Interactomics: Basics and Applications

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Lecture – 30
Surface Plasmon Resonance Principles and Assays-11

In the previous lecture, we discussed about SPR Technology, especially Biacore platform to perform Surface Plasmon Resonance based experiment Dr. Uma Sinha Datta, an Application Scientist and a Trainer from GE Healthcare, gave you the basic idea for doing these experiments. Today, she is going to continue where it was stopped. In the last lecture and she is also going to provide you a hands-on session on biacore technology. So, let us have Dr. Uma Sinha Datta for today's lecture.

So, I pointed out one thing to you when you are directly immobilizing your ligand, its covalently linked right.

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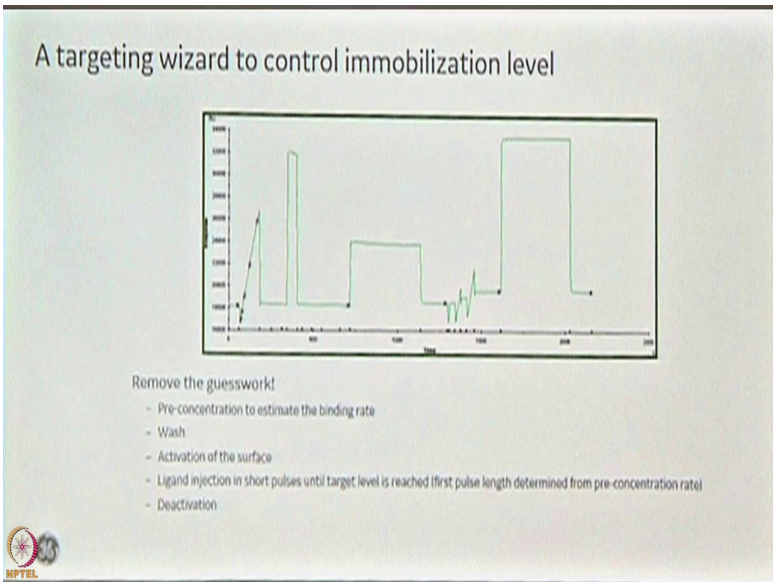

And once you immobilize it, its immobilized for you good. You cannot use the chip to immobilize anything else right. You use the same ligand to do your experiments in that, but there is a smart way to actually save your or use your chips more judiciously you know.

So, when you are actually immobilizing it and you are playing with the r u, how much to you know immobilize and you do not know how much you would like to immobilize or you are attaining some part of it. Say we decided to immobilize 600 ions, but how would you go any mobilize if you when you have to actually look at your contact time how do you attend that 600 ions right. So, there is a wizard that we have which we call it as this control wizard and when you set up your immobilization, you can say aim for immobilization ok, rather than putting a contact time for immobilization.

And when you do so, what happens is your system actually pulses small volumes say around a microliter or so on the surface and does a pre concentration for you. And with the rise in the r u, it calculates whatever you have aimed to attend around 600 r u's it will calculate and let you know in the first 6 or 5 to 6 pulses, it will let you know whether it is attainable or not. If it thinks that it is going to attend then, it proceeds to the EDCNHS step, before that it does a quick an wash to remove all the electrostatically bound, you know ligands and then you do an EDCNHS and then, you pulse your ligand based on the information that you had collected.

And then finally, you do the ethanolamine step. In case you know it decides that the concentration that you have supplied is not enough to attend your 600 r u's whether it is more or high it is going to abort your cycle post here. So, your chip is actually intact in this case and this is a very useful tool to use when we are because the chips come really expensive. There is another one which is not added here which is the CM 7, typically used for the small molecules ok. A little bit more detail on the chips.

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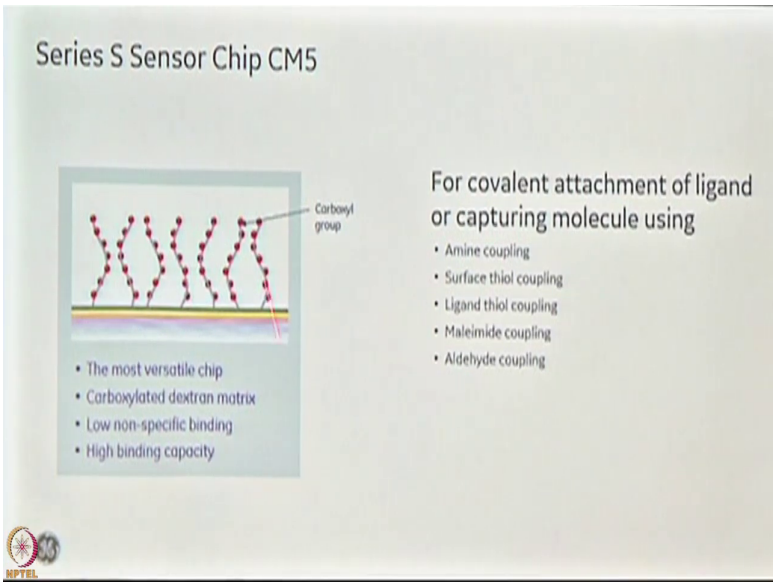

So, you know the basic structure of the chip. The chips are actually on the glass surface on which there is a gold coating, then there is a linker molecule where the dextran molecules are stuck to it and the dextran molecules have carboxyl methyl groups. And in these carboxyl methyl groups is where your covalent linking happens ok. The CM 5 chip is actually our most versatile chip you know any.

Most of your you know even now when I do my first experiment in biacore with a particular system, I would straightaway go with CM 5. If it does not work, I then go and choose a different chip because its been proved to be very very versatile to work for most proteins and it can support almost all kinds of you know covalent chemistry there.

