Chromatography technologies

Tzahi Howser

Danyel Biotech

Outline

- Introduction
- Chromatography modes overview
  - Gel Filtration / Size Exclusion
  - Ion Exchange
  - Hydrophobic interactions / RPC
  - Affinity chromatography
  - Multimodal chromatography
- Summary
- Terminology

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Introduction

Introduction

Biomolecules are purified using chromatography techniques that separate them according to differences in their specific properties: size, Hydrophobicity, Charge, Biorecognition (ligand specificity)

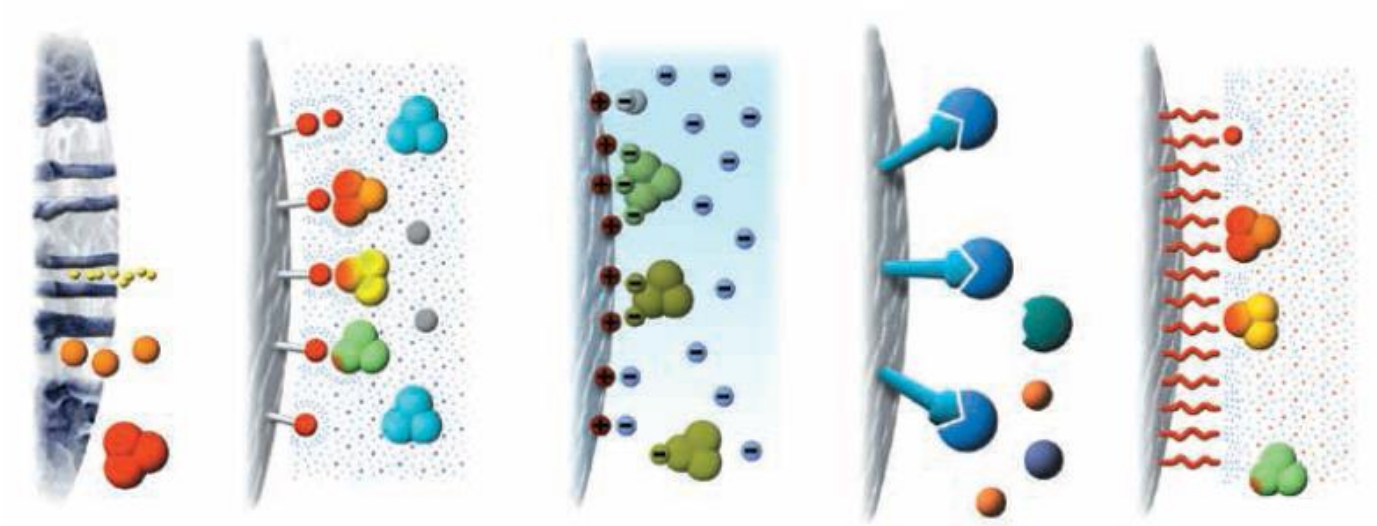

Fig I.1. Schematic drawing of separation principles in chromatography purification. From left to right: SEC, HIC, IEX, AC, and RPC.

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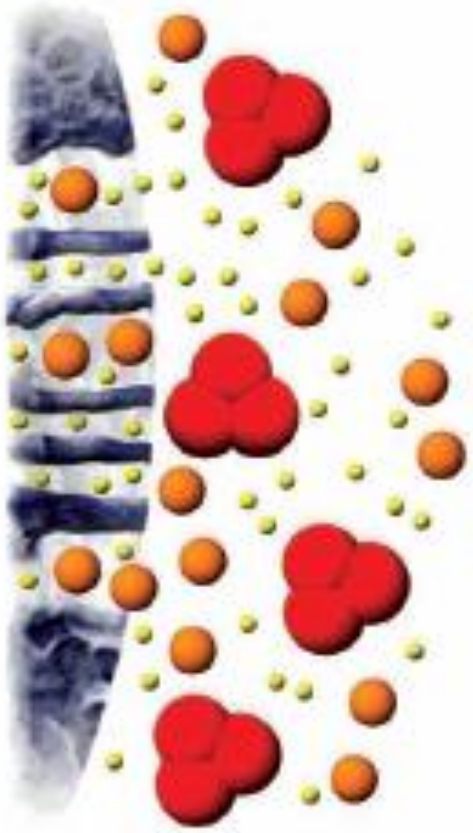
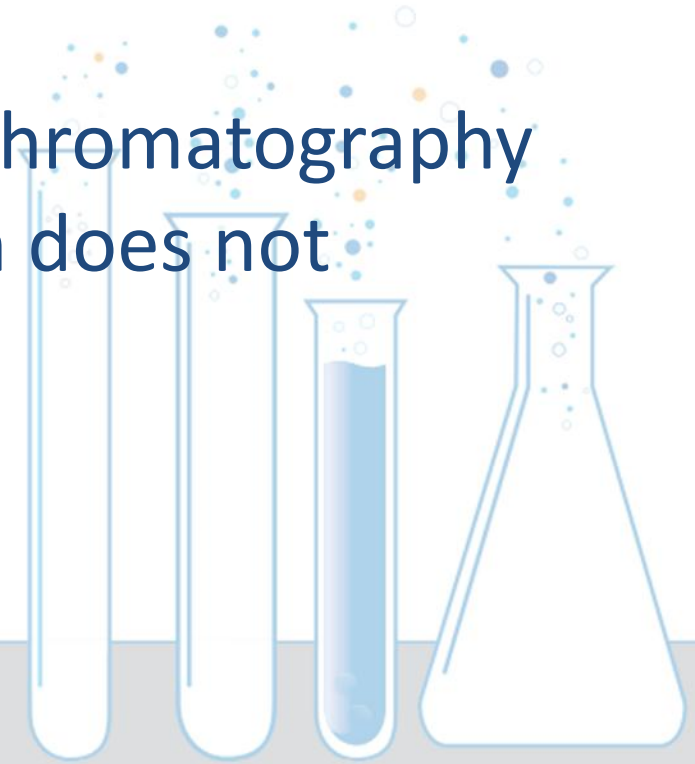
Chromatography modes overview

