# Protein Purification Part II:
## Chromatography Operations and HIC

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# Presentation Outline

- Chromatography media
- Chromatography equipment
- Column packing
- Chromatography unit operations
- Hydrophobic Interaction Chromatography (HIC) theory
- Running a HIC experiment

# Chromatography media

- The base bead is made from carbohydrate, silica, or a synthetic substance. The bead should be hydrophilic to promote favorable interactions with water.
- The bead may contain a linker arm to separate the functional group from the base bead.
- The media contains a chemical or biochemical functional group (ligand) that is responsible for the desired interaction between the protein and the chromatography media.

# Chromatography equipment

- Column
- Pump
- Monitors (absorbance, conductivity, pH)
- Chart recorder
- Fraction collector

# Column packing

- The column is cleaned thoroughly before packing.
- The chromatography media is suspended with an equal volume of the liquid that it will be packed with.
- The gel suspension is gently poured into the column to avoid air pockets in the settled bed.
- A packing adaptor may be required to accommodate the total volume of the gel to be added.
- The column may be hooked up to a pump to regulate the packing flow rate.

# Bind and elute vs. Flow-through chromatography

- Bind and elute: the molecule of interest binds to the gel.
- Flow-through: the molecule of interest does not bind to the gel.
- Note: size exclusion chromatography is a sieving method, and is neither "bind and elute" nor "flow-through."

# Chromatography unit operations

- Equilibration
- Load
- Flow-through
- Wash (one or more)
- Elution
- Regeneration
- Storage

# Column equilibration

- Prepares the column to be loaded with the protein solution.
- Define the critical parameters for the unit operation (pH, ionic strength, etc.).
- Equilibration buffer displaces whatever storage solution the column is stored in.
- Column inlet equals column effluent with respect to critical parameters.

# Column load

Dependant upon several important process variables:

- pH
- Ionic strength (conductivity)
- Temperature
- Flow rate
- Protein mass

# Column flow-through

Bind and elute mode:

- Target protein binds completely to the column. Impurities flow through.

Flow-through mode:

- Target protein flows through the column. Impurities can bind and/or flow through the column.

# Column wash

Bind and elute mode:

- Removal of non-specific binding impurities following the load phase.

Flow-through mode:

- Capture of product remaining in the load line and column following loading.

# Column elution

Bind and elute mode:

- Removal of the target protein from the column in a buffer that maintains biological activity, while maximizing product recovery and purity. May be done as a step elution or a gradient elution.

Flow-through mode:

- Does not apply.

# Column regeneration

- Chemical treatment of the column to return it to “as new” condition.
- Gel must be stable to regeneration conditions (extremes of pH, solvents, chaotropic reagents).

# Column storage

- Continued chemical treatment of the column to maintain cleanliness of column following regeneration.
- Gel must be stable to storage conditions (extremes of pH, solvents, chaotropic reagents) for extended periods of time.

# Hydrophobic interaction chromatography

- Reversible binding of proteins to a support carrying a non-polar ligand.
- Performance is influenced by pH, ionic strength, load capacity, flow rate, bed length, type of elution (step vs. gradient)
- Competition from other molecules (large and small) for non-polar binding sites.

# Non-polar amino acids

- Side chain may be a saturated alkane, cyclic or aromatic:

Alanine: R = $CH_3$

# HIC, cont.

- The ligand is non-polar, and may be saturated or unsaturated.
- A strong HIC ligand (Octyl Sepharose) has a large alkyl group (≥ 8 carbons).
- A weaker HIC ligand (Butyl Sepharose) has a smaller alkyl group (≤ 4 carbons).

# HIC Ligands

- Methyl - $CH_3$
- Propyl - $CH_2CH_2CH_3$
- Butyl - $CH_2CH_2CH_2CH_3$
- Octyl - $CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_3$
- Phenyl - 

# HIC, cont.

- An aromatic ligand (Phenyl Sepharose) offers flexibility between the weak and strong ligands, and may offer extra selectivity for proteins that have abundant amounts of aromatic amino acids (i.e. antibodies)
- Proteins may have solubility problems at certain pH values when high salt and/or high protein concentrations are present.
- Proteins will have a wide variety of surface hydrophobicity, allowing for a greater selectivity between the target protein and contaminants.

# Running a HIC experiment

| Parameter | HIC media |
| --- | --- |
| Equilibration pH | Typically around neutral |
| Load conductivity | High |
| Flow rate | Moderate |
| Elution | Decrease conductivity (step or gradient) |
| Regeneration | Base (non-silica) or acid (silica) |
| Storage | Base (non-silica) or acid (silica) |