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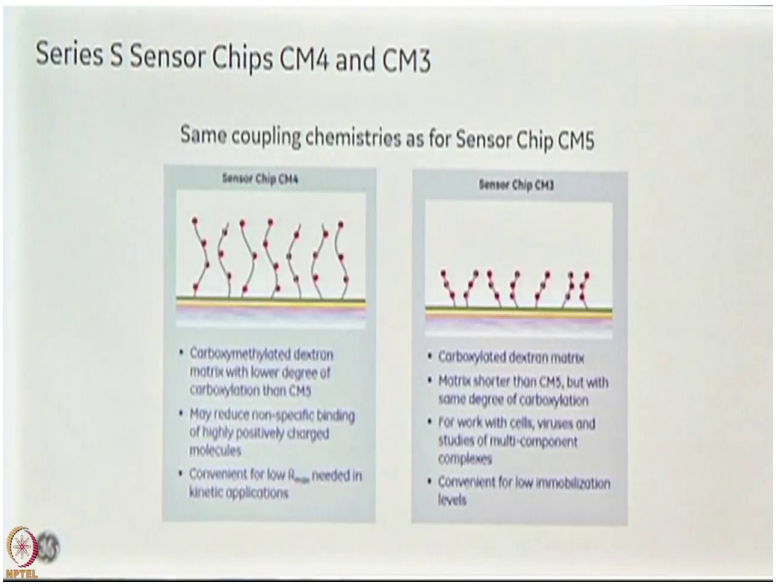

Now, a slight variation of the CM 5 is actually CM 3 and CM 4 ok. So, they are exactly the same. It is just that you know the length in one case the length of the carboxyl may dextran is the same as CM 5, but the carboxyl methylation the intent, the number of carboxyl methyl degree of carboxylation out here is less than same in CM 5. Whereas, in CM three the degree of carboxylation is the same, but the length of the dextran is actually smaller ok. So, this is used mostly to you know if you want to have work in a low r max region. You know if you have a CM 5, sometimes you know you its sometimes difficult to attend low r max.

Whereas, in this case it is much easier to attend the lower r max. The smaller size of dextran here helps to you know do your assays in larger molecules like for example, you are working with viruses or cells, where the size of your cell is quite big and you know it moves away from the surface. So, the surface Plasmon resonance will happen quite far away from the surface and where your the SPR phenomena reduces as you go far away from the surface. So, that is

where you actually use a smaller branch. So, that you can keep your proteins or your cells closer to your surface.

C1, I think we discussed about that which is the dextran is all gone, its only the carboxyl methyl groups which are stuck from the surface. In cases where your dextran is causing non specificity, you can use this kind of chip right. This is your streptavidin. This is streptavidin chip for your biotinylated protein, but remember this protein the streptavidin biotin binding is extremely high carrier. I think it is in the range of 10 to the power minus 12 or so which is actually very very close to being a covalent linkage. So, once a streptavidin and biotin links, you can never you know open it up.

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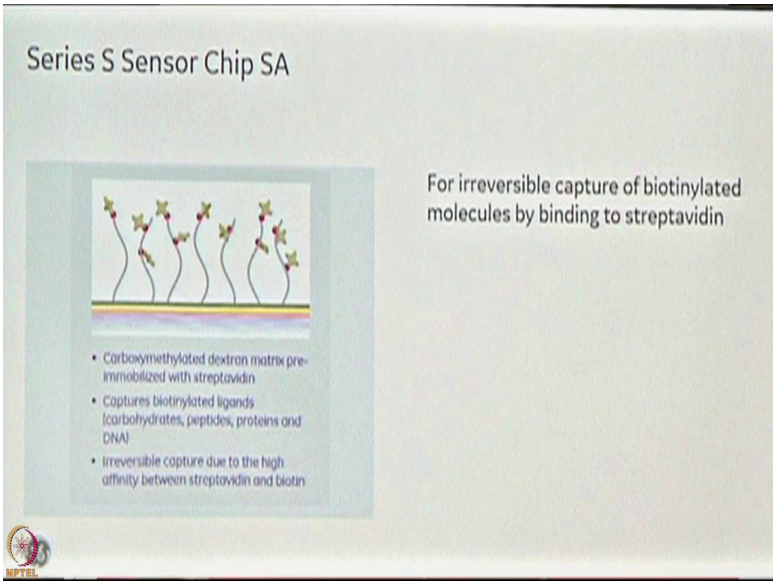

So, you have to be a little careful when you are using this chip because once bound it is good to go like a covalent chemistry.

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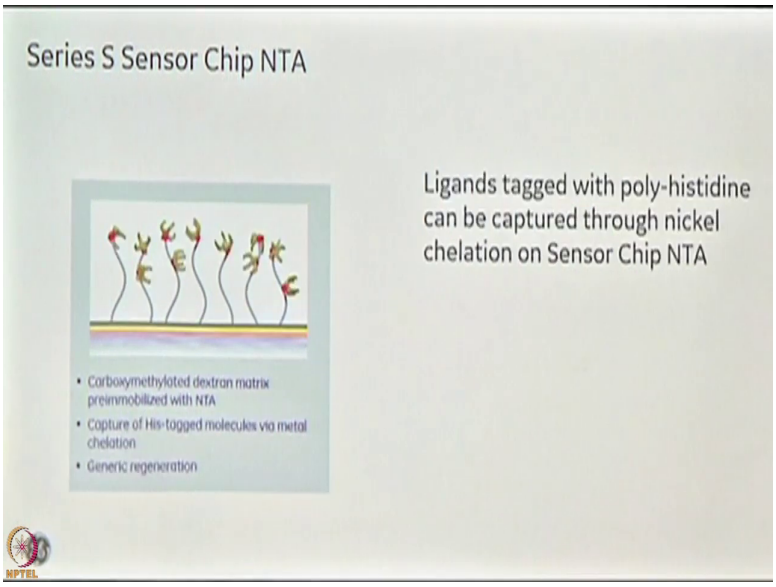

NTA; this is for the histidine tag, this is reusable. You can recharge your chip all the time with the regular you know chemicals that are available for NTA columns.