Size Exclusion / Gel Filtration

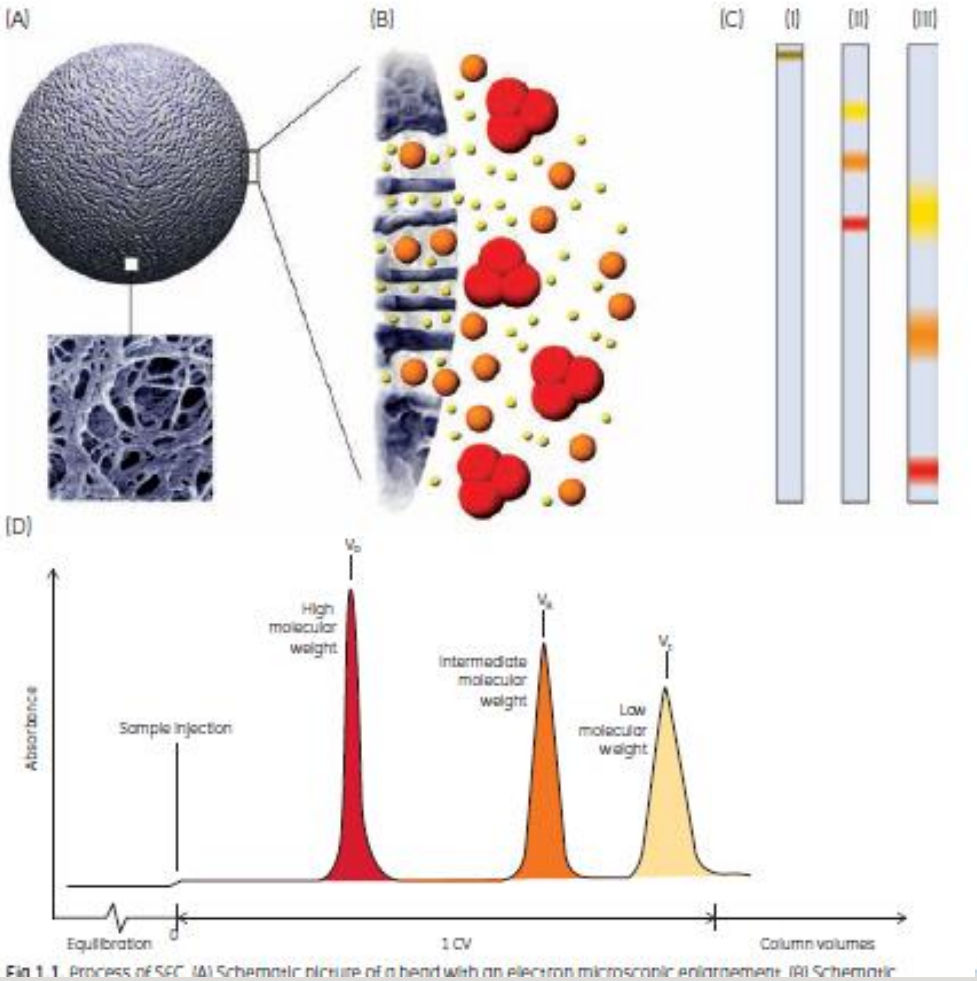


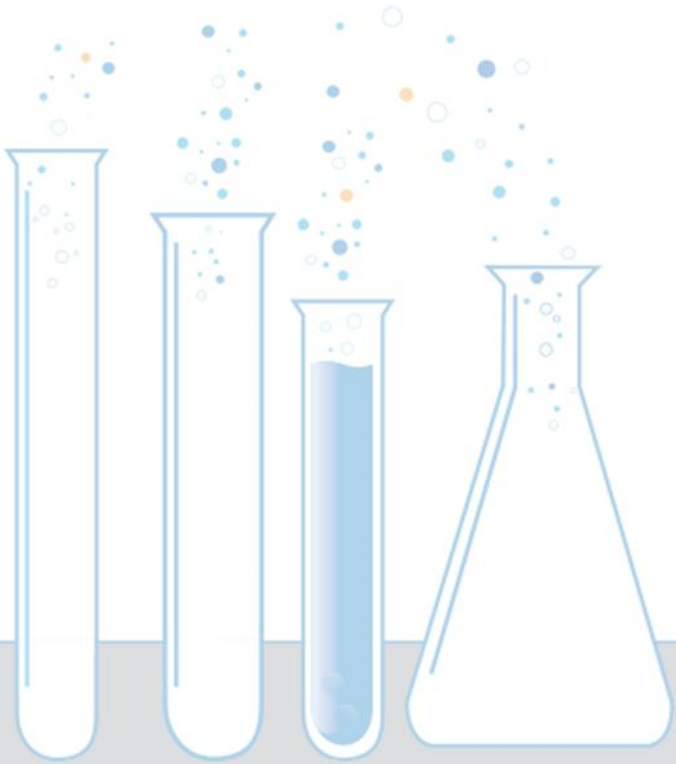
Size Exclusion (SEC) / Gel Filtration (GFC)

- SEC separates molecules according to differences in size as they pass through a SEC medium packed in a column.
- molecules do not bind to the chromatography medium so buffer composition does not directly affect resolution



Size Exclusion (SEC) / Gel Filtration (GFC)





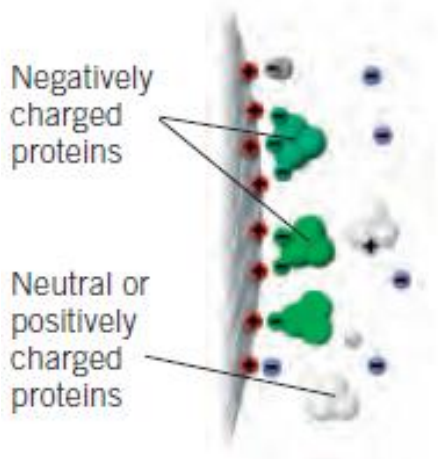
Ion Exchange

Ion Exchange (IEX / IEC)

