Experimental Biotechnology
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Lecture – 21
Gel filtration chromatography - Part2

Hello everybody this is Dr. Vishal Trivedi from Department of bioscience and bioengineering IIT Guwahati and in our previous lecture we have discussed about how to pack the column what are the different precautions you have to take while you are packing the column and what are the parameters you should consider when you are packing the columns. And now I will take you to my laboratory to show you a small demo how to pack up column and what are the different precautions you should take.

So in this particular demo the my student the Sooram Banesh is going to show you the packing of the column and how to perform the gel filtration chromatography after connecting the column and how to draw the calibration curves or the elutions of how to monitor the elution of the proteins from the gel filtration chromatography and how to draw the calibration curve or between the distribution coefficient versus the log molecular weight.

(Video Starts: 02:05)

In this video we will demonstrate how to perform gel filtration chromatography or size exclusion chromatography there are various methods are available in chromatography to separate different types of biomolecules. For example if you want to separate based on the size or shape it is called of gel filtration chromatography which suits the most. If you want to separate the molecules based on charge then you can go for the ion exchange chromatography.

So these are various methods that are available but in this video we are mainly focusing that the gel filtration or size exclusion chromatography. What is gel filtration chromatography there is 2 phases in this process one is stationary phase another one is mobile phase. Stationary phase mainly a matrix cross linked matrix for example we can use dextran or another name of dextran is Sephadex this is highly cross linked glucose molecules or we can use agarose this is also crosslink and we can use polyacrylamides.

But in this video we are showing Sephadex g-75 this is the stationary phase we are using. So in this matrix it contains beads which having small pores. So if you want to separate a mixture of molecules starting from 1 KDA to suppose 200 KDA. So the small molecule which is 1 KDA between permeates through or diffuses into the pores in the beads and the bigger molecule having the 200 KDA it will exclude it from the retaining give that portion of pores.

So it will a look first and the smaller molecule will retain them and we have to give a sufficient buffer to a look that one. So this is the overall concept of the gel filtration chromatography it can be widely used in separation of proteins, peptides or oligonucleotides. So in this video we will show you how to tap the column first and what are the buffers required for gel filtration chromatography.

There are 2 kinds of columns available. One is pre packed columns and another one is column material which can be used for the packing of the column here to show you how to pack a column and also we have pre packed column. Fit the bottom of the column that should be a sintered filter in pre packed columns which will forbid going of the beads through outlet. So here if you see in a pre-packed columns there is some sintered filter bottom of the column in both the ways.

Outlet and inlet both contains this sintered filters, inlet which is mainly from buffer inlet if there is any particles which will obstruct the flow they will be separated on top and also the outlet one which can be used preventing passage of the beads through outlet. So here we do not have exactly sintered filter but we can use piece of cotton. So just put the cotton, so now we inserted the cotton these are the beads we are going to use packing up the column these are the Sephadex.

So there are different materials available sucralose, sephadex, sepharose these are all derivatives carbohydrate material. So here what we do this is a overnight solid beads. So you have to take the beads and complete pack and incubate soak it in the water or the buffer. so this not solid one now after inserting this sinter filter to our column you have to watch column properly it is to 2, 3 column with distilled water.

So if anything it contains like dust or any other contaminating particles it will remove; now we directly pour the beads on top of the column if we close and observe closely you can see the settling of the columns occurred settling of the beads as we can see the beads are settling slowly. So after completely complete settling of the beads then we will put some filtered kind of thing or some piece of cotton on top of the beads.

Then we will load the sample as we can see now that packing is over. Now what we have to do is we have to equilibrate the column with the 0.05 molar of phosphate buffer. So we will just add phosphate buffer in the remove drain in the any unbound solvent. So here after equilibration of the column we load the sample after column packing you have to check the efficiency of the column so for every column the parameters V naught that is void volume in the V t total volume and V elusion volume it differs.

So for checking of the column efficiency we have to use 0.2% of total volume of the column acetone will be load. So we have to observe the elusion at 280 nanometers from this we can calculate number of theoretical plates N. So the maximum number of the theoretical plates means the more efficiency for calculation of any unknown protein molecular weight we should know what is the V t, V naught and V.

V t is that total volume of the column means the buffer occupied in the space of beads and also the buffer in between the beads so that will be total volume the void volume is the buffer in between the beads emission volume is where elute suppose we are eluting protein so it washed volume it is empty that is called as emission volume. So for estimating the void volume we can use blue Dextran.

