**Introduction to Proteomics**
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**Lecture – 21**
**Chromatography Technologies**

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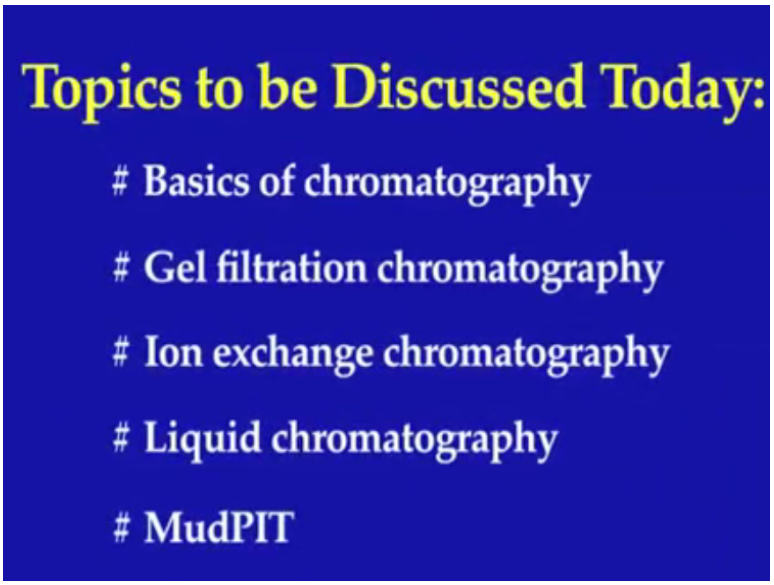


Welcome to the proteomics course. Today we will talk about protein purification and peptide isolation using chromatography methods. The chromatography techniques have always been considered prominent for the separation of enzymes and proteins. Protein purification by conventional chromatography is usually achieved by combining chromatography methods such as gel filtration, ion exchange and affinity chromatography.

From complex proteome it is challenging to purify a protein in the single chromatography step. Therefore, sequential prefractionation steps involving different modes and types of chromatography methods are becoming necessary for proteome level analysis.

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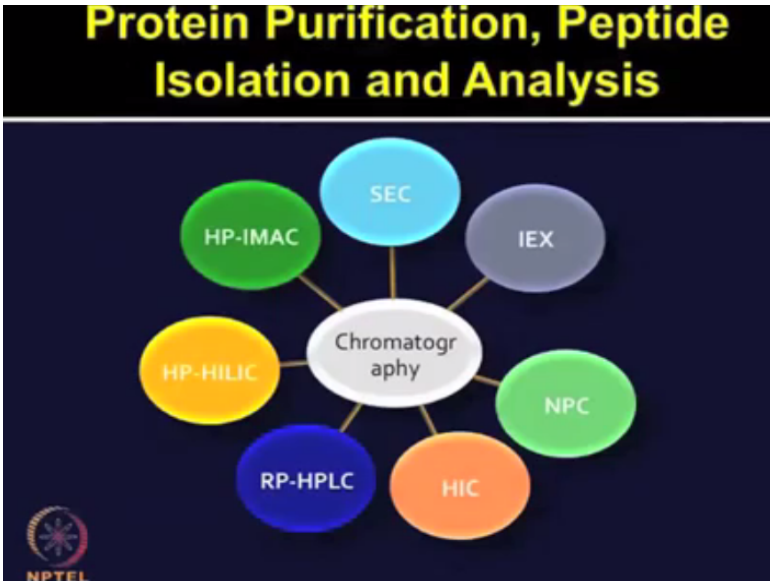


Protein purification, peptide isolation and analysis: It can be performed by many methods. But there are certain chromatography methods which are commonly used such as Size Exclusion Chromatography (SEC), Ion Exchange Chromatography (IEX), Immobilized Metal Affinity Chromatography (IMAC), Normal Phase Chromatography (NPC), Reverse Phase – HPLC or RP – HPLC, Hydrophobic Interaction Chromatography (HIC) and Hydrophilic Interaction Chromatography (HILIC).

These are only few chromatography methods. But there are many more which are also used for different type of applications.

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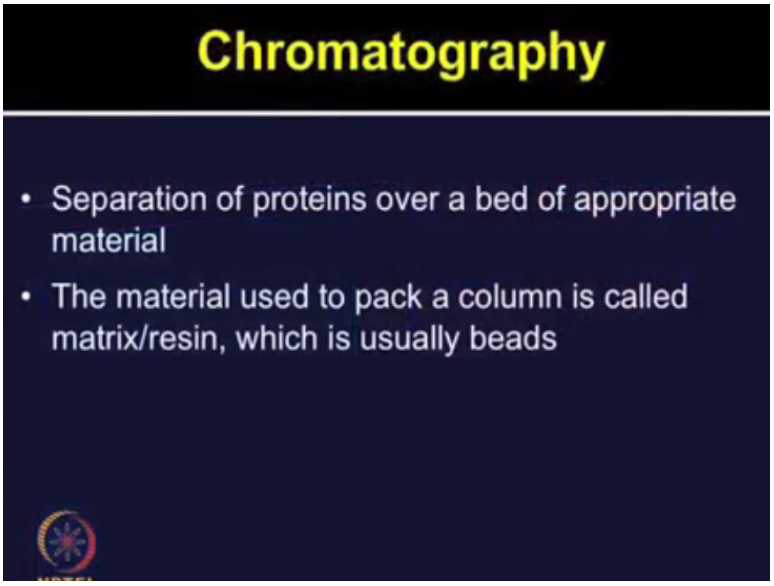

So let us talk about what is chromatography? So chromatographic methods involve separation of proteins over a bed of appropriate material. These materials are usually packed in a given column and known as matrix or resin. So these matrix and resins they are usually beads with or without attached chemical groups. Binding and interaction of proteins with the column matrix is an important feature of chromatography.

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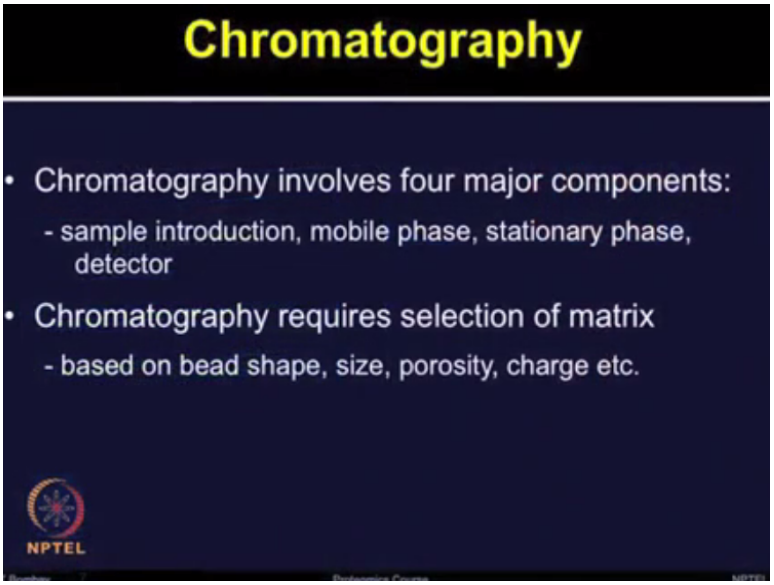


The chromatography technique involves four major components: an inlet for sample introduction, the mobile phase, stationary phase and a detector. Each type of chromatography requires a very educated and informed choice of matrix considering the bead shape, size and porosity. In addition to the functional group type, the charge, distribution and density as well as elusion conditions such as the pH, the ionic strength and gradient shape.