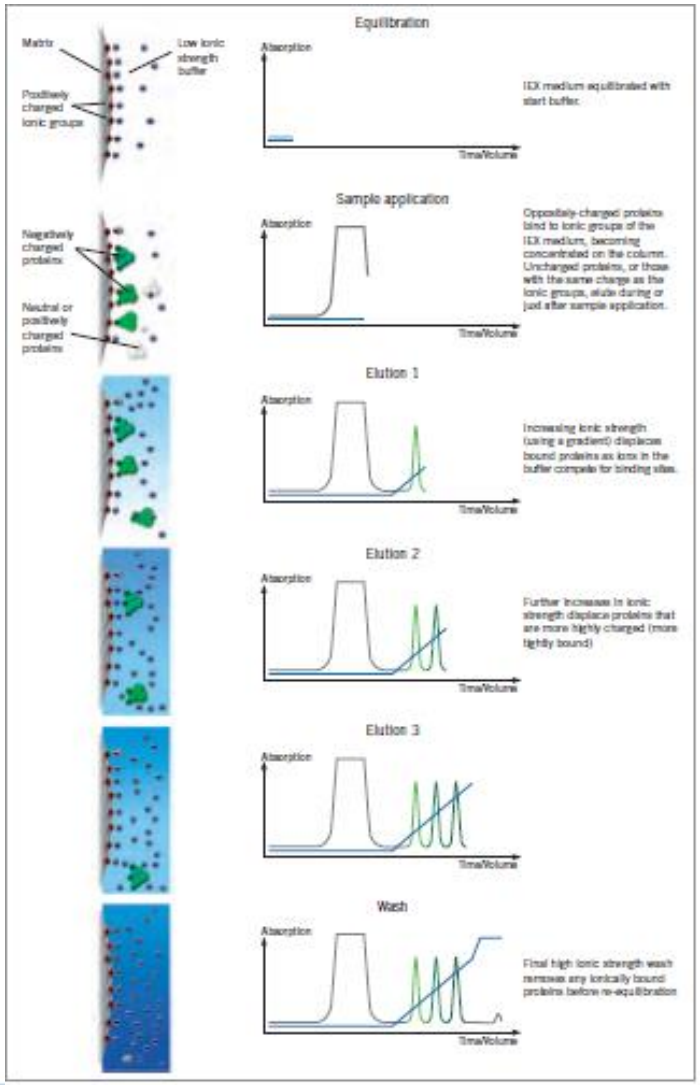
Ion exchange (IEX) chromatography can separate molecules or groups of molecules that have only slight differences in charge.

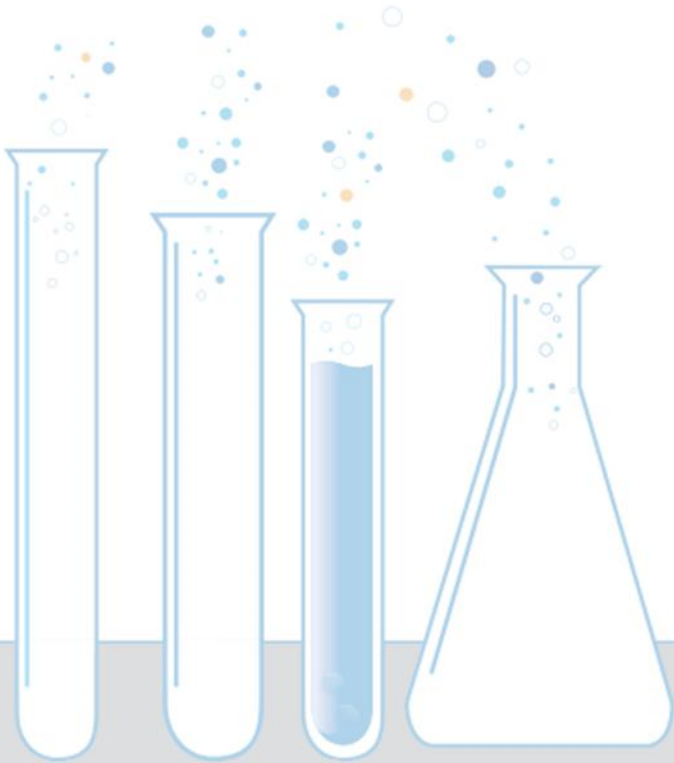
Separation is based on the reversible interaction between a charged molecule and an oppositely charged chromatography medium.

Changes in the buffers salt content facilitate the bind and elution



Ion Exchange (IEX / IEC)





Ion Exchange (IEX / IEC)

Isoelectric point (pI) – The pH on which the net charge on a protein is “0”