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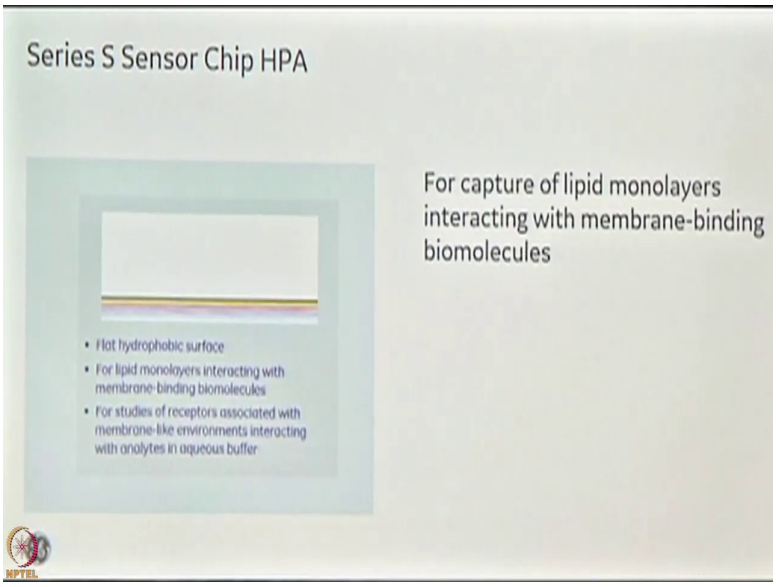

HPA, this is for hydrophobic interactions. You can coat your hydrophobic protein you know proteins on here, but it is a flat hydrophobic thing.

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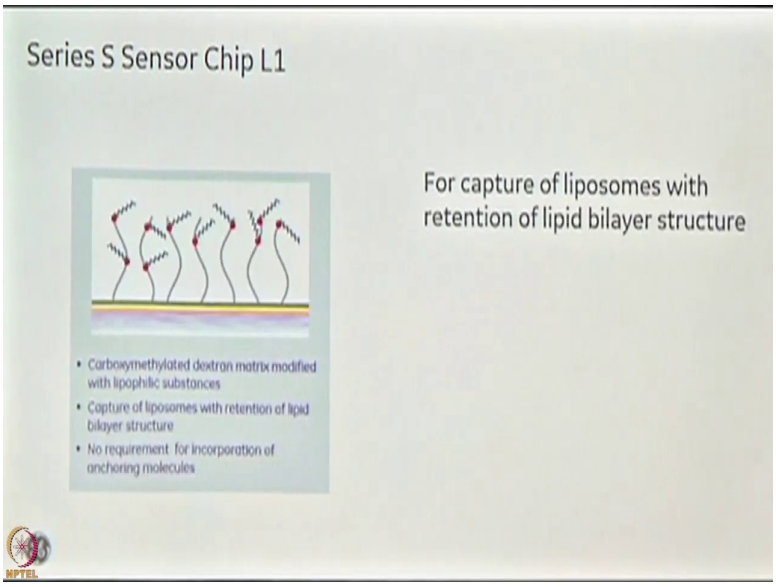

This is to make in this case, you can only make a mono layer of hydrophobic layer; whereas, here you can make a lipid bilayer for using the liposomes. If you have the liposome mixture, you can make a lipid bilayer kind of a structure on this thing and then, have the protein studies done on them ok.

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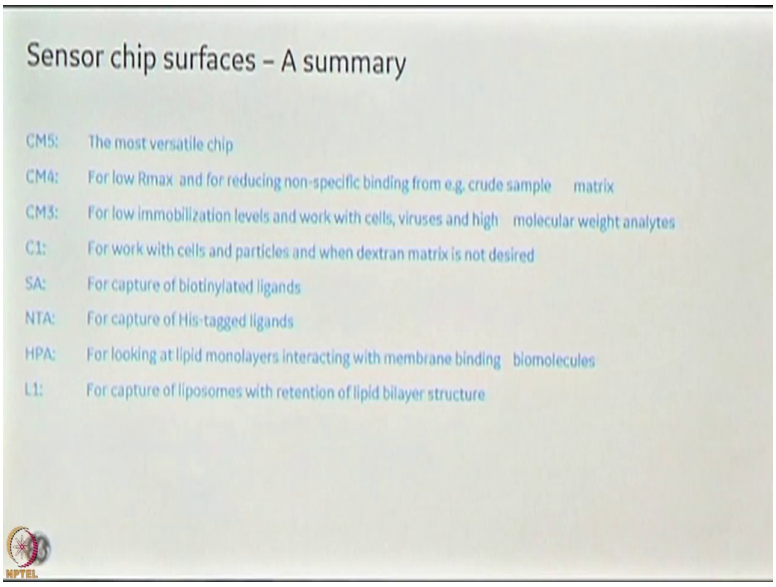

So, that is the summary of all the chips. I am not going to go through this anymore because we have talked about the various kinds of chips now, unless you have any questions, I can answer them.

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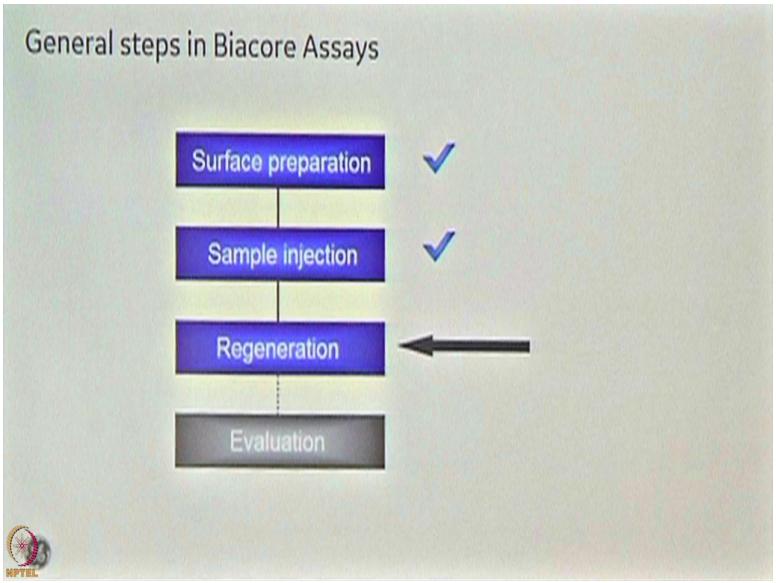

So, that was all about the surface preparation, you know what which one should you use; what is your ligand you know the Pi's that is related important, you know how you can optimally use your chips by not wasting them right and we also learnt about the r max in watch which r max you are going to work things like that.

