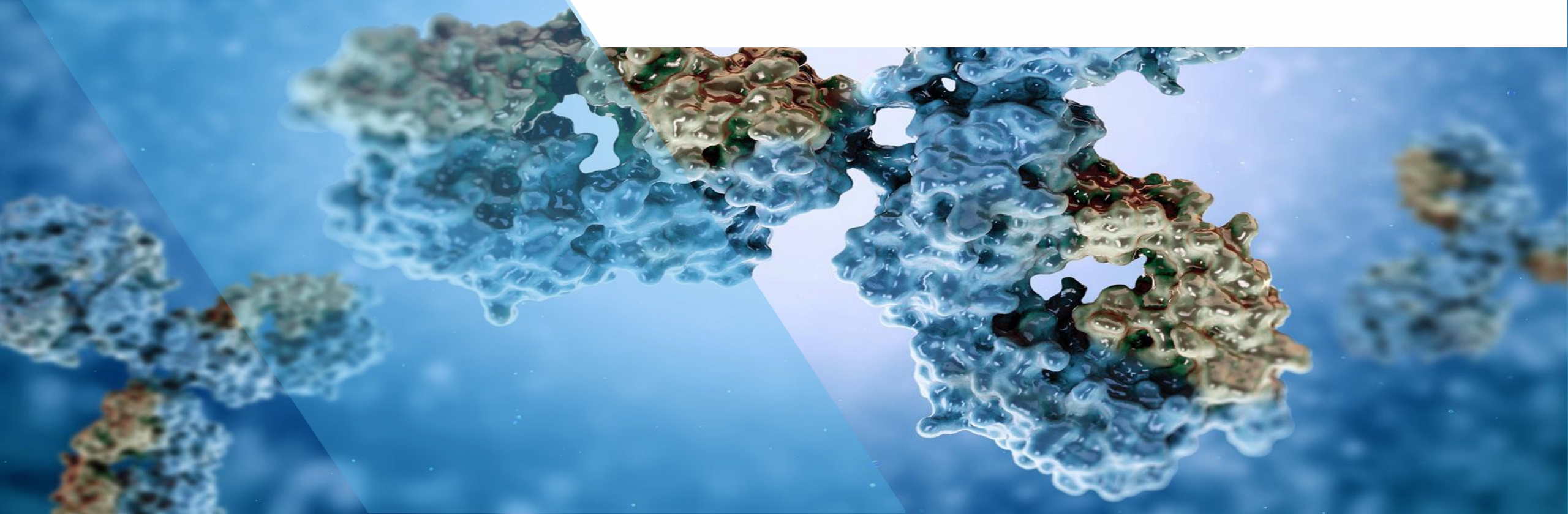
Modes of Chromatography



Learning Objectives

Outline the commonly used chromatographic techniques

Describe affinity, ion exchange and hydrophobic interaction chromatography

Know why different chromatographic techniques are used in a typical bioprocess

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Topics

- Chromatography Overview
- Types of Chromatography
- Chromatography Operations

Bioprocessing Research

The Downstream Challenge

Cell Culture Medium

Medium Supplements

Cells

Protein Product

Cell Culture Medium
Amino Acids
Inorganic Salts
Vitamins
D-glucose
Other Organics

Medium Supplements

Cells
Intact Cells
Cell Debris
HCP's
Viruses
Nucleic Acids
Lipids

Endotoxins

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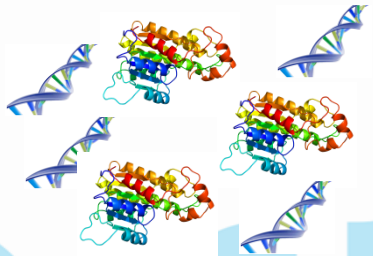
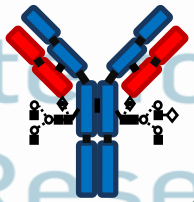
Goals of Purification

1. Remove **process** and **product** related impurities
2. Prevent **contaminating substances or organisms** from entering the product
3. Ensure the protein is in the **correct conformation** and prevent unacceptable degradation
4. Ensure the protein is **correctly formulated** at the appropriate concentration for **safe storage** and **administration**





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What is Chromatography?

Chromatography is a separation technique used to purify our protein of interest.

It is based on the affinity of the components in the mixture for a stationary phase or a mobile phase

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Topics

- Chromatography Overview
- Types of Chromatography
- Chromatography Operations

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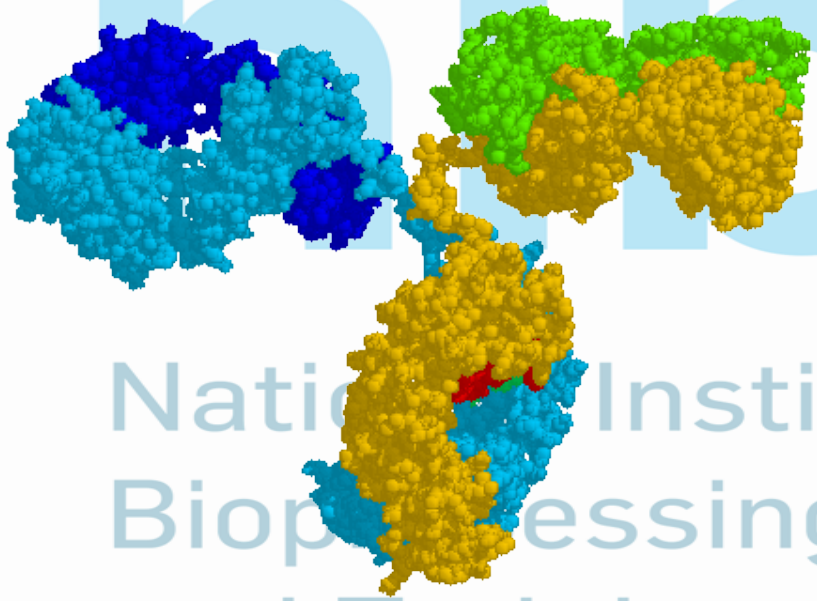
Protein Properties Determine Type of Chromatography

Size and Shape (3D Structure)

Affinity Properties (Ligand Binding)



Charge & Isoelectric Point

Density

Hydrophobicity

Solubility

Aggregation (Reversible or Irreversible)

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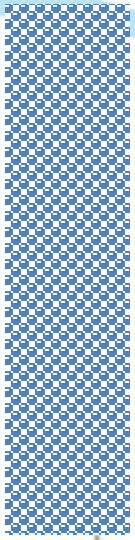
Bind & elute

Molecule of interest binds to the resin

Sample application



Product protein binds to the column, impurities are washed down to waste

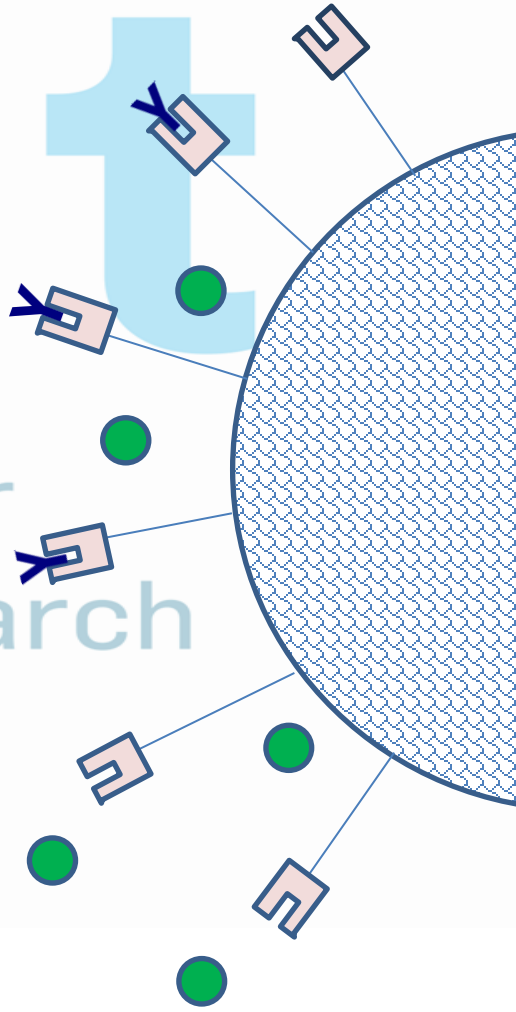
Changing the mobile phase on the column – applying elution buffer

Protein product eluted from the column

Immobilised ligand (proA)

Protein product

Impurity



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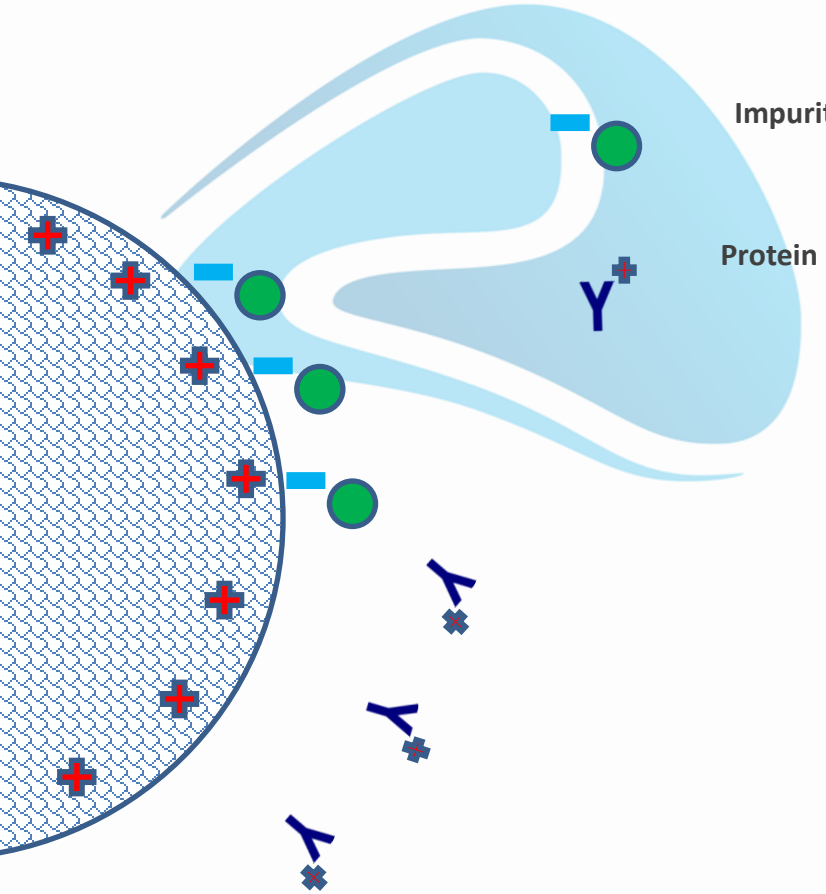
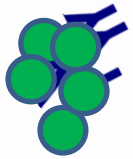
Flow through
Molecule of interest flows through the resin



