Experimental Biotechnology
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Lecture – 25
Affinity chromatography - part-3

Hello everybody this is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering IIT Guwahati, and what we were discussing, we were discussing about the affinity chromatography and in the previous 2 lectures, we discussed about the basic principle of the affinity chromatography then subsequently we have taken an example of how to generate the receptors and within that we have discussed about how to generate the antibodies into animals like rabbits or mice.

And then subsequently we discussed how to couple the receptor to the matrix and the briefly we have also discussed about how to perform the affinity chromatography what are the different steps you have to perform or what was the different steps you have to go through to get the purified protein after the affinity chromatography and we have also discussed about what are the different advantages you have compared to the conventional chromatography. So, following our discussion about the affinity chromatography, now, we are going to discuss about few more aspects related to the technique.

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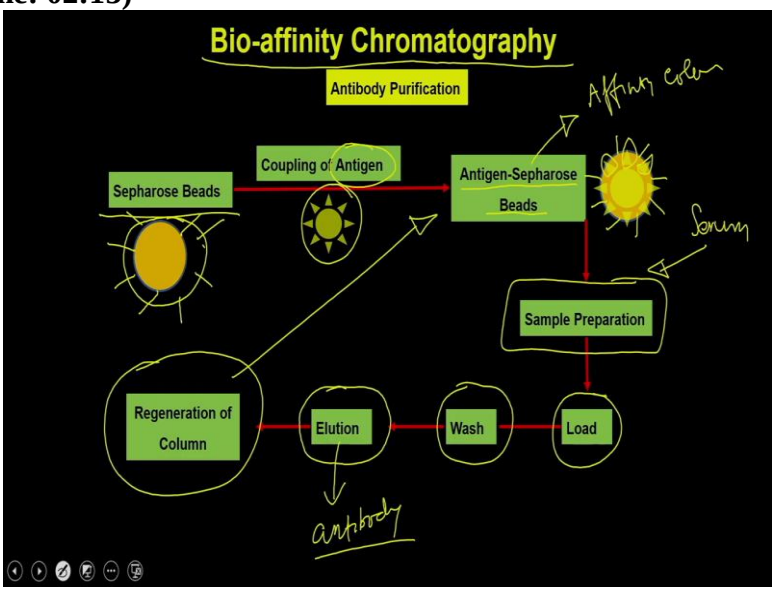

Now, the first thing what we have to discuss within the Bio-affinity chromatography is that the primary requirement. So, in the previous lecture we discuss about how to generate the antibodies and now what we are going to discuss is how to purify the antibodies, because

what you are going to get? You are going to get the serum from the immunized animals. So, serum is actually going to contain the different types of antibodies.

The antibodies against the antigen what you have injected and against which there will be a very high quantity of antibodies, but the animals are also going to be exposed to the natural Antigens. For example, it may happen that the animal is getting some kind of infectious organisms or other kind of thing and in general also since, we are not keeping the animal under the sterile conditions.

The normal foreign particles are going to be enter into the body and that is why the immune response in the animal is going to be a mixture of the antigen what you have injected plus the antigens, what he is getting naturally during the course of its stay in the animal house. So, because of that from the complete mixture of the antibodies, which are present in the serum, you have to purify the antibodies of which are being developed against the antigen.

So, the first step is that you have to purify the antibodies utilizing a series of steps. So, what you are going to do is you are going to take the sepharose beads. So, sepharose beads are going to be having the, you know the functional groups. So, we can have the different types of functional groups or sometime if the sepharose beads are not having the functional groups, you might have to perform the chemical reactions to activate them and to generate the functional groups.

After that you have to incubate that with the antigens and subsequently what will happen is the antigen is going to be coupled to the beads and you are going to develop the antigen bound sepharose beads which are actually going to work as the affinity column. So, all these beads are going to have the antigen present onto their surface and there will be additional sites which you are going to block at the end of the coupling reactions with the help of the nonspecific proteins.

For example, in previous lecture, we have discussed that you have to utilize the glycine to block the additional activation sites and then you have to prepare the sample for the serum which means you are going to get the serum from serum you have to prepare the sample. So, that you will be able to apply this sample onto the column and then you have to wash the column.