Anion Exchanger / Cation Exchanger

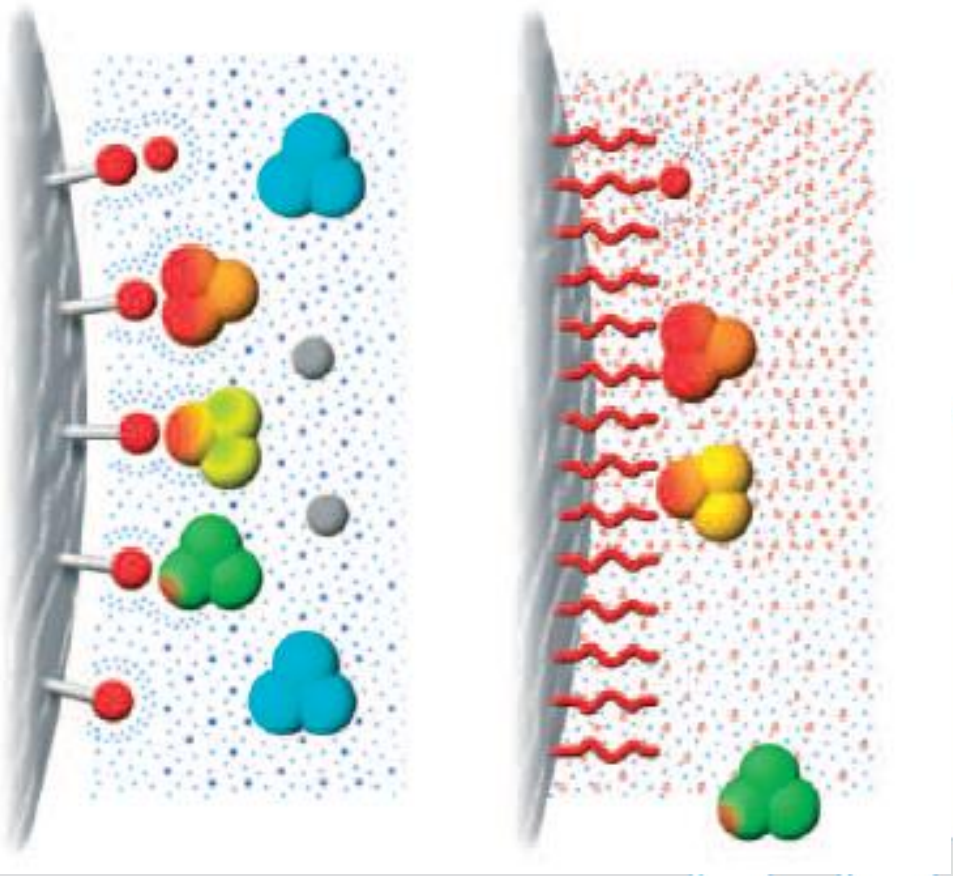
Strong Ion Exchanger – Non pH dependent

Weak Ion Exchanger – pH dependent

Loading buffer – low salt content

Elution buffer – High salt content

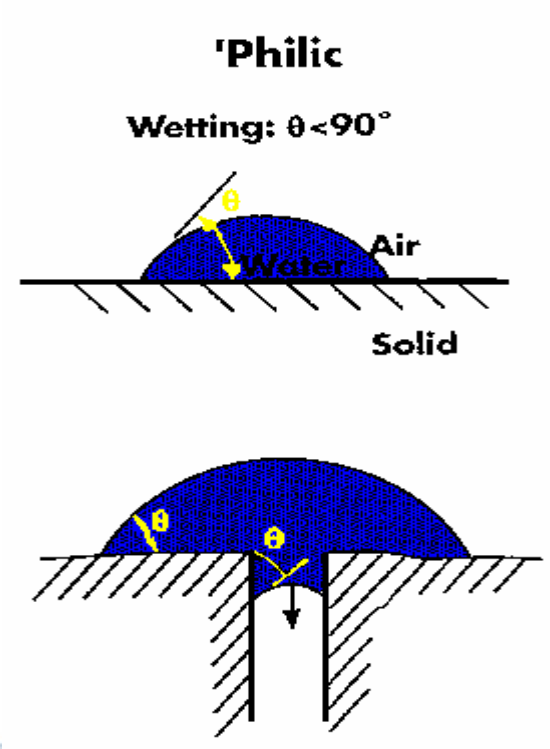
Hydrophobic Interactions and Reverse Phase

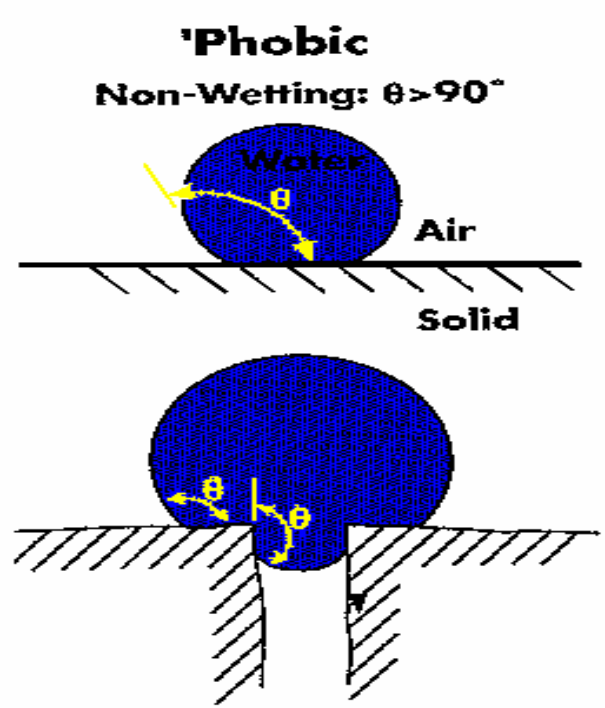


Hydrophobic interactions (HIC)

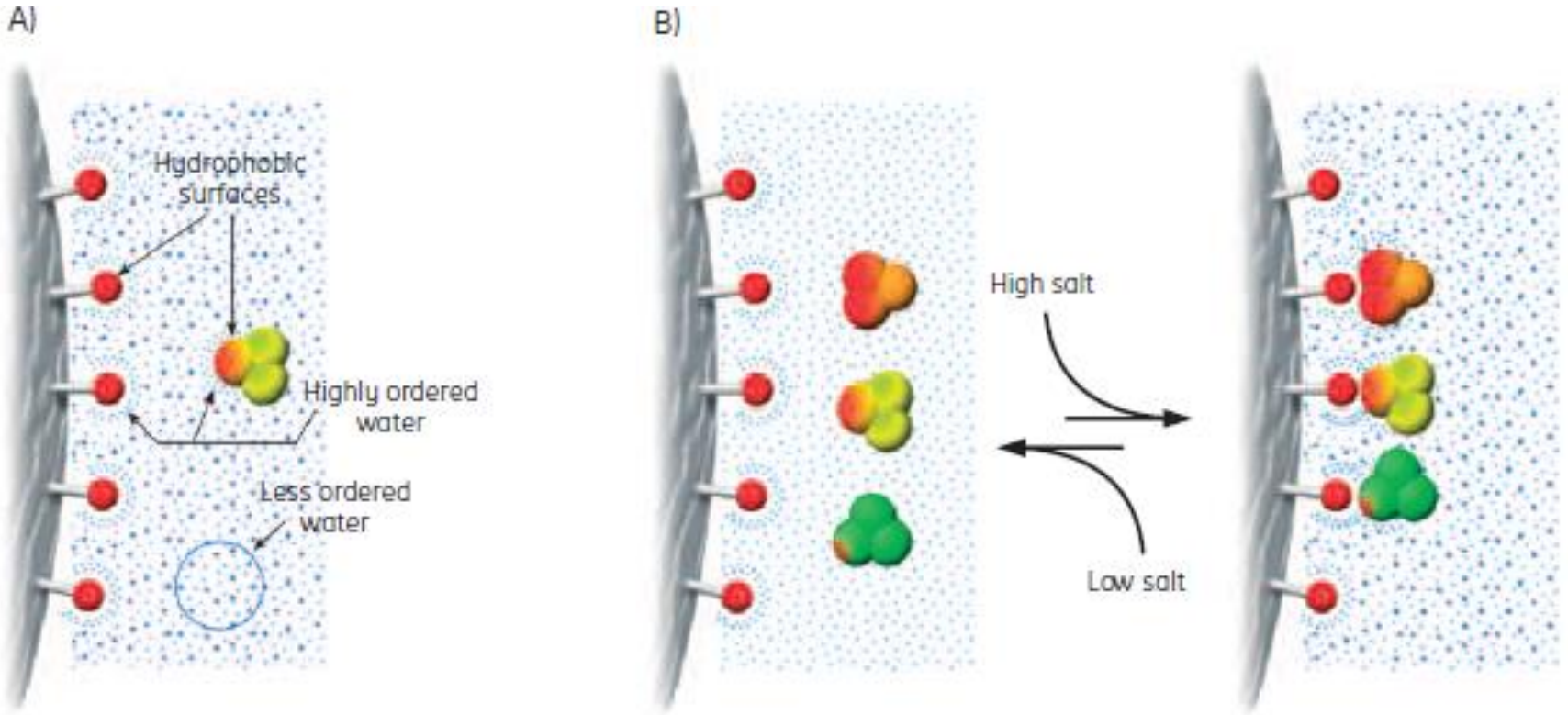
HIC separates proteins according to differences in their surface hydrophobicity.

There is no universally accepted theory on the mechanisms involved in HIC.





Hydrophobic interactions (HIC)



Hydrophobic interactions (HIC)

Different molecules have different hydrophobicity

The longer the “C” chain is the more hydrophobic is the molecule.