So, the next one is sample injection. This is rather a simple thing. It is just that you know you are passing your analyte over the ligand that you have prepared. Now, if you remember we talked about that SPR is actually a refractive index measure right. The change in mass causes a refractive index change which you are reading and so, difference in concentration in your buffer would also actually change your response unit that you are reading right. So, what we call it is actually a bulk effect.

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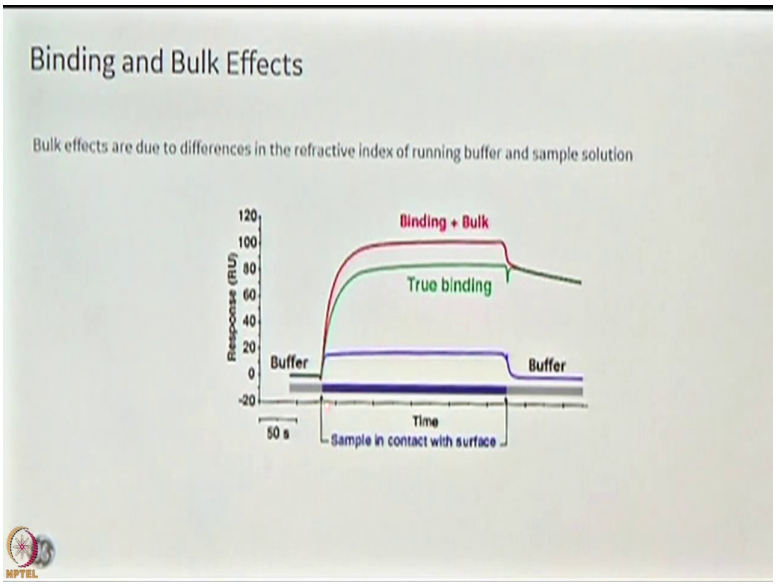

You know the when you are passing your analyte, your analyte is in a particular buffer the buffer also gives you some response unit ok. So, if you look at this graph, here the blue one is just the buffer running without anything ok. So, it has a teeny bit of response unit. The red one is actually your, you know binding as well as your bulk you know.

So, the you are looking at a response unit which you have received after binding as well as you are looking at the bulk. If you subtract these two, you actually get your true binding. So, all SPR experiments should have 0 concentration which is subtracted and the buffer needs to be exactly the same. So, whatever is the running buffer or the 0 buffer has to be used to make the sample right.

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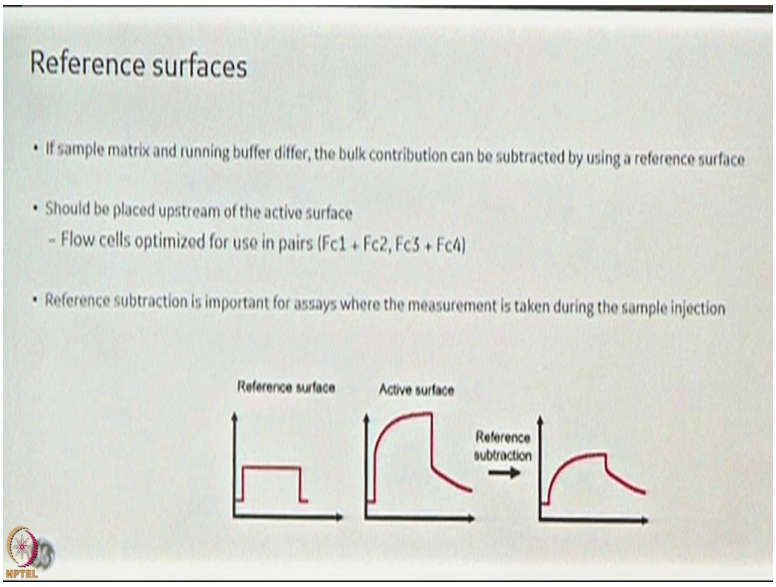

And then, you use a reference surface this is to remove non specificity of your analyte with the surface right, when you are passing your analyte over the ligand, somebody asked me I think from the venue that how do you make sure that it is binding to the ligand and not to the surface right. So, you have another blank surface which is just before your active surface and there you actually see, if there is any nonspecific binding that is happening with your dextran and you subtract that, which we call it as a reference surface. So, when your analyte passes to the reference surface you see a certain pattern of response unit.

This is your active surface and when you subtract here, this is actually your you know the real binding minus the nonspecific binding. In some cases, if you have too much non specificity, it might interfere with your binding. In those cases, you are supposed to use other additives like detergents or there is something called an NSB reducer which is nothing but a free of carboxyl methyl groups which are mixed with your analyte to you know reduce the non-specificity ok.

The three different ways to produce or make your reference surface. There are various ways you can have a reference surface.

The first one is unmodified; you do not have to do anything ok. In most cases that works. But if you are not happy with that un unmodified, you can also treat it exactly how you immobilize. So, you activate and deactivate without passing the ligand. So, you are treating the surface exactly the same way that you have treated your active surface ok. So, the first one is unmodified; the other one is activated and deactivated and the third variety can be you can have a dummy ligand, a knockout ligand which is knocked out of binding that is also possible ok. So, if you have it if you have the luxury to have that, you can also have that another way of doing the surface.