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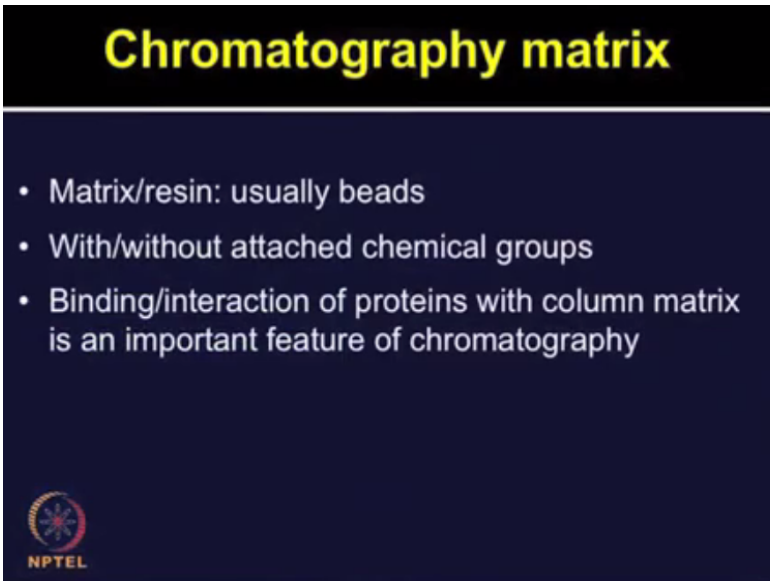


(()) (03:55) just talked the chromatography involves the selection of right matrix. These matrices are very important for binding and interaction of proteins and they can determine the kind of achievement that we can achieve from these kinds of chromatographic methods.

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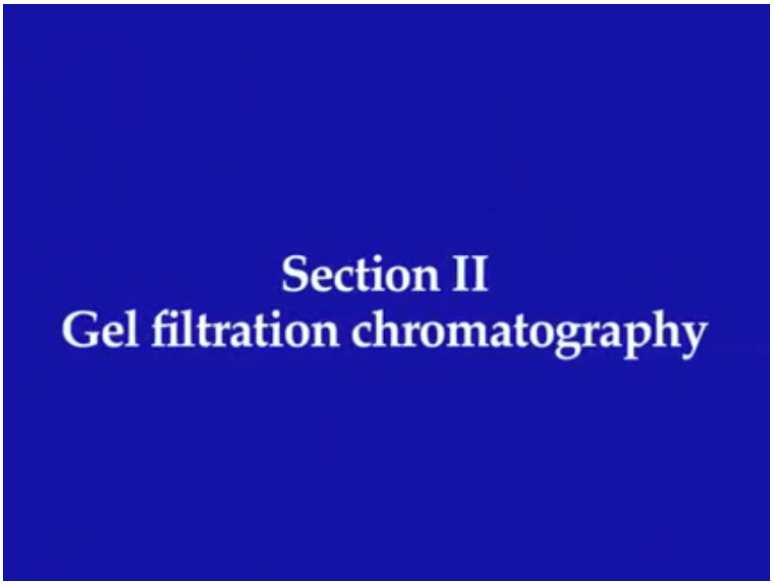


So let us move on to more specific chromatography technique such as Gel filtration chromatography. The Gel filtration chromatography; it separates proteins on the basis of difference in size. It is used to separate a protein of interest from protein mixture that is larger or smaller in size. If proteins are having similar sizes, then the gel filtration or size exclusion chromatography is not an appropriate choice for doing the protein purification. So this method is

also used for various proteomic applications when there is need to remove contaminants such as salts and low molecular size detergents.

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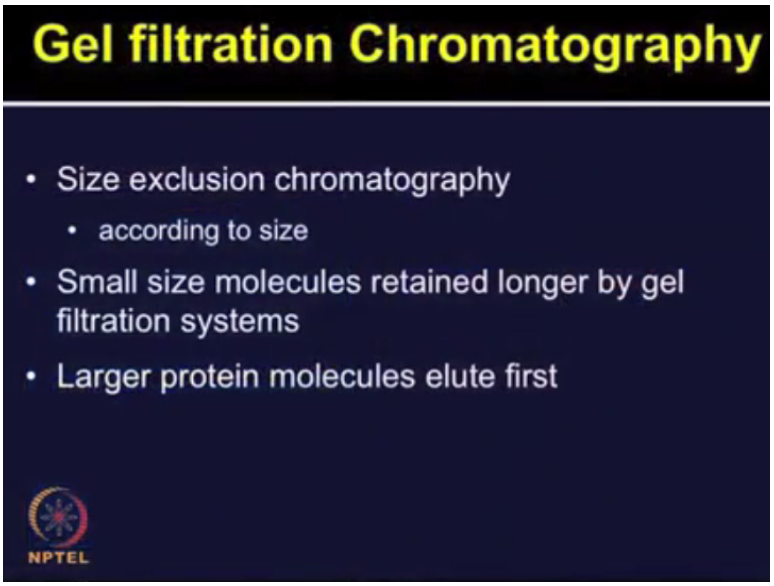


So Gel filtration chromatography is also known as Size Exclusion Chromatography. It means the molecules are separated according to the given size. The small molecules are even if you are separating small molecules such as salt both will be retained longer by the gel filtration system and larger molecules such as proteins will elute first because they cannot enter inside the porous these beads and they can be used to separate proteins based on the size.

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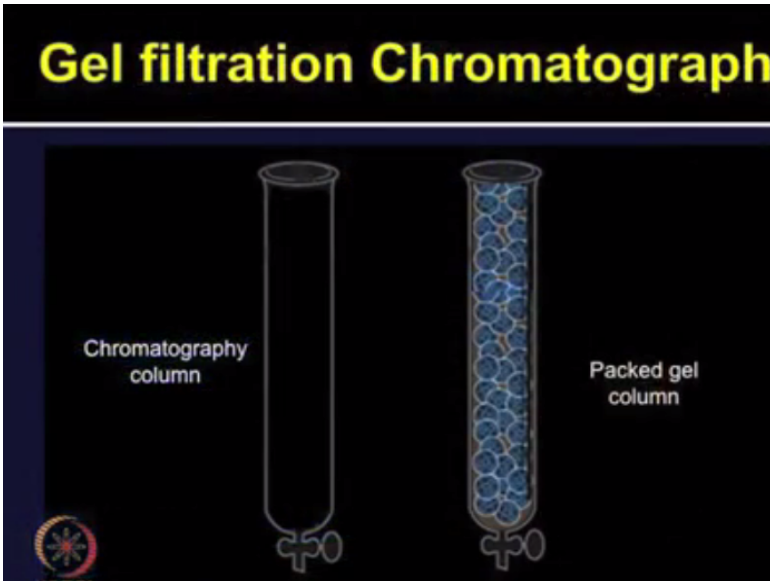

The gel filtration column is composed of porous beads which are made from polyacrylamide, dextan or agarose. Now these columns are packed with hydrated porous gel matrix.

