Interactomics: Basics and Applications
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Lecture – 29
Surface Plasmon Resonance- Principles and Assays-I

Today's lecture will be delivered by Dr. Uma Sinha Datta. She is a Global Training Manager at GE Healthcare Dr. Uma's research experience includes molecular virology, molecular and structure characterization of segmented RNA virus, early detection kit both for immuno diagnostics and RT-PCR. She is an expert in Surface Plasmon Resonance Technology especially GEs Biacore certified trainer and the incell analyzer GEs high content analyzer.

In the next two lectures, Dr. Uma will explain the concept of Surface Plasmon Resonance Technology by using Biacore technology platform. She is going to provide you not only basic understanding of how SPR works and how the Biacore technology platform works, but also a brief overview of application is possible and how to process the data analyze data and interpret in meaningful manner. So, let us have Dr. Uma Datta Sinha's lecture today.

So, you all had chance to look at the Biacore. We talked about some of the an SPR technologies, how it actually determines the interaction based on the Surface Plasmon Resonance Technology, right. You know what is the association phase, what is the dissociation phase, regenerations required, right to run the cycles.

So, post that you know I would like to talk about a little bit on the assay development part. You know when you are ready to do your or startup with your Biacore experiments, what are the things that you would like to you know take care, you know when you have to interactance and you would like to see the interactions, what are the things that you would like to optimize you know which one should go as a ligand, which one should go as an analyte, you know various other things what would be the reference service like you know things like that.

So, let us start. So, this is the basic an assay development. Before I do that I would just I would like to spend a minute to talk about the organization that I work for.

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So, I am part of GE healthcare and I work for a small group called fast track. Fast track is actually group which offers services to the customers. There are two paths to it, first is the process development the other is their training and education and I am currently the global fast track training manager. So, I take care of all the trainings that happen globally. And fast track, like I said it is a global organization it is located strategically over the world and you know we do all kinds of process developments on and training.

So, coming to the objectives of the lecture. So, like I said we will talk about on the assay development part. What you would do when you first would like to set up a Biacore assay?

You know, what are the optimizations that you would like to do? What you know things like which one to.

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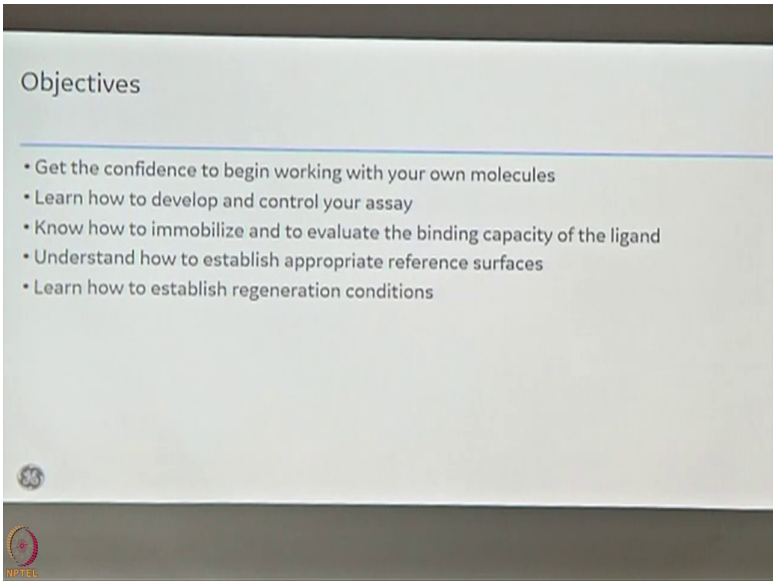

First of all how are the you know first assay how does it should look like, then which one can be a ligand, what can be your analyte, what are the different reference surfaces that you can do you know. And very important also we talked about regeneration how do you optimize the regeneration condition because you know if your regeneration is not perfect your runs typically do not go so well, right.