Certain some sample considerations not I mean very I mean they are very critical, but also very logical. I mean you know the sample has to be homogeneous, you cannot have particulate matter or you know they will create spikes, dirty sensorgrams. Eventually, difficult to evaluate right. The quantity of analyte also has to be extremely you know it is very important, particularly in certain applications like kinetics, the concentration needs to be determined very accurately.

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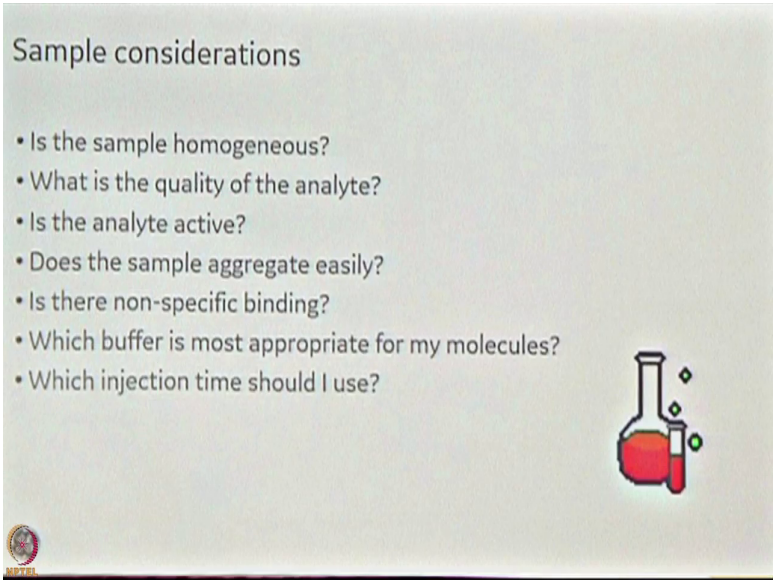

Wrong concentrations will land up giving you know different values ok, you need to make sure your analytes are active. They are free of aggregates. If it is aggregated, then you get higher response than usual which is not right ok.

And buffers, they that is again what buffers to use you know that is also very very important because sometimes you know your interactants may require certain metals, you know for binding to happen that is something that you need to find out from literatures whether you want to add some additives to inhale you know promote your binding. So, these are somethings that need to be considered and also injection time, we spoke about how much of time does it take to run a full cycle right.

And it is a very generalized question because it depends on your association and the dissociation that we understood right. So, if it is a very quick associator and a dissociator, you

would obviously need a smaller time and if you have a very slow association and dissociation; obviously, the time needs to be increased ok.

Buffer requirements; the buffers that go in should be 0.22 micron filter. They are of course, available to buy from the, but it is not a big deal, I mean you can make it yourself as long as your chemicals that you are buying are on good quality and just pass them to through a 0.2 micron. Do make sure you have P20 which is a detergent in the and it is a nonionic detergent.

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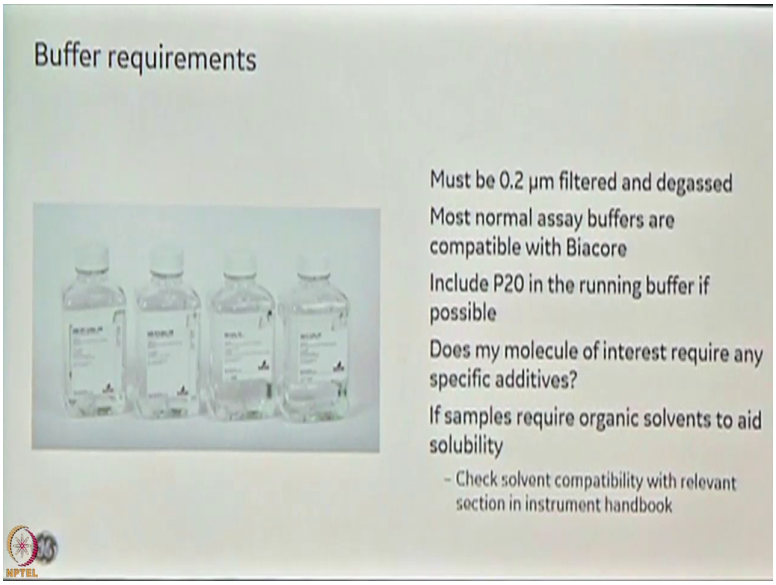

Because you do not want any ionic detergent running over the surface that can foil your things. It is important to have a detergent to remove the stickiness because the proteins are quite sticky and they stick to your flow cell and you know for the maintenance of your system, it is you know important also. In some cases where you do not see binding, you might want to

remove the P20 and do your experiments and see if it is working; but make sure after that you run through a good you know maintenance cycle.

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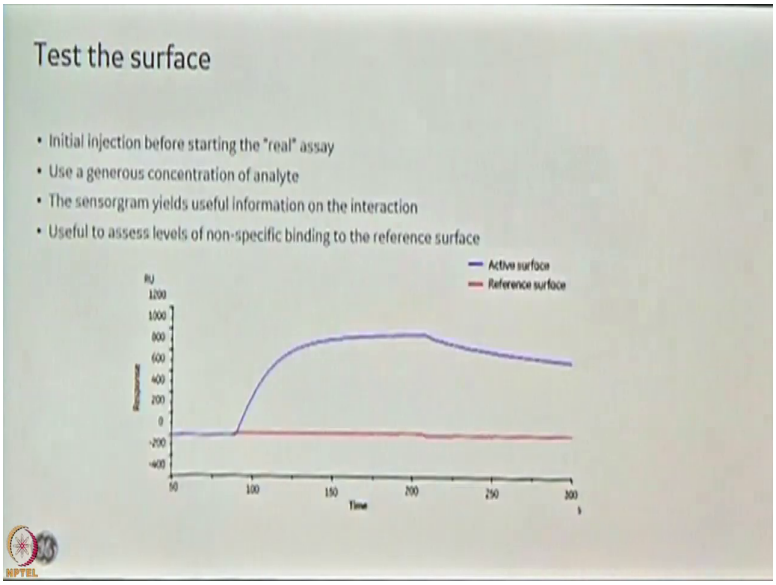

What is P20?