So, that the unbound out of loosely bound nonspecific proteins are going to be eluted or the non-specifically bound antibodies are going to be washed away and then you have to perform the elution of the antibodies of your interest and then after the elution you are going to get the antibodies, which are going to be developed in animal against this particular antigen. Then you have to regenerate this antigen bound column so that you can be able to perform the chromatography for the second round. So, let us discuss all these steps in detail to understand how to perform the antibody purifications utilizing the affinity column prepared by you.

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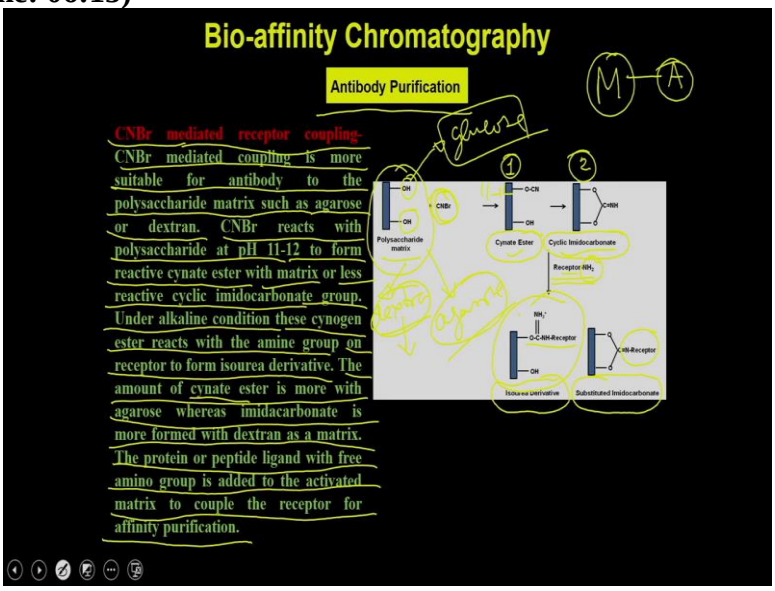

So, in the first step you have to do coupling reactions. So, we have discussed about many of the coupling reactions in the past, but for the antibody purification, you can also use the CNBr mediated coupling. So, the CNBr mediated coupling is more suitable for antibody to the polysaccharide matrix such as the agarose or the dextran why it is so, because the polysaccharide matrix normally contains the OH as the functional group which are present onto that.

So, this OH is coming from the glucose which is present or which has been part of these polysaccharide matrix then what happen is when you add the CNBr which is actually called as the cynogen bromide. So, once you react the CNBr with the polysaccharide at pH 11 to 12 it actually forms the reactive cyanide ester with matrix or it forms 2 complexes. One very highly reactive cyanide ester or the less reactive cyclic imidocarbonate group.

So, once you add the polysaccharide matrix you add the CNBr at pH 11-12 it is actually going to form the valid highly reactive cyanide ester or it is going to form the cyclic

imidocarbonate whatever the intermediate is going to form once these intermediates are being generated, then under the alkaline conditions, these cynogen esters react with the amine groups present onto the receptor to form the isourea derivatives.

So, once you are going to develop the cyanide ester you can add your antigen which actually going to contain the free amino groups and as a result, the free amino groups are going to be coupled to the matrix with the help of the isourea derivatives or in the case of cyclic imidocarbonate it is going to produce the substituted imidocarbonate and the receptor is going to be coupled to the matrix.

The amount of cyanide ester is more with the agarose whereas the imidicarbonate is more formed with the dextran as a matrix. So, when you react this CNBr to the polysaccharide matrix, so, polysaccharide matrix could be of the dextran origin or it could be of the agarose origin depending on whether you are taking the dextran or the agarose the cyanide ester or the cyclic imidocarbonate are going to be developed or going to be generated in the different proportions.

So, if you are taking a dextran as the matrix, the cyanide ester is going to be more or cyanide ester is going to be less whereas, if you take the agarose designer dusted is going to be more but irrespective of whether you are developing this intermediate or this intermediate it is not going to interfere for the coupling reactions, because both of these intermediates are going to react with the free amino group and ultimately it is going to give you the subsequent complex.

The protein or the peptide ligand with the free amino group is added to the activated matrix to couple to the receptor for affinity purifications. So, after this you are going to get the matrix where the antigen of your interest is going to be coupled and now, our affinity column is ready to be used for purification of the antibodies.

