Downstream Processing
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Lecture - 29
Chromatography (continued)

We will continue with the topic of Chromatography. Chromatography is the most important downstream in bioprocess, because it can help one to separate unpurified proteins. It can be used for us separating and purifying the metabolite and very small molecules and peptize and so on actually.

So, chromatography is the most important and downstream and that is why, we are also going to spend several classes in the area of chromatography and as I talked about before there are different types of chromatography, and we having slowly spending more time on each one of them. Chromatography is like ion exchange, chromatography is a like hydrophobic interaction, then chromatography like a size exclusion or gel permeation.

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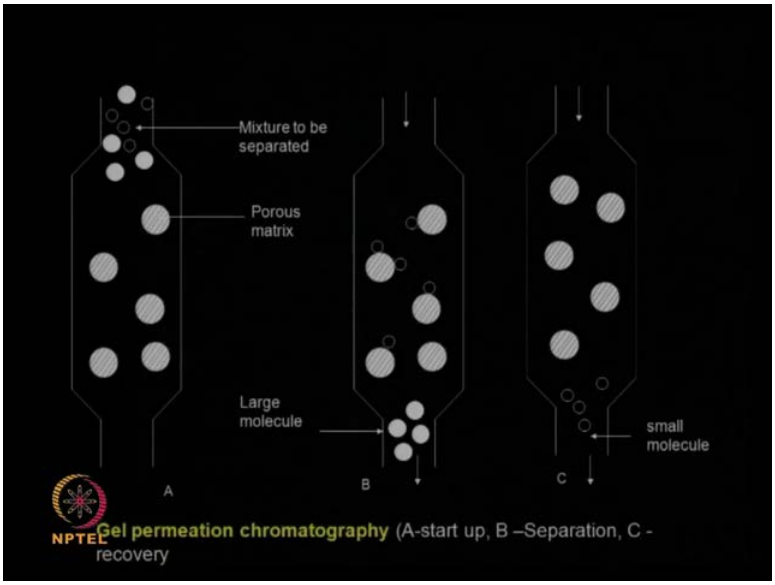

So, we shall spend some more time on this gel permeation chromatography is also called size exclusion chromatography, because it is based on size. It is almost like a filtration, because filtration is based on size, it separates small particles from large particles using a medium. Same thing happens with that chromatography of a gel permeation

chromatography as well. So, it separates small molecules, that means small proteins from large proteins and based on the size.

So, what do you have here in this particular set up is we have porous matrix and there are pores are ascend in the solid. So, small molecules like small metabolites or small proteins or small peptize enter this porous matrix, where as the large one that means, large size molecule or large molecule weight molecules, do not have any interaction with the porous matrix, so they start travelling faster. So, you will find large molecules or large molecular weight compounds coming out first, whereas a small ones or entrap inside this porous matrix, so they take much longer time to come out.

So, you separate routines based on their size, the big ones come out, first the small ones come out later. So, this is how it works, if you look at this picture you can see that mixture of the large on the small proteins are fed, the small proteins start interacting with the porous matrix. So, the large proteins come out first and then later on you collect the small proteins like the small molecules. So, this is the principle of a gel permeation chromatography.

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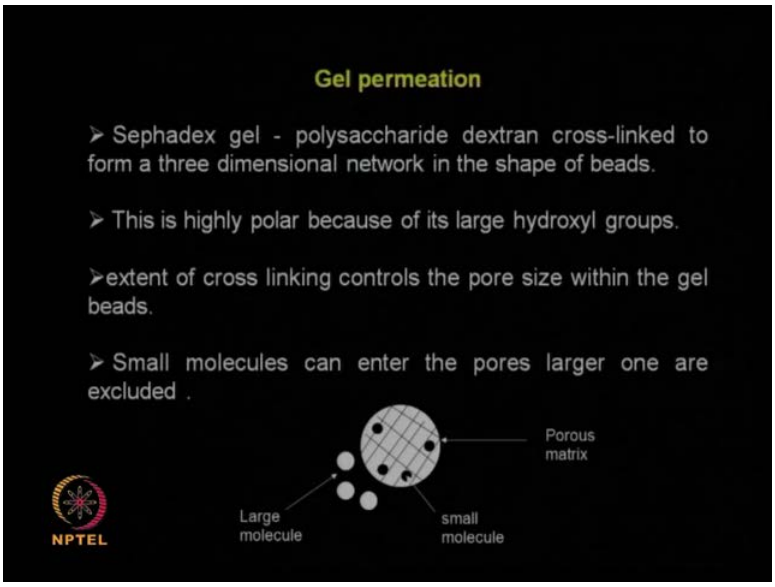

So, generally we use sephadex gel type of column, it is got a polysaccharide dextran cross-linked to form a three dimensional network in the shape of a bead. So, at they are always in a shape of a bead and it is got a three-dimensional network and lot of pores are there. And it is a highly polar, because you have several hydroxyl groups present inside

and the higher is the cross-linking the pore size becomes smaller, lower is the cross-linking the pore size is large. So, we can control the pore size by modifying the cross-linking.

So, when I control the pore size, I can use it for different types of a filtrations. So, basically what happens here, you have a small molecules entering inside the porous matrix and the large molecules do not find excess into the pore, so they the just travel down the tube.

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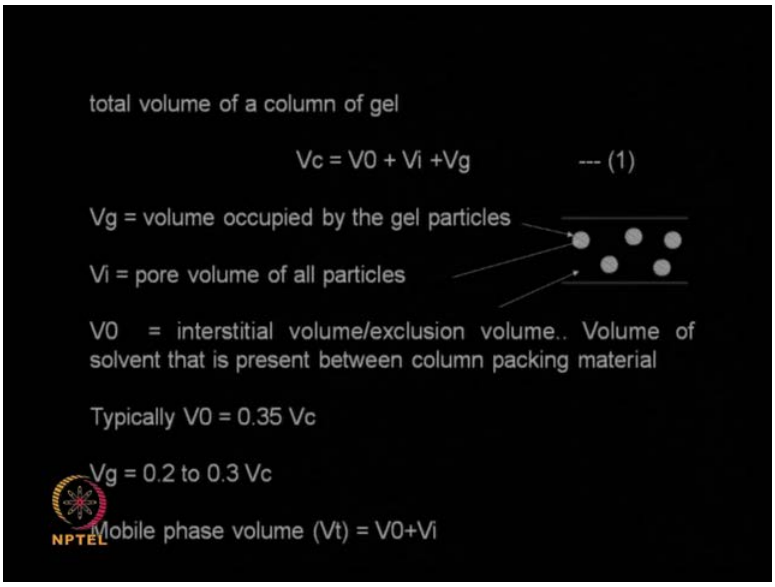

So, the total volume of a column of a gel, so if you look at the total volume, there are many parameters and one needs to consider. So, the total volume of a column of a gel is made up of 3 volume V naught V i and V g, V g is the volume occupied by the gel particles. So, the gels particles are actually, occupying certain volume, but you need to keep in mind when the solution is flowing in the gel get swollen. So, the volume occupied the gel when it is really dry visa-vise the volume occupied when it is inside the column when it is swollen will be very different.

So, V g is 1 term, next term is V I, that is the pore volume of all the particles, because as I said these sephadex beach or gel or highly porous. So, that will also have certain in the volume and then interstitial volume that means, the volume and we have which is not occupied by the gel particle, equal if external wide volume or exclusion volume. So, there are 3 terms here. The volume occupied by the interstitials between this beach the

pores ascend in the beach and the volume occupied by the gel particles, so all these 3 make up the total volume of the column of a gel.

Now, typically this V naught as I said that is the interstitial volume is about 33 35 percent of the total volume of the pack column that is the external porosity is the order of 30 33 35 percent. That is what it is actually, where as if you look at V g that is volume occupied by the gel particle that will about 20 percent 30 percent of this V c that is the total. So, the remaining is what is the pore volume actually.

Now, if you look at the volume occupy by the mobile phase, mobile phase will be occupying all these empty spaces, that is V naught plus mobile phase will also be present inside the pores, that is the V i. So, the total volume of the mobile phase inside the column will be V naught plus V I, V g is the actual volume of occupied by the gel particle. So, 1 needs to have some idea about, what is the swelling capacity of the gel, what is the volume of the pore and what is the packing density. So, all these parameters will affect my total volume of the column.

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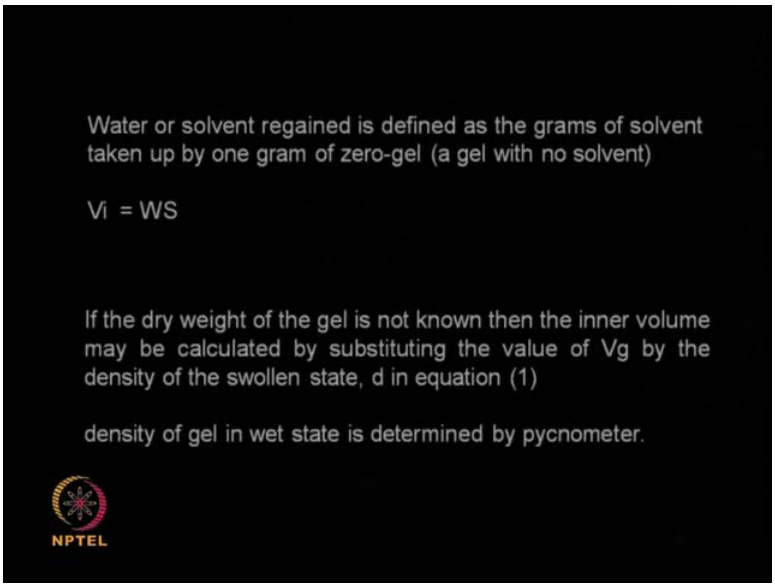

So, as I said the gel when it is dry will have certain volume and when it is swollen and in because of water or solvent coming in it will have another volume. So, that swelling part comes into the picture actually. So, how do you measure that, you can do it experimentally in the lab, you can take the gel particles, you can dry it thoroughly in an oven and see what is the volume occupied by that.

So, that is one way of looking that the gel volume actually. So, the water or solvent regained is defined as the grams of solvent taken up by 1 gram of 0-gel, 0-gel means a gel does not have any solvent that means, the it is very, very dry. So, the dry weight of the gel is not known then the inner volume may be calculated by substituting the value of V g.