P20 is a detergent. It is a nonionic detergent. Sorry, I do not have the full name. So, once you have you all immobilization ready, you pass your sample exactly that the experiment that you did today in the lab, you know you did a 0 concentration, you did a low concentration and you did a high concentration. You need to test the surface exactly that way because the first thing is you do not want to use huge amount of analyte which is which could be very expensive for you to get it actually to set up so much of run and then, you realize your ligand you know ligand was not proper or your surface was not right.

Right; so, you test the surface once before you setup big kind of experiment. So, you run two different concentrations, low and a high. First, you get to see the shape of the curve which gives you a lot of information like we discussed, you know the shape of the curve you get to know and you get to see that exactly what you were expecting, you have got that.

The other thing is you see a dose dependency right that a low concentration, in higher concentration, you will see a dose dependency. The third point is you have also calculated your r max and based on that you have had immobilized your ligand and with that are you getting your r max; are you getting your r u or not.

So, these are very very important things to keep in mind when you are setting up your assays because if you do not, if something is actually not falling in place, it is its time to check right there rather than you know setting up the whole thing and going ok. So, that was all about the sample injection you know. So, the last one is your regeneration right. After your association dissociation, before you go to the second cycle, you actually find out the regeneration which is stripping, removing all your analyte from the bound ligand ok.

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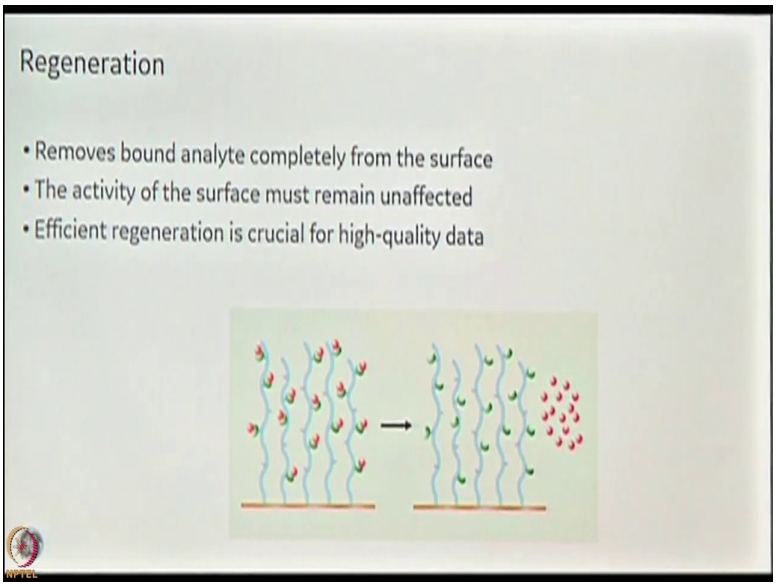

I think you saw this. It is just removing all your bound analyte from the ligand.

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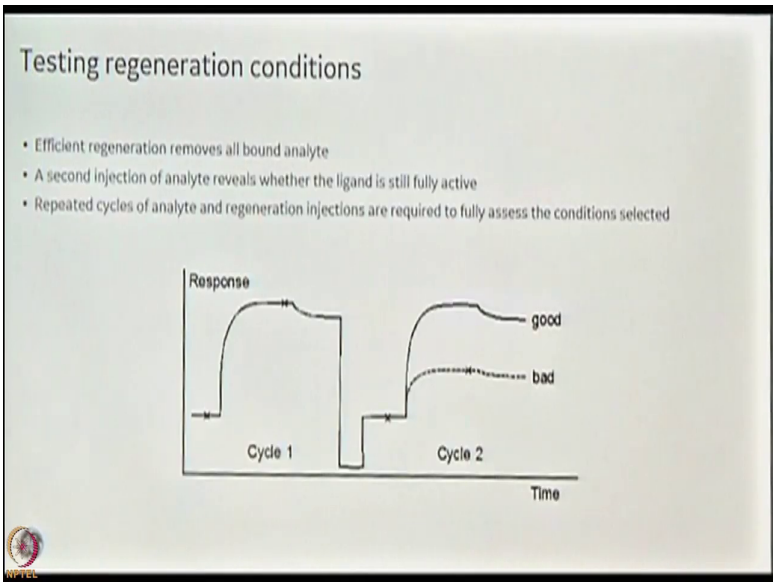

So, there is a way to check whether your regeneration conditions are fine or not ok. So, if you may have taken the same 50 millimolar glycine pH 2.5 right and you have done a regeneration and you see. So, you run the first cycle, you get your curve right and you do a regeneration with your desired regeneration condition.

Then, you run exactly the same cycle again, the second time. If your regeneration cycle is good, then you are supposed to get the same response unit. Because you are using the same concentration of analyte ok. If you do not, if it goes down; then it is not optimal, but at this point you do not know whether it is with this one cycle you do not know whether it is it was harsh or it was mild right. You just know that it was not optimal, your regeneration was not fine.