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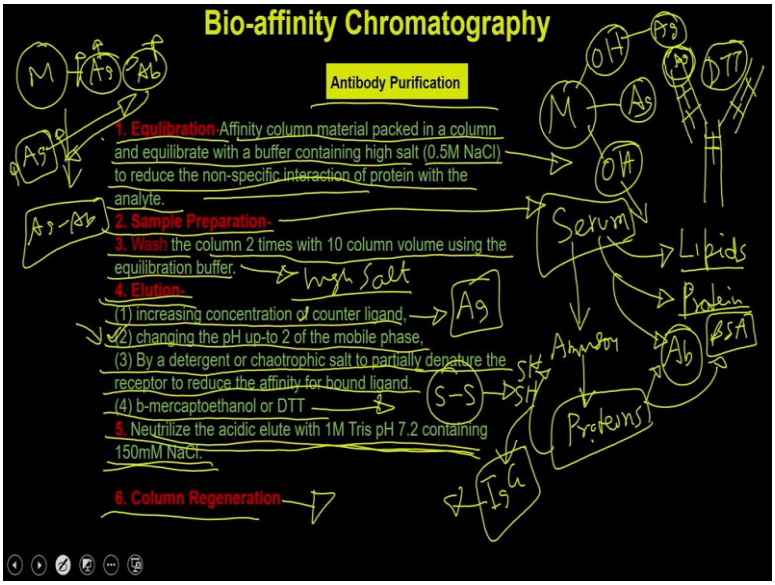

So, the antibody purification has multiple steps. The first step is the equilibration of this metal the matrix bound antigen complex antigen column. So, you take the affinity column where the Anti matrix is having the antigen coupled and then you pack it into a column and equilibrate with a buffer containing very high salt which means, the salt which contains the 0.5M NaCl.

This is only because you want to reduce the non-specific interaction of the protein with the analyte which means you do you want to disrupt the ionic as well as the hydrophobic interactions. So, that the non-specifically proteins which are present in the serum should not react with the matrix because, as you see that matrix is also containing the OH as the functional group.

So, most of the OH might be used for forming the cyclic Ester but or may be involved in coupling with the antigen. But some of the OH will still be pending on this OH can easily participate into the ionic interactions or it can make the interaction with the polar seduce present onto the some nonspecific proteins. So, that is why you have to keep very high salt concentrations to avoid or to reduce the non-specific interactions, then what you have to do? You have to prepare the sample.

So, your sample in this case is serum and serum is actually going to be processed in such a way that you have to remove the some of the proteins. So, what you have to do is because serum also contains the very small quantity of lipids. Serum is also containing the other kinds

of proteins like BSA, which is actually been present in the bovine serum but you can also have the HSA if you are taking out the blood from the humans.

So, irrespective of you are going to get the albumin protein which is actually going to be the maximum protein present in serum and all these proteins are not useful, because apart from that you are also going to have the antibodies. So, you are only looking for the antibodies not the lipid or the other proteins. So, what you are going to do is you are first going to treat this serum with a ammonium sulphate.

So, that it actually going to give you the protein because ammonium sulphate is going to precipitate the all the proteins which are actually belong either going to be the antibodies or either going to be the serum albumins after that you can do the another round of the ammonium sulphate precipitation so that it only gives you the I g G fraction of the serum and that actually is partially going to concentrate the samples number 1.

So, if you go through with this process of the aluminium sulphate fractionation of your serum. The first step you are going to remove the lipids which are actually going to interfere in the purification step because it is going to reduce the flow rate and as well as it is going to non-specifically bind to the matrix. The second step when you do the another round of ammonium sulphate precipitations you can be able to selectively collect the I g G fractions.

After leaving the BSA and other protein fractions and that is how you are actually going to concentrate the samples you are only going to have now the I g G fraction from this fraction, you can actually be able to perform the affinity chromatography utilizing the antigen bound matrix and that actually is going to give you the more yield. Now, next to what is you are going to load this I g G containing fraction and load it onto the column.

The loading is simple because, if you are using the chromatography system, then you can use the loop or you can use the syringe to inject the sample onto the column. If you are doing it in a manual method for example, if you pack it into a column and then use the manual mode, then you can just load the sample with the help of the pipette after the sample loading, you can do the washing.

So, you wash the column 2 times with the 10 column volume using the equilibration buffer, which means you are going to wash with the high salt buffer so that the all proteins are going to be removed and then once all the non-specific proteins are going to be removed because of this washing step then you can do the elution. The elution in the affinity chromatography where you would like to purify the antibodies can be done in multiple ways.

