Experimental Biotechnology
Prof. Vishal Trivedi
Department of Biosciences and Bioengineering
Indian Institute of Technology - Guwahati

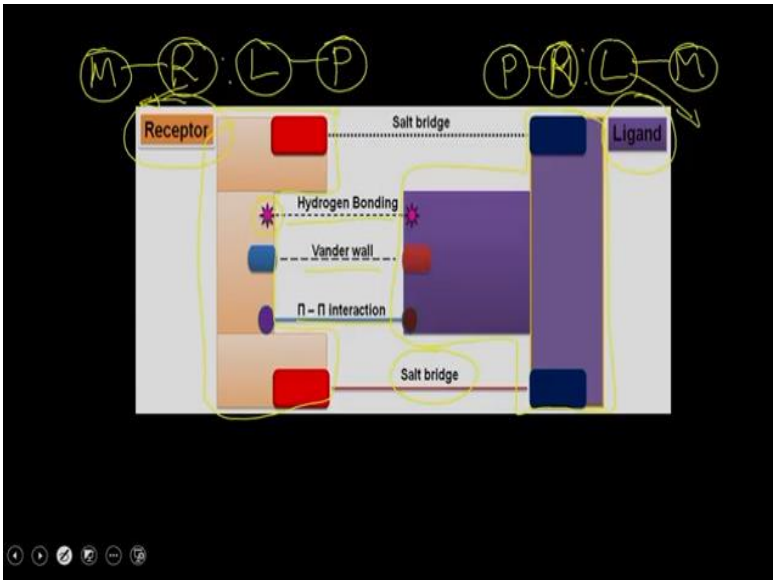
Lecture - 24
Affinity Chromatography - Part - 2

Hello everybody this is Dr. Vishal Trivedi from department of biosensors and bio engineering IIT Guwahati. And in this module we are discussing about the affinity chromatography. And in the previous lecture, we discussed about the basic principle of affinity chromatography, what are the determinants are playing a crucial role by bringing the receptor and the ligand together?

And we have also briefly discuss about the different types of matrix what you can use. So, in a typical matrix what you have is, you have the coupling of the receptor or the ligand the matrix and then you can use the couple the cognate pair for example, in the case of matrix you can couple the ligand or in the case of ligand you can couple the matrix to the protein of your interest and then subsequently you can be able to utilize them into the purification utilizing the affinity chromatography.

So, now, in today's lecture, we are going to discuss about the different aspects related to the generation of the receptor or the ligand and how you can be able to couple them to the matrix with the help of the different types of the chemistry what has been developed to couple the ligand or the matrix to ligand or the receptor to the matrix and how you can be able to utilize them for purifications?

(Refer Slide Time: 02:33)



So, as we can have discussed last time that the receptor or the ligands are actually are the making a pair because the receptor or the ligands are sharing the several interactions for example, you have the first thing is the receptor is having a 3 dimensional conformation which is exactly complimentary to the ligand. So, this ligand is going to fit into this receptor and then you have the other kinds of interactions like the hydrogen bonding vander waal or the pi-pi interaction.

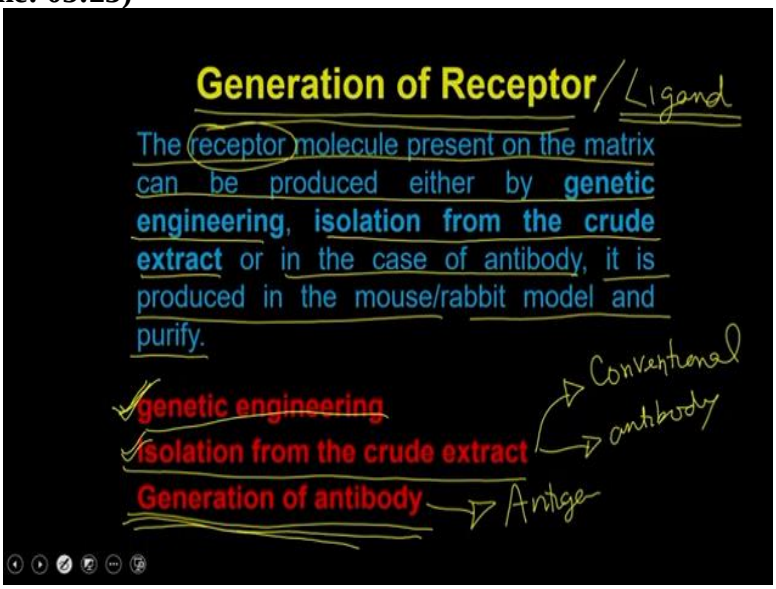
And in the some cases you may have the salt bridges interactions between the receptor and the ligand. And now, if you want to design the affinity chromatography and would like to purify the protein or the analyte of your interest, you have the 2 choices either you can use the receptor or to the ligand to make the pair with the matrix which means you can couple the ligand or the receptor to the matrix and if you use the receptor to be coupled onto the matrix then you are going to produce the protein along with the ligand.

Similarly, if you couple the ligand to the matrix then you are going to generate the protein to the receptor. So, that receptor and ligand are going to make the interactions. Now, the question comes under what conditions you are going to prefer to use the ligand or the matrix? The sole purpose of any chromatography technique is to make the purification as easy as possible as economically viable which means it is should not be very, very costly to perform and the other parameter is that it should not require the extensive infrastructures.

So, in many cases, whether you use the receptor or the ligand depends on the considering these parameters. So, in few cases you are going to couple to the ligand or in few cases you

are going to couple to the matrix the receptor to the matrix. Now, let us discuss how you can be able to generate the receptor or the ligand, and how you can couple them to the matrix? So, that you can be able to utilize them into the different types of chromatographic techniques; for example, whether it is a bio affinity chromatography or the pseudo affinity chromatography.

(Refer Slide Time: 05:23)



So, generation of the receptor, although we are saying the generation of the receptor, but it is more or less like the receptor or the ligand which is very, very the same thing actually. So, the receptor molecule present onto the matrix can be produced either by the genetic engineering, isolation from the crude extract or in the case of antibodies, it is produced in the mouse or the rabbit model and purify.

So, you have the 3 options to generate the receptor or the ligands, you have the genetic engineering, so, you can actually genetically clone the gene which is responsible for a particular type of receptor and that is how you can actually and then subsequently you can purify that receptor by using the recombinant DNA technology, you can put some tag or some other kind of features and then you can be able to purify the receptors using the other chromatography techniques.