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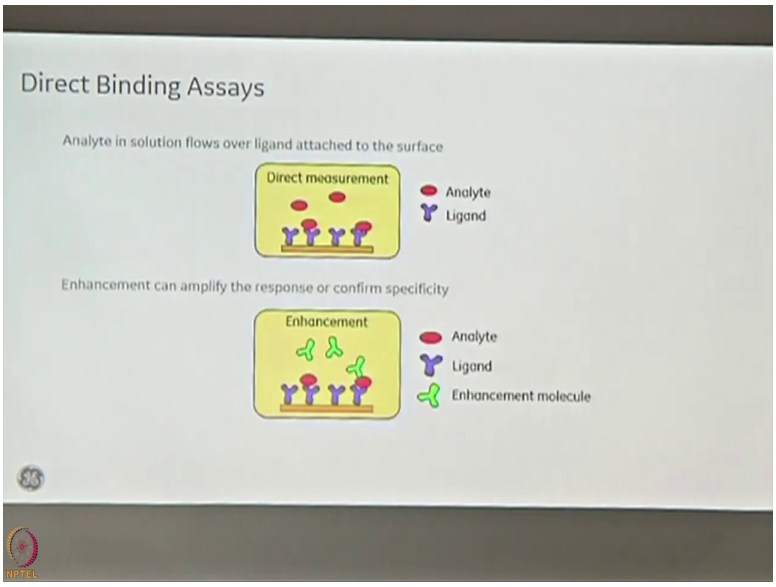

So, the first, so let us look at some of the assay formats. The first one is the direct binding assay format which is very simple you know your interactant is actually immobilized on the surface like you can see here. So, we talked about in Biacore that you have a ligand analyte, right. Whatever goes on the surface is called the ligand and whatever is flown on the flow cell is called the analyte. So, in the direct binding your ligand is actually covalently linked to the surface and your analyte is moving on top, right.

You also have another direct format, it is nothing we call it an enhancement or it is also similar to the capture method, right what we were talking about. So, the first the capturing molecule is immobilized. Then you actually bind your ligand and then your actual analyte comes and binds, right. So, this is we you can also call this an enhancement molecule, sometimes if your if

you are treating this as a direct binding. If this molecule is extremely small then you can use an enhancer you know a specific molecule that it binds to read it.

In the direct binding cases, your sensorgrams would look something like this.

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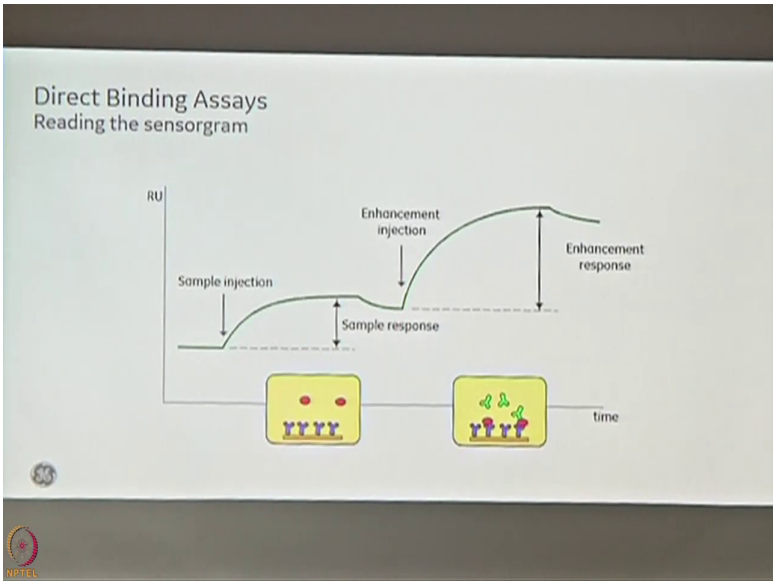

In this type you this is where your baseline is, this is your association phase and this is your dissociation. And if you are looking at and after that you can actually do a regeneration for this type only. But if you are looking at this enhancement what you do is actually first this is where your red molecule is binding to your ligand and then your enhancer is binding. So, that is your final response that you look at, right.

