% 0M to 100% 0.5M

- This can be used to determine the binding affinity of proteins to the matrix

Lecture 10 – Size Exclusion Chromatography, Affinity Chromatography, Purification Tags

Size Exclusion Chromatography

- Separates proteins based on size
- Principle: movement within liquid phase through a stationary porous phase (cross-linked porous polymer)
  - Larger molecules can pass through the column faster → smaller ones spend more time in st phase
- Stationary Phase → Liquid inside the beads
- Mobile Phase → Solution outside the beads



- V_0 → void volume (non-bead volume)
- V_e → elution volume, related to size of eluted molecule
- V_t → total column volume
- $V_t - V_0$ = bead volume

Fractionation Range (FR)

- The lower/upper limit of proteins that can be separated by a particular stationary phase → unique for each resin
- Proteins below FR are in the V_t
- Proteins above FR are in the V_0
- Anything within the FR is between these volumes

Determining M_R

- Similar method to gel electrophoresis
- Run unknown protein in the column alongside known proteins to generate a linearized curve of standards

Applications of Size Exclusion Chromatography

- Purification
- Determination of M_R
- Desalting a protein solution → salt/low M_R contents eluted
- The process is fast, but only works for small volumes, it also dilutes the protein of interest

Affinity Chromatography

- Separates on basis of binding affinity to column matrix (has specific ligands)
- Buffer is the mobile phase
- Proteins with affinity to ligand will bind
- Protein with no affinity for ligand will not bind → washed out in mobile phase
- Bound proteins eluted by solution containing: high [ligand], high [salt] → outcompete/breaks interaction

Example 1

- ConA agglutinates RBCs
- Binds to glucose/mannose → stationary phase has mannose → ConA will bind to it

Example 2

- Serine Protease (Thrombin)
- Binds to Sepharose that has Benzamidine attached
- Mobile phase → buffer to promote interactions
- Elution buffer → competitive ligand or pH change (specific vs. non-specific elution)

Relies On

- Bio-specific ligand covalently attached to chromatography column
- Coupled ligand must retain specific binding affinity for target molecule
- Binding between target and ligand must be reversible

Affinity Purification Tag → Fusion Proteins

- Proteins have structural domains that fold independently and have discrete function
- Domains can be added to protein to alter their characteristics → a purification tag is one of these domains

- 6x His-Tag → interacts with cobalt/nickel ions
- MBP → interacts with maltose (amylase)
- GST → interacts with reduced glutathione

- Affinity tags can be at N-terminus near promoter or C-terminus near termination sequence → encodes the domain that will bind to a specific ligand during affinity chromatography → N-terminus tags interfere less with DNA-binding sites
- The recognition site must be outside the protein to ensure that when it is cut it leaves protein intact

6X-Histidine Tag

- Polyhistidine moiety → 6 His residues
- Rarely affects protein structure

IMAC → Immobilised Metal Affinity Chromatography

- Binds to a metal chelator → Nickel-Nitrilotriacetate (Ni-NTA) column under native and denaturing conditions (it is not a protein tag, a protein tag will denature)
- Column can be washed with high [imidazole] to elute the protein
- Protease can be added before or after elution to remove 6x-His Tag

Maltose Binding Protein Tag (MBP)

- Encoded in pMAL expression vector by bacterial malE gene on the N-terminal site

Pros

- Maintains target solubility + folding; transports to periplasmic space with leader sequence

Purification

- Binds to amylose (maltose) linked matrix
- Eluted by adding maltose through the column → outcompete ligand in column

Glutathione S-Transferase Tag (GST)

- Binds to reduced glutathione (GSH) → this is a very strong binding
- GSH is used as the ligand and also used to elute (in excess to outcompete)
- GST-fusion proteins also maintain solubility
- Protease added <u>after</u> elution

Lecture 11 – Immunospecific Purification, Purification Calculations

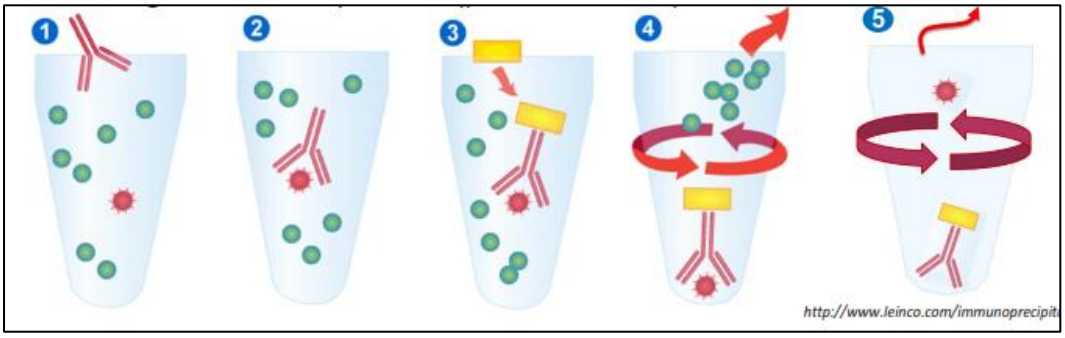
Immunospecific Separation of Proteins

- Antibodies →Immunoglobulins produced by lymphocytes in responses to foreign molecules (antigens)
- Recognise and bind antigens (proteins) at specific epitopes
    - N-terminus → hyper-variable region → antigens bind here
    - C-terminus → constant region → IGBPs bind here (e.g. Protein A or Protein G)

Immunoglobulin-Binding Proteins (IGBPs)

- Precipitate bound antigens directly from solution via immunoprecipitation
- Can be covalently linked to agarose, Sepharose, PAGE, magnetic beads
    - Allows for column or batch purification

Immunoprecipitation



1. Mix protein sample (antigen) with soluble antibodies
2. Incubate antigen-antibody interaction
3. Add Protein A/G to form insoluble antigen-antibody-protein complex
4. Centrifuge to pellet complex and draw off supernatant

5(a). Elute the antigen using a high/low pH buffer or a change in [salt]

5(b). Centrifuge again to collect supernatant containing unbound protein

Antibody Attachment

- The antibody can be attached to something to facilitate its collection or the protein purification process

1. Sepharose/Agarose Beads
    I. Add coupled bead-antibody to protein mixture (or cell lysate)
    II. Incubate to form bead-antibody-antigen complex
    III. Centrifuge, draw off supernatant → repeat multiple times
    IV. Add buffer to separate protein, centrifuge and draw off protein supernatant

2. Magnetic Dynabeads
    I. Add protein to coupled magnetic bead-antibody mixture
    II. Use a magnet to pull beads to bottom of vessel
    III. Draw off supernatant
    IV. Resuspend proteins and elute, draw off protein supernatant