The first and the easiest way of that you increase the concentration of the counter ligand which means you can easily add the antigen. So, what will happen the antigen is if you add the antigen, what will happen is that you have the B matrix where the antigen is being coupled. So, this antigen is going to bind the antibodies and once you add the same antigen, which is actually going to be the free flow.

What will happen is this antigen is going to competite to the antibodies and because the amount of this antigen is going to be very high compared to the antigen which is bound to the matrix, the antibody is going to bind this antigen instead of binding to the matrix and that is why the antigen antibody complex is going to be released from the column in a those dependent manner in a very regulated manner.

So, if you have a very small quantity of antigen, nothing will come out and if you have very high quantity of antigen which is actually be good enough to competite with the antibody which is bound to the antigen coupled to the matrix, then the antibody is going to be released from the column and it is going to be freely available for your further applications. The second is you change the pH.

So, if you change the pH up to the 2 like if you bring it to the acidic environment, what will happen if it is partially going to denatured the antigen as well as it is partially going to denature the antibodies and if you do so, the antibody is going to lose the affinity for the matrix bound antigen and it is going to be released as soon as you do the illusion with the help of the PH then you have to add the alkali or you have to add something.

So, that you will be able to change the pH to 7.4 because, if you keep the antibodies in pH 2 for a very long time, it is actually going to be permanently damaged and then you will not be able to utilize an antibody because ultimately the antibody is should have the 3 dimensional

confirmations and 3 dimensional structure intact so, that it should have the affinity for the antigen.

The third is you can use the detergent or the chaotropic salt to partially denatured the antibody to reduce the affinity for the bound ligand end and the fourth is you can use the b-mercaptoethanol or DTT. So, you know that the whether methanol or DTT are actually breaking the disulfide linkages, which means it is actually converting a disulfide linkage to the SH which means it is actually reducing the disulfide bond and giving you the sulfhydryl groups.

So, you know that the antibody structure is being maintained by multiple disulfide linkages between the light or the heavy chain and even the complete antibody structure which is going to be stabilized by the multiple disulfide linkages. So, if you add the DTT or b-mercaptoethanol, it is actually going to break all these disulfide linkages. So, once you break the disulphide linkages or disrupt the disulphide linkages.

You are actually partially going to denatured the antibody and as soon as you denatured the antibodies, it is actually going to incapable of binding the antigen by the variable region and because of that, it was actually going to be released from the column but as I said, just like the pH this process is also very, very detrimental and it is not good for the antibody. So, you have to oxidize the antibody as soon as it comes out into the fractions.

And you have to remove the DTT or beta mercaptoethanol very quickly so, that you will be able to renature the antibodies. So, that it the antibody will acquire its original three dimensional structure that the fourth is you can utilize if you are eluting with the help of the PH change, you have to eventually neutralize the acidic elute with the help of the Tris pH 7.2 containing 1.5 milli molar NaCl.

Now, once you have done the purification of antibody from the column, then you have to regenerate the column new definition of the column is required that you should wash the column with a very, very high salt contribution then you have to wash it with the chaotropic salt, so, that you should remove the pre existing all the antibodies which are being coupled to the antigen onto the matrix or in general which are going to be coupled to the matrix in a nonspecific manner.

So, that it should you should resume the flow rate and it also going to remove even there will be any remaining lipids and other contaminating factors which are present into the serum and now binding to the matrix or to the bind ligand or bind antigen. So, after that this column is going to be freshly available for another round off antibody purifications.