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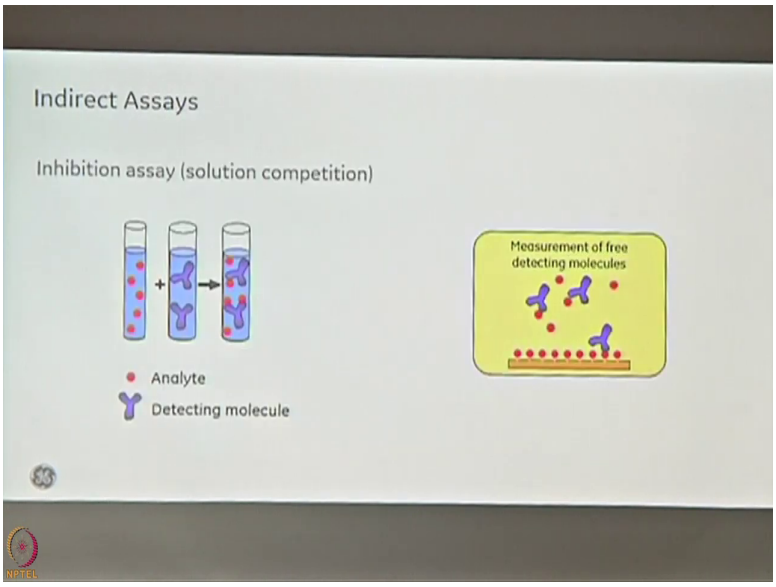

There are certain indirect bindings that you can do, where we use it we utilize the competition. So, the binding does not really happen on the surface, the having the binding happening on the in the solution, right. So, this is called the solution competition.

What you do is here you mix the analyte and the detecting molecule in a particular portion. The analyte is kept constant were in the increasing concentration of detecting molecule, and you mix them. So, with the increasing concentration of detecting molecule what will happen is you will with increasing concentration of detection molecule you will have free detecting molecule in the solution, right.

And then when you put this mixture here, so with the increasing concentration of analyte you actually see drop in response unit, right. So, it is actually a reverse you know with increasing.

Usually, in the direct binding with the increasing concentration of analyte you see increase in response unit here you are seeing just the opposite, right.

This is another one, it is a similar format. So, here you are actually having a competition in the term that you are having a competition in the solution. The bound ones, the bound ones will not come and bind here only the free detecting molecule are available to come and bind, right.

Now, here when you are mixing it with. So, this is where we call it is a surface competition the competition is happening at the surface. You have the analyte as well as you attach or link your analyte with a competing analyte, in the sense its a high molecular weight analog where you link it with and the key is the high molecular weight analog when you attach it the size of this should be considerably larger than your analyte. You mix them, here they actually in earlier case the binding was happening in solution, but here of course, there is no binding.

When you put this mixture on your surface where your you know ligands are bound you see the binding only because of your competing analyte, ok. When these analytes are so small and negligible, you do not get to see your binding. So, here to with increasing concentrations of your analyte you actually see less and less of binding. So, it is again a reverse plot, right.

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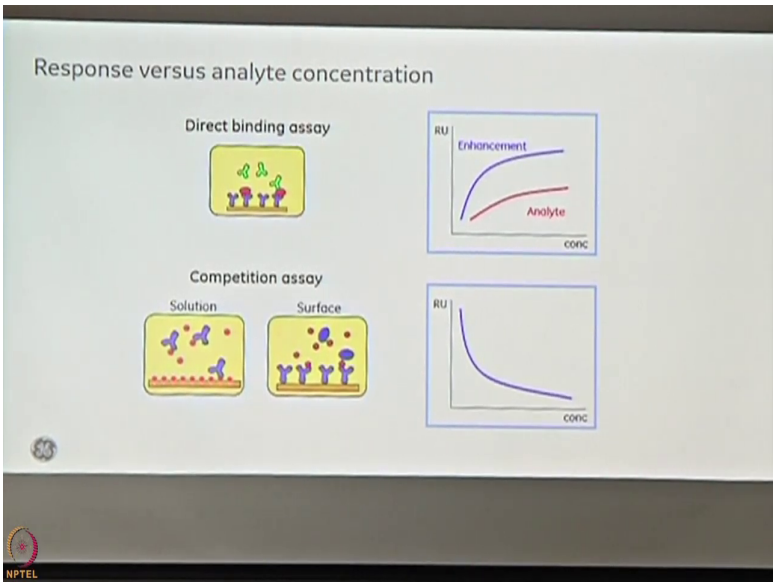

So, if you summarize the response versus analyte concentration in direct binding you see increase in RU with analyte whereas, in indirect you actually see a decrease in binding with your. So, these are basically two types of assay.