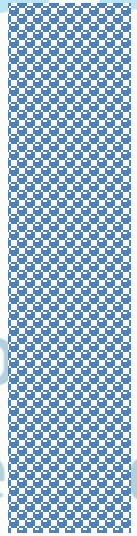
Impurity with the net negative charge

Protein product with net positive charge

Sample application



Impurities are held within the column



Changing the mobile phase on the column – applying buffer (e.g. wash buffer) to remove impurities from the resin

Product protein flows through the column and is collected in the bag/ vessel

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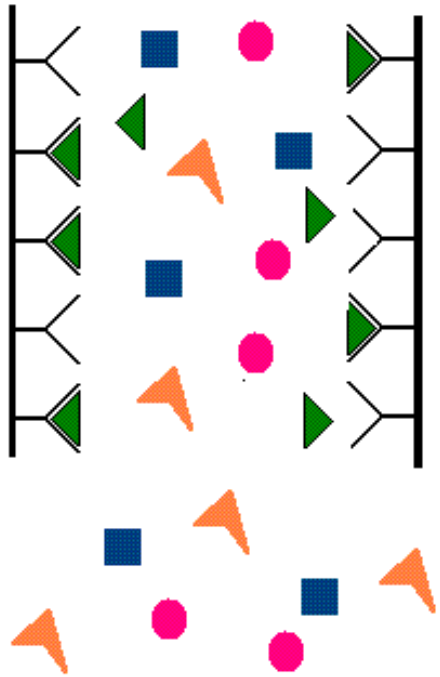
Affinity Chromatography

Purification of a biomolecule on the basis of its biological function or individual chemical structure

Reversible interaction between target protein and specific ligand attached to a chromatography matrix

Can be used to separate:

- Active biomolecules from functionally different forms
- Isolate pure substances present at low concentration in large volumes of crude sample
- Remove specific contaminants



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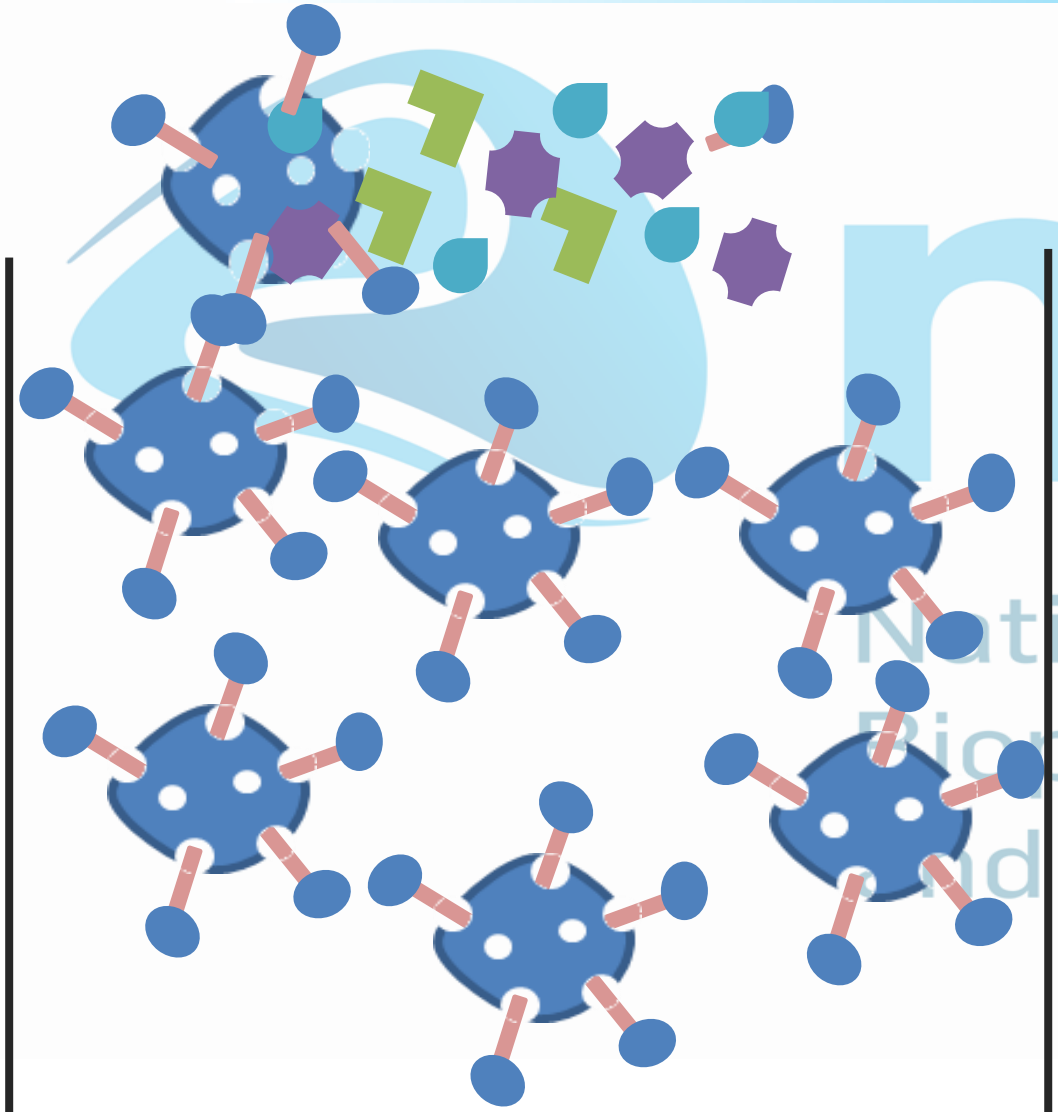
Affinity Chromatography Principles



Vendor supplies specific affinity matrix whereby a ligand is attached to an inert matrix e.g. agarose

Many copies of the ligand will be attached to the matrix.
A set amount of which is then packed into a column
(may be pre-packed by vendor)

A liquid containing your molecule of interest,
as well as many impurities, passes over the matrix

Molecule of interest binds to ligand – impurities flow away

Molecule of interest is detached from ligand

Can be re-used for multiple batches

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Ligand

The ligand is defined as a molecule that binds ‘lock and key’ to a protein

The selection of the ligand for affinity chromatography is influenced by two factors:

1. The ligand must exhibit specific and reversible binding affinity for the target substance(s).
2. It must have chemically modifiable groups that allow it to be attached to the matrix without destroying binding activity.

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Mechanism of Action

Binding of the target molecule to the ligand can be a result of:

1. Electrostatic interactions
2. Hydrophobic interactions
3. Van der Waals' forces
4. Hydrogen bonding

Interaction is based on the solution the protein is in

To elute the target molecule, the interaction can be reversed, either specifically using a competitive ligand, or non-specifically with **pH, ionic strength or polarity changes.**

The coupled ligand must retain its specific binding affinity for the target molecules and, after washing away unbound material, the binding between the ligand and target molecule must be **reversible** to allow the target molecules to be removed in an active form.