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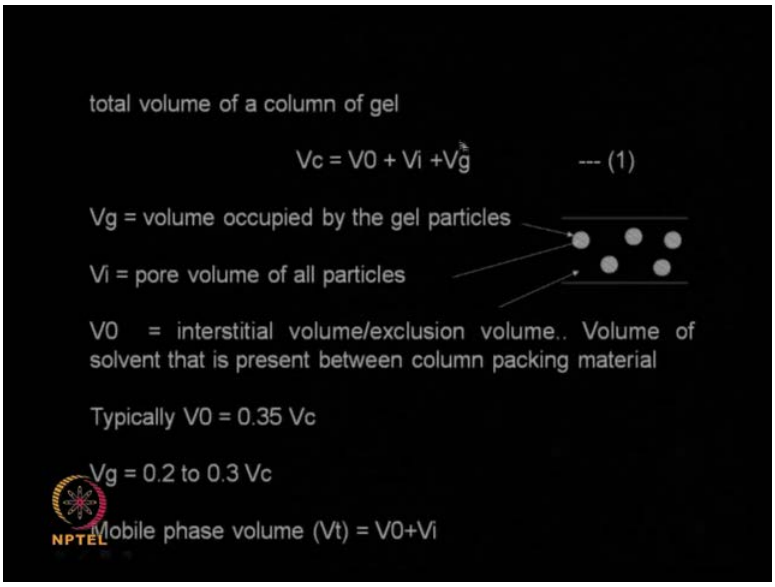

That is V g is the volume occupied by the gel particles.

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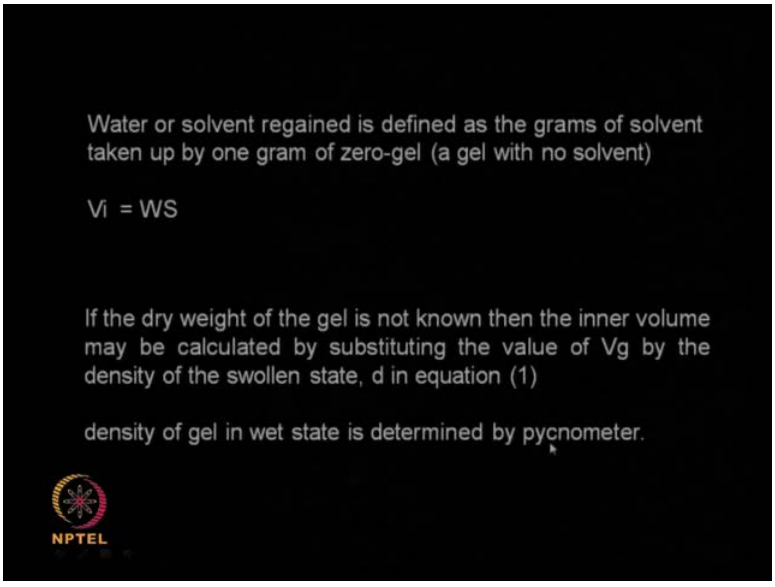

When it is completely swollen by the density of the swollen state and the density of the gel in wet state is determined by an instrument called pycnometer. So, 1 can find out what is the density, when it is in the swollen state and we can find out the volume occupied by the dry particles by completely drying these gels in an oven and then looking at the displacement of water or some other solvent, which does not allow the gel to expand. So, that will give us an idea about the volume occupied by the gel in a dry condition.

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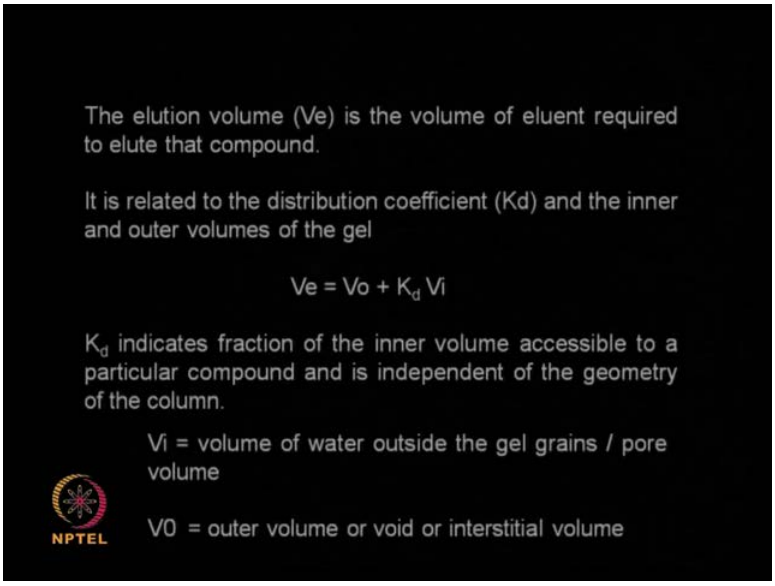

There is something called elution volume, that is the volume of the eluent required to elute a compound. So, what do we do in a gel permeation chromatography, we inject our solute in the feed at time equal to 0 and then we are adding an eluent. So, the solute travels through the column and finally, it comes out. So, the elution volume is the volume of this eluent required to move the solute from the inlet addition injection point right to the outlet.

So, now these depends upon, so many factors, it depends upon the interaction between the solute and the gel particle, it depends on the volume of the interstitials, it depends on the volume of the pores and so on actually. In fact, that is what this particular relationship is going to give you. So, the elution volume V is the function of distribution coefficient, distribution coefficient is like partition coefficient or the interaction between and solute absorbed vis-à-vis the solute presents in the liquid phase.

So, the elution volume V e is given by V naught plus K d into V i, V naught is the void volume I as I said or interstitial volume that is the excluded volume, now V i is a volume of water outside, the gel grains that is the pore volume. So, you have solvent present in the interstitials, solvent present inside the pore right. Now those are the 2 terms coming in V naught and V i.

Now, there is a term called K d, K d is something like a distribution coefficient, that means distribution between the inner volume and the outer volume. K d indicates fraction of the inner volume accessible to a particular compound. So, K d depends upon that geometry of the column, it depends upon the geometry of the packing and so, on actually. So, K d can be large, K d can be small, K d can be 0, K d can be 1 and so, on actually, depending upon that my left hand side varies that is the elution volume.

So, this equation tells you what is the volume of eluent required to elute out a solute from the inlet to the outlet. Now, this is the function of the 2 volume V naught, that is the outer volume or the void volume and V I, which is the pore volume and there is another term called K d, which is the distribution coefficient. Now K d depends upon the geometry of the column geometry of the packing and so, on actually. So, for different values of K d the elution volume can change. So, K d can be 0 or K d can be 1 or K d can be in between 0 and 1 and so, on actually.

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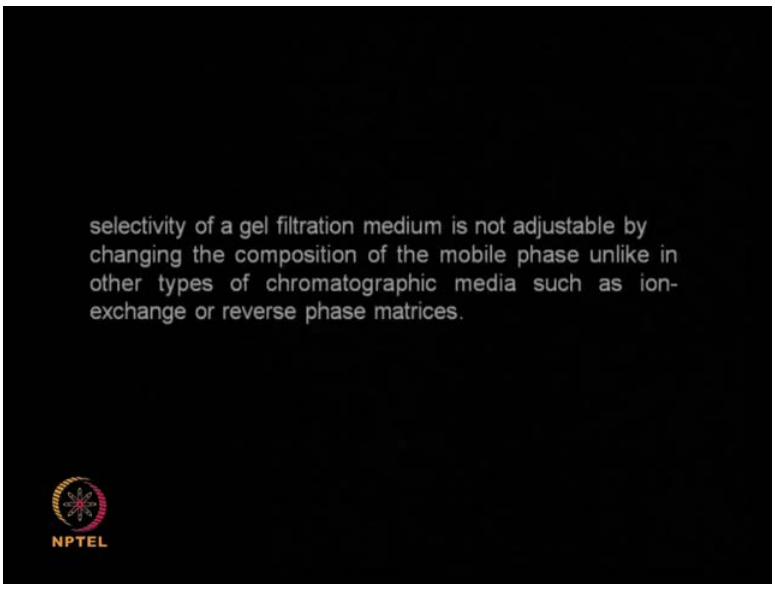

Now, selectivity of a gel filtration medium cannot be adjusted by changing the composition of the mobile phase. So, in a gel filtration unlike your ion-exchange or reverse phase type of chromatography or we cannot change the selectivity pattern by changing the medium, because gel filtration depends upon the size of the solute, it does not depend upon any other parameter just the size.

So, by changing the composition of the medium, we cannot change the selectivity pattern, if you want to change the selectivity pattern, we have to change the beads. We have to change the porosity of the beads, we have to change the pore volume of the beads, that is only way where as, if you take a other chromatography like a ion-exchange or reverse phase chromatography, I can change the composition.

For example, if we take a ion-exchange chromatography, I can change the ionic strength of the medium. Thereby I can either enhance the interaction of the solute molecule with the stationary phase or I can reduce the interaction. Same thing with reverse phase and I can change the hydrophobicity of the solvent carrier medium and by changing that I can enhance the interaction between the solute and the stationary phase or I can reduce the interaction.

Where as in gel filtration we cannot do that, because gel filtration is based only on the sizes, it is almost like your normal filter medium. So, gel filtration based on only on the size. So, the interaction happens between the solute and the stationary phase gel. So, the stationary phase gel means, the size of the gel, the porosity of the gel, the pore size of the gel. So, that is the very very important difference between a gel filtration or a gel permeation or size exclusion chromatography vis-à-vis, other type of chromatography like ion-exchange or reverse phase chromatography.

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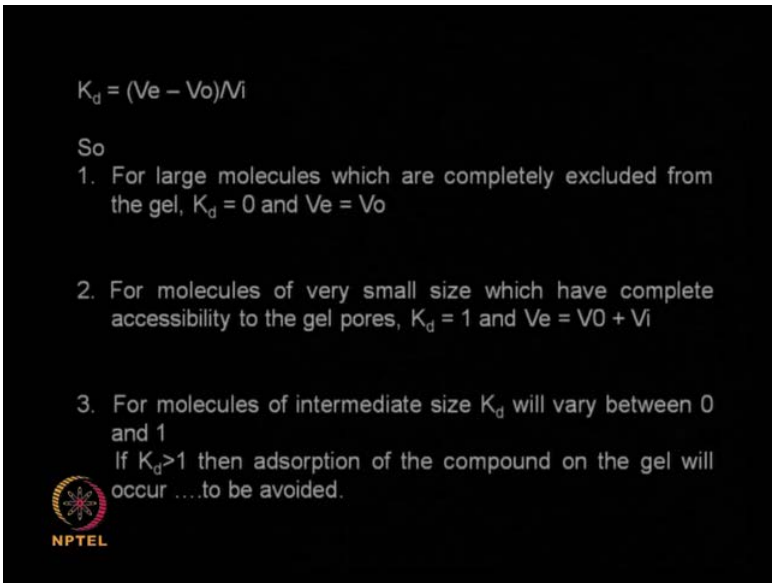

Let us again go back to this particular equation, where we said elution volume is a function of a the pore volume and the interstitials volume and the term called K d, that is a distribution coefficient. Now if we rearrange the equation, we get K d equal to V e minus V naught by V i. Now there could be 3 different situations, 1 can consider if the molecules are very, very large; that means, a molecules cannot enter the pores and it completely excluded from the gel. So, we can consider K d is equal to 0. So, that is the situation, where molecules are very large.