Student: So, on the direct in assay is there along with the ligand the rate constant somewhere is or is not it getting on the rate of the (Refer Time: 09:05).

That is, right. So, you probably will not get the rate constants there. You can only do only certain applications like concentration determinations.

Student: I see.

That is a very good point, yes. So, these are different various formats that are offered, but then again it is limited to what applications you are using too.

Student: (Refer Time: 09:26).

Coming to the general steps of Biacore assay I think. I do not need to explain that you are all now quite comfortable with this you understand this, but we will talk about now in detail on the surface preparation by you know, so surface preparation. So, when you have set of two interactomers, right, you would like to use it in Biacore to see and bind it, right.

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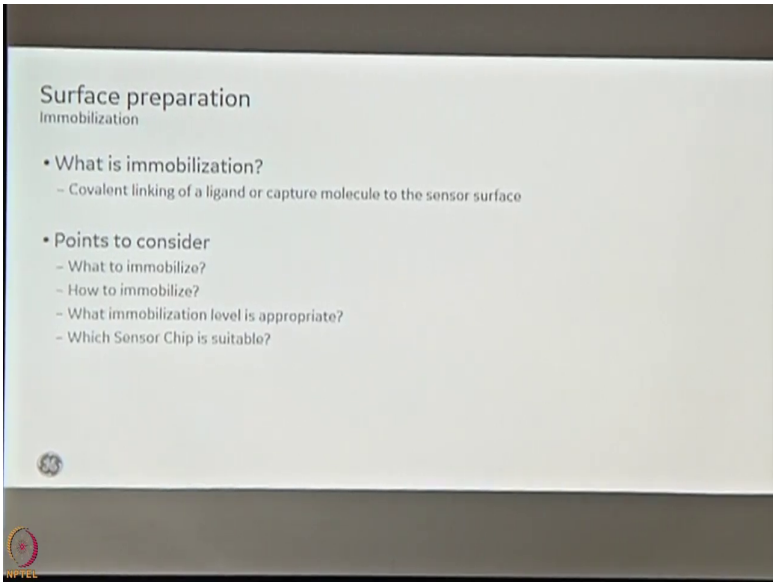

So, you need to first understand why you would like to, which one you would like to choose as a ligand, which one you would like to choose it as a analyte things like that, ok. So, first of all what is immobilization? We all know, right. How do we stick it to the surface? We

covalently link it, right. It is not just any attraction its covalent linkage, right. So, once you immobilize that is you know immobilize for good you cannot strip it out, ok. So, when once you immobilize something on the surface you cannot strip it out, right.

So, points to consider you know, first when you have it which one you would like to immobilize, then how you would like to immobilize, which chemistry are you going to use, whether you are going to use the direct binding approach, you are going to use the indirect binding approach all these things, right. What is the immobilization level you would like to use? Because when different applications require different immobilization techniques amongst, right.

So, for example, in kinetics you know adding too much of ligand is extremely detrimental you do not get a right kinetic data. Your ligands have to be mobilized very low. Whereas, if you are doing a concentration analysis you need to have a high ligand concentration. So, these knowledge is very very important, and which sensor chip is suitable for your assay. We talked about various sets of sensor chip like you know CM 5, CM 3, C 1 when you have a lot of non-specifity hydrophobic for HPA you know. So, all these things NTA for a nickle tagged if you have something like that, ok.

What do we mobilize? We come back to the question of which one to immobilize. So, the first thing that you would like to look at is actually the molecular weight of the interaction, which one should you would you think should go as a ligand if you have two interactants. And one of them is large, and one of them is small, which one would you like it to go as a ligand?

Student: Small one.

The small one absolutely, because you can use the larger one as an analyte, so you get an use a you know higher response you know. But having said that with the Biacore T200 the sensitivity it offers, even if you have a smaller you know small molecules can still be used as an analyte, but you do see some amount of background you know a noise. But then it is still

enough to do you know kinetic analysis, but not with the earlier versions like x one 100 where the sensitivity was not as high with the T200.