So first we have to cover top with the piece of cotton then we will load the blue Dextran after loading sample loading will start collecting the buffer in loaded completely eluted that will give the void volume. So now we loaded the blue dextran we will add the buffer then we will elute, so as we can see the blue dextran is passing down we have to replenish buffer continuously and we start collecting the eluted volume. So often complete edition of the blue dextran we have to measure the volume and that volume gives us void volume.

Now the blue dextran completely eluted it is around 80 to 90ml. So now will got void volume. Now what about a total volume, total volume consist of packed volume of the beads total packed volume of the beads this so is around 25ml of up beads are there. So that means total volume is 25ml. So with these values after filtering the protein suppose if you are using some unknown protein you will have to calibrate with for known proteins.

Then you have to construct a calibration curve between the partition coefficient which is calculated with elution volume subtracted with void volume divided by total volume subtracted void volume that will give partition coefficient and on excesses you have to take log molecular weight once you plot you will observe some correlation based on that you can calculate unknown proteins molecular weight.

So this is the gel filtration column attached to rotate the purification system. So here it will show you we will inject the blue dextran and BSA. Show the pattern how they are eluting this blue dextran is void volume of data and BSA gives HL is elusion pattern. So if you run fewer proteins with a known molecular weight we will get the calibration curve with that we can calculate unknown proteins molecular weight.

So this column is equilibrated as we can see here. When we introduce it into buffer after removing the 20% ethanol and water also so we can see this one corresponds to blue line corresponds to 280 nanometer which is relevant to protein one. So we can see we have there is a initial spike but gradually it the line the curve flattened so that means there is no contaminants and now the column is ready to inject the protein.

So what we will do we will inject the protein and we will show how to inject protein and so then we will show the pattern they are eluting. So here in end the program so we will start the new program system flow will take 0.5ml per minute insert flow path column position at 1 and a downward flow insert monitors we need 3 different wavelengths 215 for peptide one, 254 for nucleic acid, and 280 for aromatic amino acids.

And we have to set the alarms also. We will set this 3 and this one 01 complete system pressure 3 and this one 01. So we will inject the protein now and will say how it suits, this is the port where we are going to inject the sample. So once we will inject this one and execute inject. So this is not pattern of injected components this one corresponds to the blue Dextran and it gives the void volume of 8ml in as we can see here it corresponds to 8ml.

So there is no proper resolution between BSA at the blue dextran this corresponds to 8ml which is blue dextran and this one is 9.2 to 9.5 this corresponds to the BSA. So once this is finished we have to run another one column of in buffer to remove any other proteins and after that we will keep it in water. So to remove any kind of salts if present that will keep it 20% ethanol will run at least one carbon volume.

So that directly we can use to preserve the column after that we have to with the 20% ethanol complete system. So that there is contamational bacterial if you left for few days and so this is all about jet filtration chromatography. So we will show you how to analyze the results. So once the filtration run is over you have to analyze the results. So this is the software we will use for the evaluation purpose.

So we have to open the chromatogram which you want to analyze. So we already opened this is the chromatogram we ran recently. So we have to analyze peaks. So peak integrate option is there. So just say which one you want to analyze 280 nanometer one or 215 we have only 281. So that is let us say unless. So as we can see it gave the retention volumes of the peaks and also the area and the height of the peak these values can be used for constructing calibration curves this one belongs to blue Dextran and this one is for BSA.

So in a summary in this video we showed how to run gel filtration we showed manually how to pack the column with the beads and also connecting through the instrument. So hope this will help to improve your research

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So in this demo the Banesh has discussed many parameters and many aspects related to column chromatography. Now once you pack the column one of the crucial parameter is that how you

are going to do the quality testing or quality checking on to your chromatographic column before you start using it for your experiments. So one of the crucial parameter is that you should be able to know the different type you should be able to calculate the different parameters related to a gel filtration column and that those parameters should be with the standard columns.