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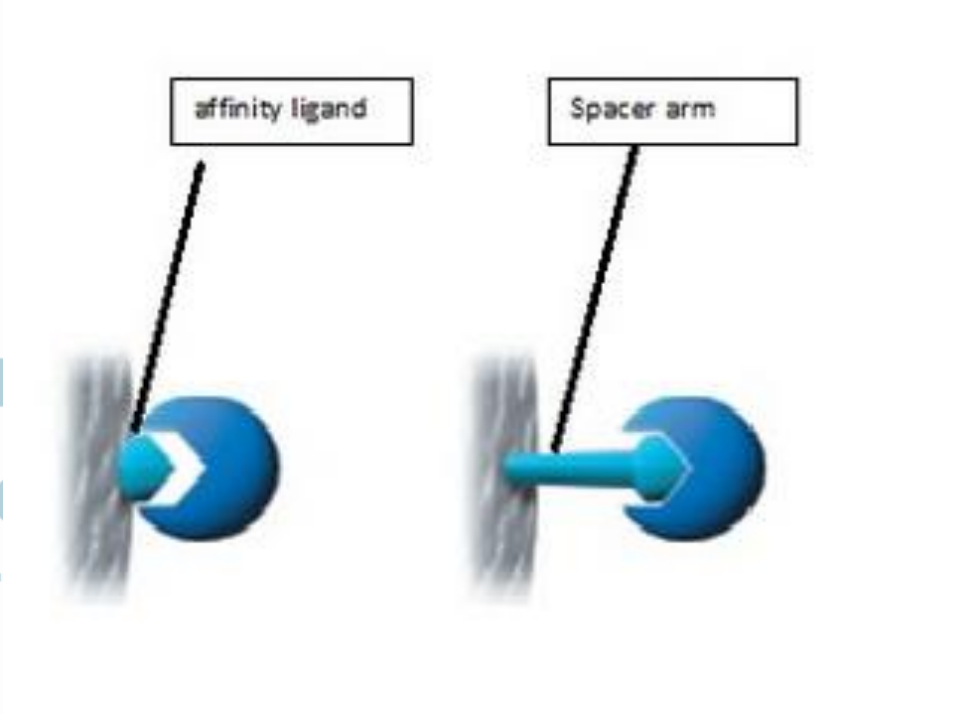
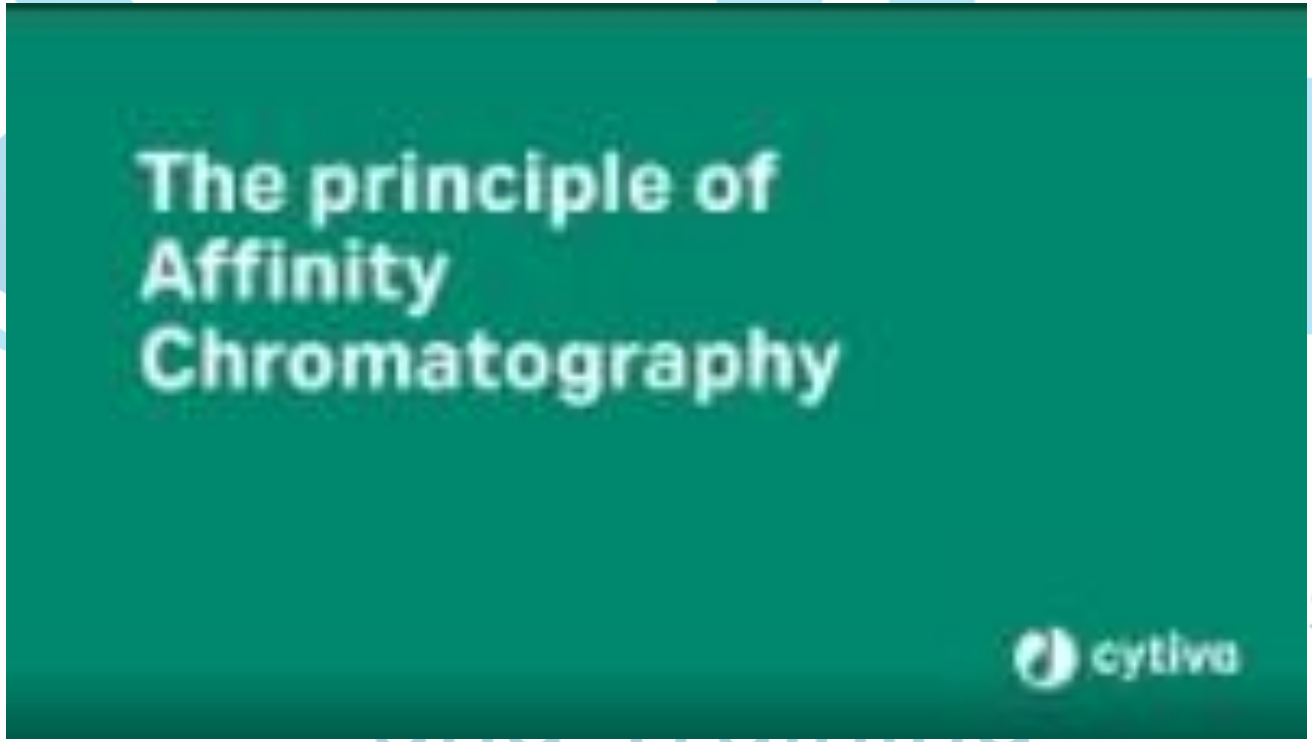
Spacer Arm

A spacer arm is used to **improve binding by overcoming steric hindrance**

- The binding site of a target protein can be deep within the molecule
- An affinity medium with small ligands directly on the bead have low binding capacity
- The ligand is unable to access the binding site of the target molecule.
- The spacer arm is a **carbon chain** interposed **between** the **ligand and supporting phase**

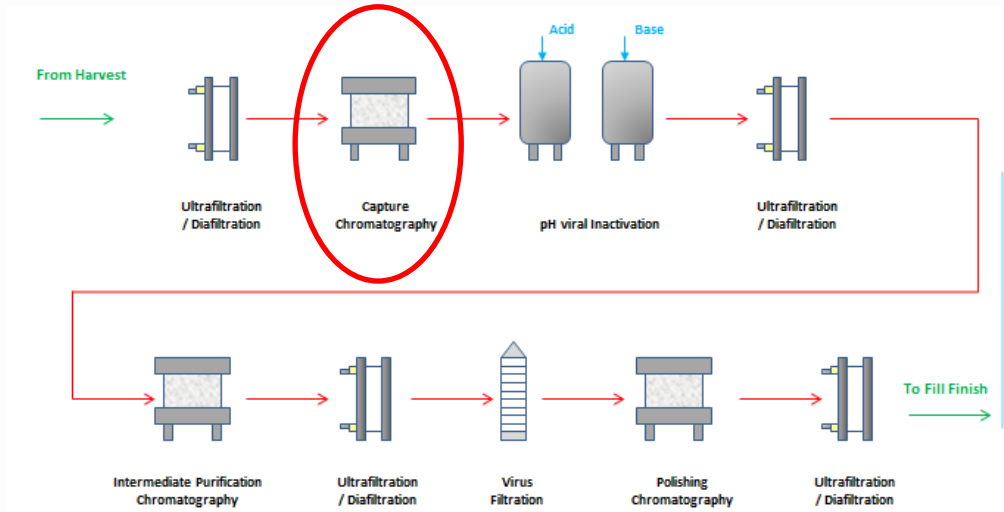


Affinity Chromatography Animation



https://www.youtube.com/watch?v=B__n4buDycs

Affinity Chromatography at Downstream



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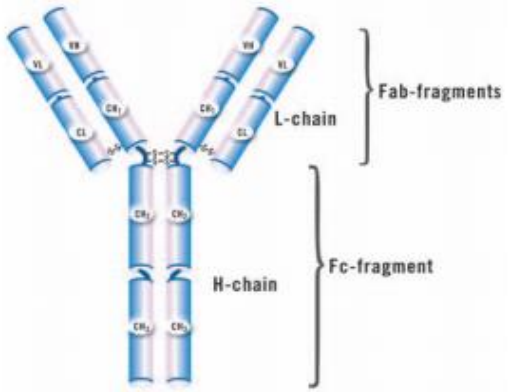
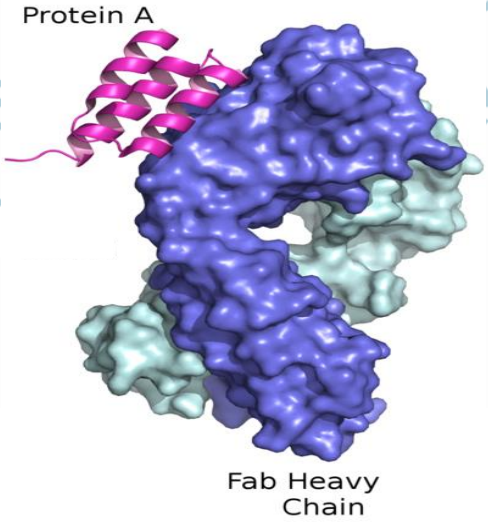
- Affinity chromatography is used for capture where the objective is to quickly adsorb the protein of interest from the crude sample and isolate it from critical contaminants such as proteases.
- An example of affinity chromatography is protein A.

Protein A

- Protein A is naturally found in the peptidoglycan layer of some bacterial cell walls
- Protein A reversibly binds to the Fc region of antibodies
- This is a defence mechanism of bacteria to prevent antibodies from destroying them in the bloodstream
- When a company is producing a mAb, they can exploit this naturally high-affinity between protein A and antibodies





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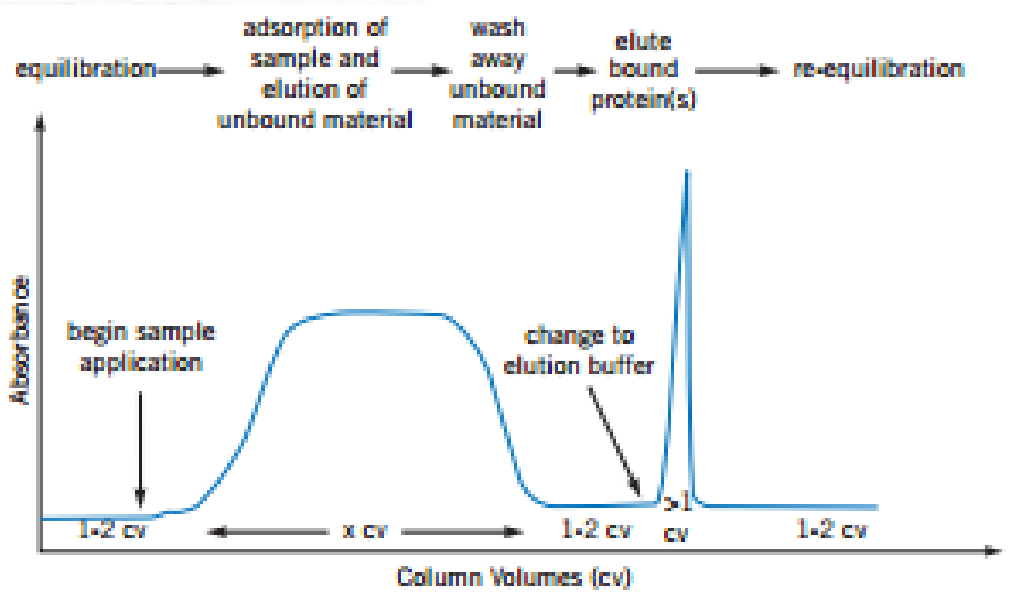
Protein A: Binding and Elution

- The binding of IgG to protein A occurs at neutral or alkaline pH values (~pH 8.0).
- A decrease in pH alters the binding sites, reducing their affinity, or it causes indirect changes in affinity by alterations in conformation.