Tagging of the interactants. If you have a tag you would; obviously, like to use a capture method to put it in the as a ligand, right. Functional groups, the few things, functional groups and binding activity of the immobilize. They go together actually because if you are using a functional group to immobilize which is actually in the active pocket then you know you destroy the activity then you know then there is no point, right. So, you need to have some amount of information of you know the functional group that you are using and that is not being used to you know immobilize it on the surface, right.

Purity, the most pure one should go as a ligand you know. You cannot have a impure ligand. The more junk you immobilize the data becomes more dirty. You can still use it as an analyte you know, so of course, again in when I talk about analytes being impure, it is only limited to applications like binding experiments. If you have to do kinetic you cannot have; you cannot afford to have a impure analyte, but if you are doing a concentration analysis, if you are doing a binding it is fine to have a slightly impure analyte.

Valencies, the more number of valencies, where do you think it should go it should? You know the; so, like an antibody and an analyte or so you used anti-beta 2 microglobulin today, right and beta 2 microglobulin as an analyte. Which one did it go on the ligand?

Student: Anti valency maam.

The anti, the valency was 2, you put that on the surface, right. So, but if you had done the opposite and, ok. So, the question is if you have a antibody on the surface and have an analyte on the flowing on the surface it is actually a one is to one binding because we call it as you know the binding is considered with respect to the analyte. But if you have the antibody on the on flowing on the thing and your analyte the same detecting molecule is on your surface then it becomes bivalent analyte, right the bivalent analyte. So, your mode would change, ok.

pI of the protein. pI of the protein I would like to slightly stop by out here and we will explain it in greater detail one in the upcoming slides because that you will see is an extremely important when you are immobilizing your ligand, ok.

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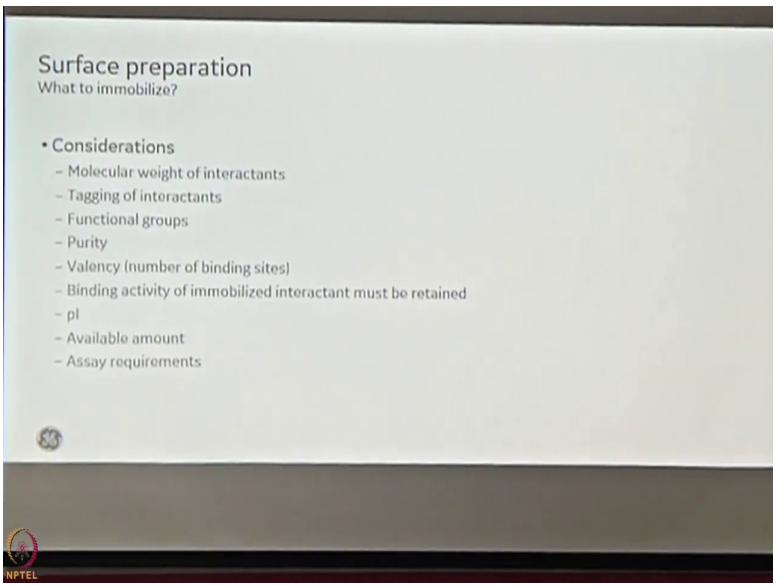

Amount of available; obviously, if your amount is very available is very little then you know you would rather use it as a ligand because when you are using it as an analyte you have to you know run various number of cycles leading to more number of you know requirement of analyte. And then assay requirements. Of course, assay requirement is very very important, what actually do you want to get out of the result that is very important to.

Surface preparation, I think you saw the slide. You know a surface can be prepared in two ways, one you directly immobilize the ligand, right in the other one you actually capture the

capture your ligand. So, in capture you are actually capturing molecule is actually immobilized directly on the surface.