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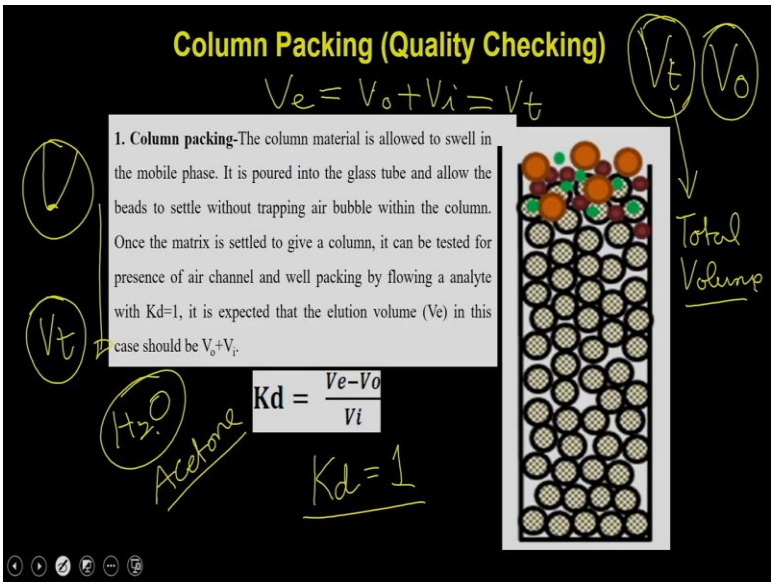

So what are the different parameters so if you pack a gel filtration column you have the multiple parameters for example you are going to have the V t you are going to have the V o. So these are actually the 2 parameters you can be able to calculate for your column and that should actually come. So one of the easiest ways of calculating the total volume, now one of the so how to calculate the total volume?

If you remember when the K d is equivalent to 1 the V e is equivalent to the V o + V i which means that if I take a molecule and V o + V i equivalent to V t actually. So if I have to calculate or if I want to calculate the V t of a column what I have to do is I have to take a molecule which is immiscible to the aqua solvents and that can be detectable using the standard spectroscopic methods. And that should be present at t center or at the end of the poll.

Because that is the molecule who is going to travel all the way up to the column and it is going to give you a travel distance which is equivalent to the V t. So what are these molecules these molecules are going to be the smallest molecule possible. So in this gel filtration

chromatography what is the smallest molecule the smallest molecule is water. Because that is the smallest possible right you have the water which is often molecular weight of 18 Dalton.

But if you add the water you will not be able to distinguish the water which is present in the mobile phase versus the water which is you are going to inject into the column. So because of that you have to take a molecule which is of the smallest in range but that should be immiscible or should be detectable from the water. So in that case what you do is you take any organic solvent for example you can take the acetone.

So if you take the acetone and if you inject very small quantity of acetone into the column the acetone is going to be go and filled at the bottom of this pore and then it will take the total travel length of the column and it will give you the V t of the column. So you are going to have the V t which is being calculated experimentally. Then you can also be able to calculate the V t of a column simply by measuring the volume of the beads by theoretical method.

So the theoretical V t should be closer to the experimental V t. which you are going to calculate after flowing the acetone if that is the case that actually will ensure that you have pack the column nicely and the column is free or the defects.

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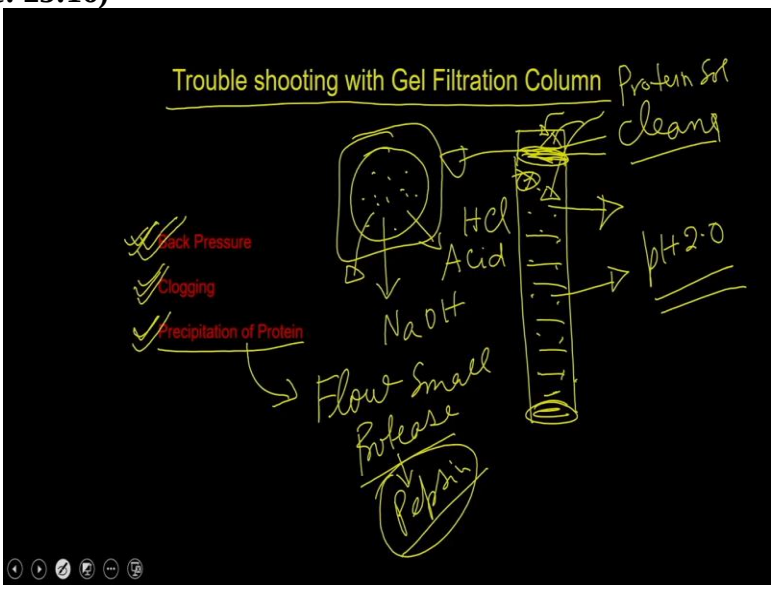

Now once you have packed the column you are also going to face different types of problems when you are performing the gel filtration chromatography. So what are these problems first

problem is you are going to see a back pressure. So when you run the columns they actually did because irrespective of whether you want it or you do not want it that all the columns are actually having a filter on top.