Loading buffer – High salt content

Elution buffer – Low salt content – non polar solvent

Reversed Phase Chromatography

Highly Hydrophobic Matrix

Elution performed in non-polar organic solvents

Mostly eluted proteins are denaturated due to harsh solvent conditions.

Examples: C-4, C-18

Affinity Chromatography

Affinity Chromatography

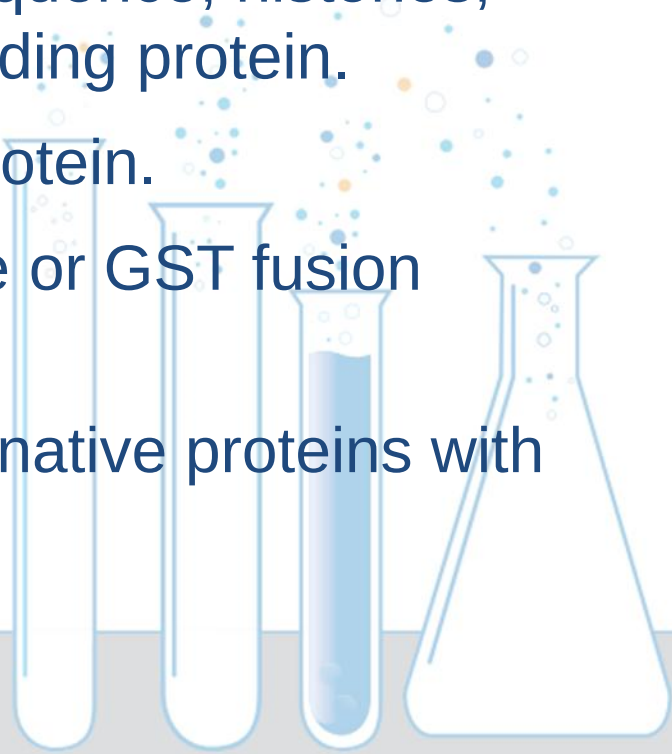
Affinity chromatography separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatography matrix.

The technique offers high selectivity, hence high resolution, and usually high capacity.

Affinity chromatography is the only technique that enables the purification of a biomolecule on the basis of its biological function or individual chemical structure.

Affinity Chromatography

- **Enzyme** - substrate analogue, inhibitor, cofactor.
- **Antibody** - antigen, virus, cell.
- **Lectin** - polysaccharide, glycoprotein, cell surface receptor, cell.
- **Nucleic acid** - complementary base sequence, histones, nucleic acid polymerase, nucleic acid binding protein.
- **Hormone, vitamin** - receptor, carrier protein.
- **Glutathione** - glutathione-S-transferase or GST fusion proteins.
- **Metal ions** - Poly (His) fusion proteins, native proteins with histidine, cysteine and/or tryptophan residues on their surfaces.



Affinity Chromatography

Binding: buffer conditions are optimized to ensure that the target molecules interact effectively with the ligand and are retained by the affinity medium as all other molecules wash through the column.

Elution: buffer conditions are changed to reverse (weaken) the interaction between the target molecules and the ligand so that the target molecules can be eluted from the column.

Wash: buffer conditions that wash unbound substances from the column without eluting the target molecules or that re-equilibrate the column back to the starting conditions.

Ligand coupling: covalent attachment of a ligand to a suitable pre-activated matrix to create an affinity medium. Pre-activated matrices: matrices which have been chemically modified to facilitate the coupling of specific types of ligand.

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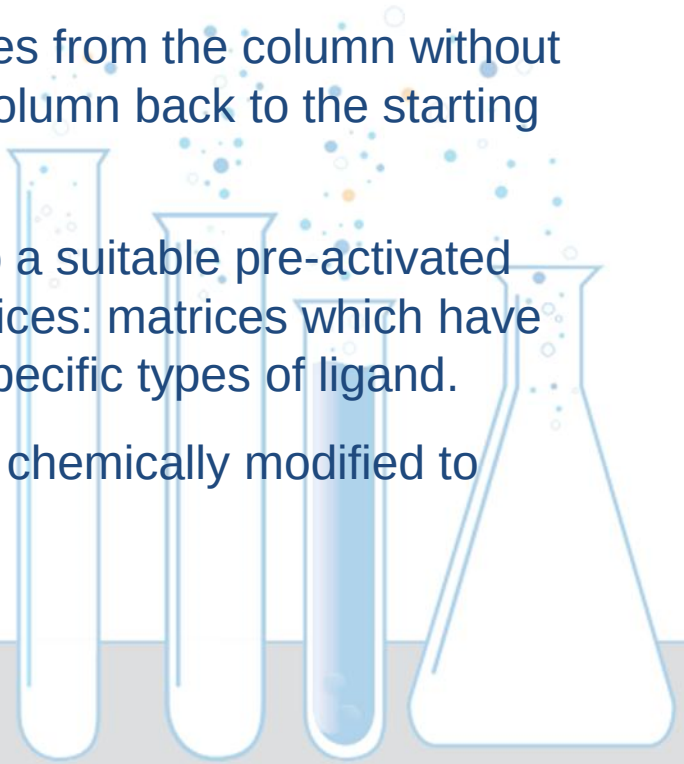


Affinity Chromatography

Matrix: for ligand attachment. Matrix should be chemically and physically inert.

Spacer arm: used to improve binding between ligand and target molecule by overcoming any effects of steric hindrance.

Ligand: molecule that binds reversibly to a specific target molecule or group of target molecules.