The other option is that, if the protein is not or the gene is very difficult to clone or it is not been isolated so, far, but the protein is available, then you can be able to isolate the protein from the crude extract that can be done simply by either using the conventional chromatography techniques like ion exchange chromatography or gel filtration

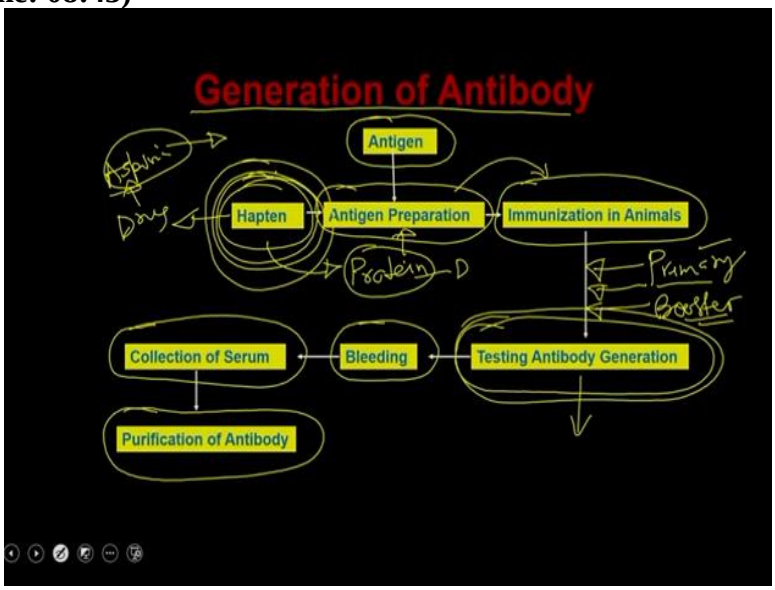
chromatography or hydrophobic interaction chromatography or that can also be done by the antibodies, which means, suppose I want to make the, you know insulin receptor or I do want to isolate the insulin receptor.

So, I have 2 options either I will break open the cells, I will prepare the lysate which is actually going to contain the insulin receptor, and then I will go with the fractional you know fractionation of that particular lysate with the help of the different types of chromatography techniques, ion exchange chromatography, hydrophobic interaction chromatography, gel filtration chromatography, and then ultimately I am going to get the pure insulin receptor.

The second option is that I can just use the antibodies, which is directed against the insulin receptor, and then I can just take out the insulin receptor from the crude mixture. And that is how I can get the receptor and that can be used for subsequently to for the other affinity chromatographic techniques for example, I can use the insulin receptor to simply purify the insulin from the blood. The third is that you were the places where you are actually going to use the antibodies.

So, antibody is actually making a pair with antigen. So, you can be able to generate the antibodies which is maybe against any receptor or ligand or you know, so, it can be generated against the antigen see, the recombinant DNA technology, the genetic engineering and the isolation is already what we have discussed or the genetic engineering is what we have discussed in other courses. So, we are going to discuss about the generation of the antibody.

(Refer Slide Time: 08:43)



Now, the generation of antibody is a multi step process, where you are first thing what you require is the antigen. So, antigen is the is a proteinaceous substance which is actually be present which is the external agents or it is something which is going to cause the immune response into the particular animal or the animal. So, antigen could be immunogenic or the non immunogenic so the antigens, which are non immunogenic are called as the hapten.

So, in those cases where you are working with the antigen, which are coming into the category of hapten for example, the drug molecules, so, if you are trying to generate the antibodies against the for example, the aspirin. So, if you are trying to generate the antibody against the aspirin or the chloroquinone or artemisinin or any other paracetamol, in those cases, the antibody is not going to be developed or these molecules are non immunogenic or they are not going to cause any immune response into the animal.

So because of that they are fall under the category of the hapten. So, in those cases, what you are going to do is you are actually going to convert a hapten into a immunogen simply by coupling that to a protein and because of that the hapten is also going to be convert into a immunogenic molecule and then that complex can be used as antigen and that complex can be prepared or can be used for immunize the animals, irrespective of whether you are working with the hapten which has been converted by coupling it to a protein to antigen.

Or you have the natural antigens, which are the protein of very, very high molecular weight then process these antigens, so, that they will be good enough to you know immunize the animals. So, we will discuss that what are the different procedures or processes you have to do when you want to process the antigens, so, that it will be ready for the immunizations and then you are going to immunize the animals with the help with these antigenic preparations.

And once the animal is going to be immunized, it is going to be immunized 2 times, one is the you are going to put the primary injections and then you are going to give the booster injections and that actually is going to create or develop the antibodies into the animal, once you antibodies are been developed, you can be able to collect the blood of the animal and then from the blood you can be able to collect the serum.

And then from the serum you can be able to purify the antigen or purify the antibodies at this stage where you are actually going to see the antibodies in the blood you can be able to detect

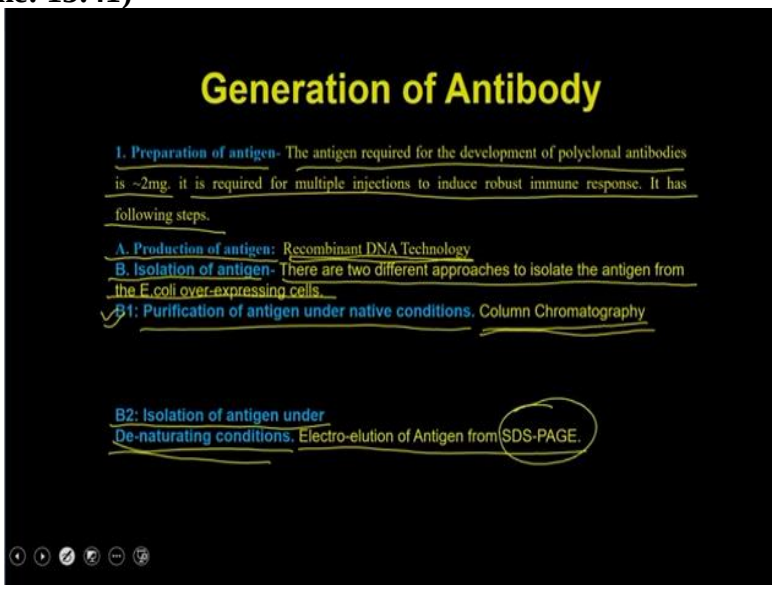
these antibodies because then only you will be deciding whether I should give the additional booster dose or whether I should just go ahead with the this particular level of antibodies in the blood and start collecting the blood.

So that is the whole scheme through which you can be able to generate the antibodies in the animal. So, let us summarize this first you have to do is you have to prepare the antigen. So, whether the antigen is the hapten or the proper antigen, which is like bigger proteins, if it is a hapten you have to couple that to the protein so that it will become the protein like protein drug complex and that is also going to be processed for the antigenic preparations.

So, that it will be ready to be a given for the injections into the animal. And once you are going to immunize the animals, you are going to immunize the animal 2 times, one is the primary injections and then the secondary injections with the help of the primary and the secondary injections, you are going to generate the antibodies, then you are going to test the antibodies with the help of the ELISA.

And once you see that the antibodies are being developed with the help of the ELIZA, then you are going to collect the large quantity of the blood from the animal and subsequently you are going to collect the serum from the blood and that blood serum is going to contain the antibodies. So you can be able to purify the, your antibodies or the antibody which you have developed against this antigen and that you can use subsequently into the affinity chromatography. So, let us discuss these procedures in detail.

(Refer Slide Time: 13:41)



So, the first is the preparation of the antigen. So, the antigen required for the development of polyclonal antibody is approximately 2 milligram it is required for the multiple injection to induced the robust immune response and the preparation of antigen has the following steps. The first is that you have to generate or the produce the antigens. So, do you have the 2 options 1, you can use the recombinant DNA technology which means you can actually be able to clone the antigens.

And that is how you can be able to produce the antigen in large quantities and purify. The second option is that you can be able to isolate the antigens. So, there are 2 different approaches to isolate the antigen from the E.coli overexpressing cells. For example, you can purify the antigen under the native conditions. So, even if the antigen is been produced by the recombinant DNA technology, the isolation of the antigen is having the 2 options.