So, if K d equals to 0 then V e equal to V naught that means, the eluent volume will be exactly the equal to the interstitials phase that is all. Because you are as solute does not interact with your solid phase material that is solid gel. Now if you have very very small molecule then k d is equal to 1, in that case we have V e equal to V naught plus V I, now V naught nothing but the interstitials phase and V i is your pore. So, this is the total volume occupied by the continuous phase solvent, but generally we may have K d varying between 0 and 1. So, K d can varying between 0 and 1 in that situation, your eluent volume will vary between V naught and V naught plus V i.

So, if K d is very very large is greater than 1 then what will happen then absorption of the compound and the gel will occur. The compound will get absorb then it will not get desorbed this gel filtration, it just doing a filtration job, it is not doing a absorption process 1 needs to keep that in mind. So, gel filtration is only acting as a filtration

equipment, it is not acting as an absorbent. So, we do not want K d very large greater than 1 and if it is. So, then the solute get absorbed on the gel then it is almost like a absorption column rather than a gel filtration column.

So, generally we will have a these three situations K d equal to 0 that means, for very very large molecules. The molecules are completely excluded from the gel K d equal to 1 that is very very small molecules. So, they are accessible completely accessible to the gel pores then we have K d between 0 and 1. So, that is the general case. So, in each case, we will have the volume of the eluent required and in terms of V naught, that is the interstitials or the void volume and in terms of V i that is the pore volume that means, the volume occupied in the pores.

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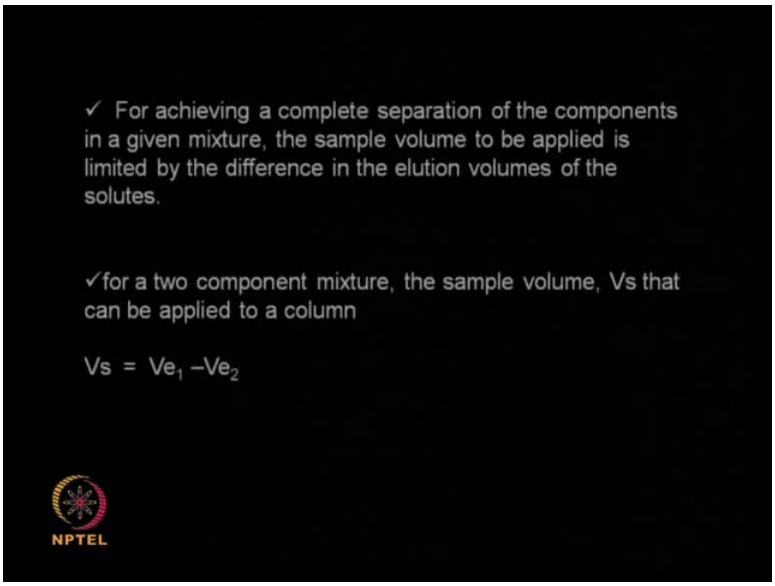

So, for achieving a complete separation of the components in a given mixture, the sample volume to be applied is limited by the difference in the elution volumes of the solutes. So, you need to keep that in mind, now suppose I have a 2 component of mixture. So, I have a mixture containing 2 components 1 and component 2. So, I should know, what is the elution volume for component 1, I should know what is the elution volume for component 2 and that difference is called the V s and that is what we should apply for achieving a separation.

So, V s that is the sample volume require will be the difference in the elution of the solute 1 and 2. So, if the elution volume for solute 1 and elution volume for solute 2 or

almost equal then your V s will become very, very small, what does that means, we are not able to achieve a good separation, V s becomes very, very small, if the elution volume for component 1 and elution volume for component 2 are very rashly different then this difference also increases. So, V s also becomes very, very large.

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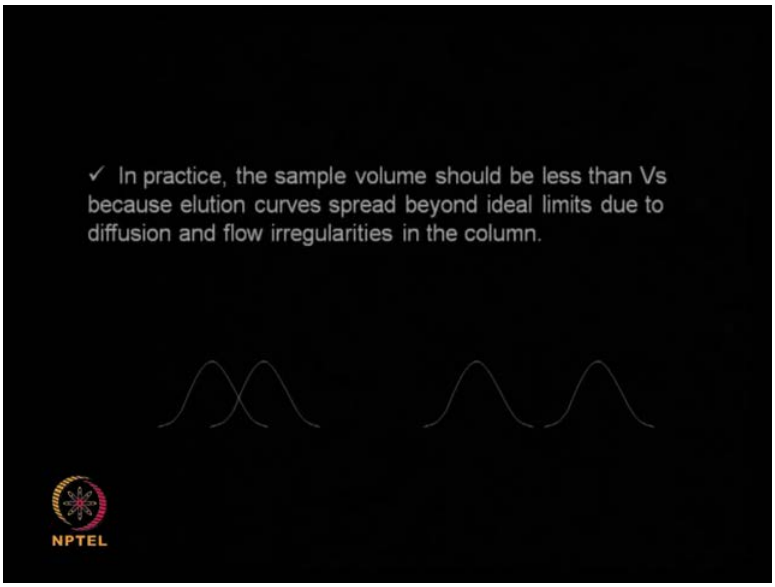

So, but then we need talking consider point like a flow irregularities, the packing has not of the gel is not homogeneous, it is lot of heterogeneity there, it is going to be diffusion limitation and so, on. So, instead of achieving a good separation like this, we may end up with the separation like this. So, here you can see there is a overlap in these 2 places and you are going to have a impurity of a component 1 and component 2 happening here, where as if you look here, they are very, very good separation here.

So, we need to consider what is elution volume for component 1, we need to consider what is the elution volume for component 2 and if they are vastly different then we can achieve good separation. But then here need to consider the other heterogeneity of our column packing, heterogeneities of the flow irregularities diffusions and so on actually. So, all these factors make your system non-ideal and you may end up with the overlap of these 2 components and where as you would like to achieve like this.

So, imagine if you have mixtures of a several component, each 1 having different elution volume, you may end up having lot of overlaps of several of this piece. So, we need to

do quite a lot of trial and error optimization. So, that you get good separations of a the various components in your g p c or gel permeation phase chromatography.

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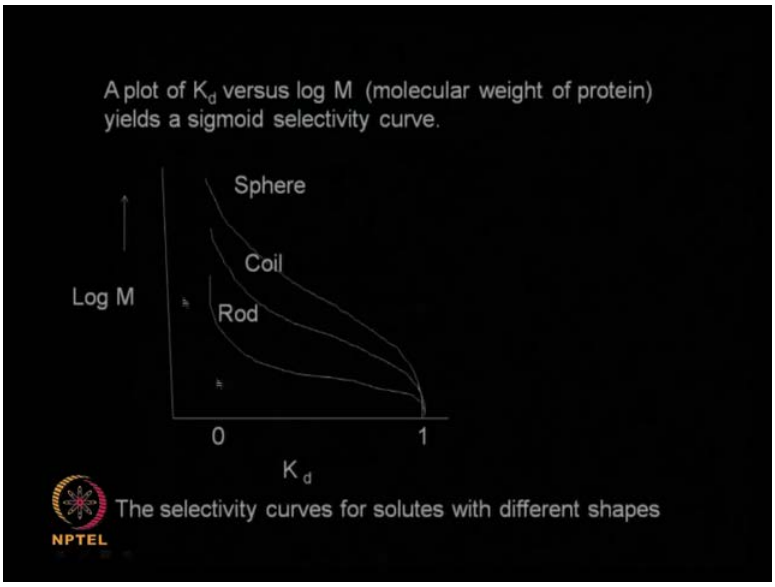

Now, I mention that K d is a function of a the size on the shape of the gel and also the type of a solute and a type of a that tube, which we use and so, on. So, this particular plot tells, you how does the molecular weight of the protein and behaves as a function of K d for different shapes.

So, if you have a spherical shaped material. So, logarithm of molecular weight and K d will appear like this, where as if your protein is like a coil then it will appear like this. If it is like a rod that means, 1 of the dimension is much smaller, where as other dimension is very, very large then you are going to have a relationship of this form. So, the logarithm of M versus K d will appear like this.

So, generally if the proteins or spherical in shape, you are going to have a lidar range in which you can operate, where the logarithm of molecule weight and K d could be linearly varying in this range and a if it is a spherical coil then rod the as a relationship for the type of a graph changes quite dramatically. So, generally we can take the middle range, which looks quite linear. So, this range you can develop a relationship between log M and K d of this form.

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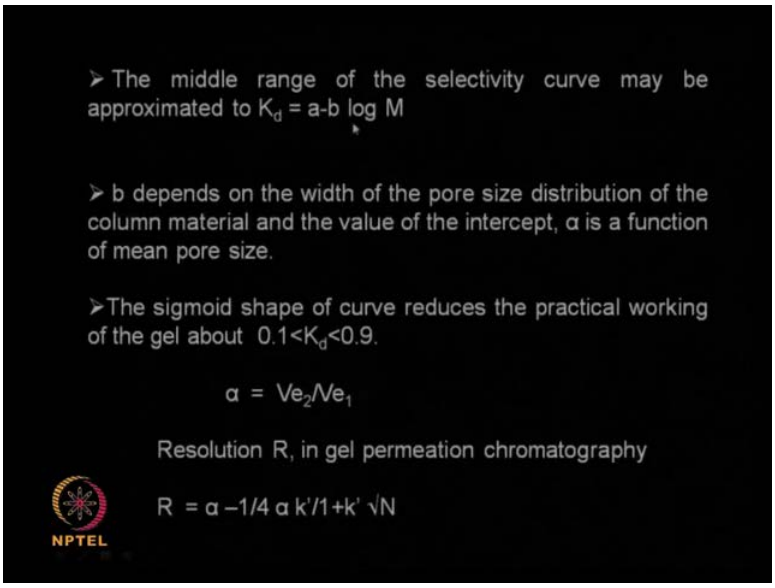

K d equal to a minus b log m. So, this is a linear relationship and we can calculate a and b, if you have certain standard draft. Now, b depends on the width of the pore size distribution of the column material and the value of the intercept and alpha is a function of mean pore size. So, b depends on the width of the pore size distribution of the column material and the value of the intercept alpha is a function of mean pore size, so because of the shape of this curve.