Ref: http://www.gelifesciences.com/

Advantages vs. Disadvantages

Advantages

- High specificity
- High selectivity
- High recovery
- Purity increases of 1000-fold

Disadvantages

- Ligand availability
- High cost
- Often exhibit poor stability
- Ligand leakage

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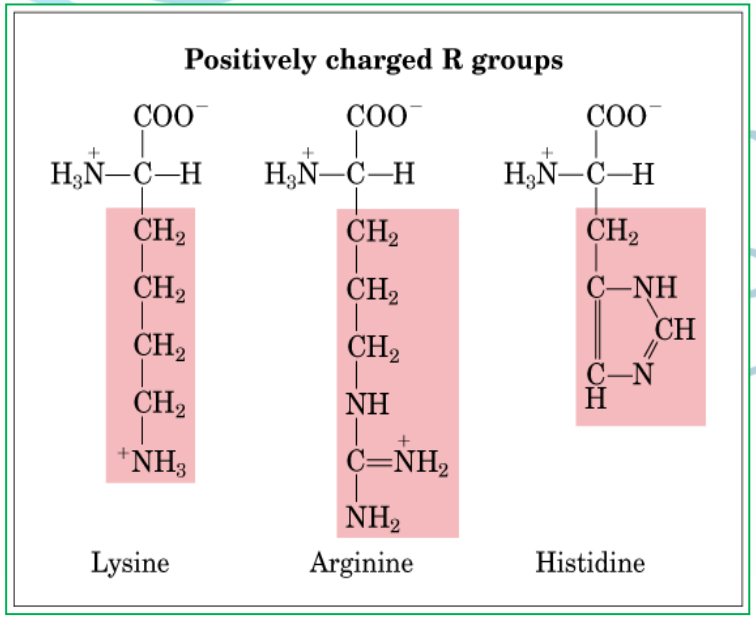
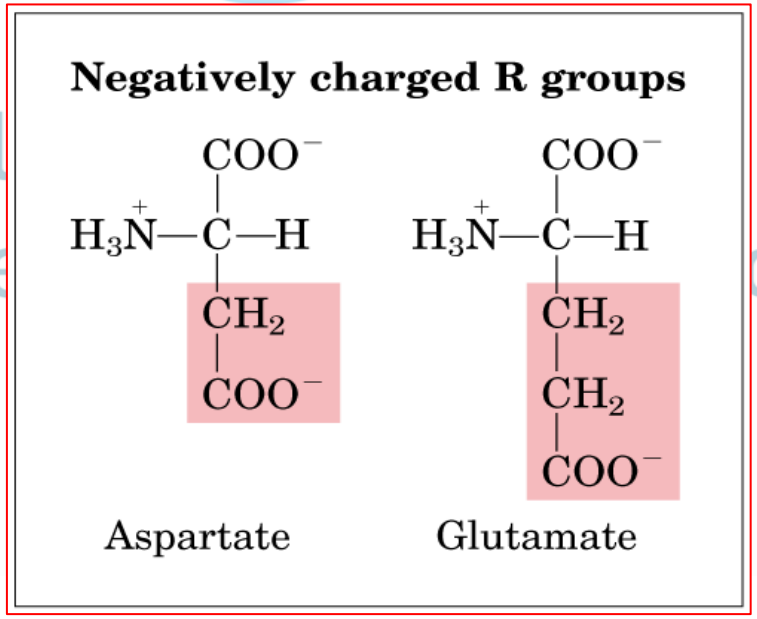
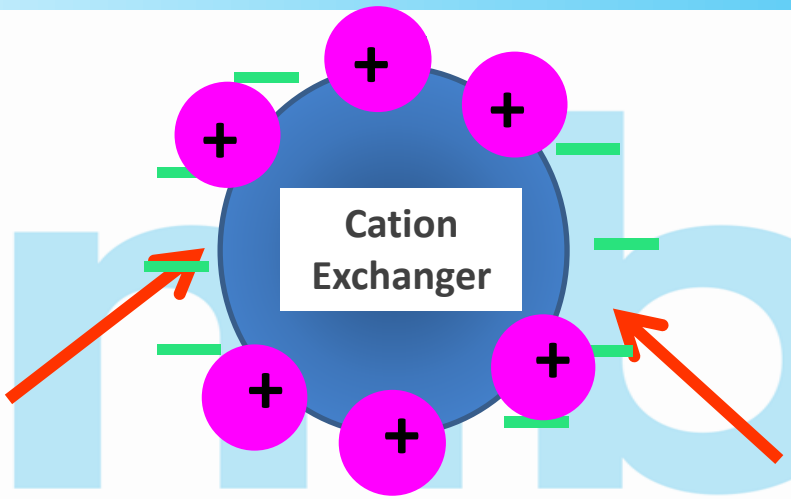
Proteins Carry a Charge

- All proteins are made up of different combinations of the 21 amino acids
- Some of these amino acids possess side groups (R groups) that are either **positively** or **negatively** charged.



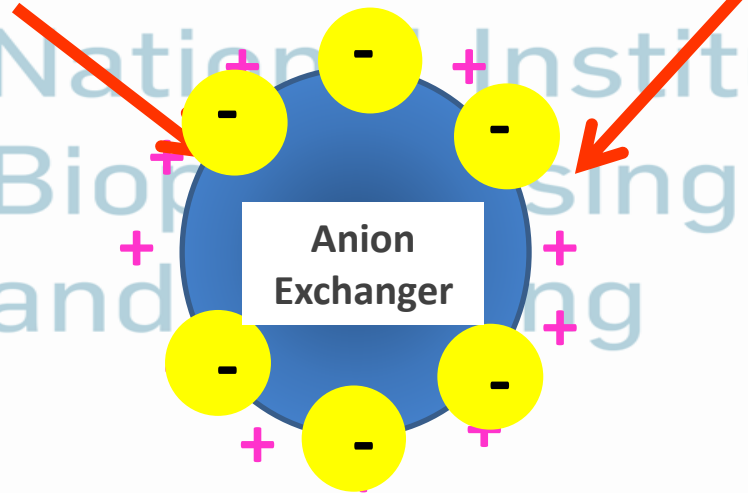


Ion Exchange Chromatography



Chemically &
physically inert Matrix

- / + charge attracts
target molecule



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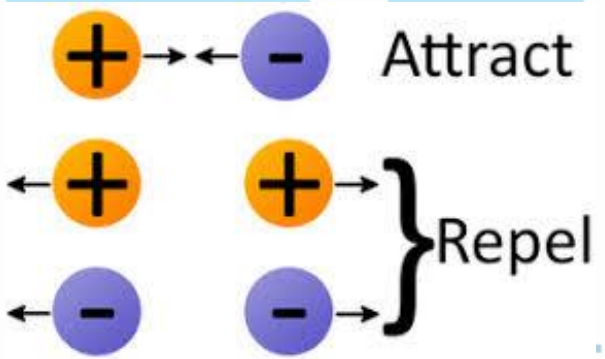
Ion Exchange Chromatography

IEX separation uses reversible interactions between charged molecules and oppositely charged Ion-Exchange resin



Cation Exchange

- Uses negatively charged resin
- Binds molecules with net positive surface charges

Anion Exchange

- Uses positively charged resin
- Binds molecules with net negative surface charges

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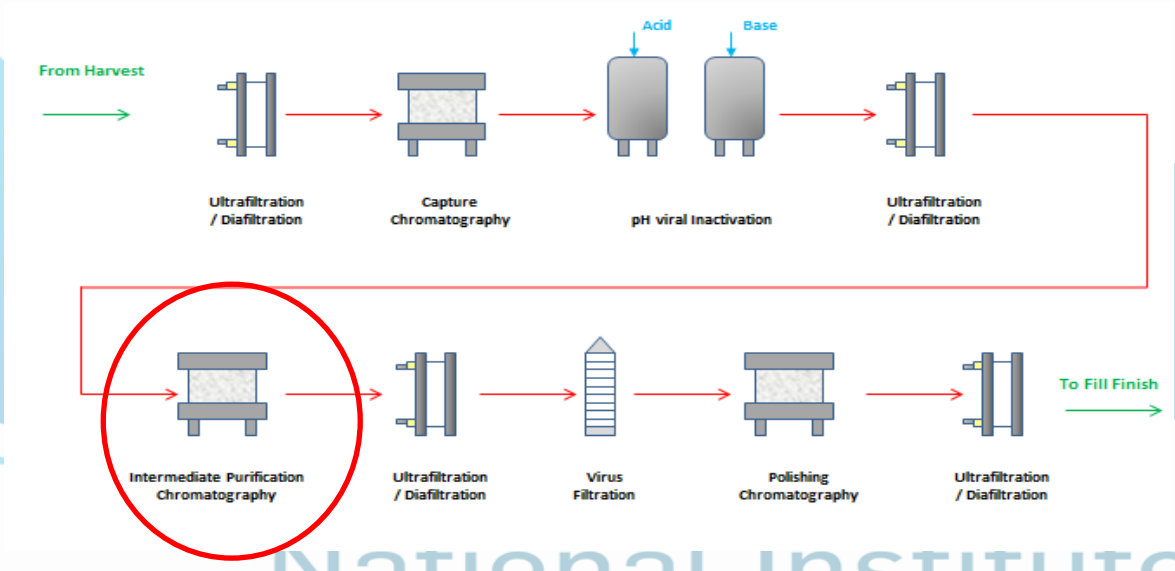
Ion Exchange Chromatography Animation

https://www.youtube.com/watch?v=rPsJo0jHJ7g

Ion Exchange Chromatography at Downstream



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Ion exchange chromatography is most commonly used for intermediate purification.