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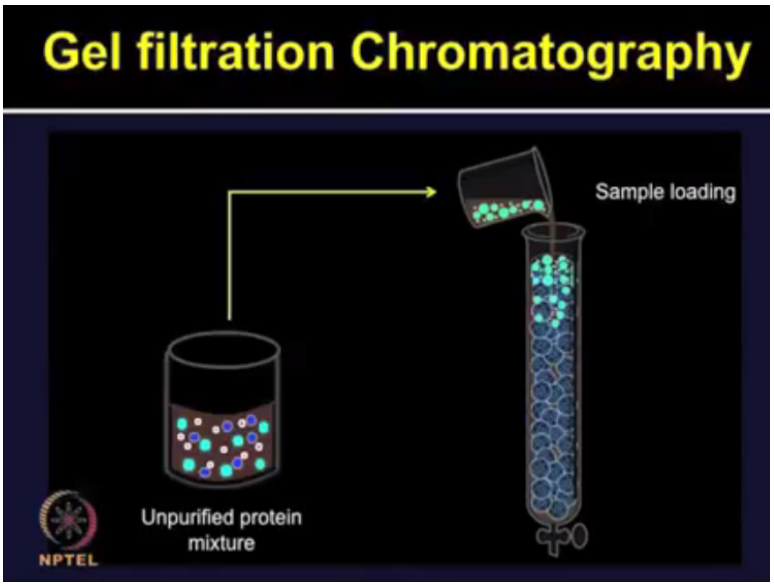


The protein sample which contains a mixture of unpurified proteins of different size is then loaded on this column.

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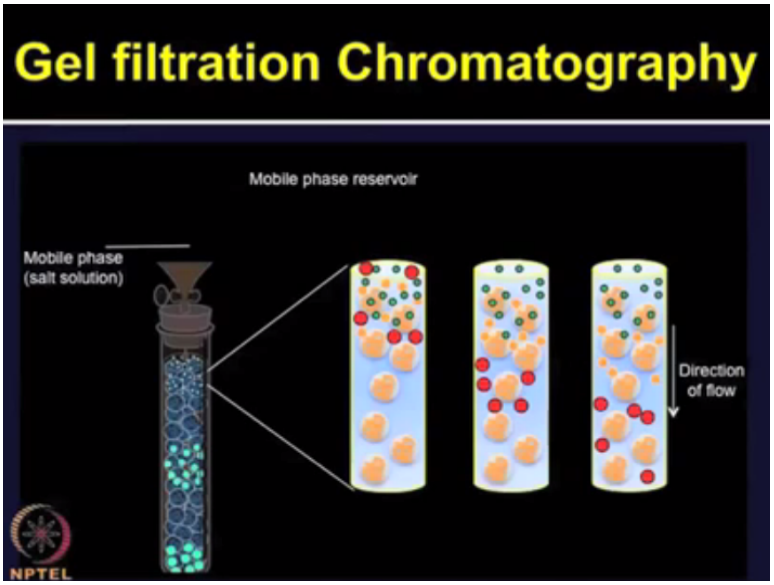


Now when protein sample is applied to the column, the small protein passes over the beads of the column while the large proteins are excluded. Therefore, this technique is also known as molecular exclusion chromatography. The beads of different pore sizes are increasing retention

time by adjusting the increased column length or decreasing the flow rate can be adjusted to achieve higher resolution of proteins.

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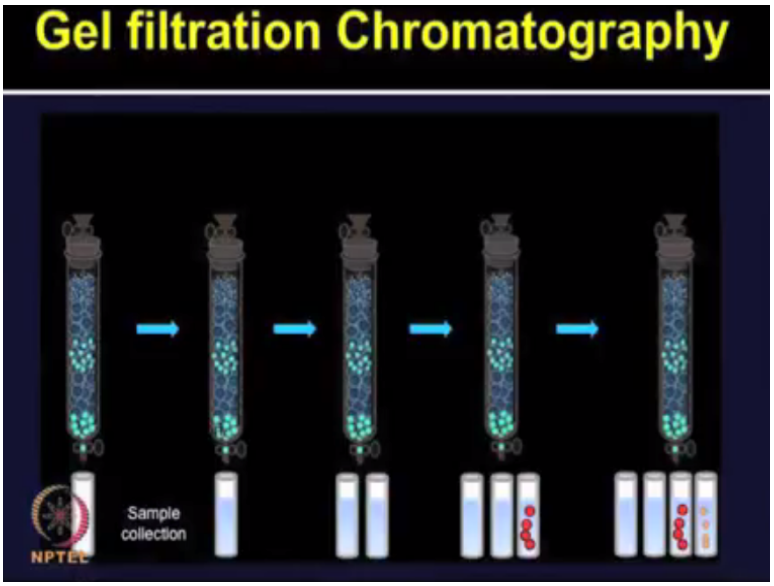


After these steps the fractions are collected and analyzed for the protein content. So you can expect that larger proteins will elute first and then the small molecules will be eluted later. So gel filtration chromatography is useful for removal of every contaminants doing these (()) (07:25) steps as well as for buffer exchange. However, its drawbacks are its low capacity, broad distribution of pore size and its small sample volume to be analyzed.

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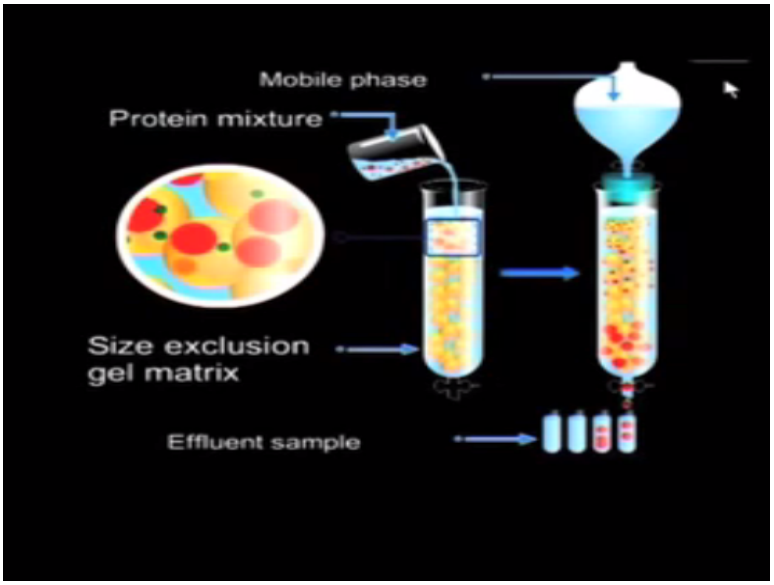

So let me describe the gel chromatography technique in following animation. Let us first see the definition of few components. Size exclusion gel matrix: The matrix filling the gel filtration column consist of a highly hydrated polymeric material commonly dextan, agarose or polyacrylamide. The protein mixture is a mixture of unpurified proteins of different sizes which is applied on top of the column.