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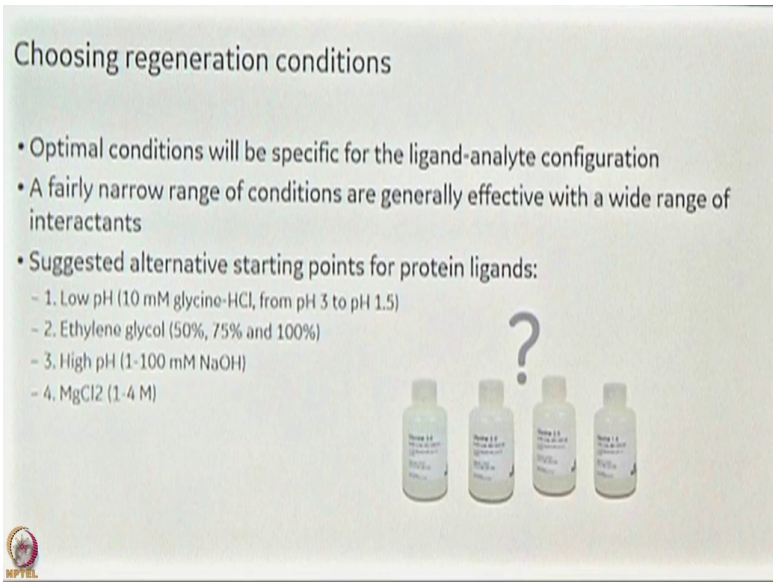

So, you need to do couple more things actually. So, before that lets look at some of the regeneration buffers that we supply. But again, these are very generic buffers you can have them yourself. The regeneration can be low pH ranging from pH 3.5 to 1.5. It can be salt sodium chloride, it can be detergents, ethylene glycol ok. So, various things typically similar things that you use in downstream processing also.

So, some guidance to find out whether it was the regeneration you know how to find out the ideal condition and there is again a scouting wizard that you can use to check, you know which is your ideal pH condition right. So, what you do is a minimum run 5 cycles of your you know use the same analyte concentration, the same pH and you run 5 cycles of it ok. So, what you are seeing out here is the analyte binding response and at the same time, you need to see your baseline ok.

How your baseline is? If you do not if your baseline is increasing that is some definitely something is stuck on the surface right. If your baseline is either stable or going down; that means, it is too harsh right. So, let us look at this situation where is this one is 5.5. So, you have run 5 cycles with its 3, 2.5, 2 and 1.5 ok; 3, 2.5, 2 and 1.5.

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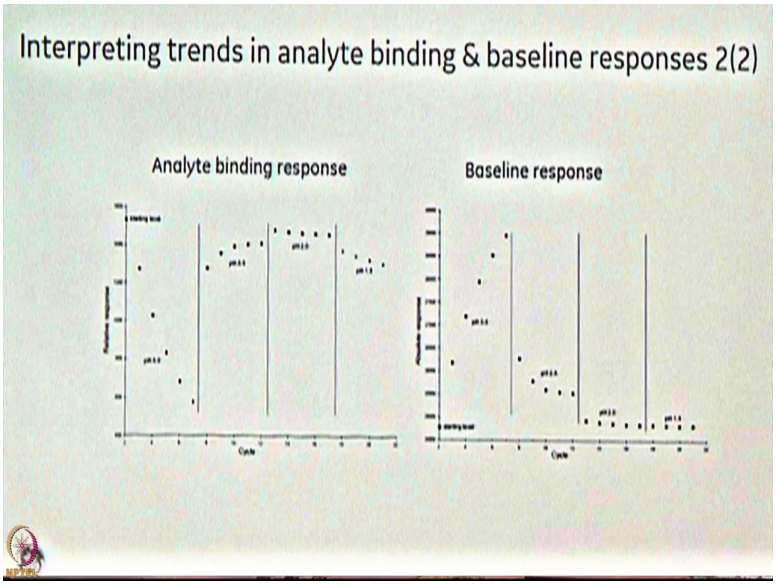

So, these are the red regeneration that done and you are looking at and an analyte binding here and the baseline response here. If you say at 3, what is happening? The analyte binding is dropping and your baseline is increasing. It is a clear case of what? It is clear; it is too mild right because your baseline is increasing. It is truly, it is definitely, it is very mild.

You are not removing all your ligand; as a result, your baseline is increasing right. And this is dropping because your ligand is not available to bind in the next cycle ok. And when you go to 2.5, you see a slide here there is a slight increase and then, here there is a slight decrease. This

is probably slightly are not explainable because you know in earlier because it was running just after this.

So, some spillover from 3 pH 3 is still there. So, not yet you do not know the conditions yet, but if you look at pH 2, both are stable kind of like a actually ideal place where you are keeping both your analyte binding as well as your baseline perfect right. So, meaning that you are stripping out everything and each after each regeneration you are binding the same amount.

But if you look at 1.5, your baseline is stable; but your binding is dropping right. So, when it is too harsh, not necessarily it is going to strip out your ligand. So, your baseline typically remains stable, but your binding goes down. So, that is your harsh condition. So, this is again a very good way to find out your you know regeneration condition.

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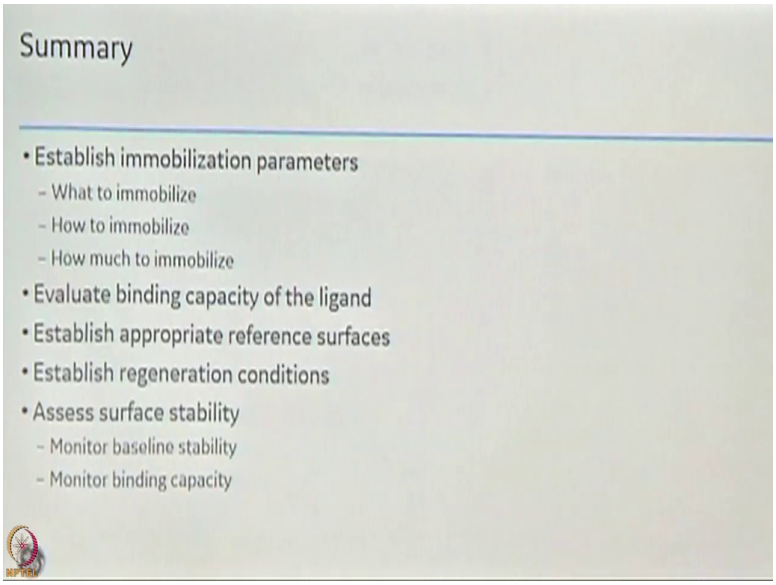

I think that is you know the end of the talk. So, we talked about some you know ideas to you know how do you do your immobilization; what are the things that you need to take care before you run your samples; your regeneration conditions. I think it will give you a good start, when you start with your Biacoresis. Thank you. If you have any more questions, I am happy to answer. Yes sir?