This removes most of the significant impurities such as proteins, nucleic acids, endotoxins and viruses.

Advantages vs. Disadvantages

Advantages

- Ligand availability
- Inexpensive
- High recovery

Disadvantages

- Low specificity
- Sample must be applied at low ionic strengths and controlled pH (may require a buffer exchange step beforehand)

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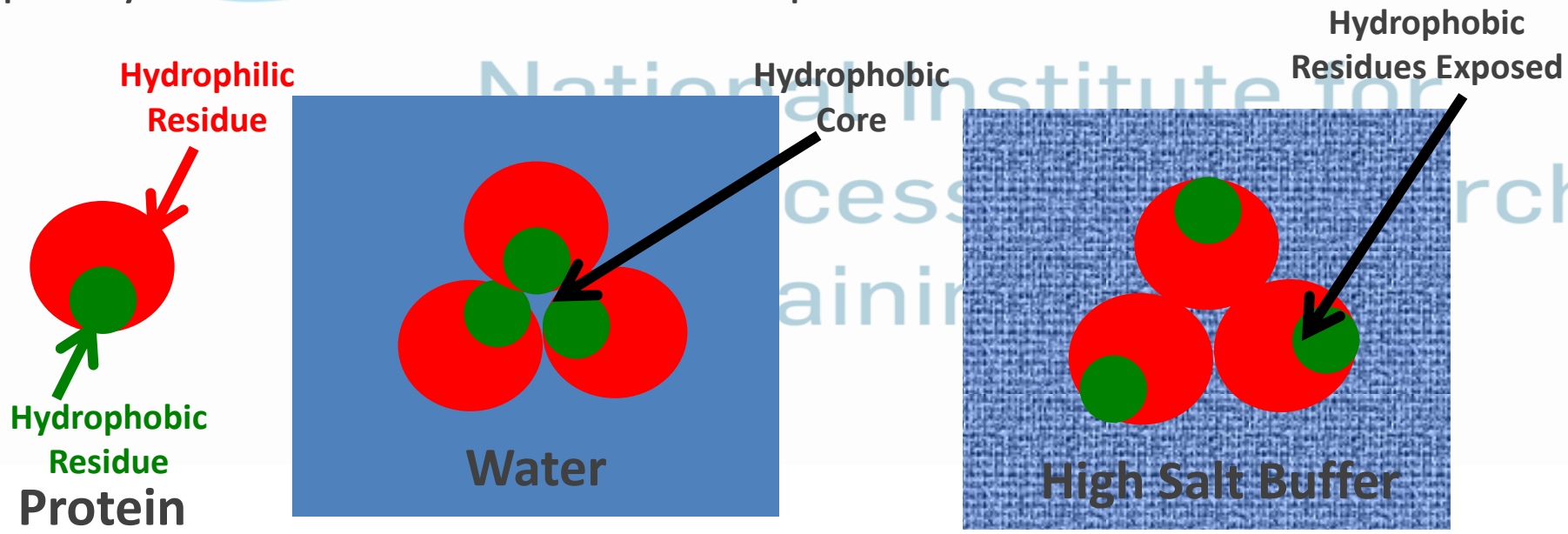
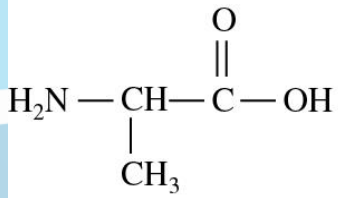
Hydrophobic Interaction Chromatography

The three-dimensional structure of a protein is a result of intra-molecular interactions as well as interactions with the surrounding solvent.

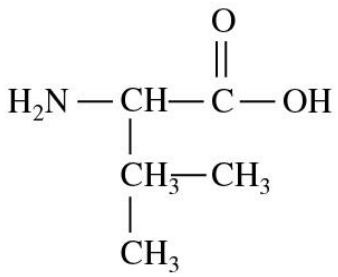
In the case of readily soluble proteins, this solvent is water, and hydrophobic side chains are therefore typically driven to the interior of the protein.