One is that the purification of the antigen under the native conditions were what you are going to do is you are going to overexpress the antigen into the E.coli expressing cells and then you can be able to perform the different types of column chromatography to purify the antigens, so, under these conditions the antigen is going to be functionally active and that can be used for immunizations.

The second is that the isolation of antigen under denaturing conditions, which means, so, there are that what happened is in some cases the antigen what you are overexpressing the E.coli is not soluble, which means it is not present in the supernatant and because of that, this antigen cannot be purified utilizing the conventional chromatographic techniques. So, in those cases you are going to isolate the antigen under the denaturing conditions.

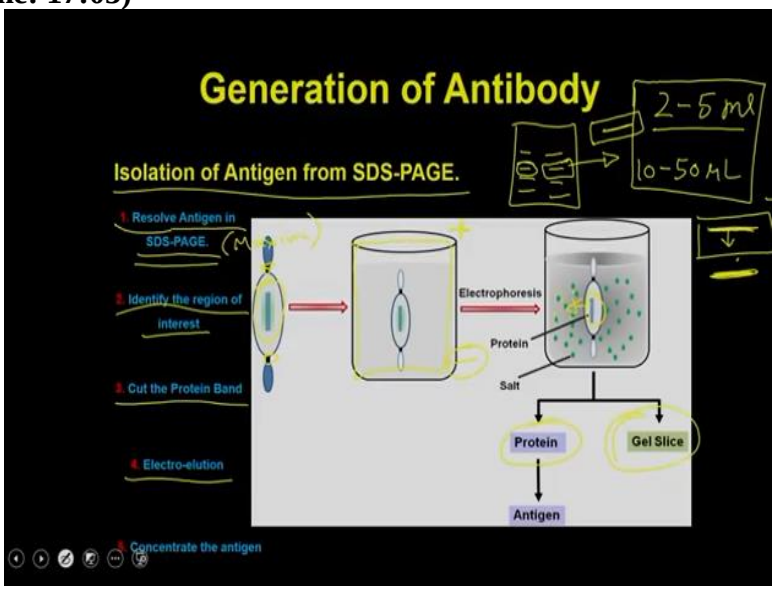
The second is sometimes the antigen is very, very produced in a very, very small quantities. So, if you use the conventional chromatography you are actually going to lose the protein at every step for example, I think we have discussed that in the previous lecture that if even if you use a conventional chromatography the you might lose more than 50% protein. So, if your protein production in the E.coli cells is very, very low.

And you are using the conventional chromatographic techniques, the overall yield is going to be so low that you will not be able to get the enough quantity of the antigen to immunize the animals. So, under any of these conditions, you can be able to purify the antigen under the

denaturing conditions, because, even if you have a protein which is present in the native conditions or the denaturing conditions, the overall immune response does not vary, because even if you have a protein under the native condition.

It is going to be denatured while you are going to process it for the injections, so, it does not it hardly matters. So, in the isolation of antigen under the denaturing conditions, what you are going to do is you are going to do the electro elutions of the antigen from the SDS page. So, let us see how you can be able to isolate the antigens from under the denaturing conditions using the electro elutions.

(Refer Slide Time: 17:03)



So, isolation of antigens from the SDS has the multiple steps, the first is that you are going to get the antigens overexpressing cells, then you are going to prepare the lysate and then these lysate has to be resolved onto the SDS page. So, in this case, you are not going to use a mini SDS gel for you are going to use is a very large SDS page. So that you will be able to load somewhere around 2 to 5 ml of lysate normally, in a typical SDS page, what you load is somewhere around 10 to 50 microliters.

Whereas in this case, we are going to load 2 to 5 ml of lysate. So, it is going to be a big gel and so you have to run a maxiprep gel. So, this is going to be on our maxiprep gel, and then what you have to do is you have to identify the region of your interest, which means if you run the SDS page, you are going to get different types of bands and then you have to identify the region where your antigen of interest is present. And then what you have to do is you have to cut this region and bring out the protein band.

So then you have to cut the protein bands. Once you got the protein band which is present in the gel slice, you will be able to do the electro elutions and in the electro elution, what you are going to do is you are going to take this gel block put it into a dialysis bag and then you put it into a beaker and the, in the beaker you are going to add or you are going to perform the electrophoresis. So, you are going to connect both the positive and negative electrode across this beaker.

So, what will happen is that the, because you are going to do the electrophoresis the protein band is going to start migrating from the gel block. So, you can imagine that even if I have a protein block into the gel block, and if I put the electrophoresis which means I am going to put the negative on top and positive onto the bottom, this protein band which is negatively charged, because it has the bound SDS is start going to migrating and after some time the protein is going to come out into the solution.

So it will migrate and then eventually is going to fall into the outside buffer and then what you do is you just collect the outside buffer and that outside buffer is going to have the proteins whereas the gel slice is going to be present inside the dialysis bag and then from here you can be able to just concentrate and collect the antigens. So this is very, very simple procedure and this is very easy to perform and it is very, very you know.

So, it is very routinely being used to isolate the antigens which are very difficult to overexpress or which are not soluble or all other kinds of conditions what we have discussed. So, to explain you this procedure more in details, I would like to take you to my laboratory and then I will show you a demo, where we are going to perform these steps and so, that it will become more easy for you to follow these steps in your laboratory and you will be able to isolate the antigens under the denaturing conditions.

Today, we are going to give you a demo about the electro elution. So, electric elution is a technique through which you can be able to isolate the antigen from SDS page. So, you can see this is a typical SDS page through which you are interested to isolate this particular band in ideal situations, you are supposed to not stain these gels, but since we are just showing it for the demo purposes, we are going to show you with this particular gel.

So, before you start the experiment for the electro elutions, what you require is you require SDS page where you might have dissolved your sample or the protein you require the blade so, that you will be able to cut the your protein of your interest, then you require a dialysis membrane you require a beaker full of water and then you also require the rubber bands so, that you can be able to tie up the dialysis membrane. So, let us start the, this gel elution electro elution experiments.

So, in the step 1 what we have to do is we have to first see that the, what is the band what you have to isolate? So, for that purpose what you have to do is you have to first check the protein is the site of the protein or the antigen where the site is present. So, for example, in this I have we have run the purified protein and this is a major band what we have to isolate. So, what you have to do is you have to you know cut this particular region of that SDS page.

So, what you can do is simply go with the lane for example, if I want to run isolate this particular protein band, so, what I will do is I will just first cut the SDS page from the side like this and then I can just cut from the top and bottom and then your you can be able to remove this particular gel block and that should be good enough for a eluting this particular protein or this particular antigen if you are interested to isolate the protein for a very, very large concentrations.

Then in that case, what you can do is you can simply merge all these wells and you can run a very large quantity of the protein. So, once you have cut the block and you know your block is ready for the electro elutions, then the in the second step what you have to do is you have to prepare your dialysis membrane. So, this is a typical dialysis membrane what we use in the laboratories. So, what you see is it is actually a plastic kind of thing. So, it is not a plastic but it is look like as.