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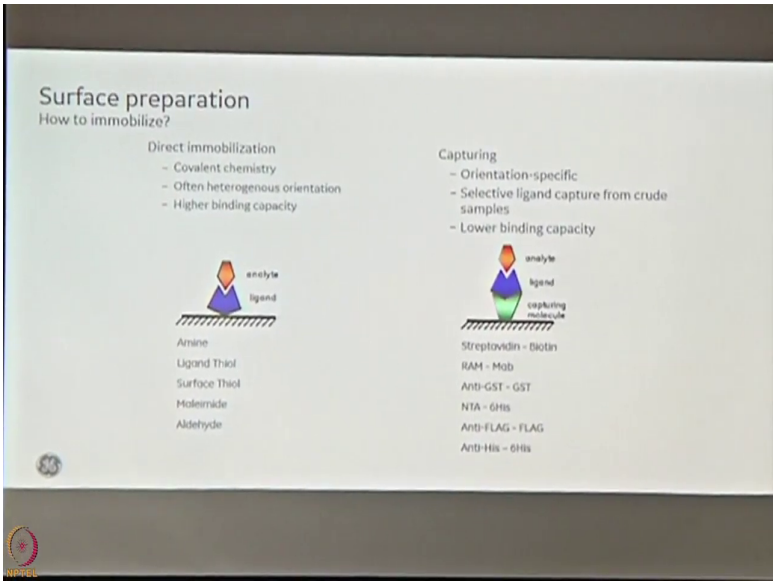

Now, the difference would be is that you know in this case you lose the directionality of your ligand like we were discussing. So, it is actually immobilized at random using you know any of the free amine groups or the thiol groups on the surface. Whereas, if you are using a capture molecule you maintain a directionality of your you know ligand (Refer Time: 16:24) if you if your assay requires so.

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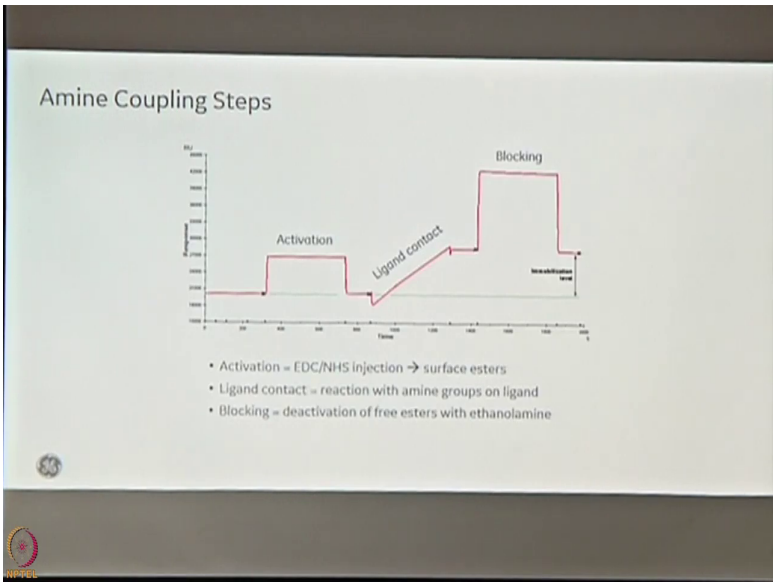

This one that you are looking at is actually the step of your immobilization. So, how do we mobilize is like you know first we activate our surface using EDC, NHS, and do not ask me what is the full form because I really cannot remember ever. The EDC NHS, what it does it actually activates your surface into the carboxyl groups into a reactive ester group, ok. And that is where you are seeing the EDC NHS is being pushed.

After that you know, at this point your surface is actually activated and then you push your ligand, with which has the free amine groups or the free other groups. And at this point all your proteins are getting covalently attached or linked to the surface, and then finally, you do a blocking with ethanolamine.

This blocking is to block all the activated ester groups which are not, which has not formed a covalent link because if you do not block at that stage when you are actually passing your

analyte they may come and bind there, right. So, this blocking step is extremely important. And the difference from here to here is actually your immobilization level, ok.

Choice of immobilization strategy, it will depend on your ligand again. Amine coupling is very widely used. You know in most cases you know more than 90 percent of the cases we typically use amine coupling, particularly in proteins when we are talking about because this huge amount of amine groups available in most proteins, right.