Mobile phase: The proteins are eluted out of the column by using a suitable mobile phase that carries the protein out for elusion. For gel filtration a salt solution of appropriate strength is commonly used, so that it will not have any effect on properties of the proteins being purified. Solvent or buffer system is often used in other types of chromatography. Effluent sample fractions: The solution leaving the column is collected in suitable size fractions. Initial fraction will contain only the mobile phase while later fractions will have purified proteins.

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Let me give an analogy of gel filtration with sieve. This process is very similar to separation of very particulate matter from food grains using a sieve. The larger grains remain behind the sieve while the smaller sand or stone particles pass through them and are removed. In keeping with this the gel filtration technique is also commonly referred as molecular sieve.

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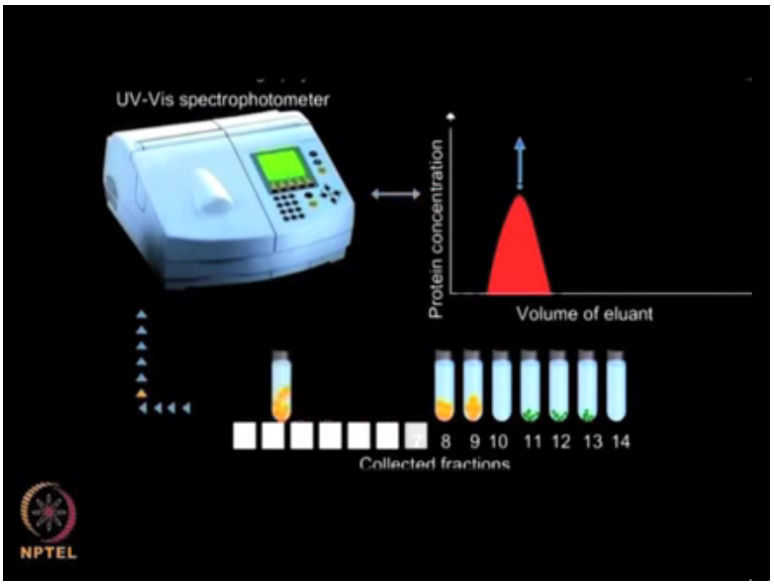


Now let me show you how this process works. So first the (()) (09:46) which is suitable for the required protein separation. This packed gel matrix is then loaded with the protein sample containing a mixture of unpurified proteins of different sizes. The column is then eluted with a salt solution of appropriate concentration. Large proteins that cannot enter the pores of the gel move down through the interstitial spaces at a faster rate and are eluted first.

The smaller proteins move in and out of the pours thereby taking longer time to be removed from the column. The fractions of appropriate sizes should be collected and analyzed for their protein content. The larger proteins eluting first will be present in the initial fractions while the smaller proteins which elute out later will be present in the later fractions.

Once all these fractions are collected then it can be analyzed for their protein content by using a UV visible spectrophotometer. Now analyze the protein content by using UV visible spectrophotometer at 280 nanometres. Once all the absorbents values are recorded a graph of eluant volume versus protein concentration can be plotted. Highest molecular weight is in the beginning and lowest molecular weight towards the end.

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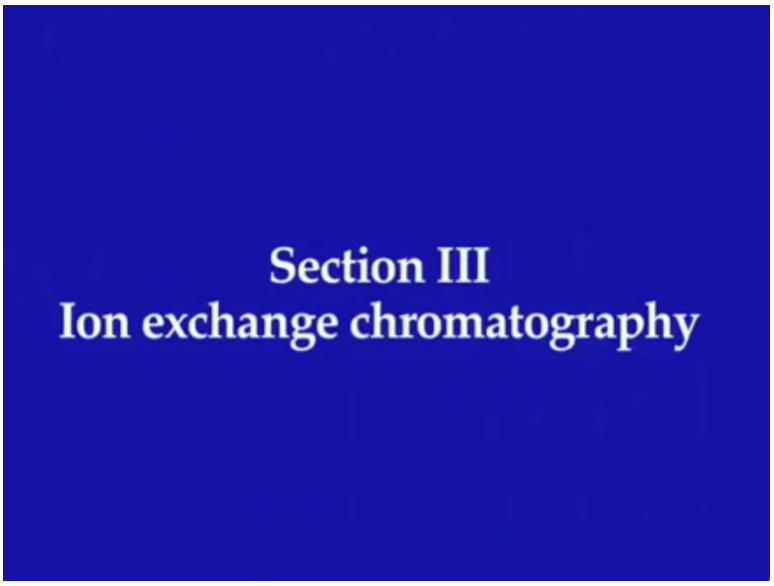


Let us now move on to next chromatography method which is ion exchange chromatography. This is one of the most versatile chromatographic separation methods which relies on differences between number of charges and distribution of charge groups in defined pH and solvent conditions.

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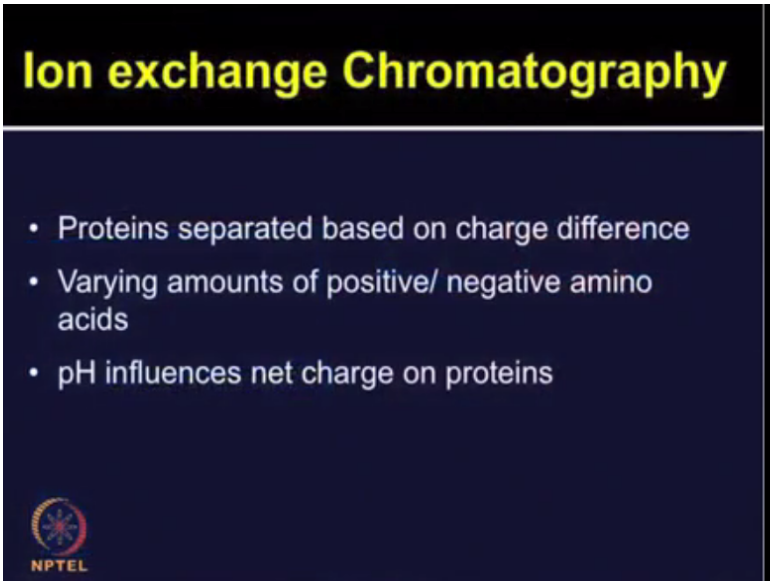


In ion exchange chromatography the proteins are separated based on charge difference. The proteins with overall negative charge will interact with positive charges or the vice versa. So by varying the amount of positive and negative amino acids and even pH influence the net charge on proteins.

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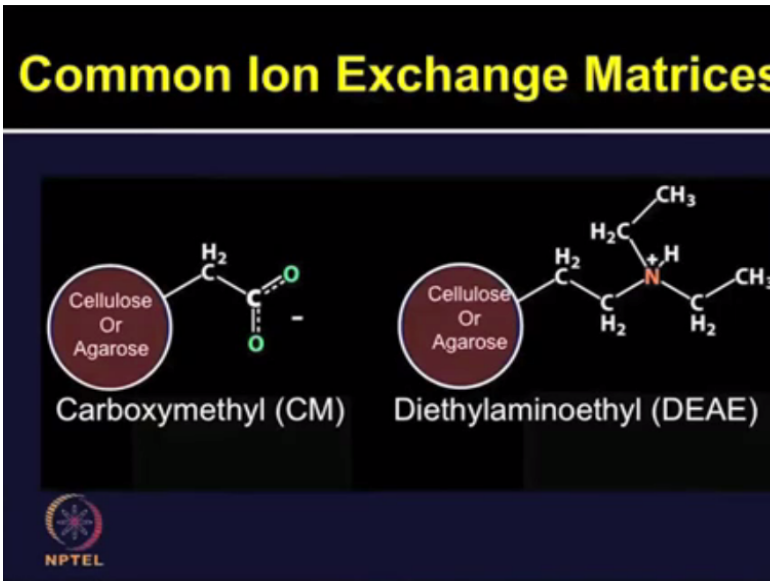


So in this slide some of the common ion exchange matrices are shown like Carboxymethyl (CM) and Diethylaminoethyl (DEAE). So when a desired protein is positively charged the cation exchange chromatography should be used. When a desired protein is negatively charged the anion exchange chromatography method should be used.