And then you have the filter at the bottom and in between you have your material being packed. So this column this filter is always getting the some kind of proteins or other kind of factors are being stuck to these filters and because of that you are actually going to have the back pressures. So what you have to do you have to clean these filters with the help of you have to use some cleaning agents and you can use spill you know you can remove these filters and clean it.

So that actually will take care of the back pressure problems then you have the clogging of these columns which means the proteins are going to be present onto these filters and those filters are actually having the small pores through which only the water can be able to pass through. So if you have the proteins getting through to pores proteins also get bind to these pores and that how you are actually going to have the clogging.

If you have the clogging problems then what you have to do is take out these filters and treat it with the acid or you can treat it with the N a OH are the alkalis so in the acid you can treat it with a HCL when you treat it with the HCL or the N a OH the proteins which are present into these pores are going to be damaged. And that is how it is going to be clear off. If that is not could enough you can still be able to treat it with the organic solvents as well.

Which are not going to affect the filters and that is how it is going to clean your filter and then you can put the filter back and it should definitely going to take care of the back pressure as well as the clogging problems. The third problem is that when you are going to see the precipitation of the protein so what happens is sometime when you load a protein suppose you are doing some experiments.

And you do not know how the protein is going to behave when it will go to the gel filtration column. So suppose I loaded a protein solution. And as soon as it enters into the gel filtration because one of the thing which happens in the gel filtration is that it dilutes the protein solutions.

So once the protein solution is been diluted it precipitates because the protein is not stable or some other issues.

So once the protein gets precipitates it actually is going to distribute throughout your column instead of being localized into the few beads or few pores because of that irrespective of whether you and another issue is first of all it is going to be distributed throughout the column The second is it is actually going to be bind to the pores. So it is irreversibly going to stuck to some of the beads.

So what happened is if that happens this column is no longer be useful because the protein is going to be keep eluting from this column irrespective of whether you have run it for 1 column volume or 2 column volume the second is because the protein got stuck into the beads it is useless. So in that case what you have to do is you have to flow the small quantity of protease so if I want to do that what I will do is I will take the pepsin.

I will equilibrate this column with buffer which is maintaining a pH 2 and then I will inject the pepsin and let the pepsin to go and incubate and then what will do is will take this column take out from the chromatography system tie it up and keep it in room temperature which means I will leave it on my bench and what will happen is this pepsin will going to be diffused because as soon as you are not connecting it to the machine.

The protein is going to diffuse because even if the pepsin is present in the first layer of your column it will come out from the beads it will diffuse and it will diffuse throughout the column. And ultimately what will happens the pepsin is going to queue up these proteins and they will be able to degrade. So it will degrade all the proteins that are being stuck or what has been present in the different parts of the column.

And that is all it is actually going to remove these precipitated proteins irrespective of whether you have the backpressure problem you have the clogging or you have the precipitation of the proteins and you are either working with the filters or you are doing the protease treatment. Once

you are done with this you have to recalibrate this column to utilize this column for monitoring the molecular weight of the unknown proteins.

So that is very mandatory that whatever the treatment you do you have to because all these treatment are actually disturbing the packing of the columns. And that is why it is actually going to change the void volume and total volume and all of the parameters. So that is why it is important to recalibrate and recalculate the relationship between the kav versus log molecular weight.

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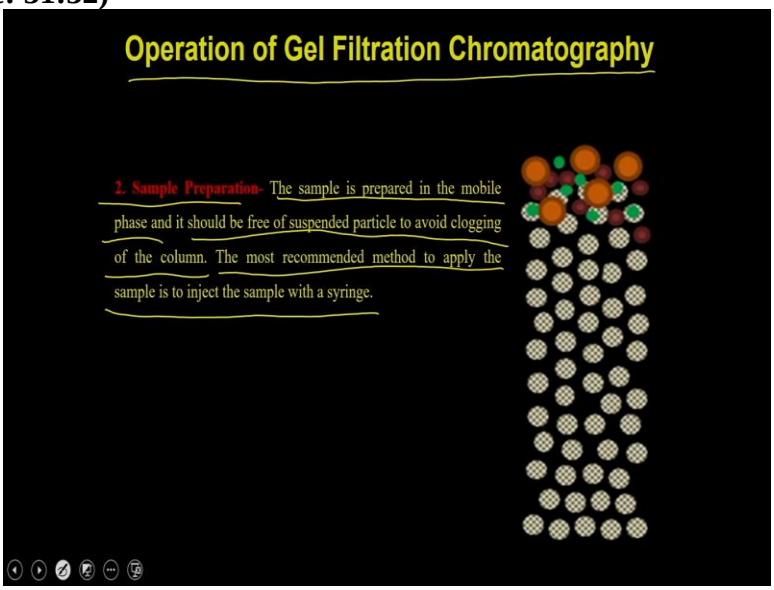

