Purification by Chromatographic Methods

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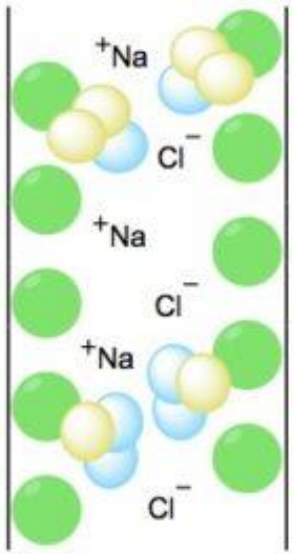
FE 361 Enzymology

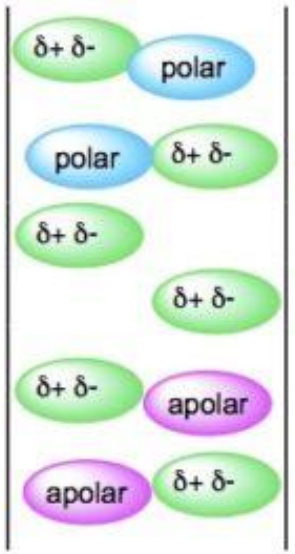
- Biomolecules are purified using chromatographic methods that separate according to differences in their specific properties.

Property	Technique
Size	Gel filtration
Hydrophobicity	Reversed phase chromatography
Charge	Ion Exchange chromatography
Ligand Specificity	Affinity chromatography
Isoelectronic Point	Chromatofocusing

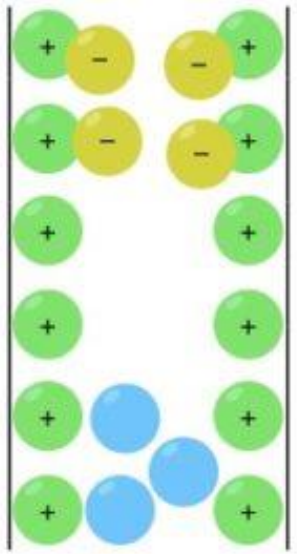
Chromatography is the separation of a mixture of compounds into its individual components based on their relative interactions with an inert matrix.



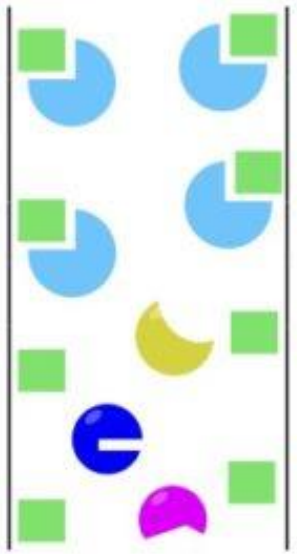
Hydrophobic Interaction
(hydrophobicity)



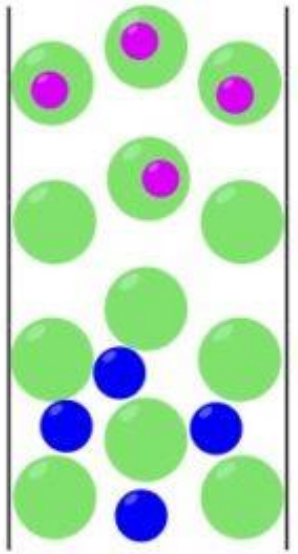
Normal-phase
(polarity)



Ion-exchange
(net charge)



Affinity
(specific binding)



Size-exclusion
(molecular size)

Ion Exchange Chromatography

- Ion Exchange chromatography separates molecules on the basis of differences in their net surface charge.
- Proteins are separated based on charge differences.
- Ion exchange chromatography (or ion chromatography) is a process that allows the separation of ions and polar molecules based on their affinity to ion exchangers.

- Remember: Proteins (enzymes) have a net charge in solution. The charge of protein (enzymes) depends on pH of the solution and isoelectronic point of the protein. Each protein has its own unique net charge versus pH relationship.