So, it has 2 folds actually are the 2 membrane 2 slips actually, but they are you know, stick to each other. So, before you start what you have to do is you have to just cut the dialysis membrane and what you can see is that it is actually stick to each other. So the both the ends are stick to each other. So, to get this dialysis membrane to be work in a working conditions what you have to do is you have to put this dialysis membrane into a beaker and then you fill this beaker with a water.

And then you first put this into microwave so that it can warm up. So while this is this dialysis membrane is going to be warm up what happened is that the 2 sheets of the this particular dialysis bag is actually going to be removed from each other because when the water is going to boil, it is actually going to remove the surfaces what has been used to adhere these 2 membranes and as a result.

It is actually going to give you a bag in which you can be able to place your dialysis your as the band but we have just got from the SDS page and then we can be able to do the electro elution. So, now, what you see is that I have boiled the dialysis membrane and now dialysis membrane is you know, all the both the layer of the dialysis membrane is been removed. Now, what you can see is that the bag is ready for putting the, you know the membrane or this SDS block.

So, what you can do is you can simply open this and then you place this block inside the membrane inside this pack very carefully so, that you should not damage the protein and once this has been put inside then what you can do is you can just put the rubber band from both the ends or you can just put the rubber band on this side and you have to be very careful that it should not be you know leaky or should not cause any problems.

So, when you put the rubber band on one side and then you put the rubber band on the other side and in between what you can do is you can just simply fill a small amount of the buffer into this so, that your protein will be in the water instead of and by doing so, you can also check that there is no leakage actually, and because it there will be a leakage protein is going to come out from here instead of going via the electro elutions.

So, you can just add you know, 1 or 2 ml of your buffer and that should be good enough and then you can do is what you can do, you can close it from the top as well with the next rubber. So, if you are want you can even use the you know, you can use the dialysis clips as well. So, that you know, but if you can use even the thread as well for the purposes only you know, the you can just close this so, that you know you can make a bag like this.

Now, you can see that, you know band is inside. And what I will do is now what you have to do is you have to just submerge this into a horizontal gel upgraters, fill it with the buffer, and it does not matter what buffer you use, but it should be conductive. So that the current should

flow and then what you can do is you can just close this and connect it to the cathode and anode. So, you will connect to the black and the red, and now you just run it on 100 volt.

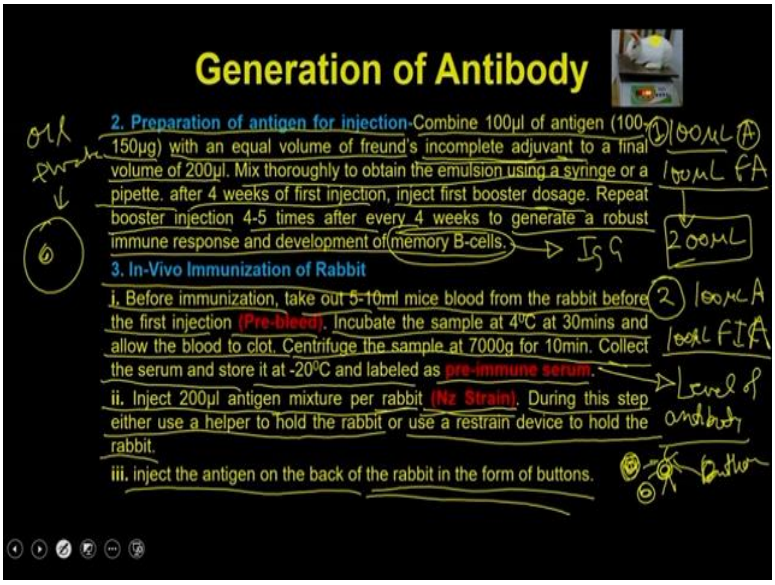
So, you can just run it on a 80 to 100 volts. And what we will see is now that the electrophoresis is going on, and when the electrophoresis will be going on what will happen is that this protein band is actually going to start travelling into this particular SDS page. So, because we have cut the SDS page, at this end, the protein band will run into this direction, but after this actually, there is no gel actually.

So, what happened is the protein is actually going to fall outside the gel and because outside a we have already covered it with the dialysis membrane, so it will actually fall into that particular liquid what is present into the dialysis membrane and now your protein or the antigen is going to be present into the that particular solutions. So, now we are going to continue with this electro elution or this electrophoresis for at least for another 1 or 2 hours, and then we are going to see whether there will be you know, electro elutions or not.

And then after that, what we are going to do we are going to remove the rubber bands, we are going to open the dialysis membrane from one end, we are going to collect the supernatant. And then you can just concentrate that supernatant. And it is actually going to give you the protein of your interest. So, this is all the few steps what you have to do if you want to do the electro elutions to isolate the antigen in a large quantity under the denaturing conditions from SDS page for the production of antibodies.

So I hope you might have understood all the steps and it could be helpful for you to advance your work. With this demo, I hope you might have learned how to electro elute the protein bands from the gel slice and you will be able to utilize this demo to extend your work or to perform the additional experiments related to this. Now let us move on to the next aspect.

(Refer Slide Time: 29:56)



So once you have prepared the end or produced the antigen in the large quantity then you have to prepare the antigens for the injections. So the preparation of the antigen for the injections what you are going to do is you are going to combine the 100 microliters of antigen where you are going to have the 100 to 150 micrograms of protein with an equal amount of freund's incomplete, freund's complete adjuvant to a final volume of 200 microliter what you have to do is you have to take the 100 microliter of the antigen.

And 100 microliters of the freund's adjuvant and you have to take the freund's complete adjuvants, then it is actually going to be 200 microliters and these 200 microliter you have to mix thoroughly to obtain an emulsion using a syringe or the pipette. So, once you mix them, the freund's adjuvant actually contains the detergent and it actually contains the oil and it also contains the mycobacterium tuberculosis cell wall.

So, freund's adjuvant is actually going to stimulate the immune response and it is actually going to activate the system so, that the system is ready to take up the antigen. But the since you are making the emulsion where the oil and the water is going to mix together and because of that the antigen is going to be trapped into the small vesicles. So, what happen is when you take the oil and you will mix it with the water, it is actually going to make the emulsions where your antigen is going to be trapped within this emulsion.

So, because of that, the antigen is going to be released very slowly from the site of action and that is the purpose by for which you are actually producing this emulsion. So, that is the antigen should be keep immunizing your animal for a very, very long time, because what

happened is if the antigen is going to be cleared from the side of your injection very soon, then you are not going to challenge the animal for a very, very long time.

So, that antigenic response will not go to the very large chunk of the B cell or T cell it was going to spread to very small population of the B cell and T cell and because of that, the overall immune response is going to be very, very less and why we are adding the mycobacterium tuberculosis cell wall because the mycobacterium tuberculosis cell wall is very immunogenic.

So, when you inject the primary injections, where you have the freund's adjuvant and which actually contains the mycobacterium tuberculosis cell wall, the cell wall is actually going to activate the system so, that the large amount of the immune machinery is going to be present at the site of injections after 4 weeks of your first injection, which means the primary injections you inject the first booster dose.

So, in the booster dose, what you are going to do is you are going to again prepare the antigen in the same way except that this time you are going to use the incomplete adjuvant instead of the complete adjuvant. So, in the first round, you are going to use the 100 microliters of your antigen and 100 microliters of freund's complete adjuvant; whereas, when you are going to do the booster injections, you are going to use the 100 microliters of antigens.