Now let us discuss about the operation of the gel filtration chromatography. So in the gel filtration chromatography operations the first is we have to do a column packing once the column packing is over then you have to do the quality checking simply by calculating the total volume either by flowing the small amount of acetone and the third is you have to prepare the samples.

So you have to prepare the sample in a same way that sample is prepared in the mobile phase and it should be free of suspended particles to avoid clogging of the column the most recommended way is applied to inject the sample with a syringe so that it will get into the loop and that the loop is going to inject the sample into the column.

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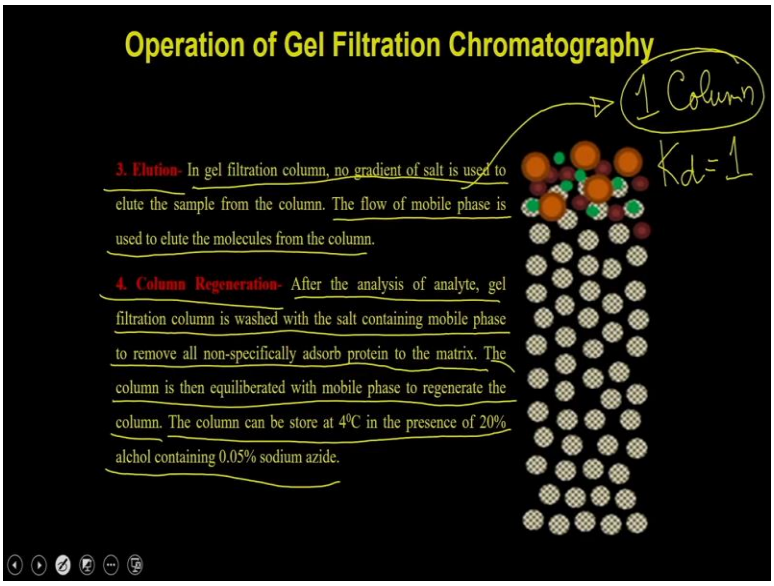

Elusion so there is no elution as such when you run the so there is no gradient of salt or something to elude the samples. Once if you flow the mobile phase in the molecules are going to be eluted from the column only thing you have to remember is that in the gel filtration column everything is going to be eluted by the 1 column volume because the K d is going to be 1 for most of these molecules and any molecule which has a K d of 1 is going to be eluted even in the 1 column volume.

So you cannot have the K d more than 1 because if you have the K d of more than 1 then that molecule is going to stuck to the column and that will not elute. So for our standard molecules those molecules which are going to follow or which are going to be separated by the gel filtration chromatography will not going to have the K d more than 1. So that is why the 1 column volume is good enough to elute the molecules from the column.

Once you are done with the chromatography you have to do a column generation. So after the analysis of analyte gel filtration column is washed with the salt containing the mobile phase to remove all the nonspecifically adsorb protein to the matrix. The column is then equilibrated with the mobile phase to generate regenerate the column if you are not planning to use the column then the column has to be stored in alcohol and it has to be preserved or it has to be stored in a 4 degree.

Now here you have to remember if you are storing a column in 4 degree and suppose you are operating this column at 25 degrees Celsius which means it is on room temperature it is always important that you store the column in 4 degree fine but if you want to operate you have to bring it in 25 degree for at least for some time and then only you should use because when you change the temperatures you are actually also going to affect the packing of the column and that eventually is going to affect the separation profile of the different proteins from this column.

So that is why the temperature has to be constant and it should be remained constant throughout you are performing the gel filtration chromatography. So with this I would like to conclude our lecture here in our subsequent lecture we are going to discuss about the application aspects of the chromatography especially designing the different types of experiments. Thank you.