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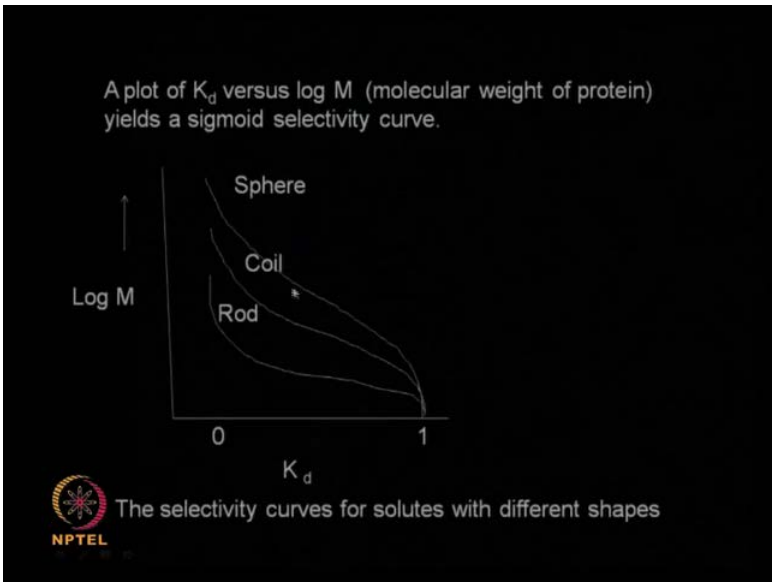

Generally, we like to operate only within this region, because within this region you have a linearity. So, this becomes almost 0.1 to 0.9.

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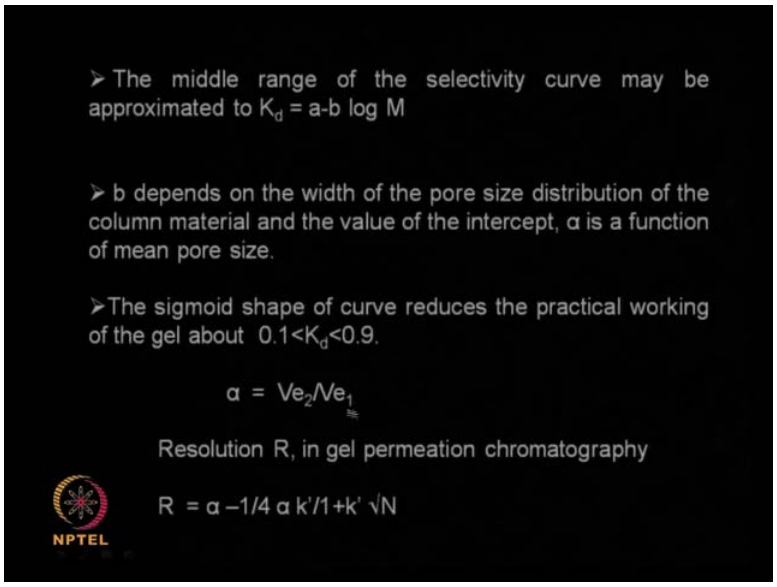

This is the range at which we a work, Now here alpha is equal to V e 2 by V e 1. Now there is some parameter called resolution that is resolution, if you have 2 components, the resolution is the separation of these 2 components. Now this resolution is given by this particular relationship alpha 1 by 4 alpha K dash plus K dash by root of N.

So, resolution will be doubled if the number of theoretical plates, N is increased by 4 times. Because you have a square root of N, which is N, where N is the number of theoretical plates, by if you keep alpha and K dash constant. It is obvious from this relationship, we can tell if I keep alpha and K dash constant will be able to increase.

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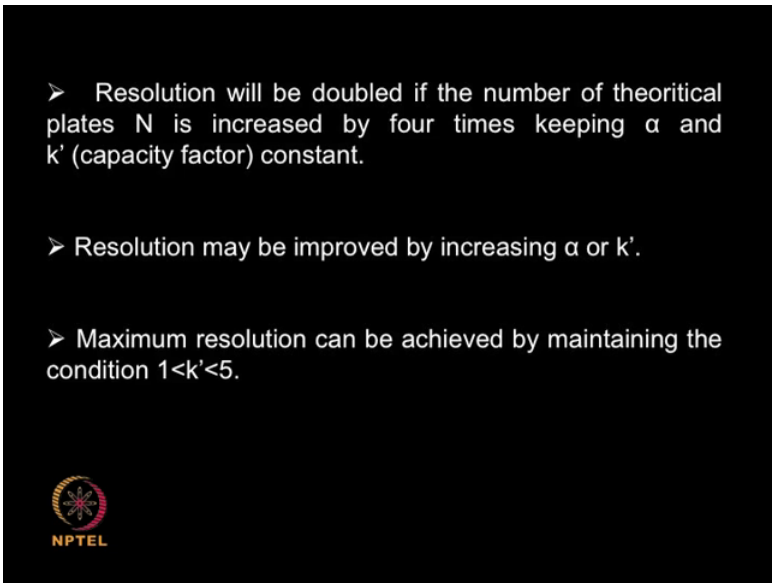

K dash is nothing but the capacity factor. So, resolution may be improved by increasing alpha or K dash. So, we can achieve maximum resolution by keeping K dash between 1 and 5. So, these this particular equation tells you, how I can improve the resolution either by increasing my theoretical plates or I can increasing the capacity factor or I can modify the ratio of the elution volume of a 1 and 2, that is component 1 and component 2 actually.

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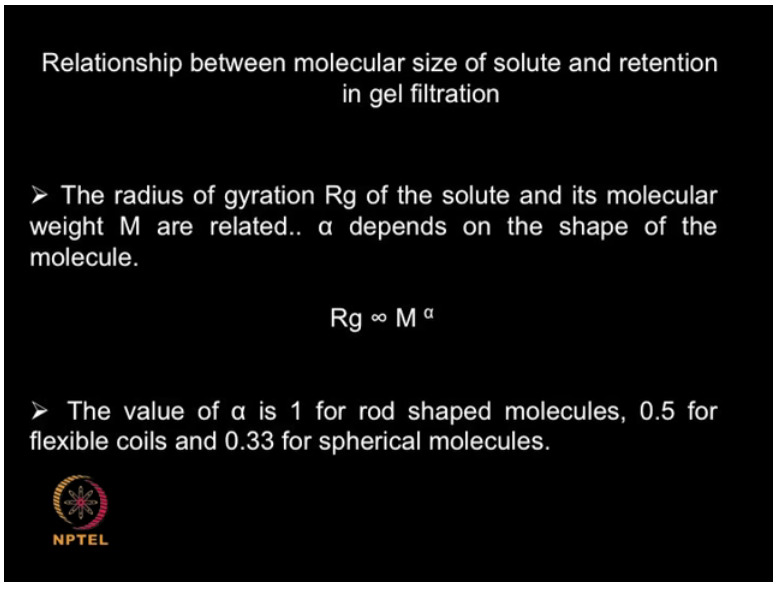

Now, we need to know a relationship between the size of the molecule or the size of the protein, which you are a elutic out with the molecular weight. So, generally it is in this particular form R g that is radius direction of the solute and M is the molecular weight and alpha is the exponent term and that depends on the shape of the molecule. So, if alpha is equal to 1 and for rod shape molecules, it is a 0.5 for flexible coil and 0.33 for spherical molecules.

So, we can see that although the size that is the radius of direction increases with the increasing molecular weight, it is not all the time a linear increase, but it depends upon the shape, if the shape of the molecule is like a rod then alpha will be 1, that means R g equal to M. If it is like a flexible coil alpha is equal to 0.5, that means R g is equal to proportional to square root of M, if it is like a spherical shape then R g will be proportional to M raised to the power 1 by 3. So, depending upon the shape of the molecule the radius of gyration changes as a function of molecular weight M.

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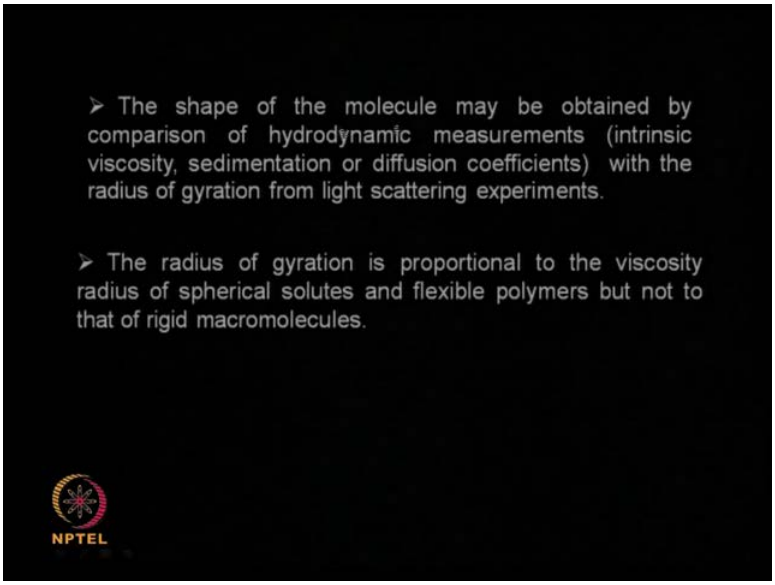

So, the shape of the molecule may be obtained by comparison of hydrodynamic measurements based on intrinsic viscosity or we can do sedimentation studies or we can do diffusion coefficients and then try to get the radius of gyration from light scattering experiments. So, the radius of gyration is proportional to viscosity radius of spherical solutes and flexible polymers, but not to that of rigid macromolecules. So, if you have

viscosity radius of spherical solutes of spherical polymers, then that will be proportional to the radius of gyration, but if it is rigid macromolecule, we cannot use that term.

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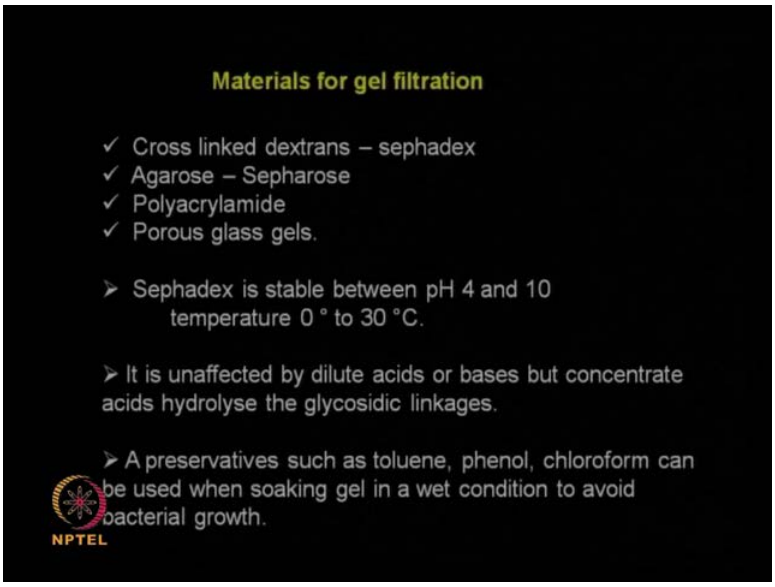

So, what are the materials used in gel filtration, we can use a cross-linked dextrans sephadex as I mentioned before, it is a highly hydrophilic then we can use agarose sepharose. We can use polyacrylamide type of gel, we can even use porous glass gel, now each 1 of them have advantages and disadvantages depending upon the type of a system, which we are studying depending upon your operating conditions, we change the gel material.