And 100 microliters of freund's incomplete adjuvants what is mean by the freund's incomplete adjuvant is that the freund's incomplete adjuvant does not contains the mycobacterium tuberculosis because now, after 4 weeks the machinary is already been under activated state the site of your antigen action injections is already is having the lot of cellular machineries.

So, now, what you do is you replace the antigen, the mycobacterium antigen and you inject your own antigens against which the immune response is now going to be developed. So as a result, in the secondary injections, the antibodies are going to be developed against your own antigen. Now repeat the booster injections 4 to 5 times after every 4 weeks to generate a robust immune response and a development of the memory B cells which are actually going to generate the antibodies.

Now what you have to do is before you do the injections, you have to you do some procedures so in vivo immunization of rabbits before immunizations, take out 5 to 10 ml of them the rabbit blood from the rabbit before first injections and that is called as the pre bleed which means you are going to take out the blood from the mouse or the rabbit and that is called as the pre bleed incubate the sample at 4 degree at 30 minutes and allow the blood to clot centrifuge the sample at 7000 g for 10 minutes.

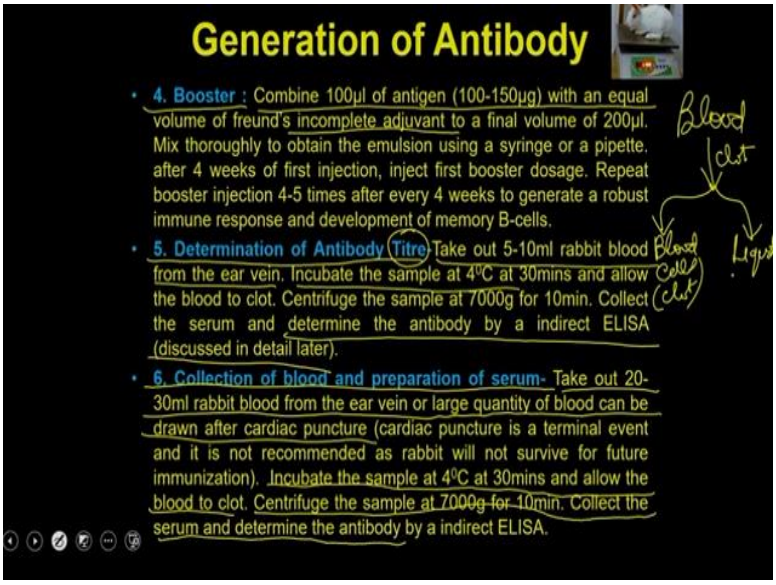
And you can be able to collect the serum and store it at minus 20 and this serum what you are going to collect before immunization is called as the pre immune serum; the pre immune serum is actually going to tell you the level of antibodies, which are already been present in the rabbit which means before the immunization which means the antibodies which are been present in the rabbit against other antigens, not the antigen of your interest.

After this the whatever the injections you have prepared either in the complete adjuvant or the incomplete adjuvant into the rabbit, which is the Nz strain cogent strain which is the New Zealand strain. So, and during this step, either use a helper to hold the rabbit or use a restrained device to hold a rabbit. So, you have to you know you have to hold the rabbits very strongly so, that you will be able to inject the emulsions, either the primary injections or the secondary injections.

So, you have the 2 choices either you ask someone to help you and hold the rabbits or you can use the restraint devices where you have to just keep the rabbits and then rabbit will not be able to move and then you will be able to inject the antigen on the backside of the rabbit in the form of the button which means if you if this is a rabbit, you have to inject somewhere on the backside of the river, the rabbit because this is a site where you have enough space and enough area which so that it will not be a problem.

And then you have to inject it in the form of button which means you have to inject at 1 site make it a button like this and then you go to some other site you inject then you go to other site. So, all these are called as buttons actually and that actually is required because the antigen is now going to you know release from these buttons on a very, very slow mode. And as a result, it is actually going to cause the robust immune response and the large production of antibodies.

(Refer Slide Time: 37:34)



Now, once your primary injection is over, then you can do a booster injections, the procedure is almost the same except that you are going to use the incomplete adjuvant versus the complete adjuvants. Once you are done with the booster injections, then you can determine the antibody titer what is mean by titer is the amount or the level of antibodies against the antigen of your interest.

So, in that case, you take out the 5 to 10 ml of rabbit blood from the ear of ear vein of the rabbit incubate the samples at 4 degree and then you collect the serum and you determine the antibody by indirect ELISA. So indirect ELIZA anyway we are going to discuss in a later module when we are going to take up the techniques related to the immunology. So, that time we are going to discuss about the indirect ELIZA and we are also going to show you how to perform the indirect ELIZA as well.

After that, once you are sure that the antibody titer is very high, you can be able to collect the blood and prepare and you can prepare the serum for that you take out 20 to 30 ml of the rabbit blood from the ear vein or the large quantity of blood can be drawn after the cardiac puncture. So you can have the 2 choices either you draw the blood from the ear when which is actually not going to be a terminal procedure.

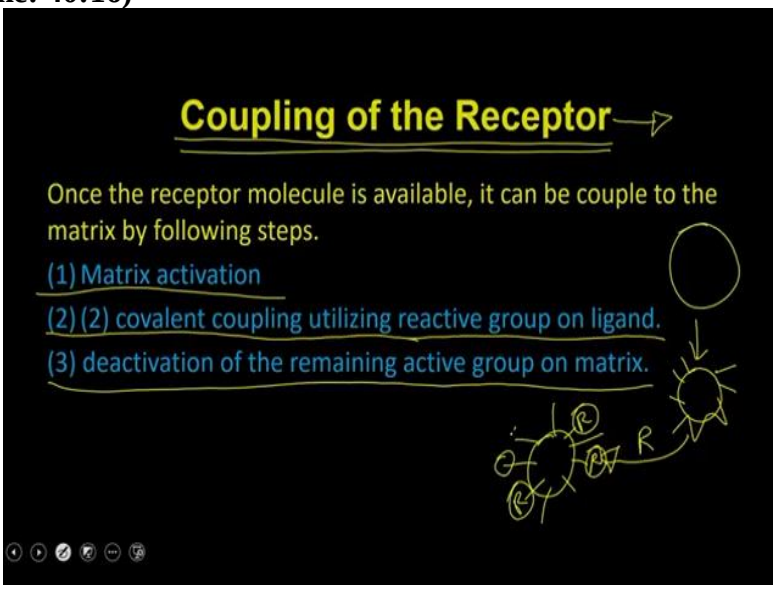
But if you are looking for a very large quantity and you are sure that the experiment is now being over and you do not need any more antibodies, then you can be able to collect the blood from the cardiac puncture, which means you can just make a hole into the heart and

that is how you can be able to collect very large quantity of the blood from the rabbit but that is a terminal procedure.

After that, it is the rabbit is not going to survive and it is going to die, irrespective of the locations then you have to incubate the sample at 4 degrees for 30 minutes and allow the blood to clot the centrifuge the sample at 7000 g for 10 minutes and then you can be able to collect because once you collect the blood and you allow them to clot so once the blood is going to be go for the clotting reactions you are going to get 2 fractions, 1 fraction where you are going to get the blood cells which is actually going to form the clot.