Affinity Chromatography

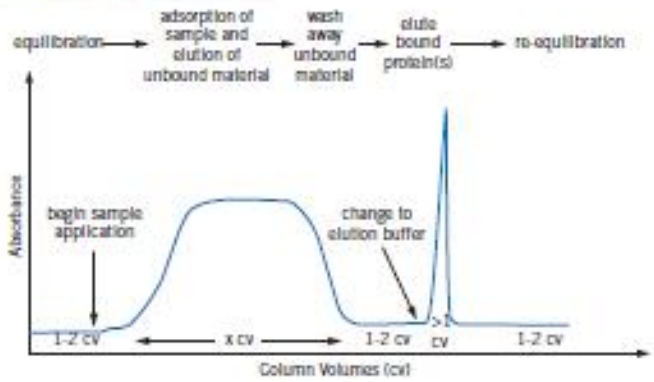
Purification steps

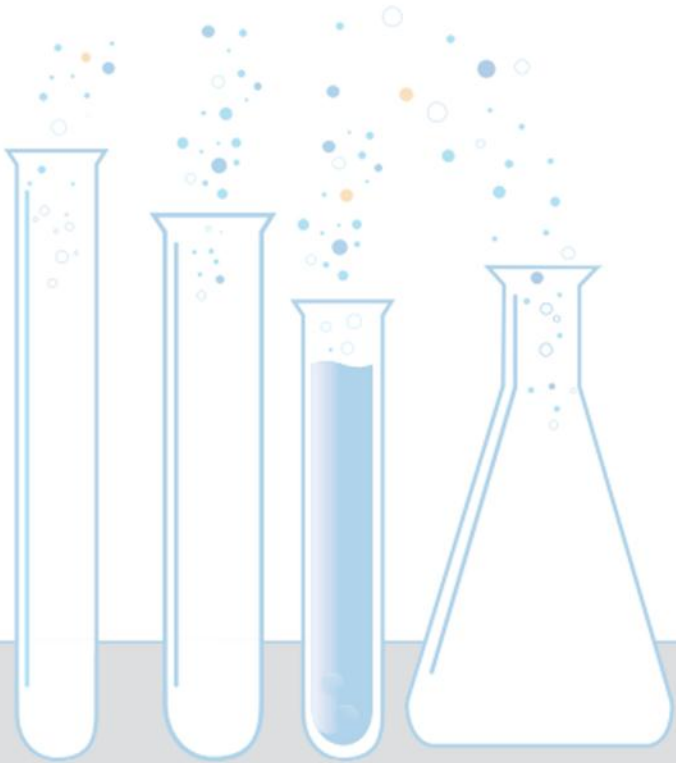
1. Affinity medium is equilibrated in binding buffer.

2. Sample is applied under conditions that favor specific binding of the target molecule(s) to a complementary binding substance (the ligand). Target substances bind specifically, but reversibly, to the ligand and unbound material washes through the column.

3. Target protein is recovered by changing conditions to favor elution of the bound molecules. Elution is performed specifically, using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity. Target protein is collected in a purified, concentrated form.

4. Affinity medium is re-equilibrated with binding buffer.





Affinity Chromatography

Salt content dependent

pH dependent

Highly specific

Ligand leakage should be monitored

Costly

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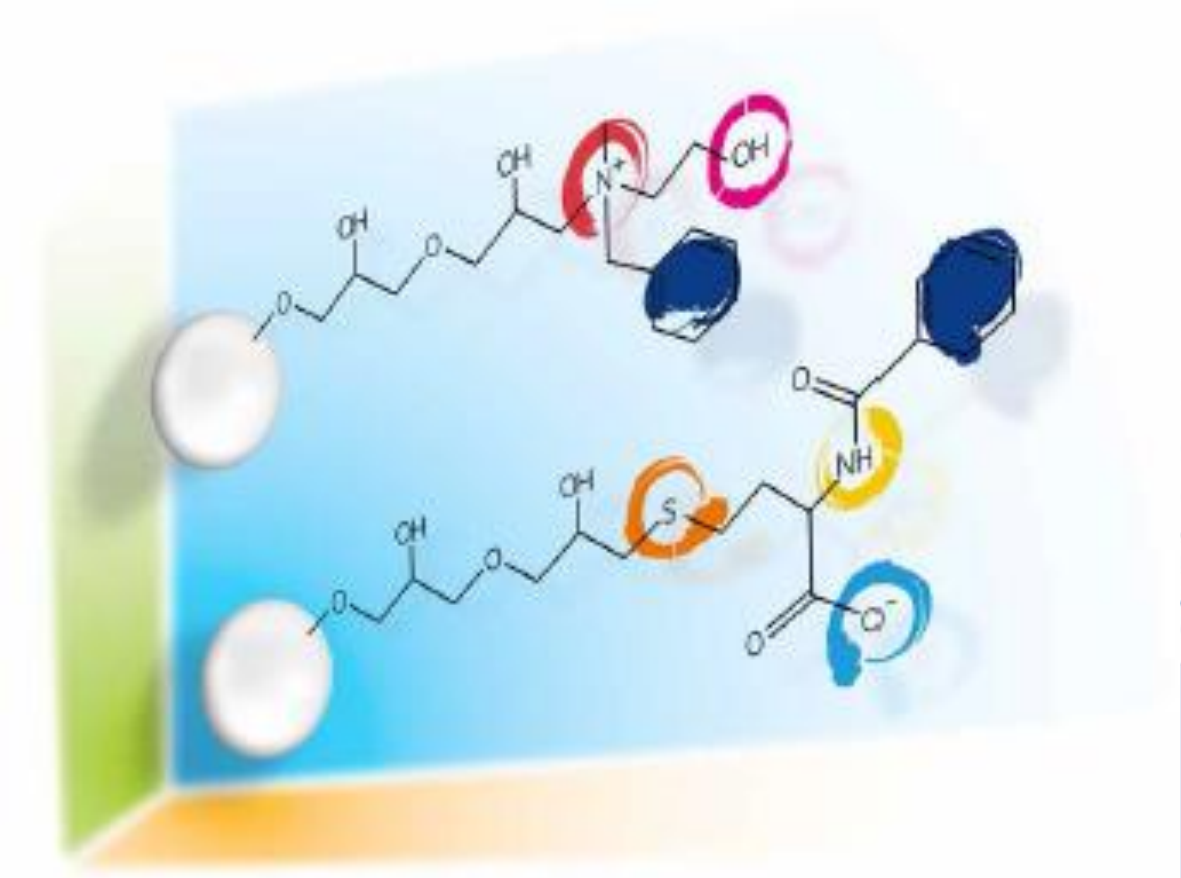
Multimodal Chromatography

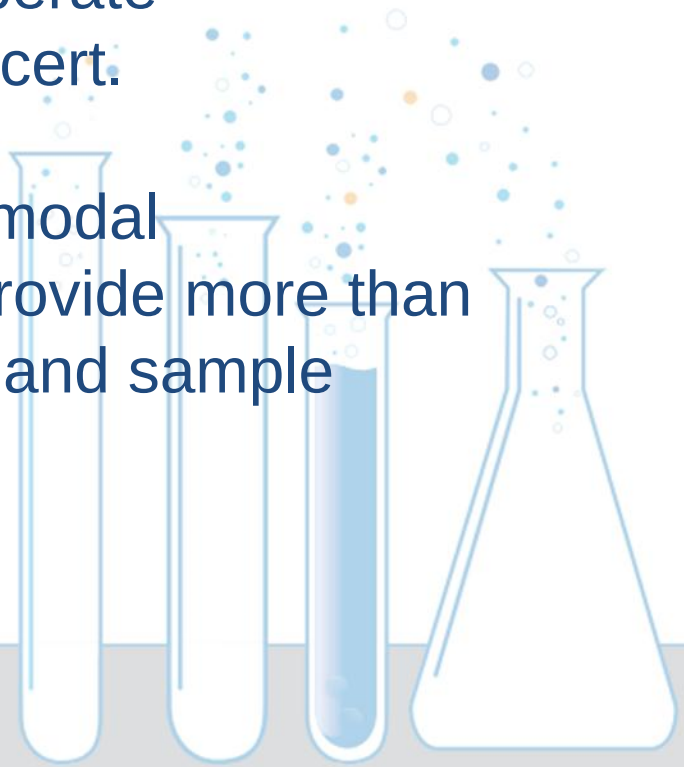


Multimodal Chromatography

The term multimodal, sometimes also referred to as mixed-mode, is broadly used in the context of an object having more than one mode of action.