Sephadex is stable between p h 4 and 10 and also between 0 to 30 degrees that is why most of the applications we use sephadex, it is unaffected by dilute acids or bases. But of course, concentrated acids will hydrolyse the glycosidic linkages, sometimes tolune or phenol or chloroform is also used while soaking, because you want to avoid bacterial growth in net condition. So, little bit of this preservative will help in reducing the bacterial growth.

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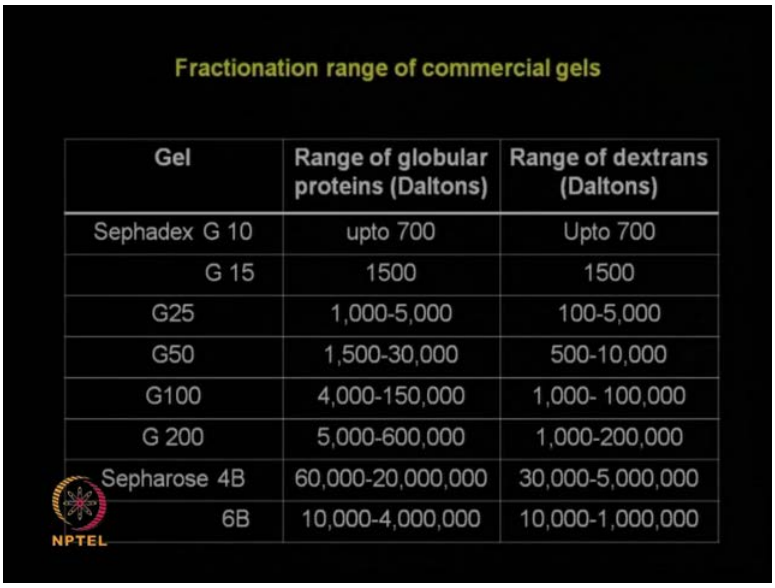

So, different types of material and each 1 of them have certain range for separations based on that molecular weight. So, if you take sephadex G 10 up to 700 dalton globular proteins, we can use it and if it is dextrans can go up to 700 Dalton.

If you take another type of sephadex that is called G 15, we can go up to 15 100 dalton globular proteins go to 25, we can work between 1000 and 5000. If we take a G 50, it can work for a 15 100 to 30000 dalton protein, if you go to G 100, we can work for 4000 to 100 15000 dalton protein. If you take G 200, it can work in the range of 5000 to 6 100 1000 dalton proteins.

If you take sepharose then it can go up to very, very high molecular weights, it can go even up to 20 million daltons and if you take sepharose 6 B, it can go up to 4 million dalton. So, you see by selecting different type of a sephadex column, we can change the operating range as against the molecular weight. So, depending upon the type of system, which you are trying to study the molecular weight, we can select different types of column.

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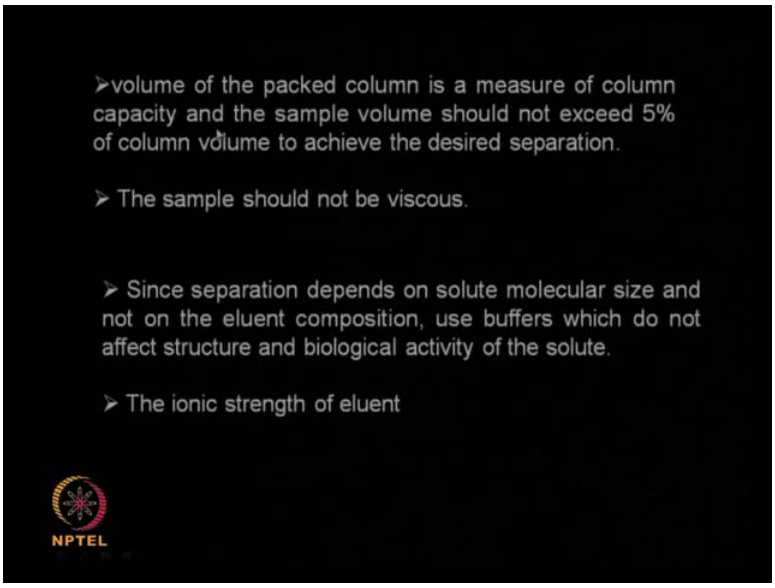

So, the volume of the pack column is the measure of column capacity and the generally the sample volume should not exceed 5 percent of this volume, this column volume. So, if I add too much of sample volume, I am going to have a complete saturation of the packing, I may have even diffusion actually. The sample should not be viscous, because if it is going to be highly viscous material the your continuous phase will not be able to transport at this sample.

Separation depends on solute molecular size and not 1 the eluent composition, you can use buffers, which do not affect structure and biological activity of the solute that is very very important. So, if I have to use buffers, I should see that the buffer does not affect the structure and the activity of the solute.

Because the whole concept of size exclusion chromatography is based on shape and size, we do not want to disturb the shape and size by adding buffer, which affects that the ionic strength of the eluent also very, very important. Because that ion ionic salts present should not again affect my the protein present inside the separation system.

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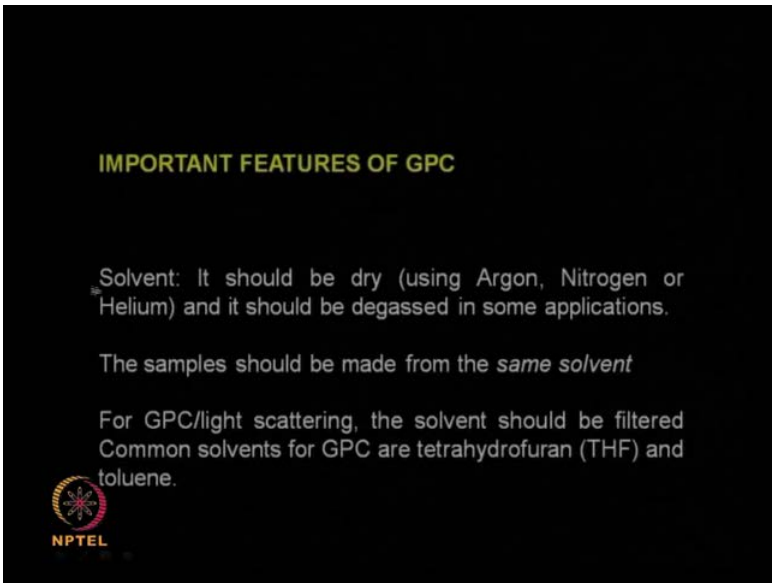

So, what are the other important feature of G P C solvent, solvent is the most important component in my G P C should be dry. So, we can use argon nitrogen or helium and again it should be degassed in some application. Sample and continuous phase solvent should be made from the same material actually, we use lights scattering type of detector the solvent should be filtered common solvent, which we use tetrahydrofuran, that is T H F for toluene.

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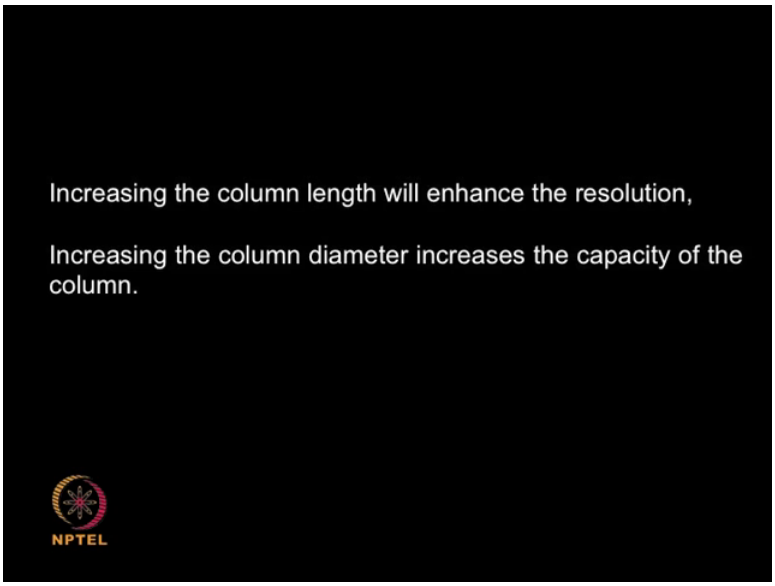

This increasing the column length will enhance the resolution, increasing the column diameter will increase the capacity of the column. So, if I want increase capacity, I will increase the column diameter, if you want to increase the column resolution, I will increase the column length. But then if you have 2 long a column, there is going to be diffusion taking place and there is going to be spreading taking place. So, there will the peak may be separating, but the entire chromatograph will be very disperse and there will be overlap.

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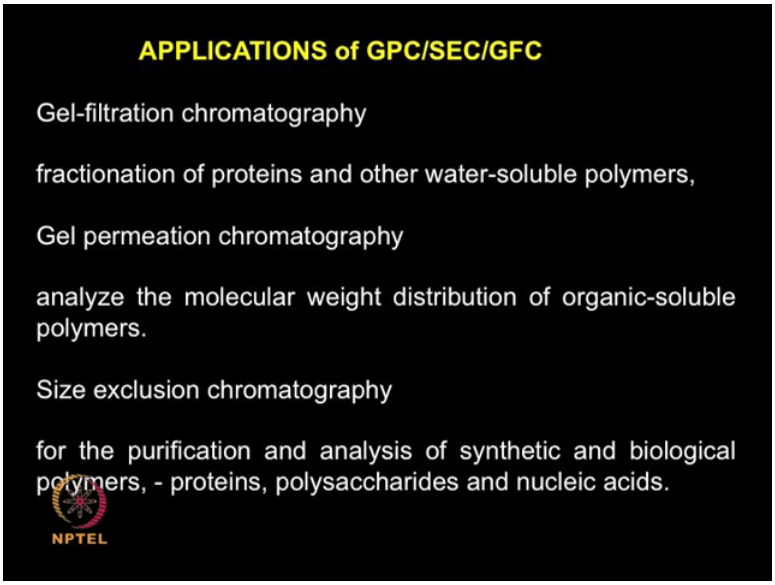

So, where do you apply this gel permeation chromatography or gel filtration chromatography or size exclusion chromatography is got several application fractionations of proteins. And other water solvable polymer gel permeation chromatography analysis of molecular weight distribution of organic solvable polymers. So, I can study synthetic polymer, I can look at the molecular weight polycarbonate, polyurethane size exclusion chromatography, I can use it for purification and analysis of synthetic and biological polymers proteins polysaccharides nucleic acids and so, on.