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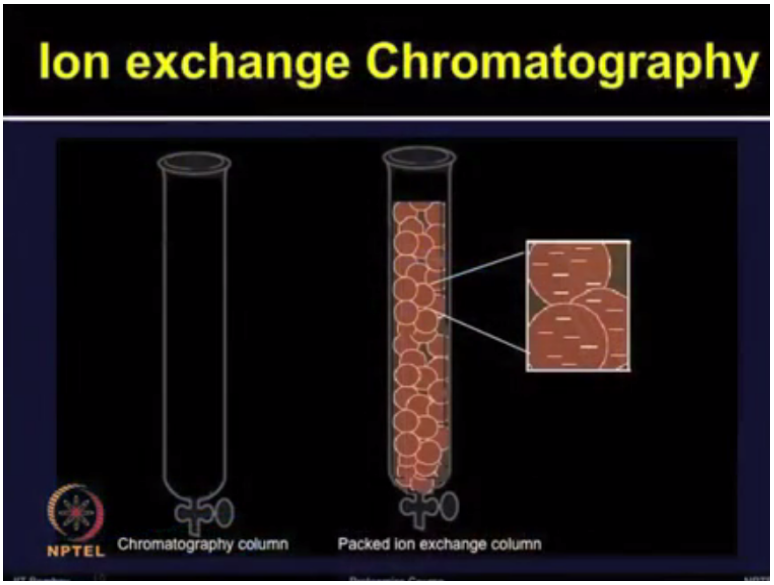


So the ion exchange chromatography the column is packed with the resin whether it is cation or anion exchanger depending upon the charge of the protein that needs to be bound to the column and purified.

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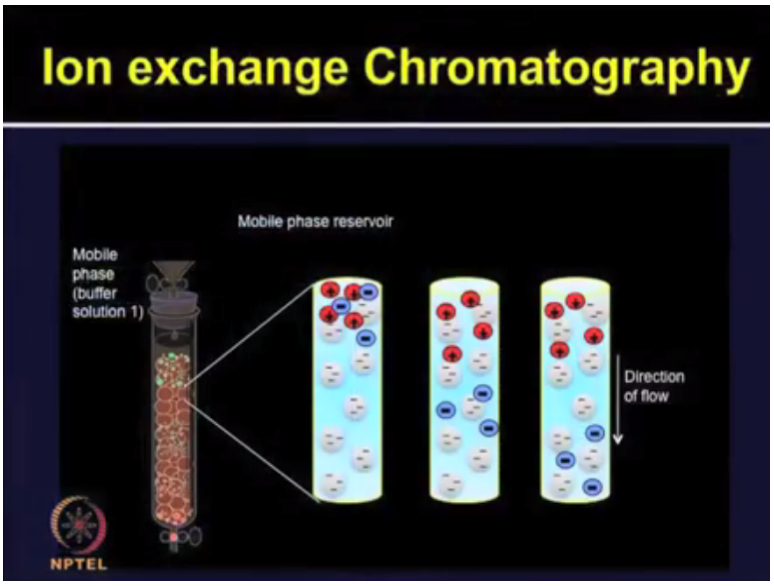


So proteins are adsorbed to the ion exchange column and then it can be desorbed by increasing the salt or altering the pH of the buffer which can change the charge on protein. So various anionic buffers such as acetate and phosphate are used for cation exchange and cationic buffers such as tris chloride or ethanol amine are used for the anion exchange.

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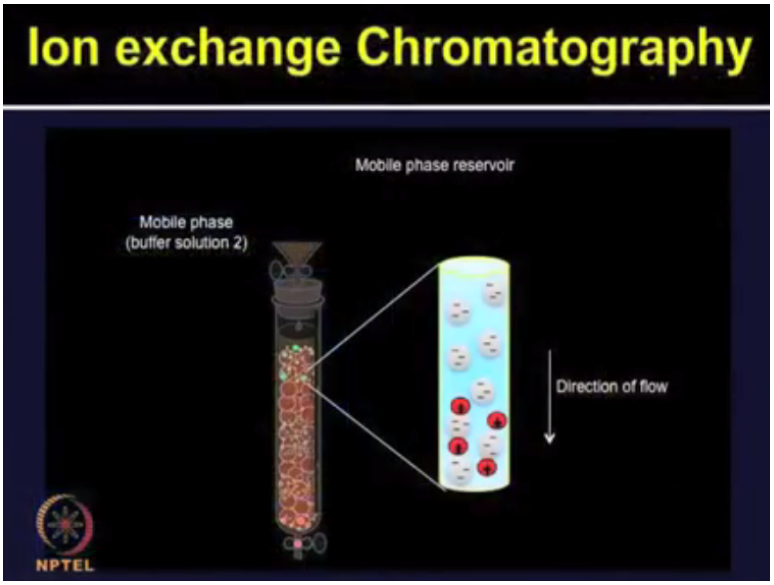


Now the buffer solution exchange so that the net pH of protein of interest can be modified at no longer bind to the ion exchange resin. Therefore, the bound protein can be eluted out as shown in this slide.

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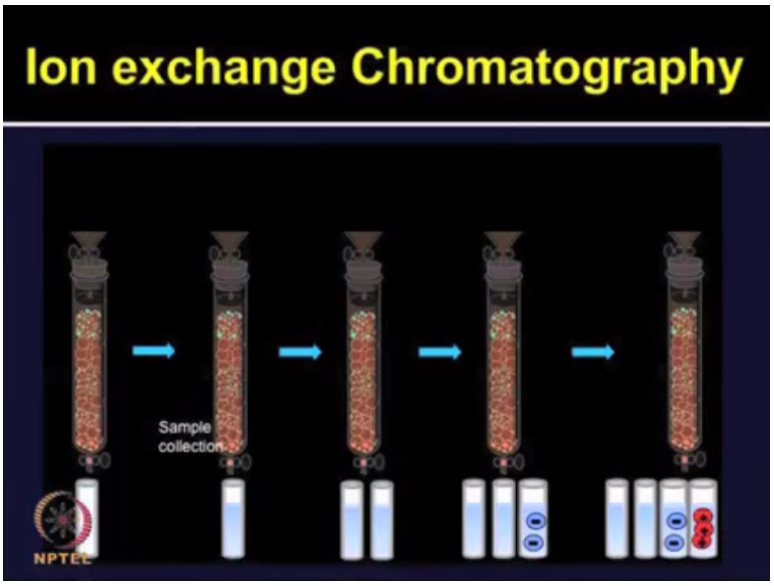


So if you have negatively charged protein which gets eluted first will be present in the initial fractions while the positively charged protein that bound to the column will be eluted in the later fraction or it will be vice versa.