And the second place where you are going to get the liquid part which means that is the serum. So, that is a yellow color serum you are going to get after the clotting reactions.

(Refer Slide Time: 40:16)



Now, once you have produced the antibodies into the rabbit, and as we have discussed all the theoretical aspect related to this particular technique, I thought of, you know, showing you how to produce the antibodies in a when you go to the animals or when you go to animal house and try to do yourself. So for this purpose, I went to the central drug research institute, and then I took the help of the institute because we do not have, you know, the animal house.

So that is how we have taken the help of the other institutes and I went there and then I have while they because they were doing dissemination and the antibody production steps. So I got the chance and then I actually made a demo video. So that where the scientists are actually explaining each and every step what we have discussed. So just now and that actually is

going to helpful for you to understand how the antibody is been produced inside the lab inside the animal house and how you can be able to immunize the animals.

And I hope that the demo video what is going to be helpful for you to understand the practical aspect of the antibody generation as well.

(Video Starts: 41:44)

I am Amogh Anant Sahasrabuddhe I work in CSIR CDRI, Lucknow and in today's demo, we will be discussing different steps involved in generation of antibodies in rabbits. So, further the first step we prepare several things like freund's complete adjuvant there it is from sigma, 3 micro emulsifying needles which has 2 capillaries with a fine needle we need antigen which is purified and filtered.

So they are no contaminations in crystalline solution of antigen then we take out some of the freund's complete adjuvant in an eppendorf and then mix them together. Since 1 since the this adjuvant is oil based it does not mix easily with a watery system like antigen is in PBS. So, therefore, we mix them rigorously, rigorously enforced for that purpose we take these 2 mix this emulsion and we use this PBS and PBS containing antigen and the adjuvant of mixing them with take out in a needle using a needle we take out in a syringe like this.

And then we fix the micro emulsifying needles into it, attach another syringe into it like this. So once you have filled your antigen and the adjuvant in this needle, push it here and then you keep pushing from one side. Keep pulling from another side. Keep pushing from one side and keep pulling from another side. So this process forcefully pushes your material among the all and the antigen to this fine needle and with that, in that process the emulsion is found emulsion can be called as water in oil or oil in water.

Because both are in the same concentration same volumes. So, you can call them in the so, it is that emulsion by this method the emulsion is formed. So, for any reference we have already prepared very much this emulsion looks like white initially it was 2 phase and then slowly it has turned into single phase, now, we can push this emulsion from one side into the another side and from another this side used to another syringe. So, this process creates very good emulsion this present separate out later, then we are ready to inject.

So, how do we check so, for checking purposes we drop one of this emulsion drop, a drop of emulsion on the water surface like this if the solution is not formed perfectly this will spread out, otherwise it will not spread so, this is a check that your emulsion is formed correctly. So, once you find that this drop is now spreading, your emulsion is actually ready for injecting, so this was the process by which you prepare the emulsion.

So this is the first step of preparing an emulsion. So, now, let us understand why we prepare the emulsion we have checked whether is emulsion is formed emulsion, now the purpose of making the emulsion because we have antigen, antigen 2 antigen you can raise antibodies, but after emulsifying them you actually make the antigen releases slowly. So, it is a systemically is kind of progression.

So, that the antigen is exposed to system in a systematic manner so, that more and more memory cells antigen is generated and that is the sole purpose of having too much otherwise if you inject antigen as if in PVS or in other working system, it will be spread out in the body and it will be cleared out by the immune system readily and no memory cells will be generated, so these are this the main purpose of preparing the emulsion.

So, now we have prepared the emulsion we have come to the animal house, this is our rabbit which will be immunized and before immunization we have to take pre immune bleed so, that we can compare later the serum and the anti serum so, now we will start how to immunize it. So, now we are preparing to immunize first important thing is in all this animal processes is we have to avoid the pain to the animal, so, for that purpose we strain, we have to strain the animal we have to inject so we strained there even in very it has aspirin.

And other one is oxovirus. So we will inject this into the emulsion into the thigh catch hold of both the legs, we need to sterilize the area using alcohol, we have to look at the thigh muscles, that should be cleaned up, cleanly visible should be cleanly visible, there are 2 kind of injection that we have then is intravenal and another one is subcutenous so we will be doing the subcutaneous injection.

This is our emulsion that we have prepared by micro emulsifying needle we have seen earlier this is the area where we would like to inject we have to take out all the air from syringe and the needle, we have taken out clean the area again and the apply some antiseptic powder here

it is betadine powder so, that the infection can develop later on just sprinkle some of it at the area of injection and then slowly release, leave the animal relaxed and it is immunized.

So take the animal in a towel or a this kind of cloth so the advantage of using this clothes for strain is the animal has its claw inside outside of this loop and then not move so, we have to restrict the movement very similar in the strain and on this cloth. Now strain the animal on this cloth, you keep the animal relaxed on this like animal straining make sure that the ear are the outside and the animal is strained properly so as to reduce its movement.

And now it is ready to bleed, we will bleed the animal on this mid ear vein ear vein and the rabbit it gets infented and the circulation is faster the vein will also expand and more flow will be there, this is the method which is normally applied, when the vein is clearly visible see this mid ear vein is big and then slightly sterilize it using alcohol and using a anti gauze needle which is big enough to give enough bleed we will prick the vein and collect the blood then stop it with this, this much blood we can collect in the bleed like this.

Now make sure there is further bleeding occurs and we will wipe out further bleeding outside using sterile water then wipe out all the blood here and then and keep wiped out all over the ear so that the vein becomes cool and gets shrunk, then check still it is bleeding keep keep it pushed until the bleeding stops. So now now I think the blood has stopped coming and now we apply some antibiotic whereas in this case it is glutamine powder so there is no further infections or inflammation in the rabbit and it also shows that this is no pain.

Now, if there is inflammation it will show some pain so it will avoid conductovers we have isolated possibly 10 to 12 ml of blood this will be reduced to half of the volume the blood the serum this is cogulated at 37 degrees for 1 hour and then we can over night so that the clot is shrunken and the serum is maximally taken off and then to this serum add preservative like sodium azide and keep under minus 20 or minus 80 as per your climate and we also test some intensity the titer of it and specificity of it using lysate, the animal is very relaxed.

It has it is very important in module that whatever the procedure, animal should be ensured not to have pain then if it has pain you can understand and we have prepared the emulsion, we injected the emulsion, we isolated the blood after giving sufficient booster doses and process and you liked this video.

(Video Ends: 56:58)

So, this demo video is probably be very helpful for you to understand the each and every aspect especially the practical aspect related to the antibody productions. Then let us move on to the next aspect that is the coupling of the receptor. So, once you produce the receptor either by the recombinant DNA technology or to isolate the antigen, the receptor from the crude mixture using the conventional chromatography or you have produced the antibodies by the, from the animals you have to perform the coupling of these receptor to the matrix.