So, you see I can use it for fractionating protein and other water solvable polymer, I can use it for a analyzing a molecular weight of a synthetic polymer, I can use it for purification of a synthetic and biological polymer protein polysaccharide that is why it is extremely popular. These G P C or S E C or G F C in and if finds application everywhere in protein purification protein chemistry in normal organic chemistry labs in polymer

chemistry lab, in downstream processing lab and biochemical engineering lab and so, on actually.

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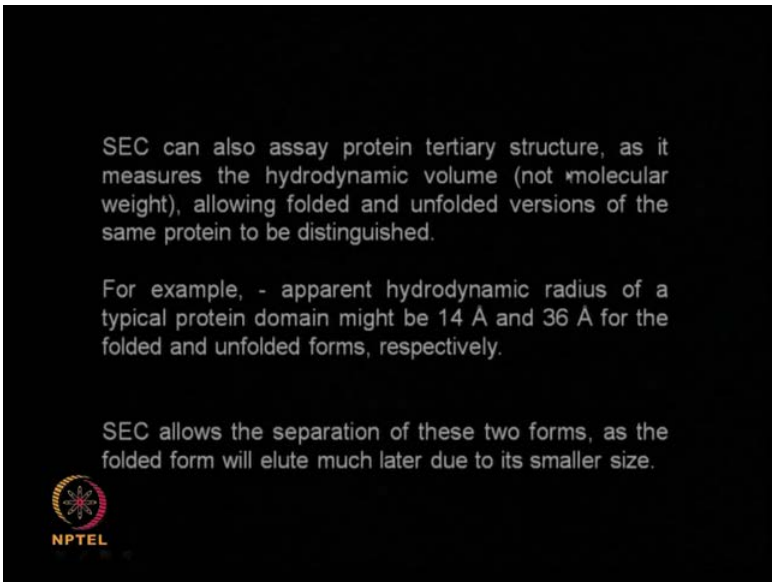

So, size exclusion chromatography can also assay protein tertiary structure, because it measures the hydrodynamic value. So, we can differentiate between folded and unfolded versions of the same protein. So, your folded protein, unfolded protein both will have different volumes, hydrodynamic volumes and will able to separate out for example, apparent hydrodynamic radius of a typical protein domain might be 14 armstrong in a folded state for 36 armstrong in a unfolded states.

So, you can get a very good separation of these 2 proteins, it can also help separation of 2 forms, you know folded form will elute much later. Because it is of smaller size where as, it is the unfolded form its larger size. So, it will come out faster.

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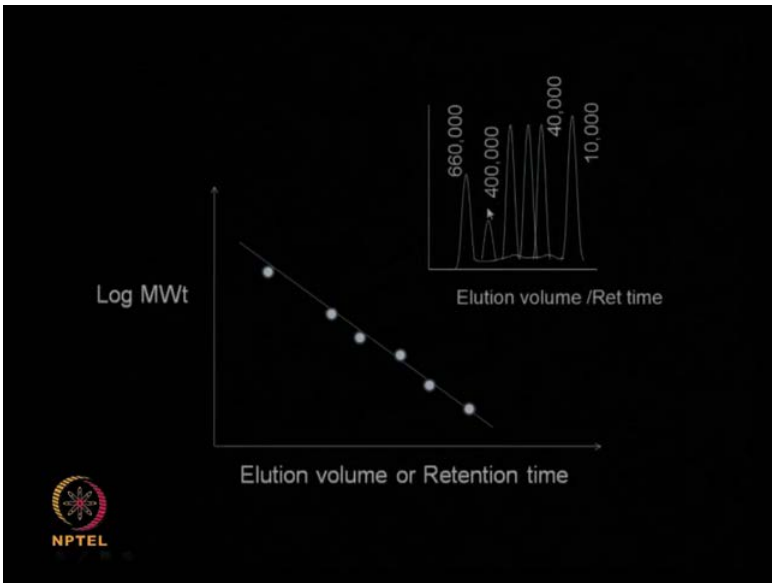

So, we can develop here standard graph between log molecular weight as a function of elution volume or retention volume time. So, the graph will look like this as the molecular weight goes down the retention time or elution volume increases. So, you have known molecular weight material compounds injected, now they commercially you will be able to get a known molecular weight of us synthetic polymer or known molecular weight of carbohydrate.

So, you buy them inject them and then get the retention time then you plot a graph between log molecular weight versus the retention time. So, you should get a nice line like this. So, if you have an unknown system, you injected find out the retention time and then you can using the standard graph get the molecular weight, this is the normal procedure, which we use. So, it is a very powerful standard graph, which you can develop with known molecular weight standards.

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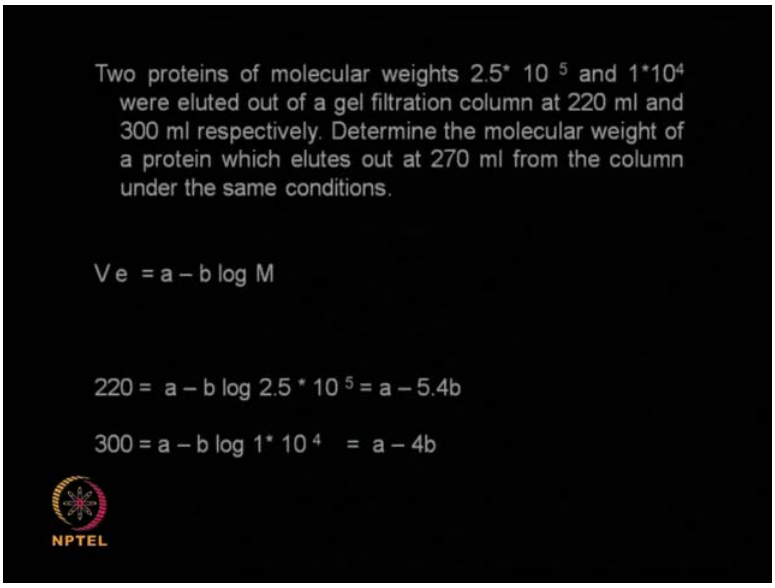

Let us look at a simple problem, I have 2 proteins molecular weight of 2.5 into 10 power 5 dalton and 1 into 10 power 4 dalton. Now they are eluted out the first one eluted out at a volume of 220 m l, 2nd one is eluted out at a volume of 300 m l. Now, I have another protein, I do not know the molecular weight of that protein that elutes out at a volume of 270 m l at the same condition.

Now, can you tell what is the molecular weight of that protein. So, we can use this particular equation, if you remember elution volume equal to a minus b log M, why do you have a negative term here. Because as a molecular weight increases, the elution volume decreases, because as the molecular weight increases the solute comes out faster.

So, the elution volume also decreases. So, we have 1 equation molecular weight up to 2.5 into 10 power 5, elution volume is 220, another equation molecular weight of 1 into 10 power 4 elution volume is 300. So, you substitute them. So, you have 2 equations, 2 unknowns a and b, 1 is this b is a slop a is a intercept, we can calculate a and b. Once you have calculated a and b for a unknown protein, we know the elution volume V e. So, we substitute and we can calculate the molecular weight of the unknown protein, it is very straight forward.

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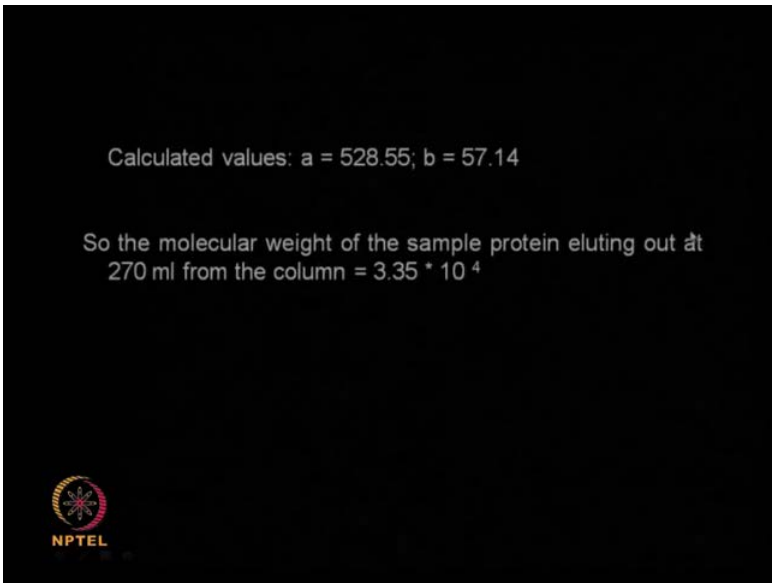

So, the values for a and b are given. So, the molecular weight of the sample protein, which is eluting out 270 m l is 3.35 into 10 power 4. So, quite a simple problem is based on elution volume is a function of a logarithm of molecular weight in a linear fashion. So, you have a linear regression relation and you make use of the relations.

So, you need to know the elution volume for two proteins, whose molecular weight is known. So, once you know that we calculate the slop and intercept of that regression relation and for any unknown system and if you know the elution volume, you can calculate the molecular weight from that regression relation. So, straight forward actually.

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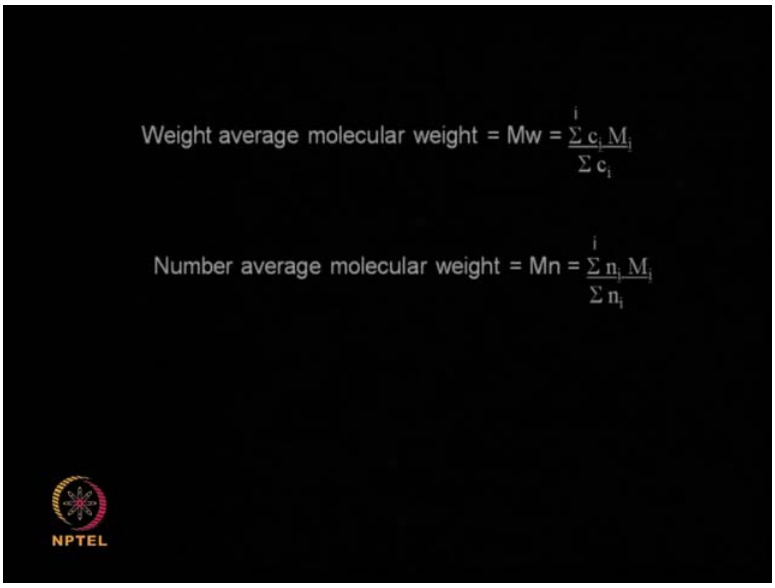

Now, in G P C, we have to consider the weight average molecular weight and number of average molecular weight. Because if you have multiple components with different molecular weights at different concentrations then the weight average molecular weight of the entire mixture is given by this particular relationship. It is a summation of concentration of species I multiplied by the molecular weight of species I.