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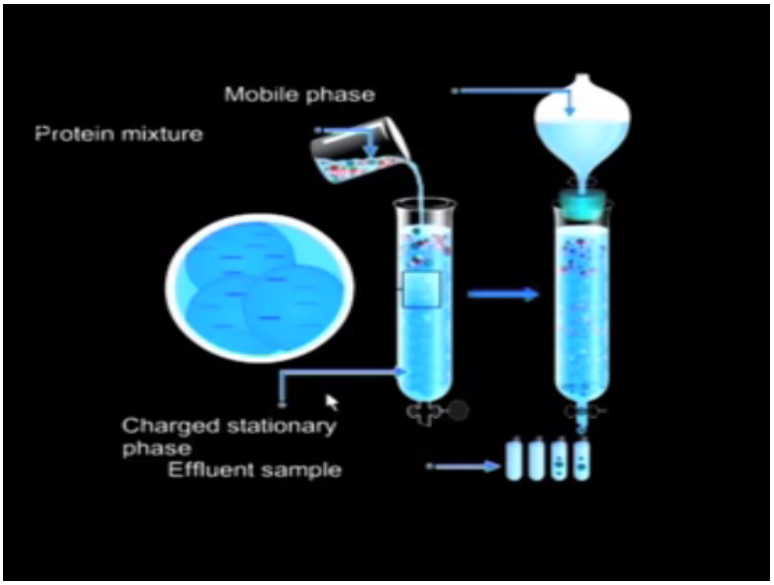


So let me describe how ion exchange chromatography works step by step in following animation. The charged stationary phase, the column stationary phase consists of a positively or negatively charged polymeric matrix which will bind molecules of the polar charge. Commonly used ion exchangers include negatively charged Carboxymethyl cellulose or CM cellulose which is a cation exchanger and positively charged DA cellulose which is an anion exchanger.

The protein mixture and purified protein mixture which consists of proteins of different net charges are loaded on to the column. The proteins having charges opposite to that of stationary matrix will bind to it while primary proteins will be eluted. Mobile phase: The proteins are eluted out of the columns by using suitable mobile phase and then samples are collected by different sample fractions. The solution leaving the column can be collected in suitably sized fractions for further analysis.

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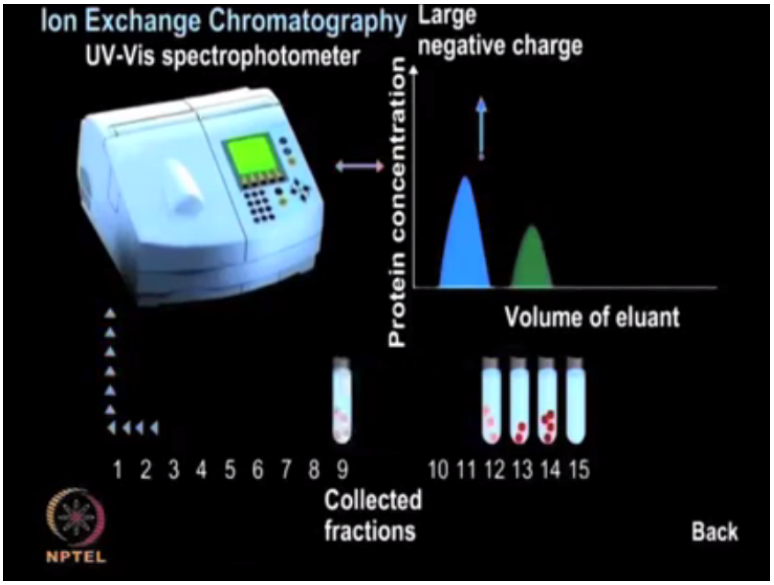


After giving you brief description of the components let me show you the process in animation. The column is packed with a suitable cation or anion exchange resin depending upon the charge of the protein that need to be bound to the column and purified. The anion exchange column is then loaded with the impure protein mixture consisting of various positively and negatively charged proteins.

The column is eluted with the buffer solution of suitable pH such that the negatively charged molecules are removed from the column while the positively charged molecules remain bound to the anion exchange resin. The buffer solution is then changed such that the net pH of the protein of interest is modified and no longer binds the ion exchange resin. Therefore, the bound protein also gets eluted out of the column in this manner.

The fraction of appropriate size must be collected and analyzed for their protein contents. The negatively charged proteins which get eluted first will be present in the initial fractions while the positively charged protein that bound to the column are eluted in later fractions. Once all the fractions are collected then the protein content can be analyzed by using a spectrophotometer.

Analyse the content these fractions for the protein content by using a UV visible spectrophotometer at 280 nanometres. A graph of eluant volume versus protein concentration can then be plotted. In this particular example the negatively charged large molecules coming first and then positively charged fractions are coming later.

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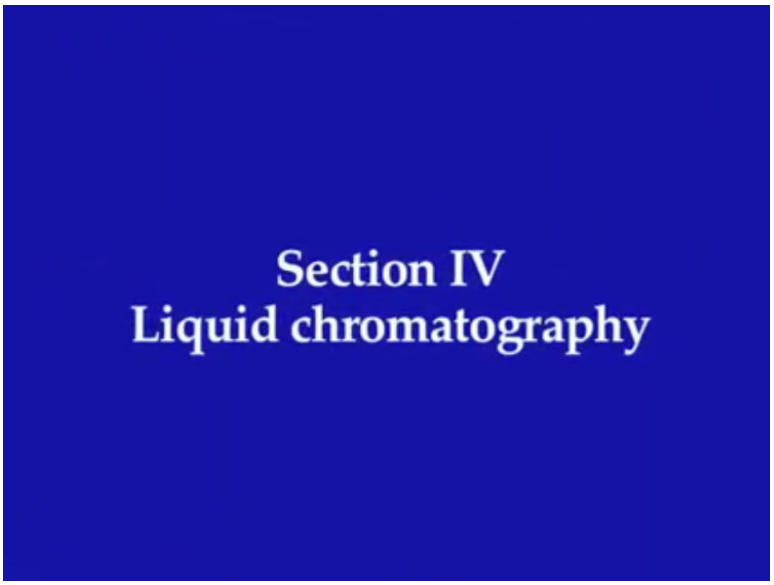


Let us now talk about High Performance Liquid Chromatography (HPLC). HPLC is a separation technique that separates molecules based on their differential adsorption and desorption between stationary phase matrix in column and mobile phase. Better separation and resolution of the components can be achieved by HPLC.