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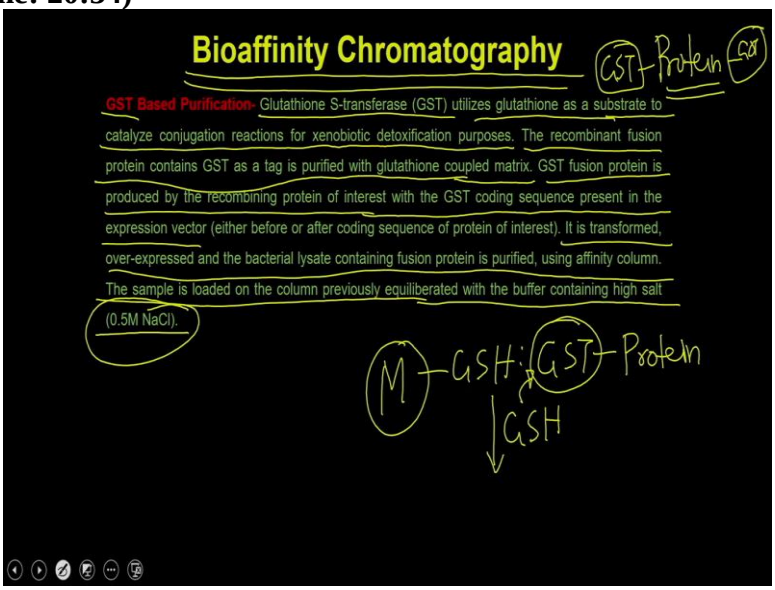

Now, let us move on to the next example of the Bioaffinity chromatography where you are not using the antigen or the antibody as a pair you are you are using the enzyme as well enzyme and the substrate. So, here you have the enzyme which is called as the GST or the glutathione S-transferase which actually utilizes the gluten as a substrate to catalyze the conjugation reaction for the xenobiotic detoxification process.

So GST is an enzyme which actually utilizes the substrate called glutathione. And that is how it is actually coupling the glutathione to the drugs and other kinds of hydrophobic molecules so that the molecules become soluble, and that is how these molecules can be removed from the body during the detoxification process. So, the DST is a very, very useful enzyme for detoxifying the drugs and other kinds of toxic material.

What we are, you know, ingesting into our body, either through the daily usage, exposure or other kinds of consumption of the food items. So, this actually can be used to because the DST has a very, very high specificity for the glutathione, this pair can be used to for the affinity chromatography, what you have to do is the recombinant fusion protein contained GST as a tag is purified with the glutathione coupled matrix.

So, in this case, what you have to do is you have to take the matrix coupled to the glutathione which is called as the GSH. And then you couple it, the GST to the protein with the help of the recombinant DNA technology. And then what will happen is this since this GST has a very high affinity for the glutathione it is actually going to make the interaction. So it is going to make the interaction and then you can utilize this simply by flowing the large quantity of GSH.

So this GSH is going to compete for the matrix bound DST and that is how the GST bound, the protein is going to be removed from the column and it is going to be elute. GST fusion protein is produced by the recombining protein of interest with the GST coding sequence present in the expression vector you can have the option of putting the GST on to the N terminus or to the C terminus.

Which means you can have the GST either onto the N terminus or to the C terminus depending on the protein of your interest is how the protein of your interest is going to respond, because sometimes when you put the GST in the front and you express the you produce the chimeric protein which contains the GST in the front and done protein of your interest.

That sometime helps even in getting the protein more soluble, because GST is a very, very soluble protein and it is a cytosolic proteins. So that how it actually helps to generate the soluble protein, but in some cases when you are adding the GST in the front of your protein of interest, it actually also interfered with the reaction of or the enzymatic reactions what the enzyme of your interest or enzyme is going to be capitalized.

In those cases, you can put the GST onto the C terminus. So, irrespective of whether you put the GST in the front or GST in the back, it is not going to affect much in terms of depreciation utilizing the GST columns it is transformed over expressed and bacteria lysate containing equivalent routine as purified using the affinity column the sample is loaded onto the column previously created with the buffer containing the high salt. So, the high salt condition is also existing here because the high side is going to reduce the non instance interactions.

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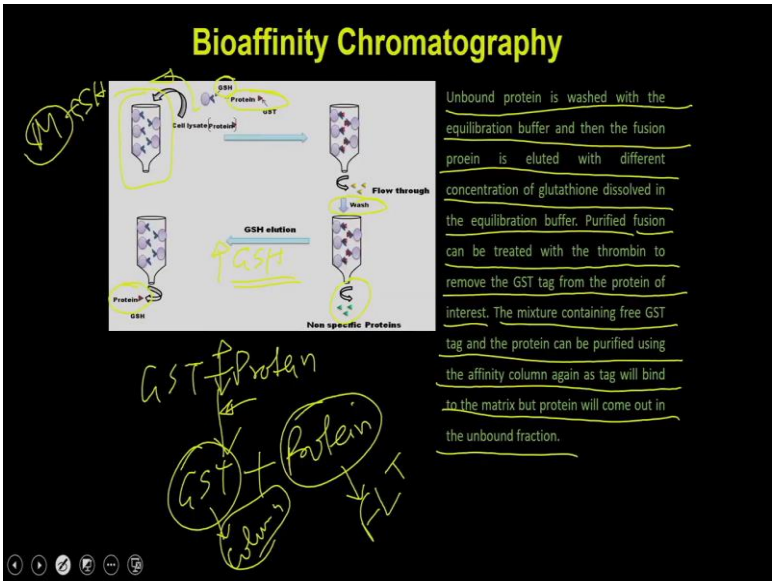