This SPR (Refer Time: 21:13) live cell?

Yeah.

So, in live cells that we have always analyze single analyte in single run.

Hm.

But if we want to use more than 1 analytes with live cells?

Yes, you can do it, but in separate runs right. So, if.

Because of it in real condition, we use that the 100s of medicines.

Right.

In that case, what is the 1 analytes, 1 drug, 1 molecule we will do so many times. But we in other things of real disease, it is more promiscuous population for single molecule.

Together?

Yes, with more than single molecule.

Yes, absolutely you can do those . So, there are ways that you can do you know multiple, whether you know one binding is affecting the other binding; those kind of studies can definitely be done absolutely. So, you have to do one after another. So, once you have your ligand you either mix two solution two things and then see the binding what is your response unit. And then, do the bindings in single analytes and then, check what is the binding if the binding has increased with mixing. These kind of things can also be done.

Choosing between the different methods which we can use for k d for solvent, putting the suggestion when one who preferably chooses the other.

So, what other methods, do you use for k d either the cell based method or the.

Any (Refer Time: 22:54).

Or maybe (Refer Time: 22:56) you can also use to find out the k d right. So, you know finding out the k d is not the crux out here. The k d is the affinity value. There are other techniques to find out the affinity value. What you and based on the k d or based on the affinity value, if you are actually rating your molecules, you might be limited in the knowledge that you have. Because two molecules can have the same k d, but they might have different association and dissociation constants because k d is actually the ratio between the association and the dissociation constant.

So, you would choose SPR, when you are actually wanting to look at the detail of how the molecule is really behaving. You know whether it is a fast associator or a slow dissociator or things like that. So, that is the key here.

If temperature affects binding from the 4 degrees centigrade to room temperature.

Yes.

So, how did you decide that temperature for ligand binding?.

So, you know in typically when you look at the papers that is published, you typically do it at room temperature. So, whenever you report a k d value, you mention the temperature at room temperature 25 degrees, 22 degrees things like that.

Now, if you are say for example, you do not see a binding at a temperature room temperature, you might lower it a little bit you know 10 degrees, 12 degrees, 15 degrees or you know to check whether it is still binding or not in that cases, you can actually do it. A lot of people want to find out the k d values at 37 thinking that it is more close to the physiological pH to look at it. In that case also you can choose a different temperature.

And you said that degassing system is inbuilt.

Yeah.

Degassing causes some cooling chilling.

No. So, the degassing actually removes all the suspended bubbles or air which is inside the liquid and you know if you do not remove them, they tend to create spikes in the response you know and you do not get a very neat. Right.

The process says that temperature drop down; it does?

It does, I am probably a little, but I do not, but it still maintains well I do not think. So, because you are your samples are kept at a particular temperature right, your analysis is happening at a particular temperature which you are actually setting. So, I mean if even if you are if you claim that you know it changes the temperature, then it is still set at the temperature where you are setting and you know you get the at least the binding at that that.

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Yeah.

In terms of association and dissociation, is it usually k off they don't know the affinity because k on is diffusion method and then the conformation changes in the environment then the k on changes?

Well, k on and k off are the property of the interactants. You do not change them typically unless you are changing the temperature, they do not change ok. So, you can only change you know increase the temperature to increase your association ok, the rate of association, you do need push it a little higher ok. But it is the property of the interactants.

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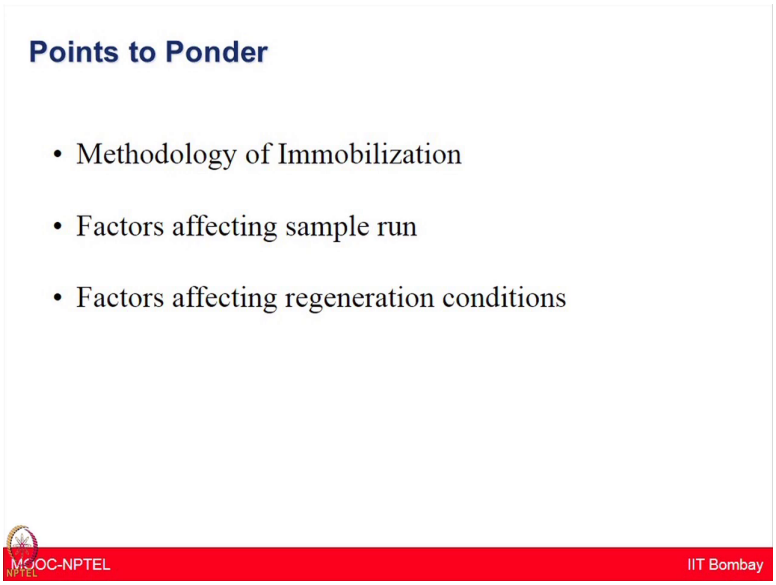

So, you know you do not change them by anything if.

I am sure by now you are familiar with the SPR based experimental workflows. You are also pretty confident about the very basic processes involved in doing these experiments till the data processing and data analysis. Please understand that if you are not very clear about experimental design and if you are not very clear about data interpretation, you may end up with lot of false positives and artifacts.

In label free biosensors one of the major consent have been how to ensure that the binding which is seen is it coming from two biomolecules or is it nonspecific binding; is there bulk effect just coming from the buffer itself or some other artifacts which are present on the this gold chips and that is where your understanding of these experiments and integrities, where Uma talked to you today because very crucial to distinguish delineate that what is the right binding and the data obtained from that the sensorgram, what says what could be an artifact which comes from the bulk effect. Some of these concepts will be taken further again.

Thank you.