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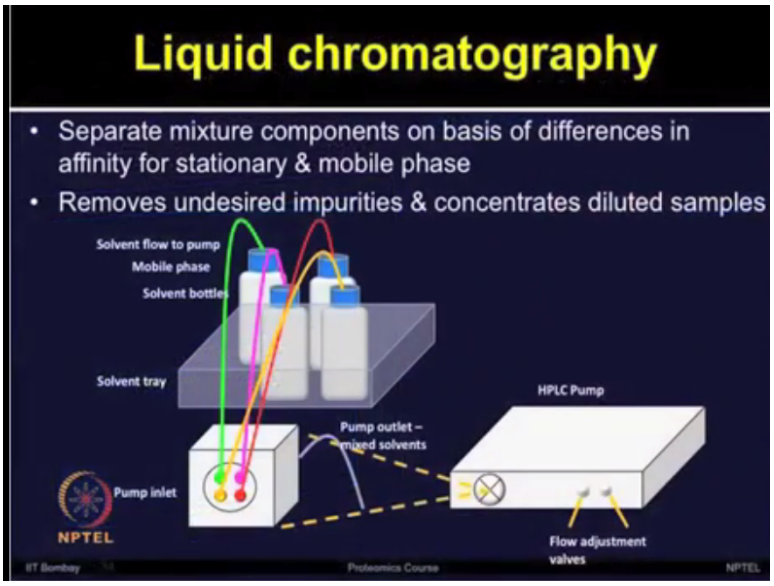


So what is liquid chromatography? Liquid chromatography separates mixture of components on the basis of differences in the affinity of stationary and mobile phase. It can remove the undesired impurities. Therefore, for various type of mass spectrometry based application it increases the (()) (19:30) and robustness for analysis of peptides and proteins.

It also concentrates the diluted samples and therefore increases the sensitivity detection of then low level proteins by using when you are applying the different type of proteomic technologies. It is mostly used for separating the peptide mixture. One of the diagram is shown here which is a typical liquid chromatography setup which consists of solvent bottles, degasifies, (()) (20:09) or ordinary pumps, the sample injected column and detectors. Different solvents can be placed in the solvent bottles depending upon the purification requirements.

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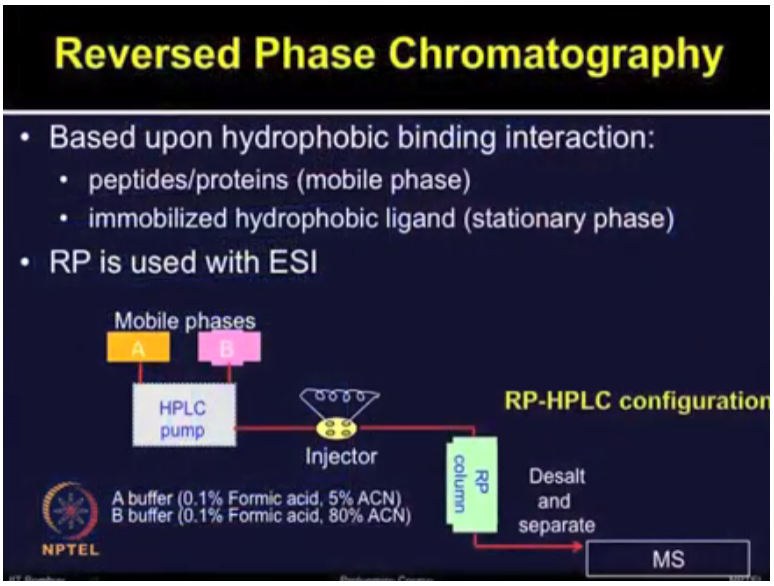


Let us first talk about Reversed Phase (RP) chromatography. The Reversed Phase Chromatography is based upon the hydrophobic binding interaction between the peptides or proteins in the mobile phase and the immobilized hydrophobic ligands in the stationary phase. In reverse phase, the stationary phase consists of the long aliphatic carbon chains which are highly hydrophobic in nature.

Molecules are bound on the column by means of hydrophobic interactions and are eluted out when the solvent polarity is modified. The Reversed Phase chromatography is mostly used with Electrospray Ionization in mass spec due to its compatibility of its acidic aqueous and polar mobile with ESI. It is also used for desalting the peptides before injecting for Electrospray Ionization.

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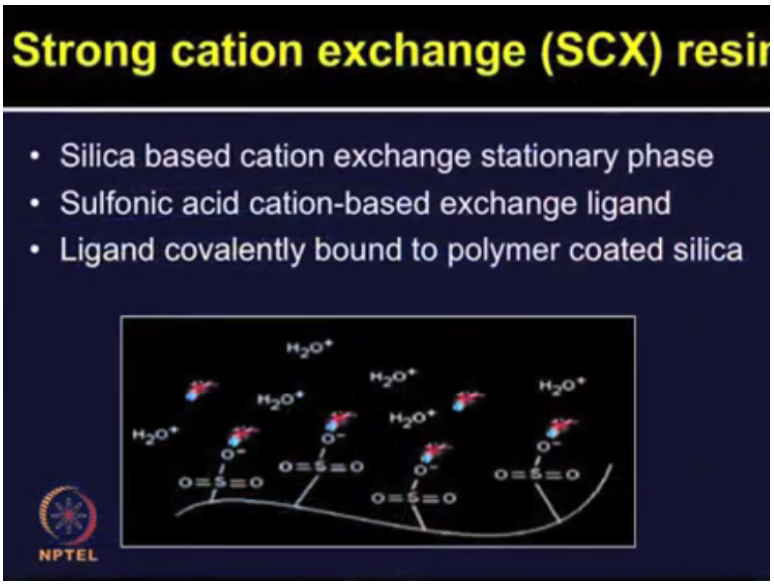


Let us now talk about Strong Cation Exchange or SCX chromatography.SCX consists of a stationary phase matrix which is made up of negatively charged sulfonic acid groups which bind the oppositely charged peptide molecules. These molecules can be eluted out using a positively charged mobile phase which binds the analyte molecules more firmly.

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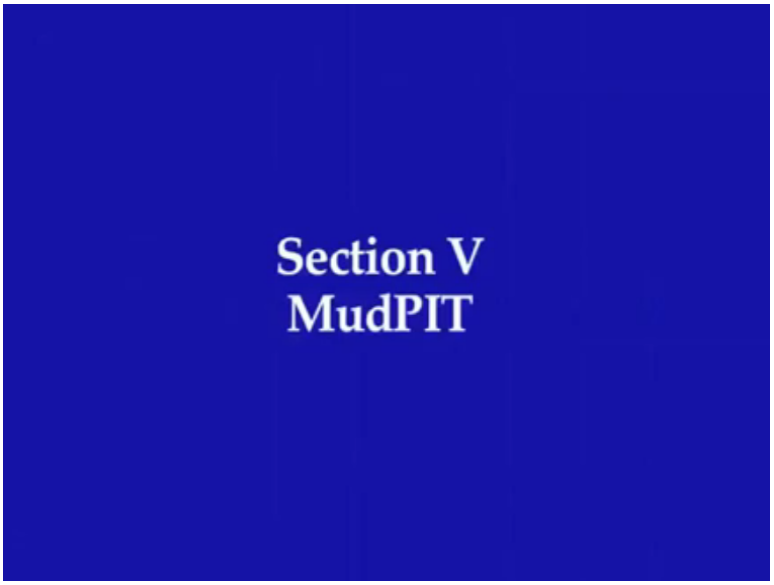


So what is MudPIT or Multi-Dimensional Protein Identification Technology? So MudPIT method combines cation exchange and Reversed Phase HPLC chromatography separation of cryptic peptides for the proteome based analysis.