So, this is what you have to generate the column where the matrix is going to have the GSH. And once you have the GSH containing columns, you can pack it and develop the column and then your flow the cell lysate what will happen is containing matrix is going to bind the GST containing proteins and then what you do is you are going to wash this column so that the nonspecific proteins are going to be removed.

And then now what you have is you have only the protein which contains the GST to bind to the matrix and then what you do is you add the GSH which is actually be the substrate and what will happen is there will be a competition between the GSH which is present on to the matrix to bind the protein versus the GSH which is present in the solution. And because the consideration of this GSH is going to be very, very high which is outside.

It is actually going to release the protein and then ultimately you are going to get the GST bound the protein into the eluent. So, the unbound protein is washed with the incubation buffer and then the fusion protein is diluted with the different configuration of glutathione dissolved in the incubation buffer, the purified human protein can be treated with the thrombin to remove the GST tag from the protein of interest.

The matrix containing free GST tag and the protein can be purified using the FET chromatographic column again, as the tag will bind to the matrix, but the protein will come out into bound fraction. So, once you got this GST bound protein, you can be able to utilize d proteases. And that protease is going to cleave off the bond between the GST and the protein and ultimately you are going to get the GST plus protein.

And now, what you do is you again utilize the same GSH column. So, once you do the same thing, the protein because does not contain the GST tag will come out into the flow through And whereas, the GST will go and bind to the column and that is how you can be able to produce the protein which does not contain the GST tag, because sometimes the GST tag is interfering in the negative reactions and sometime because of GST is a very, very big protein it also interfere in the other downstream applications.

For example, if you are interested to utilize this protein for some of the you know developing the vaccines or if you are interested to use this protein in biomedical applications or suppose, you want to utilize this protein for any other applications, then the GST might interfere and those reactions for example, if you are interested to solve the 3 dimensional structure of this protein, then GST is going to interfere.

So, you have to remove the GST simply by adding the protease which is actually going to cleave the bond between the GST and the protein and then you can just simply run the affinity chromatography where the GST will bind and the protein is going to come out from the column in a flow through and you can collect the protein.

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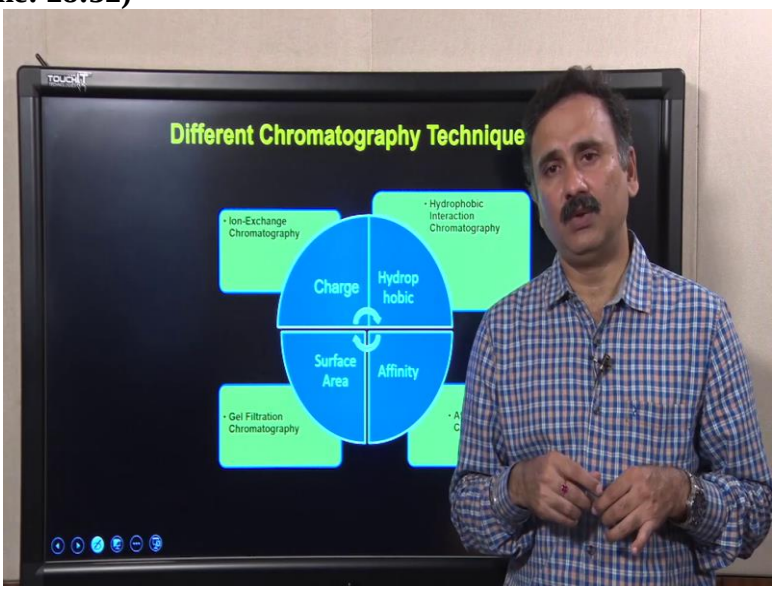

So, with this I would like to conclude our lecture here. Thank you.