These different modes of action can operate independently of one another or in concert.

In the field of protein purification, multimodal chromatography refers to media that provide more than one type of interaction between ligand and sample components.



Multimodal Chromatography

Protein property	Method	Description
Various (e.g., charge, hydrophobicity, and hydrogen bonding)	Multimodal chromatography	Separation through at least one ligand type that has more than one interaction site. Different modes of interaction can be expected depending on experimental conditions, e.g., electrostatic, hydrophobic, π-π interaction, hydrogen bonding, and thiophilic interaction. These interactions can cooperate or work independently.

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General Consideration

General consideration

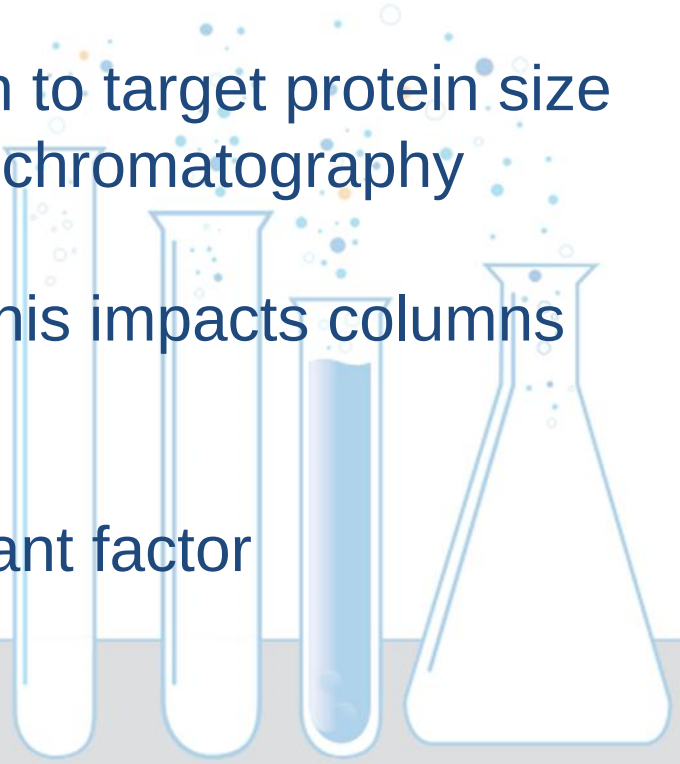
1. **Size of beads affects resolution** : The smaller the beads the higher the resolution.

2. **Size of beads affects Pressure Drop**: The smaller the beads the higher the pressure drop.

3. **Pore size** needs to be in correlation to target protein size in Affinity, IEX, HIC and multimodal chromatography

4. **Base Matrix** can be soft or rigid – this impacts columns packing

5. **Alkaline stability** is also an important factor



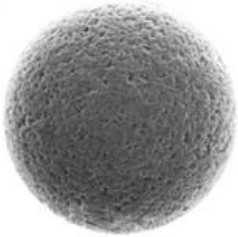
Summary

Each chromatography media…

Is a unique combination between a certain Base Matrix and Ligand

Base matrices

- Capto™
- Sepharose™
- Sephadex™
- Sephacryl™
- SOURCE™
- Etc



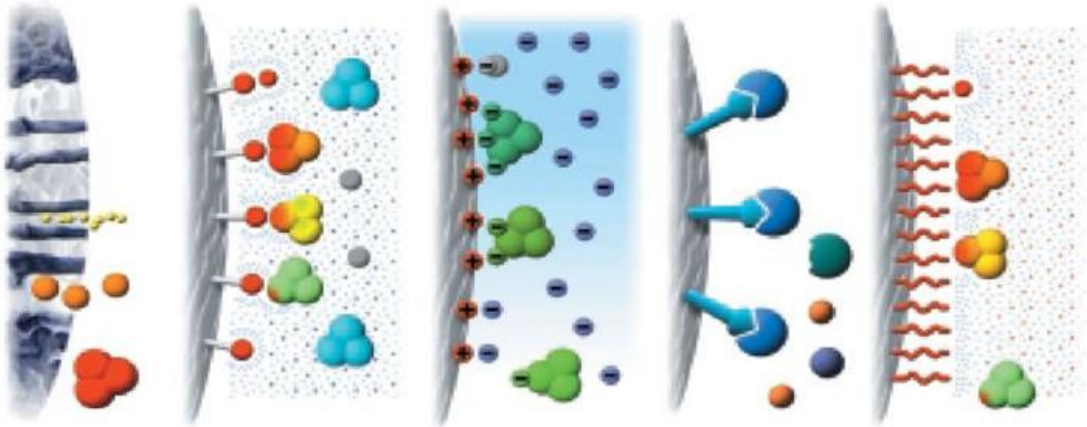
Ligands

- IEX
- HIC
- SEC
- Affinity
- Multimodal
- Etc

Batch binding

Spin columns with filter

Gravity Desalting Columns

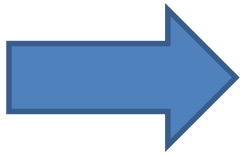


Gel filtration | Hydrophobic interaction | Ion exchange | Affinity | Reversed phase

FPLC columns

Summary

Technique	Separation parameter	Mediated By:
Size Exclusion-Gel Filtration	Size and Shape	Mw, Shape and conformation, Oligometric state
Ion Excgange	Charge	Asp, Glu, Lys, Arg, His
Hydrophobic interactions & Reverse Phase	Hydrophobic patches	Trp, Phe, Ile,Leu, Tyr, Pro, Met, Val, Aln
Affinity	Biological Function	Enzyme – Substrate Antibody - Antigen
Metal chelate (Affinity)	Affinity for metal	His tagged
MultiModal	Parameter combination	Size + IEX (Capto 700S) IEX + HIC