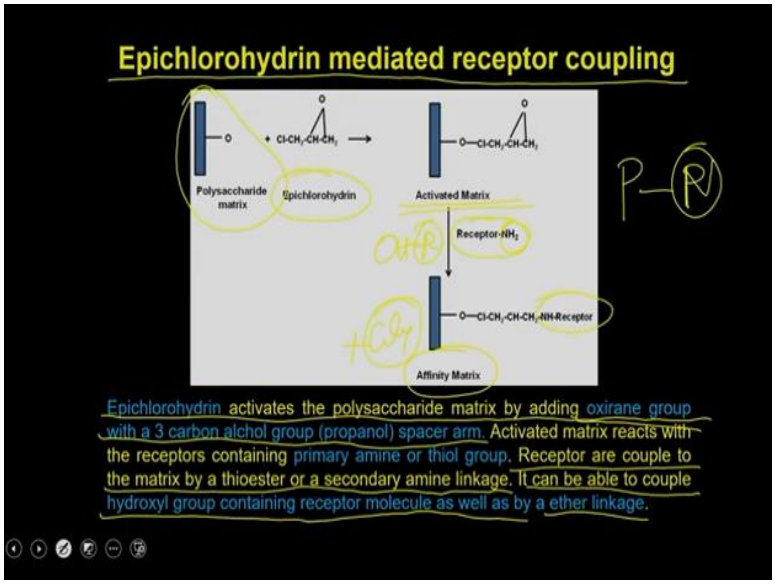
So, in the coupling reactions, you have the 3 steps, 1 you are going to first activate the matrix, then you are going to use the coupling reactions you are going to do a coupling reactions with the help of the reactive groups present onto the ligands and then you have to deactivate the remaining active groups which are present on the matrix which means, first you have to take the matrix you treat it with the chemical.

So, that it is going to be activated so it is going to be activated, which means you are going to develop some functional groups, then you are going to add the proteins or the receptor whatever; so, you add going to add the receptors, what will happen? It is actually going to take up all the receptors, what you are going to add? And that is going to be coupled, but it actually has more number of functional groups compared to the number of receptors, what is going to be coupled on to this particular matrix?

So, what you have to do is you have to inactivate these functional groups, so, that it should not take up additional molecules whatever is present in the crude mixture, because if that happens, then you are actually going to couple the 2 particular different types of molecules. One is receptor the other is the nonspecific molecule. So, because of that, you have to block these sites or you have to block these groups some way.

So, that it should not bring the additional protein molecules or additional molecules to facilitate the nonspecific binding of the antigens or other kinds of molecules, because, if that happens the affinity chromatography will not going to be very, very specific.

(Refer Slide Time: 59:32)

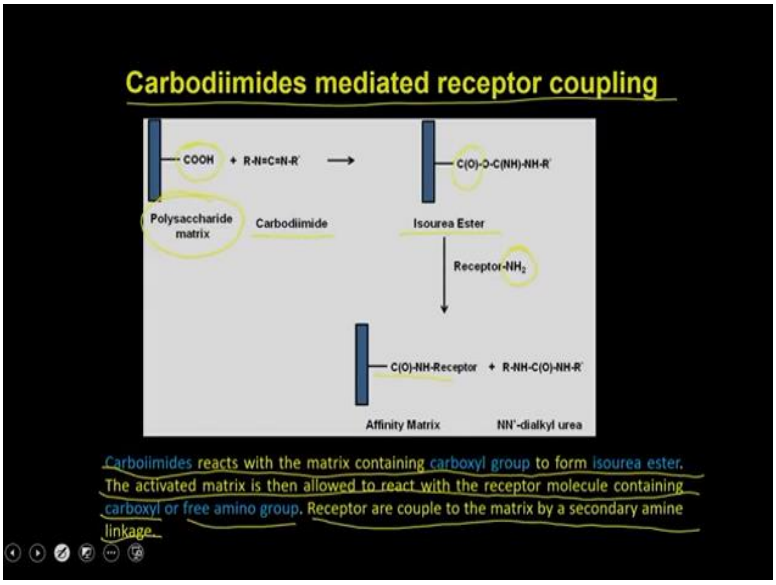


So, there are multiple ways in which you can be able to couple the receptor to the matrix. The first method is the epichlorohydrin mediated receptor coupling. So, epichlorohydrin activates the polysaccharide matrix by adding the oxirane group with a 3 carbon alcohol group, spacer arm. So, what you have is you have the polysaccharide matrix then you incubate it with epichlorohydrin and that actually is going to generate the activated matrix and this activated matrix is having the oxirane group.

Now, what you do is you add the receptor which contains the primary amine which means protein which is actually going to contains the NH group. Now, the receptors are coupled to the matrix by a thioester or the secondary amine linkage, it can be able to couple the hydroxyl group containing receptor molecule as well by a ether linkage. So, you have 2 options, either you use the receptor which contains the OH or you can use the receptor which contains the primary amines or the secondary amines.

And that actually is going to couples to the matrix which means you are going to get the coupling of the receptor to the matrix and then ultimately once your coupling is over, then you can add the large quantity of the glycine because the glycine is also having the amine groups. So, glycine is going to inactivate all the activated groups which are present onto the matrix which means all these groups are going to be blocked by the glycine and that is how you will be able to inactivate all the functional groups and that is how you will going to avoid the nonspecific binding.

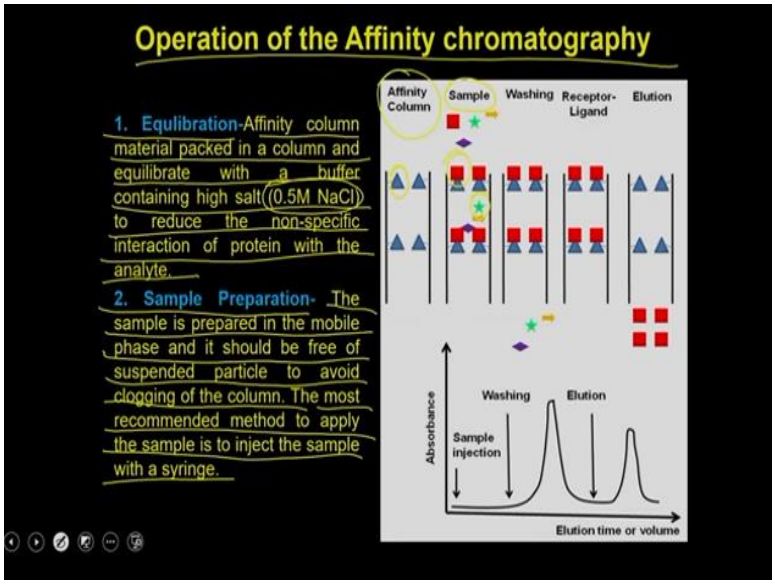
(Refer Slide Time: 1:01:30)



The second is the carbodiimides mediated receptor coupling. So, in the carbodiimides mediated receptor coupling, the carbodiimides reacts with the matrix containing the carboxyl group to form the isourea ester. The activated matrix is then allowed to react with the receptor molecule containing carboxyl or the free amino group receptors are coupled to the matrix by the secondary amine linkage, which means, the you take the polysaccharide matrix which means you can take the dextran or saccharide matrix you, which is actually going to contain the carboxyl group.

And then you add the carbodiimides; the carbodiimides is going to activate and generate the isourea esters. And then if you have the primary amine, the primary amine is going to react to this carbonyl group. And that is how it is actually going to form the amide linkage. And that is how the, your receptor is going to be coupled to the matrix and this is going to be released from the dialkyl urea is going to be released from the matrix. Now, this after this is done again here also you are going to incubate the matrix with the large quantity of glycine to inactivate the additional functional groups.