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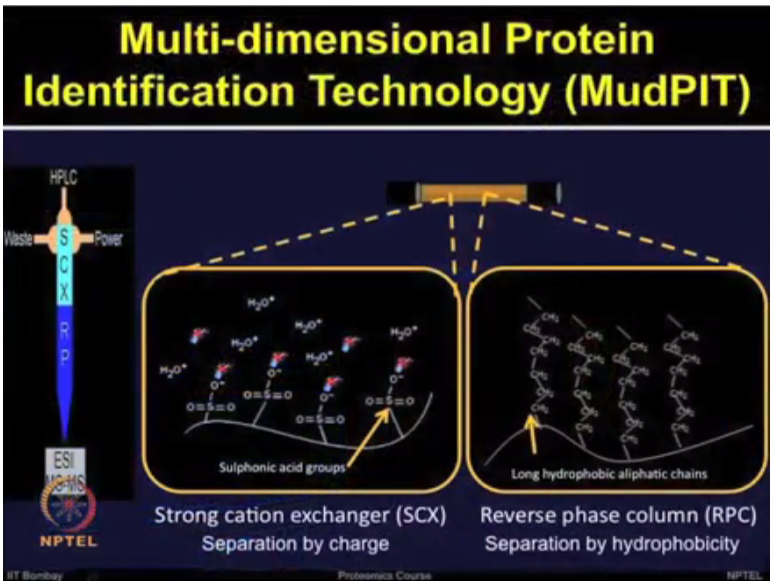


So we have already discussed 2 separate methods SCX and RP. Strong Cation Exchange is based on the attraction between the negatively sulphonic acid and positive peptide. And the elusion can be caused by addition of positively charged mobile phase. The reverse phase on the right side is based on the hydrophobic interactions between analyte and the stationary phase.

The elusion can be brought about by modifying the mobile phase polarity. The Strong Cation Exchange can be used offline and then each fraction can be analyzed by Reversed Phase HPLC followed by Mass Spectrometry. Alternatively, both Reversed Phase and Strong Cation Exchange resins can be paired into a single column and by introducing buffers in the series the multidimensional separation can be achieved.

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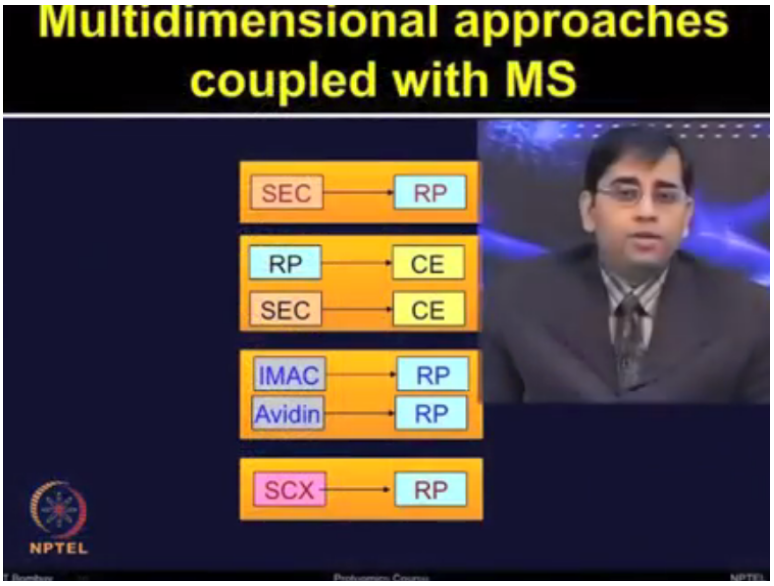


Now when application is to separate the complex proteome and analyze using Mass Spectrometry then one has to decide what type of chromatography method is applicable for their sample type. Now multidimensional approaches are usually coupled prior to the mass spec analysis and as we have discussed the certain chromatography methods which are commonly available and one has to make an educated trial of selecting what type of chromatography methods can be used for first dimension and what can be used for second dimension separation.

So we will sigh the exclusion chromatography has been used followed by the Reverse Phase. So to check the compatibility of first and second dimension based on the size in the SEC and the charge. Various types of combinations have been tried out. So let me first give you the abbreviations here and then on what property they separate proteins and peptides based on. And then I think we can talk about how these combinations can be applied.

So we have talked about Size Exclusion Chromatography which separates proteins based on the size or the molecular weight. Reversed Phase based on hydrophobicity. Then we have Capillary Electrophoresis which separate based on the charge. IMAC is affinity based interaction. Reversed Phase again hydrophobicity. Strong cation exchange based on the charge.

So these are different methods which we have discussed. Now what we are looking at, how the combination of these can be applied for proteomic investigations. So when SEC followed by

Reversed Phase has been tried the poor resolution of peptides in SEC occurs. The Reversed Phase followed by Capillary Electrophoresis or SEC followed by Capillary Electrophoresis is limited with the eluting capacity.

Now third combination of using affinity chromatography as the first separation followed by Reversed Phase has been used. But what is more popular is the Strong Cation Exchange separation in the first dimension followed by the Reversed Phase chromatography in the second dimension that has resulted into the best result for separating the peptides and it has been used for various proteomic analysis.

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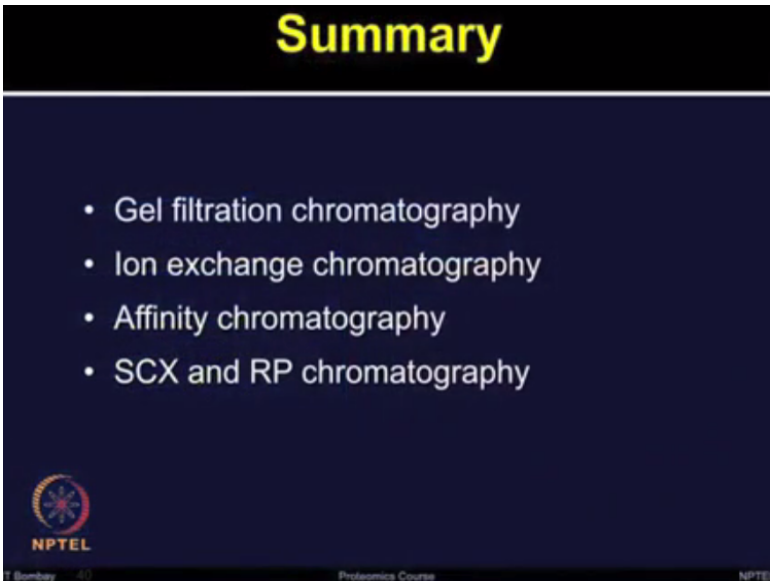


So in summary, in the last few years there has been an increased effort to develop the technologies which are capable of analysing protein expression at the proteomic scale. Some of the chromatography methods such as gel filtration, ion exchange and affinity chromatography, methods were discussed. The Strong Cation Exchange and Reversed Phase High Performance Liquid Chromatography are fundamental tools for the isolation and analysis of peptides.

The nanoliquid chromatography which makes use of C18 capillary columns has gained popularity for the proteomic studies due to their ability to achieve finite separation. So in summary we have talked about principle of different type of chromatography method commonly

being applied for the proteins and peptides and we looked at some of these chromatographic methods in more detail.