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- In a buffer solution with pH>pI of protein: protein will have a negative charge, there for a positively charged anion exchange resin is chosen to capture this protein
- In a buffer solution with pH<pI of protein: protein will carry a positive net charge, thus a negatively charged cation exchange resin is choosen.

Ion Exchange chromatography principle:

1. Binding of the portein to the fixed charges
2. Elution of the protein from the fixed charges

How ion exchange chromatography works?

1. Equilibration: Stationary phase consists of a positively or negatively charged polymeric matrix which will bind molecules of opposite charge. The column is packed with a suitable cation or anion Exchange resin depending on charge of the protein.

The first step is the equilibration of the stationary phase to the desired start conditions. When equilibrium is reached, all stationary phase charged groups are bound with exchangeable counterions, such as chloride or sodium. The pH and ionic strength of the start buffer are selected to ensure that, when sample is loaded, proteins of interest bind to the medium and as many impurities as possible do not bind.

- 2. The crude protein mixture which consists of different net charges are loaded on the column. The proteins having charges opposite to that of stationary matric will bind to it, while remaining proteins will be eluted.
- The purpose of this step is to bind the target molecule(s) and wash out all unbound material. Oppositely charged proteins bind to ionic groups of the IEX medium, becoming concentrated on the column.
- Uncharged proteins, or those with the same charge as the ionic group, pass through the column at the same speed as the flow of buffer, eluting during or just after sample application, depending on the total volume of sample loaded

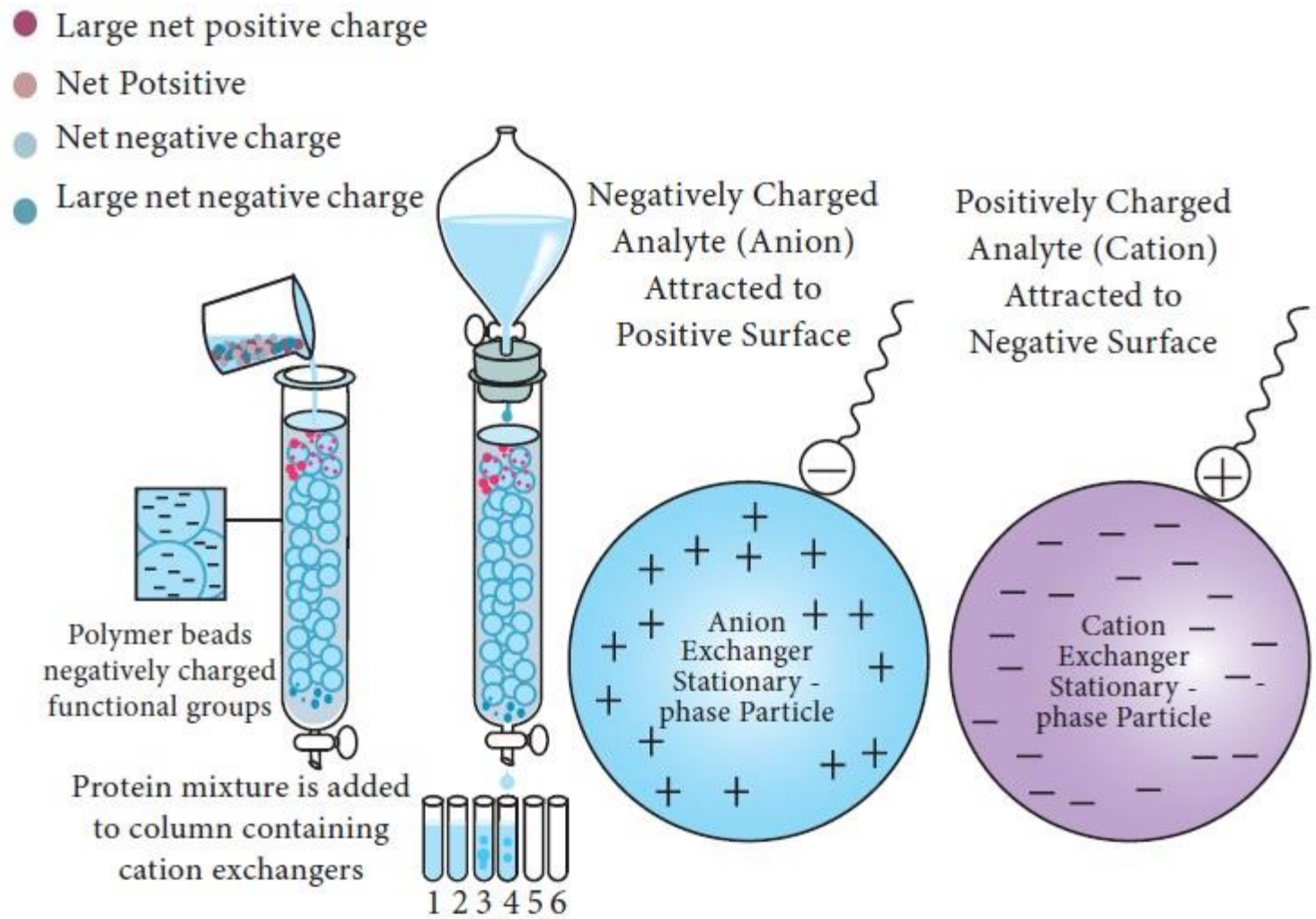
3. Elution: The column is eluted with a buffer solution of suitable pH. When all the sample has been loaded and the column washed with start buffer:

- All nonbinding proteins have passed through the column, conditions are altered in order to elute the bound proteins.
- Proteins are eluted by increasing the ionic strength (salt concentration) of the buffer or, occasionally, by changing the pH.
- As ionic strength increases the salt ions (typically Na^+ or Cl^-) compete with the bound components for charges on the surface of the medium and one or more of the bound species begin to elute and move down the column.
- The proteins with the lowest net charge at the selected pH will be the first ones eluted from the column as ionic strength increases. Similarly, the proteins with the highest charge at a certain pH will be most strongly retained and will be eluted last. The higher the net charge of the protein, the higher the ionic strength that is needed for elution

- 4. Regeneration: A final wash with high ionic strength buffer regenerates the column and removes any molecules still bound. This ensures that the full capacity of the stationary phase is available for the next run. The column is then re-equilibrated in start buffer before starting the next run

- There are two types of ion-exchange chromatography. These are cationic and anionic exchangers.
- **Cationic exchangers** have negatively charged groups, and these will attract positively charged proteins.
- **Anionic exchangers** have positively charged groups that will attract negatively charged proteins.
- Anionic exchange Chromatography should be carried out with cationic buffers.
- Cationic exchange Chromatography should be carried out with anionic buffers.

- A protein that has no net charge at a pH equivalent to its isoelectric point (pI) will not interact with a charged medium. However, at a pH above its pI, a protein will bind to a positively charged medium or anion exchanger and, at a pH below its pI, a protein will bind to a negatively charged medium or cation exchanger



Protein move through the column at rates determined by their net charge at the pH being used. With cation exchangers, proteins with a more negative net charge move faster and elute earlier.

Advantages of Ion Exchange Chromatography

1. It is one of the most efficient methods for the separation of charged particles.
2. It can be used for almost any kind of charged molecule including large proteins, small nucleotides and amino acids.
3. Ion exchange is used for both analytical and preparative purposes in the laboratory, the analytical uses being the more common.
4. Inorganic ions also can be separated by ion-exchange chromatography

Limitations of Ion Exchange Chromatography

- Only charged molecules can be separated.
- Buffer Requirement

References

- https://research.fredhutch.org/content/dam/stripe/hahn/methods/biochem/Ion_Exchange_Chromatography_Handbook.pdf. (available on 18.12.2020)