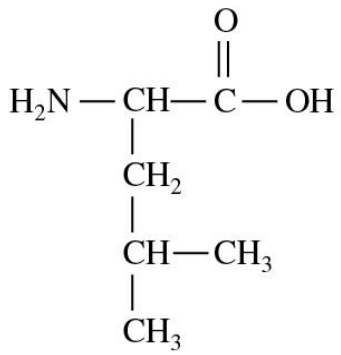
Hydrophobic Amino Acids



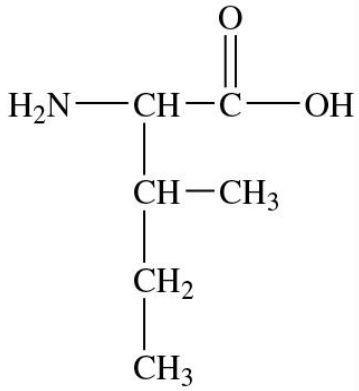
Alanine



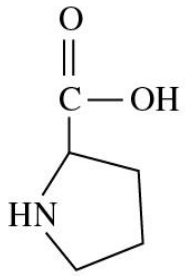
Valine



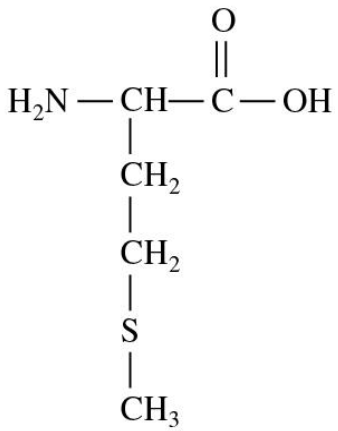
Leucine



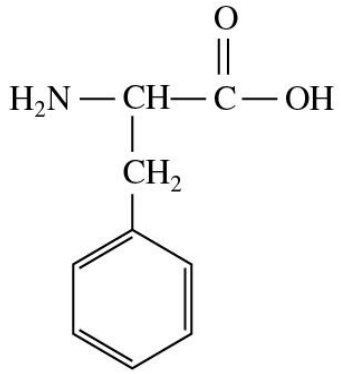
Isoleucine



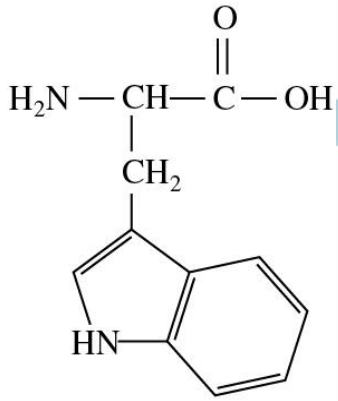
Proline



Methionine

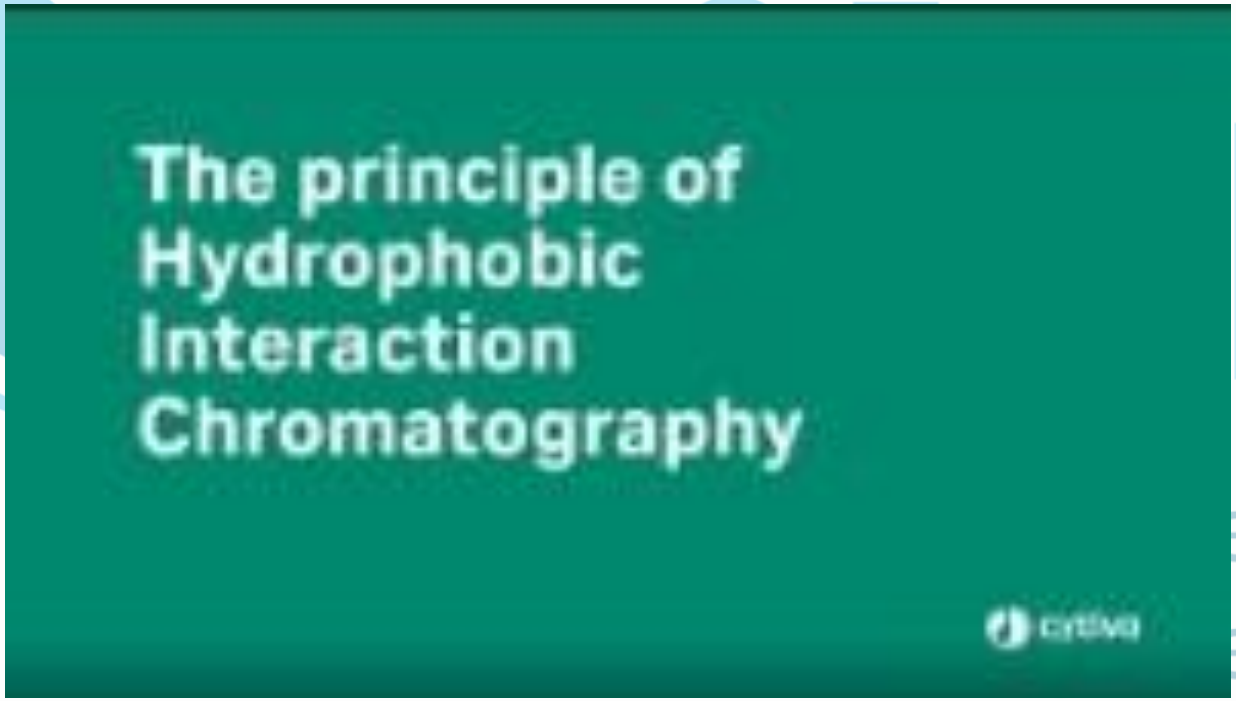


Phenylalanine



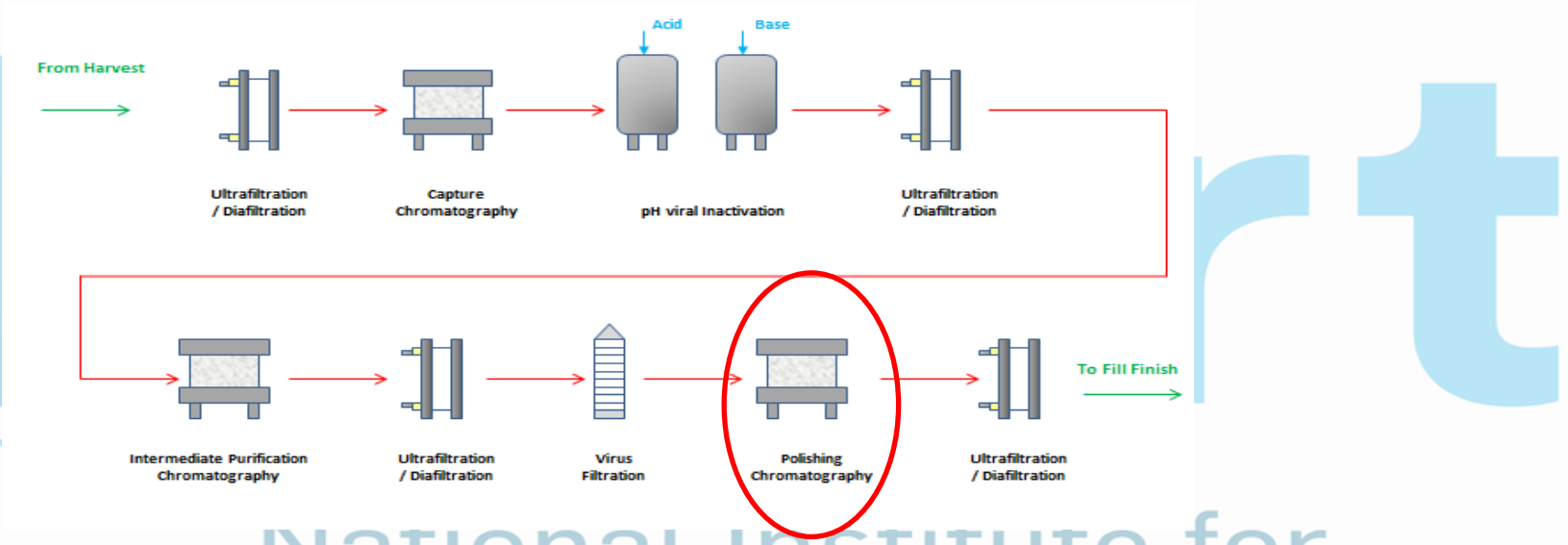
Tryptophan

Hydrophobic Interaction Chromatography (HIC) video



https://www.youtube.com/watch?v=d8P04atG9Fs

HIC at Downstream



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Hydrophobic interaction chromatography is most commonly used for polishing.

This removes trace amounts of contaminants or closely related substances such as structural variants of the target protein.

Advantages vs. Disadvantages

Advantages

- Samples with high ionic strength can be used
- Large sample volumes can be loaded
- Sample eluted with low salt

Disadvantages

- Low specificity
- High salt concentration in waste

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HIC Bind-and Elute or Flow-Through Mode

HIC can be operated in bind-and-elute or flow-through mode.

- **Bind-and-elute:** the protein product will bind to the column
- **Flow-through:** HIC in flow through mode is often used to **remove aggregates** generated in protein A purification steps for mAbs. These impurities have chemical properties very similar to the target but they will generally be more hydrophobic than the native protein.
- Therefore they bind at relatively low salt concentrations to **butyl or phenyl resins** allowing the target to flow through the column.

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Purification Strategy

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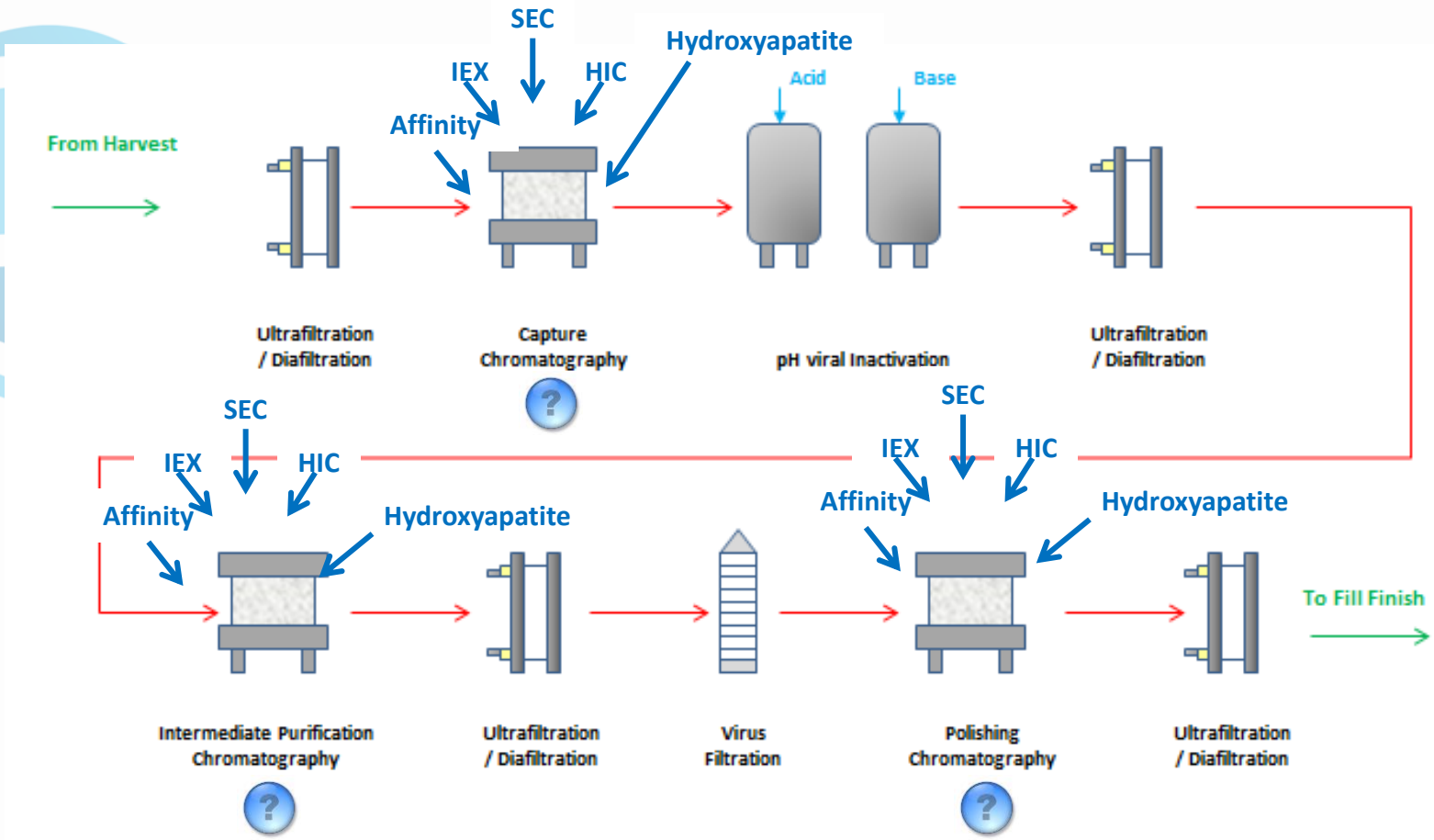
Protein purification is a lengthy and complicated process.

How do you know what chromatography technique to use?

Which Method Do I Use?



Platform Purification Of mAbs

- Most companies are now implementing a platform approach to mAb production rather than a fully generic approach.
- A platform approach is built on a foundation of process knowledge.
- Experience in purifying similar products (e.g. from the same class of molecules, such as mAbs) provides that knowledge for downstream processing platforms.
- Platform technologies facilitate rapid and economical process development and scale-up, leading to an increase in the number of clinical candidates that can be evaluated, resulting in more rapid market entry and often a reduced validation effort.