So, if you have a 3 species with vary molecular weight M 1 M 2 M 3 at different concentration, that is c 1 c 2 c 3 then what you do you do summation of c 1 into M 1 plus c 2 into M 2 plus c 3 into M 3 divided by c 1 plus c 2 plus c 3 that is called a weight average molecular weight, where c is the concentration. Similarly, you can have number average molecular weight that means, you have the number of same species having a particular molecular weight M i.

So, here M n that is the number average molecular weight is given by summation of n, that is the number having this molecular weight M i. So, summation of N i M i divided by summation of N i. So, in polymer chemistry, you have 2 types of molecular weights, one is call the weight average molecular weight and other one is called the number average molecular weight. So, this is the formula for here for calculating the weight average and the bottom one is the formula for calculating the number average molecular weight.

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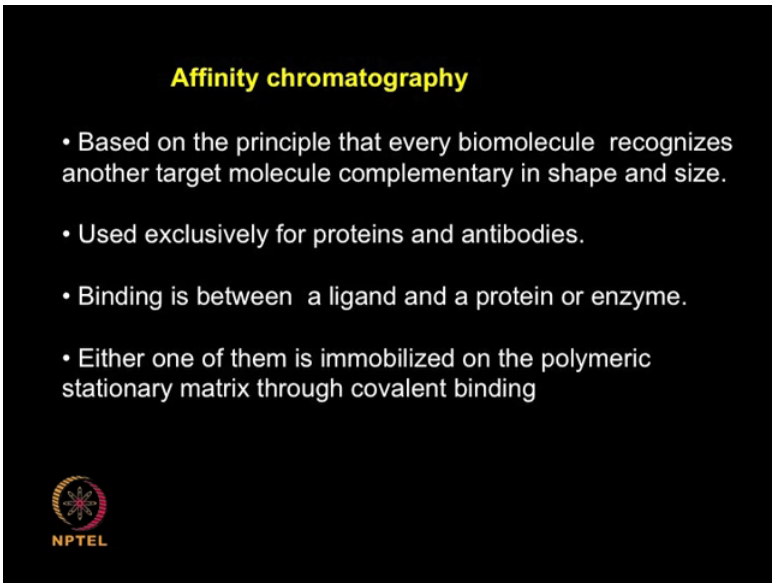

Now, let us go to another type of chromatography with. So, far we spent a considerable time on gel permeation chromatography, if you remember we in the previous classes, we talked about a the reverse phase or hydrophobic interaction chromatography then we talked about ionic interaction chromatography.

Now, we let us go to affinity chromatography this is also very powerful tool and it is based on the molecular recognition that means, if I have here protein. It will have a corresponding inhibitor, which will have exact shape and size matching with the actual site of this particular protein. So, it is based on molecular recognition. So, there is the target molecule, which is complementary in shape and size.

So, it is exclusively exclusively used for protein and antibodies. So, you can have a ligand and a protein binding, you can have a ligand and a enzyme binding and this ligand could be very, very specific to the protein or the enzyme and whereas, it will not be specific for any other protein or enzyme. So, if you take a protein, it will have a ligand, which will bind to only that protein, other ligands might not bind to the protein.

So, I can separate out ligands, which will be very specific to the protein and other ligand which are not specific to the protein will not get bound. So, we can either immobilized the protein and the mixture of ligands could be past or we could immobilize the ligand and a mixture of protein could be past. So, the immobilization happens on a polymeric stationary matrix, through covalent bonding that, what we do actually.

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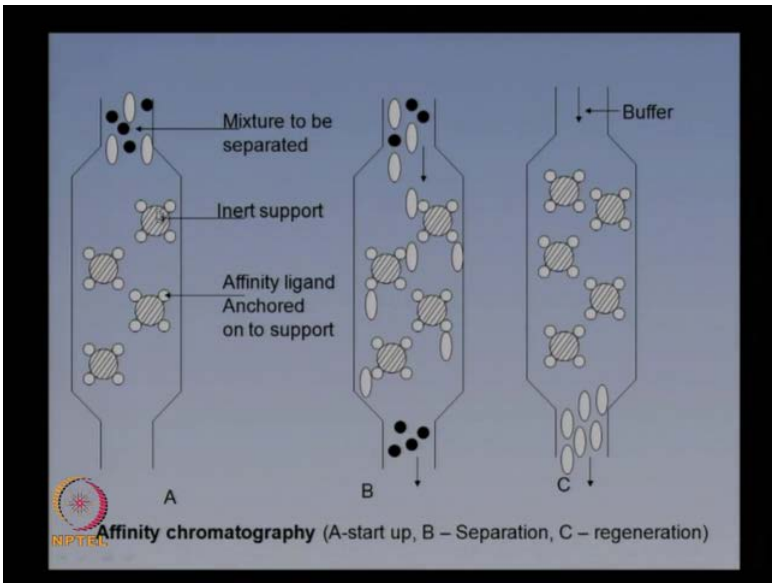

So, how does it work. So, again we have here stationary matrix and we have the affinity ligand anchored to the support. So, you have a mixture to be separated, you have components, which are complimentary to the ligand and a component, which are not complimentary to the ligand.

So, this is the mixture of protein and a only these proteins bind to the specifically to this ligand and whereas the other protein or not complimentary. So, that travel down the column without getting bound. So, what happens only these proteins are enriched the other proteins go out.

Now, how do you separate this protein, we change the buffer conditions we change the p h or we can change the temperature. So, that they get removed and this start travelling down. So, we have on the inert support certain ligands anchor to the support. So, proteins which are complimentary to the ligands get bound, other proteins travel down they get removed. So, these proteins which are now bound can be removed by changing the buffer conditions, salt conditions or p h. So, that they get removed and they get enriched here.

Now, again the column is ready for 1 more set up purification operation, this is how affinity chromatography works. So, affinity chromatography depends upon the affinity between the protein and a ligand. So, the protein could be immobilized on the support. So, we can have a mixture of ligands a flowing down only those ligands, which are

complimentary to the protein gets bound whereas, other ligands travel down or we can have ligands bound to the stationary phase.

The mixture of proteins could be passing through proteins, which are complimentary to those ligands get bound other proteins travel downward. So, you enrich protein of our interest from a mixture of protein, this is a affinity chromatography works and the immobilization is generally done as a covalent type of bonding.

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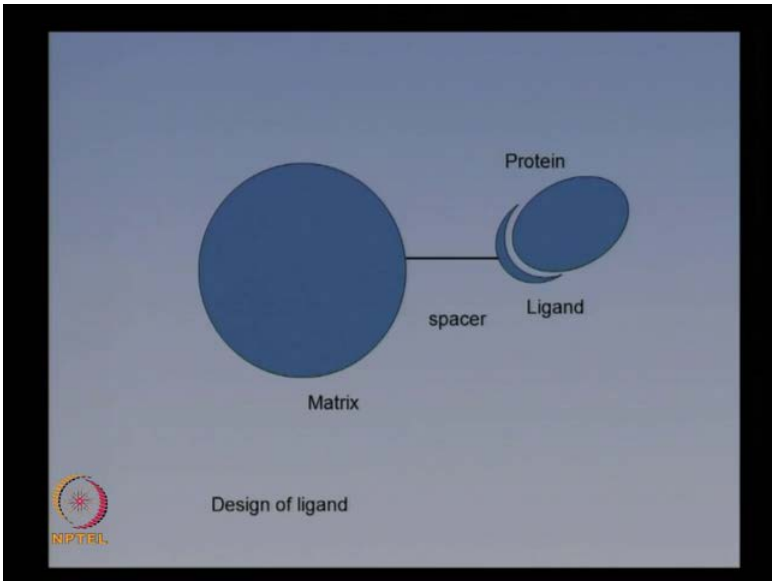

So, we have a matrix, we have the ligand the protein, which is complementary, which exactly matches in shape and size the active side of the protein shape and size matches exactly with the ligand. So, that protein gets bound here, you also have something called that spacer arm.

So, that the ligand is not very close to the matrix. So, you may have interactions of the protein with the matrix, which may disturb the selectivity pattern that is why you have a spacer arm here. These have a typical stationary phase will look like you have a matrix you have a spacer arm and then you have a ligand. So, the protein of interest will have a complimentary shape and size of that of the ligand.

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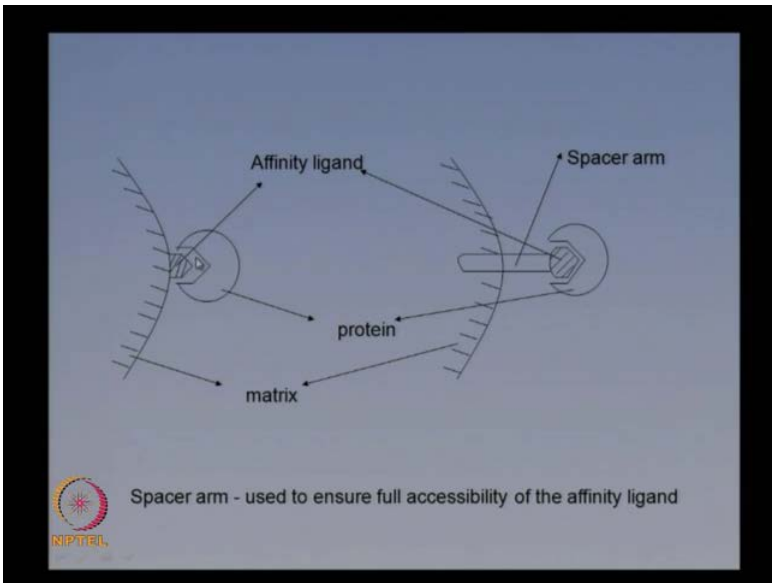

So, the spacer arm is used to ensure full accessibility of the affinity ligand, we do not want without the spacer arm the protein may come to close to the stationary phase. And it might have good interaction with the affinity ligand whereas, you see in this picture. Because you have a spacer arm the interaction is better whereas, here in this case this the interaction is very, very poor, that is why you have spacer arm here.