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Basic Terminology

Terminology

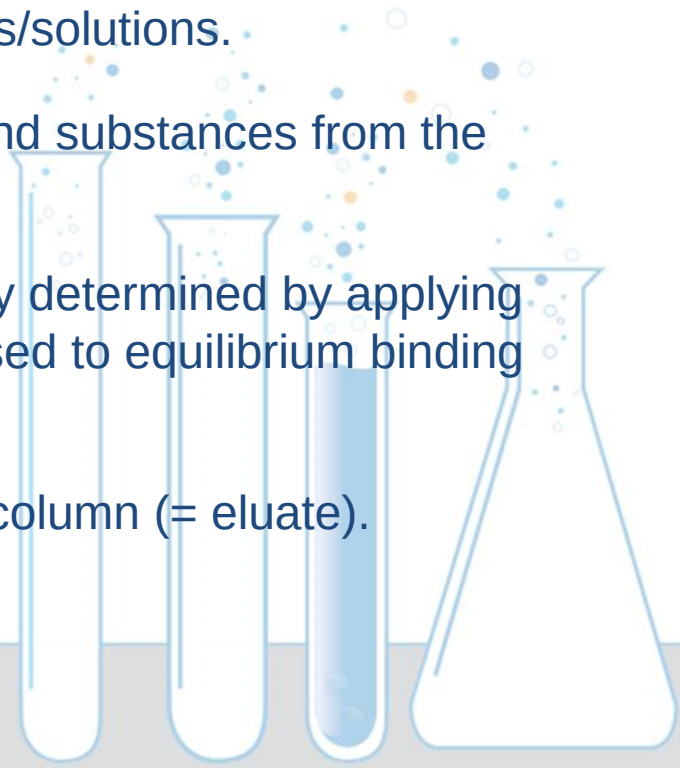
- **Adsorption** Binding. The process of interaction between the solute (for example, a protein) and the stationary phase.
- **Asymmetry (asymmetry factor)** Factor describing the shape of a chromatographic peak.
- **Backpressure** The pressure drop across a column and/or a chromatography system.
- **Binding capacity** The maximum amount of material that can be bound per ml of chromatography medium. See also Dynamic binding capacity.
- **Chromatogram** A graphical presentation of detector response(s) indicating the concentration of the solutes coming out of the column during the purification (volume or time).
- **Chromatography** From Greek chroma, color, and graphein, to write.

Terminology

- **medium/media** The stationary phase, also called resin. The chromatography medium is composed of a porous matrix that is usually functionalized by coupling of ligands to it. The matrix is in the form of particles (beads) or, rarely, a single polymer block (monolith).
- **CIP (cleaning-in-place)** Common term for cleaning chromatography columns and/or systems with the purpose of removing unwanted/ nonspecifically bound material.
- **Column** Usually column hardware packed with chromatography medium.
- **Column equilibration** Passage of buffer/solution through the chromatography column to establish conditions suitable for binding of selected sample components. For example, to establish correct pH and ionic strength, and ensure that proper counter ions or counter ligands are present.
- **Column packing** Controlled filling of the column hardware with chromatography medium to obtain a packed bed.

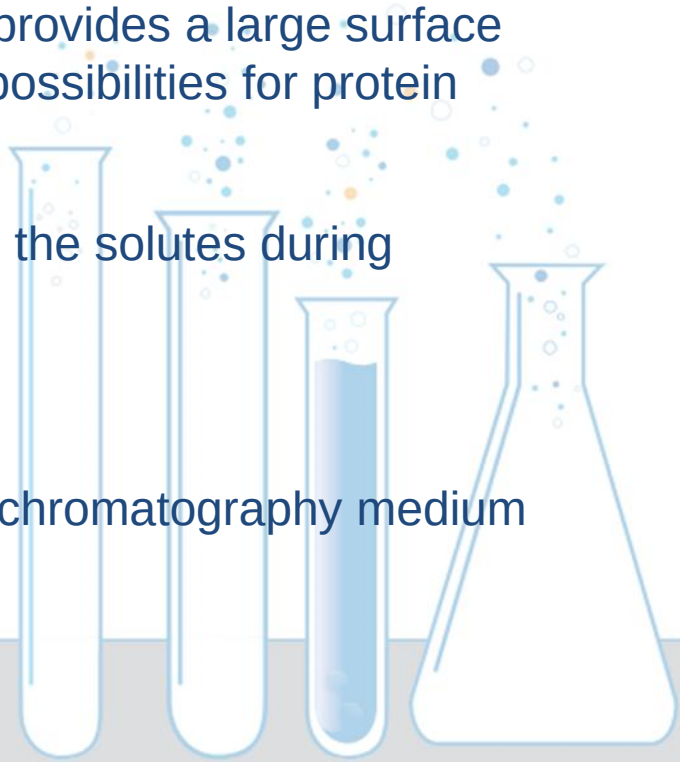
Terminology

- **Column volume** The geometrical volume of the column interior/the chromatography bed.
- **Dead volume** The volume outside the packed chromatography bed. Can be column dead volume or chromatography system dead volume. The dead volume contributes to band broadening.
- **Degassing** Removal of dissolved air from buffers/solutions.
- **Desorption Elution**. Release or removal of bound substances from the chromatography medium.
- **Dynamic binding capacity** The binding capacity determined by applying the target using flow through a column, as opposed to equilibrium binding capacity determined by batch experiment.
- **Effluent / Eluate** The mobile phase leaving the column (= eluate).



Terminology

- **Flow rate** Volumetric flow (ml/min) or linear flow rate (cm/h). Measurement of flow through a column and/or chromatography system.
- **Flowthrough** Material passing the column during sample loading (without being bound).
- **Matrix** The matrix is the nonfunctional base for the chromatography medium. The matrix has a porous structure that provides a large surface that can be modified with ligands that introduce possibilities for protein binding.
- **Mobile phase** The fluid (buffer/solution) carrying the solutes during chromatography (= eluent).
- **Pore** Cavity in a chromatography matrix.
- **Pore volume** The total volume of the pores in a chromatography medium



Terminology

- **Recovery** The relative amount of target protein that is retrieved after purification compared with amount loaded on the column.
- **Resin** The term is sometimes used instead of the more generic term, chromatography medium.
- **Resolution** Measurement of the ability of a packed column to separate two solutes (peaks).
- **Void volume** The elution volume of solutes that do not enter the pores or interact with the chromatography medium, thus passing between the beads in the packed bed.
- **Yield** Amount of target protein (or other solute) obtained after a purification step, or after the entire purification (multiple steps).