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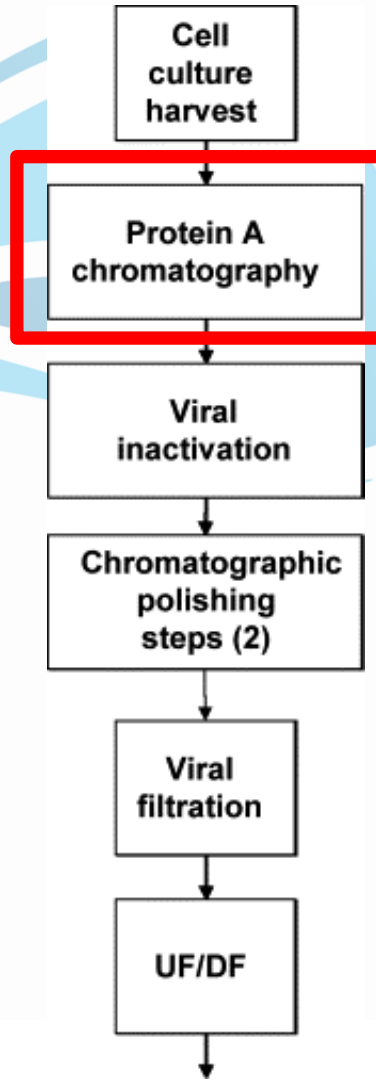
A Platform Downstream Process for mAbs



- Captures mAb from clarified feedstock, volume reduction
- Inactivation of enveloped viruses by low pH-hold step
- Removal of HCP, hcDNA, Protein A, Endotoxins
- Removes known and potential viruses by size separation
- Buffer exchange, Concentration, Formulation

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Topics

- Chromatography Overview
- Types of Chromatography
- Chromatography Operations

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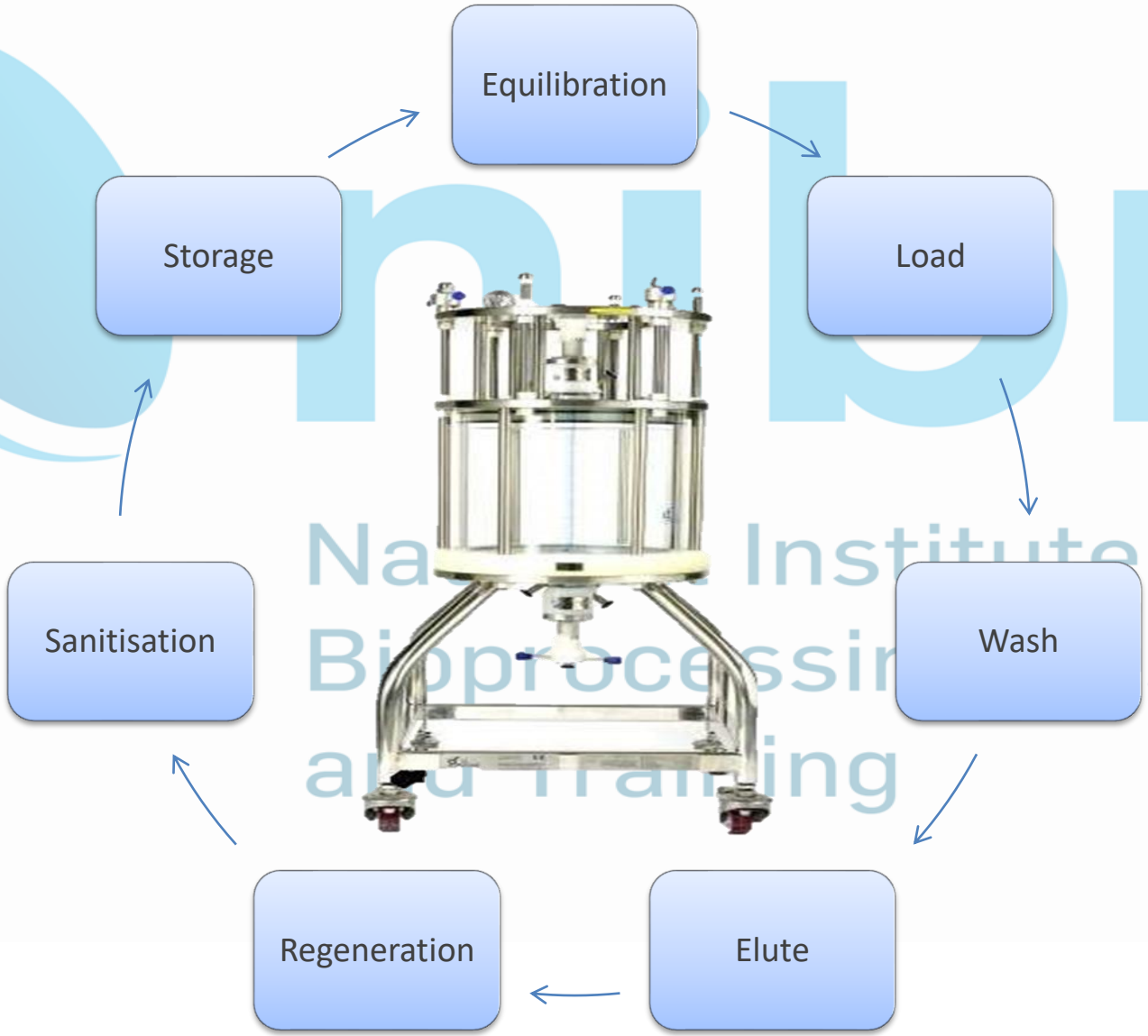
Chromatography Operations



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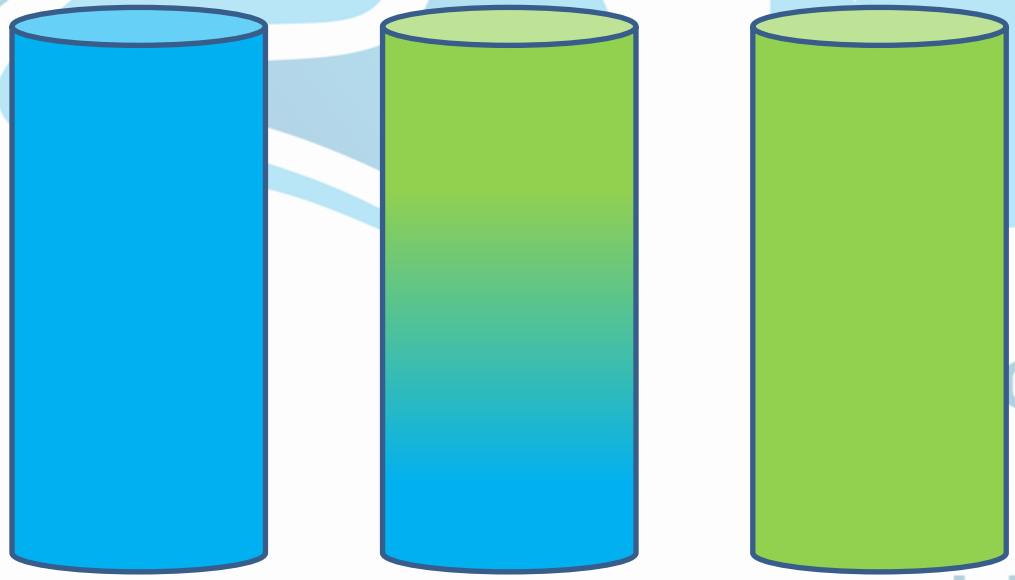
Chromatography Cycle



Equilibration:

- The column is transferred into a buffer which will be stable for the product and will encourage the product to interact with the stationary phase in the correct way.
- It may take several column volumes worth of equilibration buffer to displace all of the storage buffer.
- pH and conductivity measured at the column outlet should be identical to the equilibration buffer.

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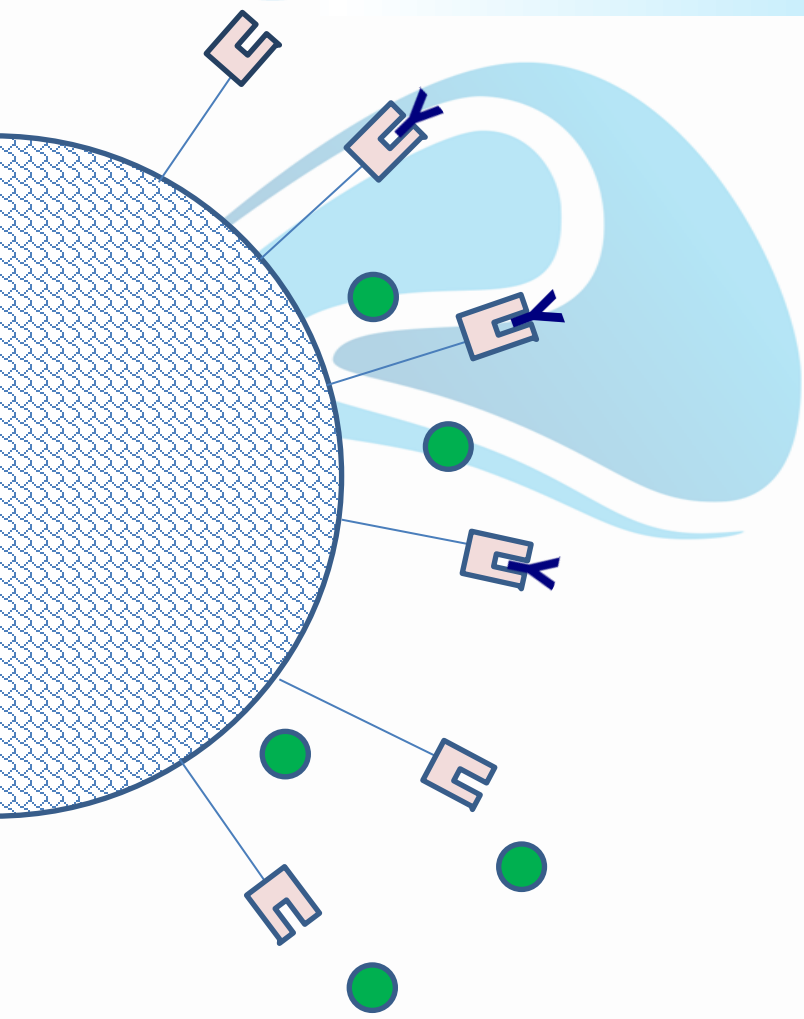
Chromatography Cycle



Loading:

- The amount of product loaded depends on the dynamic binding capacity (DBC) of the stationary phase. This can be determined at lab scale by passing a sample process feed through a known volume of stationary phase until the resin is saturated and the protein/product appears in the eluent.
- If a lot of protein needs to be processed, the batch may be split into two cycles.
- The product is applied to column at same flow rate as mobile phase.
- It is important that the products is in the correct buffer conditions to interact with the chromatography column i.e. pH, conductivity, temperature