(Refer Slide Time: 1:02:54)



Now, let us discuss about the operation of affinity chromatography and how and what are the different steps are present. So, operation of an affinity chromatography, the first step is the equilibration affinity chromatography material is packed in a column and equilibrate with a buffer containing high salt which means, around the 0.5 molar NaCl to reduce the nonspecific interaction of a protein with the analyte.

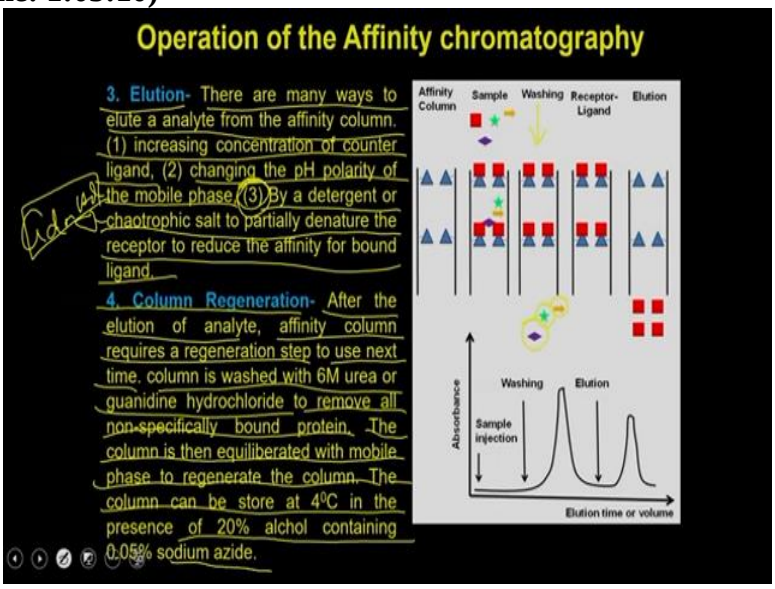
So, if you remember, compared to all other techniques, the affinity chromatography is the only technique where you are going to have the very high salt concentrations. So, that you should not you should avoid the interaction of the analytes to the matrix by the nonspecific interaction because the matrix is also made up of sugar. So, it also can provide the functional groups which were the protein other protein may also can interact by the isoelectric interactions or hydrophobic interaction or even some other kinds of nonspecific interactions.

So, to avoid that, you have to keep very high salt concentration, so that the ionic interactions and vander waal interactions or hydrogen bonding, mild hydrogen bonding should not take place, then what you have to do is you have to do a sample preparations, the sample is prepared in the mobile phase and it should be free of suspended particles to avoid the clogging of the column, the most recommended method to apply the sample is to inject the sample with a syringe.

So, the first thing is you are going to generate the affinity column which means you are going to produce the matrix then you couple it with the help of the coupling reactions, then you are going to equilibrate and apply the samples. So, once you apply the samples, your antigen is

going to bind to these receptors. Whereas, the all other antigens which are present in the sample will not going to bind and once you do the washing steps, they are going to be washed away.

(Refer Slide Time: 1:05:10)



So the third step is the washing so, in the washing step, you are going to flow the buffer which contains very high salt concentrations. And as a result, the nonspecific proteins which are or nonspecific analytes, are not going to bind. So, that is how it is actually going to remove all those proteins and then you are going to do the elutions. So, in the affinity chromatography, there are many ways to elute analyte from the affinity column.

The first is that you increase the concentration of the counter ligand, which means, you can be able to increase the antigens in a very, very high quantity. So, if you do that, the antibody will switch to the antigen instead of binding to your receptor or binding to the, your analyte. And as a result, the analyte is going to be released. The second is you can change the pH or the polarity of the mobile phase.

If you do so, then also you are actually going to disrupt the salt bridge interactions and hydrogen bonding and vander waal interactions. And that is how it is actually also going to affect the interaction or the affinity of the receptor to ligands. Then you also can use the detergents or the chaotrophic salt to partially denature the receptor to reduce the affinity to the bound ligands.

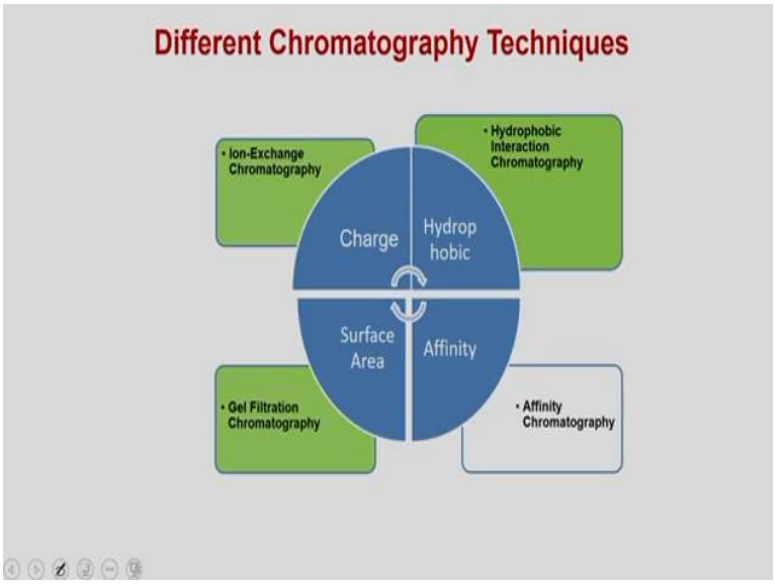
So, the third is that you can use some denaturing conditions for example, you can use the detergents or the chaotrophic salt; chaotrophic salt means you can use the guanidinium hydrochloride which is actually a denaturing agent. So, if you put the guanidine hydrochloride, it is actually going to denatured the proteinaceous receptor or the ligand and as a result, it is actually going to eventually.

Because the receptor or the ligand if they will not be able to maintain the 3 dimensional confirmations, they are actually going to lose the affinity for the counterparts and as a result the analyte which is binding to the matrix with the help of that particular receptor is going to be released from the column. But, this third method is not very much recommended, because, eventually the if you go with the third method, it is actually going to give you the antigen or the analytes in a denatured state.

The fourth is the column regeneration once you are done with the chromatography then you are going to do a column regenerations. So, after the elution of the analytes the affinity chromatography requires a regeneration steps to use for the next time the column is washed with the, 6 molar urea or guanidinium hydrochloride to remove all nonspecifically bound protein, the column is then equilibrated with the mobile phase to regenerate the column.

The column can be stored at 4 degree in the presence of 20% alcohol, which is containing the 0.05 percent sodium azide. So, once you are done with the washing step, then you can just put counterions or counter ligands and that actually is going to be compete for the bound ligand and as a result, the ligand is going to be eluted from the column.

(Refer Slide Time: 1:08:28)



So, far we have discussed about the bio affinity chromatography or we have just discussed about how to generate the receptors to prepare an affinity chromatography column and very briefly, we also have discussed about the generic steps what is present or what is being used to run the affinity chromatography. Now, in the subsequent lecture, we are also going to take up the few specific examples from the bio affinity chromatography or the pseudo affinity chromatography.

And then we are also going to discuss how to perform these chromatographic techniques? And how you can be able to exploit the affinity chromatography to solve the specific experimental questions? So, with this I would like to conclude our lecture here. Thank you.