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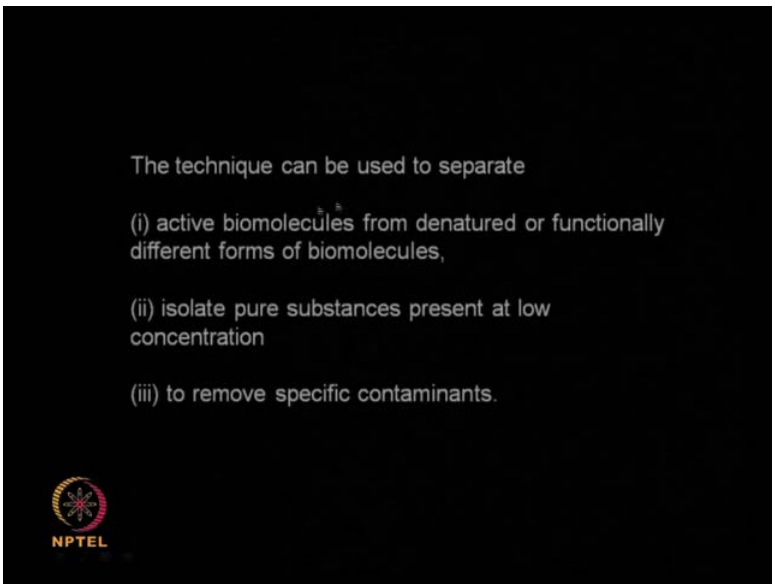

So, this affinity chromatography can be used for separating active biomolecules from denatured or functionally different forms of biomolecules, you can use it for isolating

pure substances from low concentration, we can use it for removing specific contaminants. So, you have toxic protein. So, if you have ligands, which are very specific for the protein then only those toxic proteins can be separated out.

So, affinity chromatography can be used for a wide range of purposes, it is very, very expensive, because you need to have a complementary protein or ligand and then you need to immobilize that. So, it is an expensive technique, when compare to other technique. So, generally we use it for pharmaceutical product only and not for bulk chemical product.

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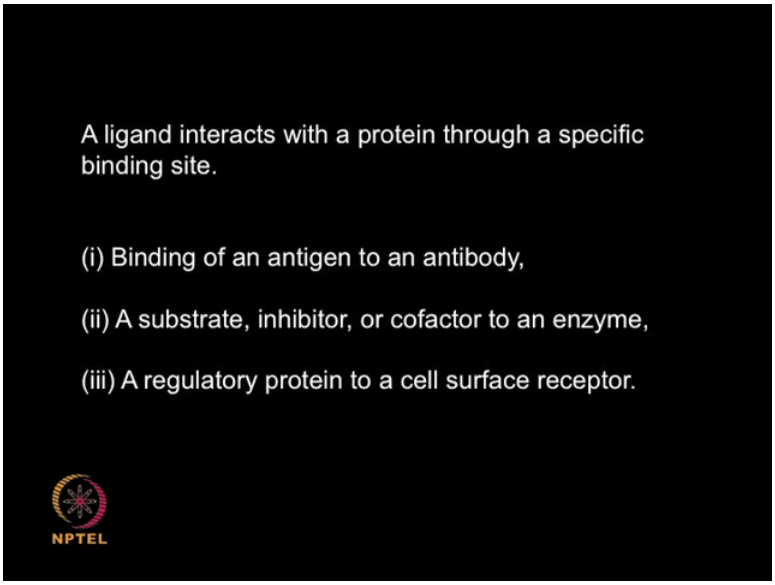

So, what happens? The ligand interacts with the protein through a specific binding side. So, you can have a binding of an antigen to an antibody, we can have a substrate or inhibitor or cofactor binding to an enzyme, a regulatory protein to a cell surface receptor. So, there is a receptor in the cell surface here is a protein, which binds to that. So, we can have an antigen antibody interaction or a inhibitor enzyme interaction or a regulatory protein cell surface interaction. So, all these type of processes may be taking place during an affinity chromatography operation.

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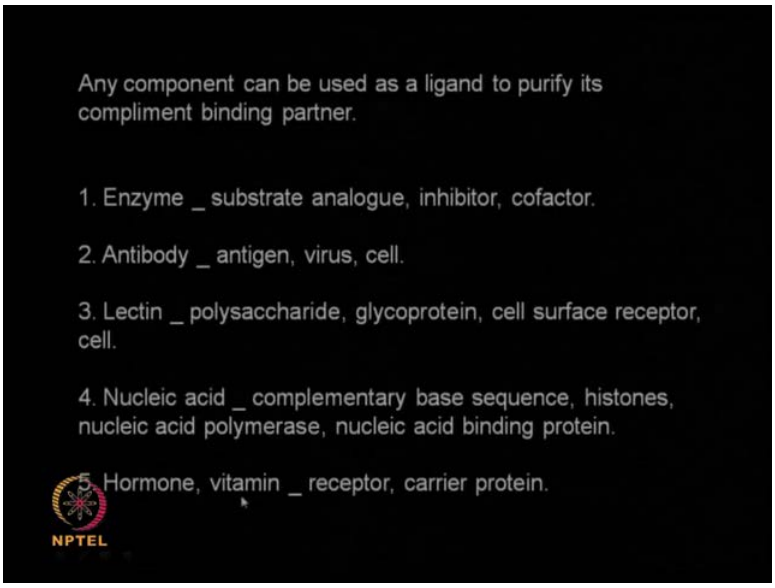

So, you can use any type of a component as a ligand purify it is complimentary binding partner. So, you have a complimentary binding partner and you have a ligand, which is immobilize. So, you can have a enzymes substrate analogue, enzyme substrate inhibitor cofactor, we can have antibody antigen, antibody virus, antibody cell, you can have lectin polysaccharide, glycoprotein or lectin cell surface receptor. You can have a nucleic acid you can have complimentary base sequences, nucleic acid histones, nucleic acid polymerase or nucleic acid, nucleic acid binding protein, if you have hormone vitamin, we can have a receptor or carrier protein.

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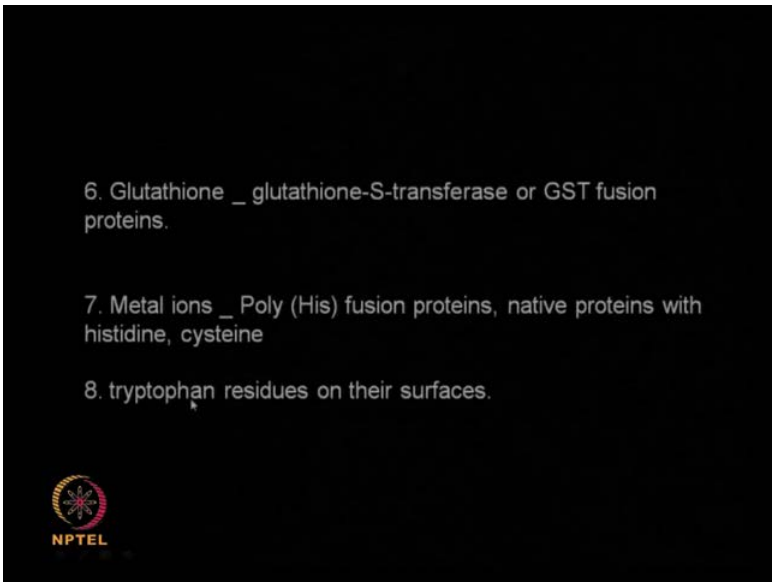

If you have glutathione, you can have glutathione S transferase enzyme or G S T fusion protein, if you have metal ions, we can have poly his fusion protein or we can have native protein with histidine or cysteine, you can have tryptophan residues on their surfaces. So, a large number of combinations of systems could be thought of. So, depending upon what you want to purify and what you want to immobilize on the surface of the stationary phase.

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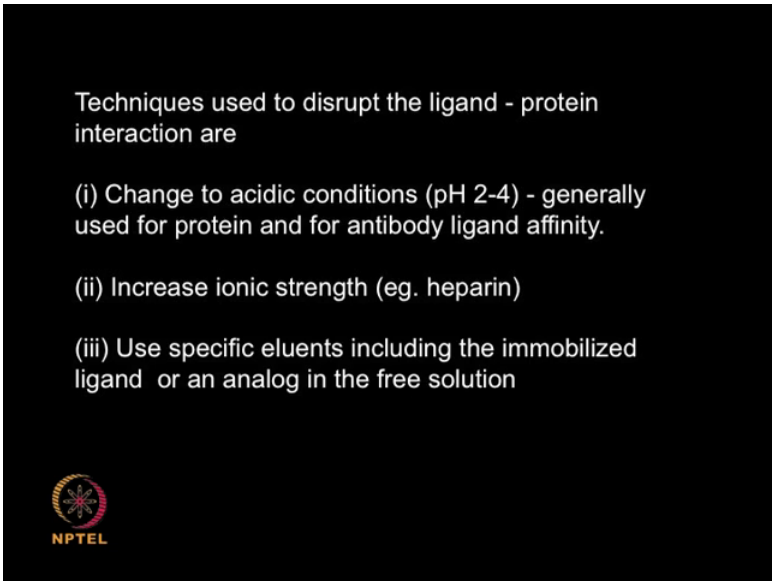

So, what are the techniques used to disrupt this ligand protein interaction. So, we have done in ligand protein interaction, now we need to dislodge it. So, that we want to collect it back or we want to regenerate the column. So, how can we do that, we can change the p h condition, we can make it more acidic, this is generally used for protein or antibody ligand affinity. So, we change the p h, so it becomes p h at 2 to 4 or we can increase the ionic strength example, if you want to separate heparin.

We can use specific eluents including the immobilized ligand or an analog in the free solution. So, we can pass your free solution, which will have the same immobilized ligand or elegant, which is an analog of in the free form. So, we can use different combinations. So, we can change the p h make it more acidic or we can change the ionic strength or we can have, we are eluent, which is analog. So, that whatever has been absorbed here removed. So, all these techniques can be followed.

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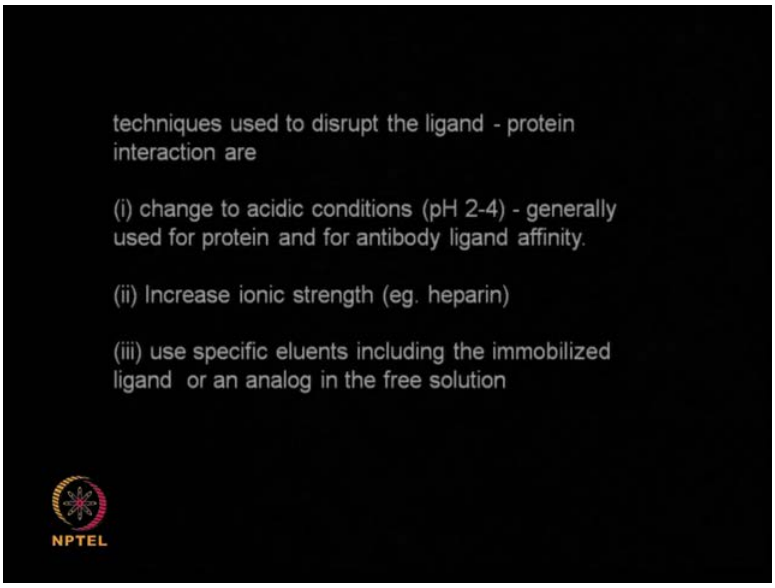

Now, we will look at further use and applications and techniques, which we need to adopt in the affinity chromatography in the next class.