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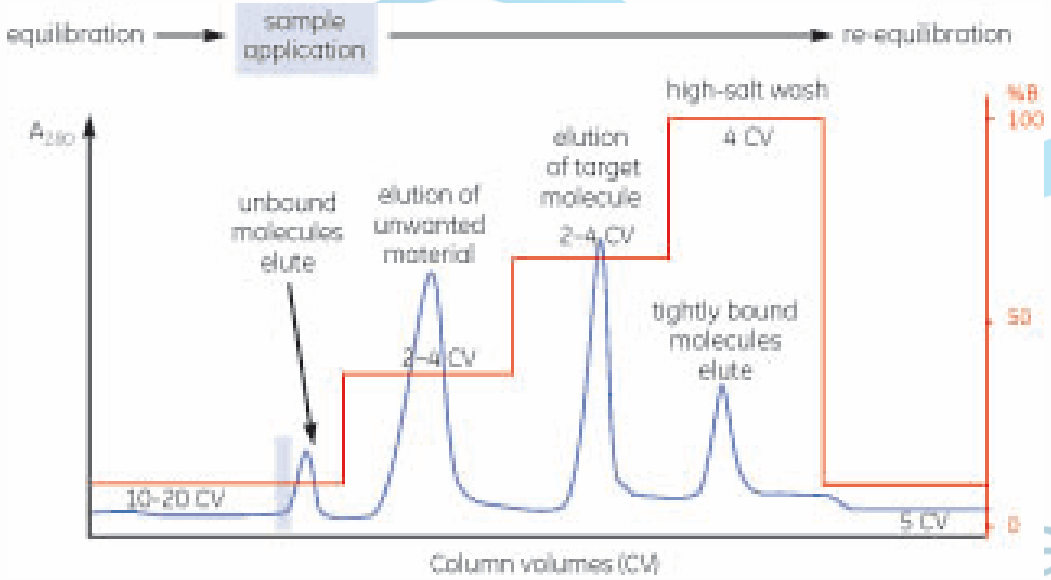
Chromatography Cycle



Wash:

- The wash step is designed to encourage anything that is not specifically bound to the resin to elute from the column
- In bind and elute chromatography, the aim is to wash away as many impurities from the column as possible
- In flow through mode, the aim is to collect all of the product from the column
- This may involve changing the conditions of the mobile phase to remove impurities which are bound to the column more weekly than the product

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Chromatography Cycle

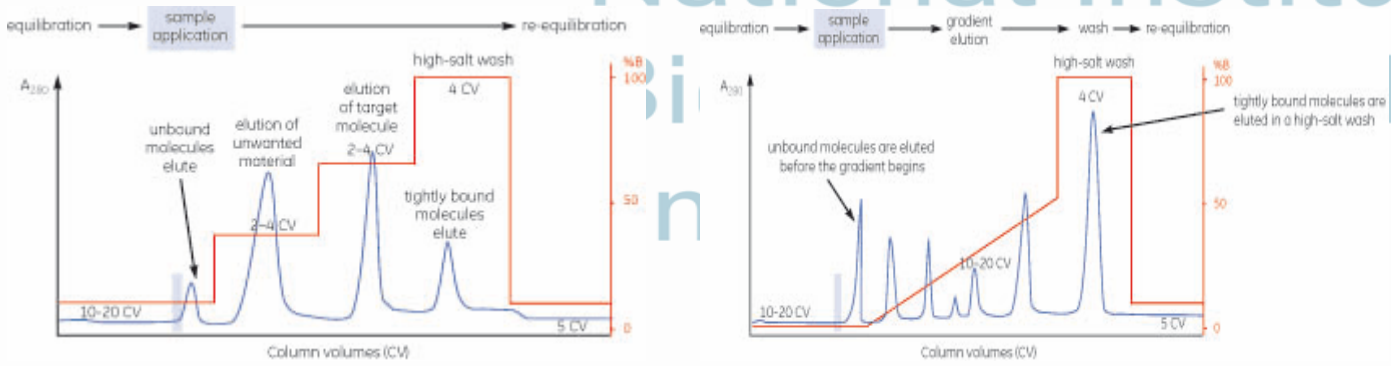
Elute

The target molecule is bound to the resin.

Change in pH (e.g. pH 2-3.5), change in ionic strength, etc. The column should be re-equilibrated to neutral pH immediately.

A compromise may have to be made between the harshness of the eluent required for elution and the risk of denaturing the eluted material or damaging the ligand on the affinity medium.

Proteins can be eluted in a stepwise, gradient or isocratic format.



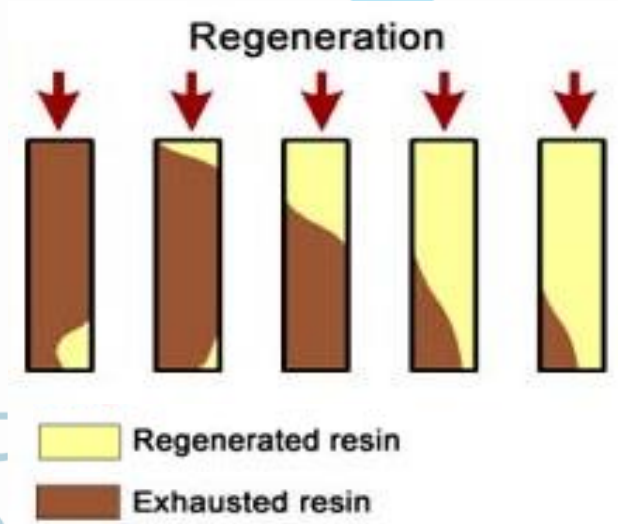
Composition of eluent does NOT change during the purification run

Stepwise Elution **Gradient Elution** **Isocratic Elution**

Chromatography Cycle

Regeneration

- Returning the stationary phase to its initial state after elution. Mobile phase is passed through the column stepwise or in a gradient. The stationary phase is solvated to its original condition.
- Regeneration can also refer to bringing back any column to its original state (e.g., the removal of impurities with a strong solvent).



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Column Chromatography Operations

Cleaning

- Chromatography is not a sterile process!
- Chromatography columns are sanitised to reduce bioburden.
- Cleaning challenges – difficult to achieve high flow rates to clean the columns; inaccessibility; damage to the resin; difficult to clean O-rings, etc; ancillary equipment such as pumps, hoses, etc. must be cleaned separately.
- Disassembly of column is labour intensive.

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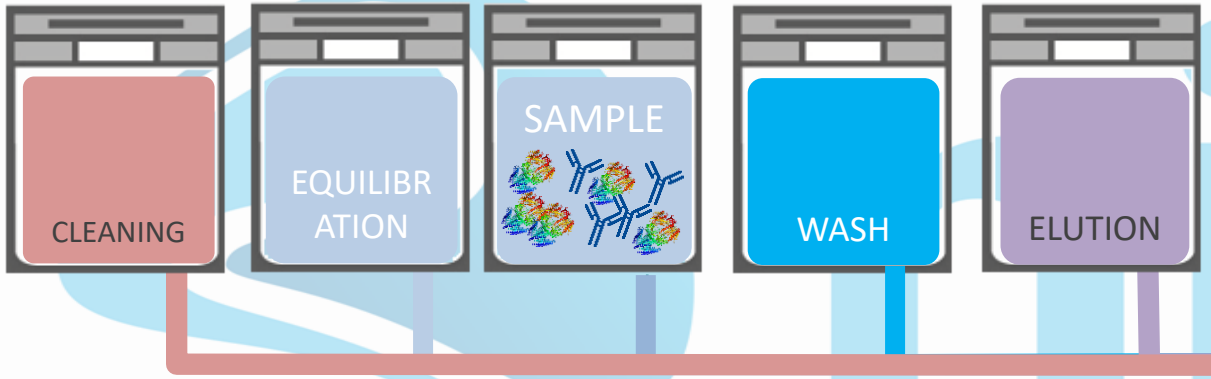
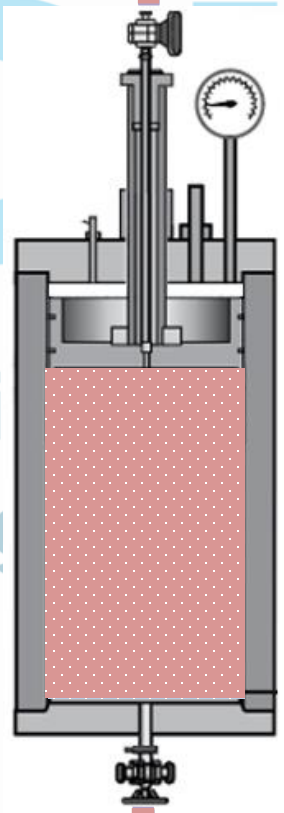
Storage

Stored in appropriate buffer/storage solution to prevent microbial growth.

Bind & Elute
Molecule Of Interest Binds To The Resin

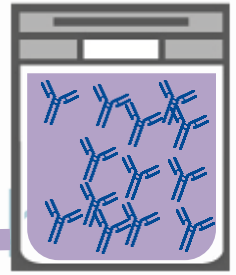


- Equilibration with loading buffer
- Sample feed
- Washing non-specifically bound components
- Elution of the target molecule
- Regeneration/ Cleaning

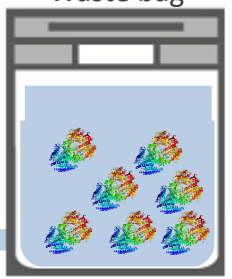


Drain

Product collection bag



Waste bag



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Sample Questions

- SAQ: explain the basis of cation exchange chromatography with reference to the key steps involved and the influence of pH on protein binding.
- SAQ: Write a note on size exclusion chromatography used as a polishing step in DSP.
- LAQ: Compare ion-exchange and hydrophobic interaction chromatographies for the purification of proteins.
  - Including:
    - ***Factors affecting protein binding***
    - ***Elution methods***
    - ***Selectivity methods***
    - ***Examples of resins used***

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Topics

- Chromatography Overview
- Types of Chromatography
- Chromatography Operations

Bioprocessing Research

Thank You

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