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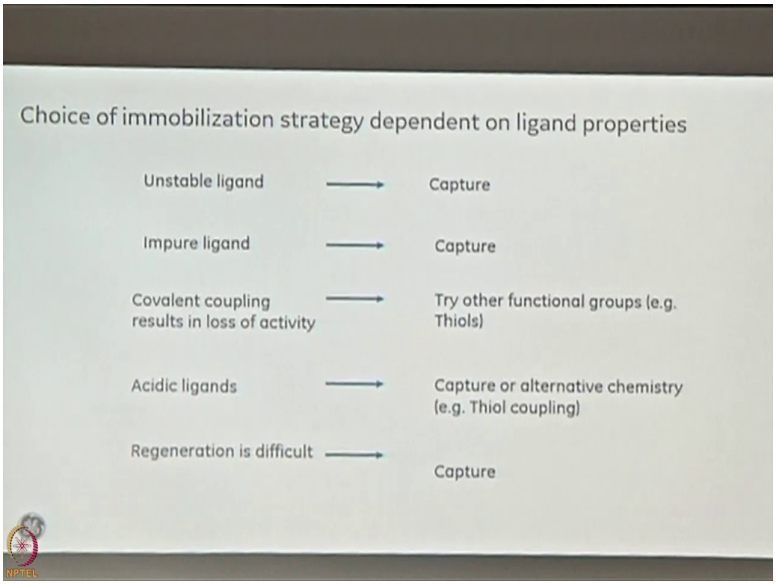

So, if we if your ligand is actually unstable then you would actually use a capture method, right. If you are using a covalent chemistry to immobilize and your ligand you know loses its activity then you would rather use a capture method.

If it is an impure ligand, like remember you we said that your ligand needs to be very very pure to be put on the covalently linked on the surface. But if it is an impure ligand then you can do is capture it, right, so you can only capture the your specific ligands.

If your covalent linking actually loses the you know results in loss of activity, say for example, you have done amine then you and it loses the activity then try and use a thiol coupling or an aldehyde coupling.

For acidic ligands typically you know capture chemistry is mostly used, and if regeneration condition is also you know difficult like if you have not found out a regeneration condition. Sometimes it is very difficult to find out regeneration condition for some cases, in those cases also you use capture chemistry, ok, ok.

Coming to the pI point which is extremely important here when you are immobilizing your ligand.

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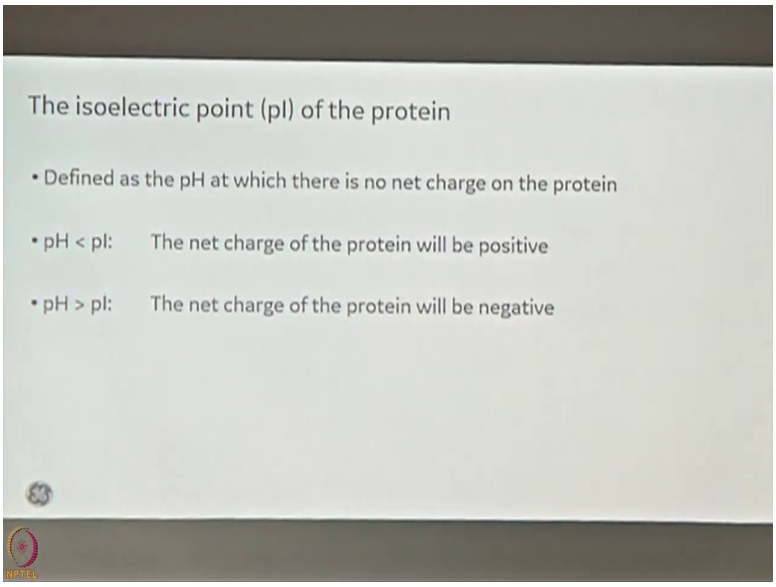

And I think I do not need to reiterate this you know. We all know what is the you know what happens when your when your protein is put in a pH less than your pI, your protein is actually positively charged, right. And if it is more than your pI your protein is actually in negatively charged. So, when we use this, it is the same thing in a more schematic thing.

Now, the pKa of the surface, the chip. The pKa of the surface is actually close to 3.5, ok. So, if you are going less than pH 3.5.

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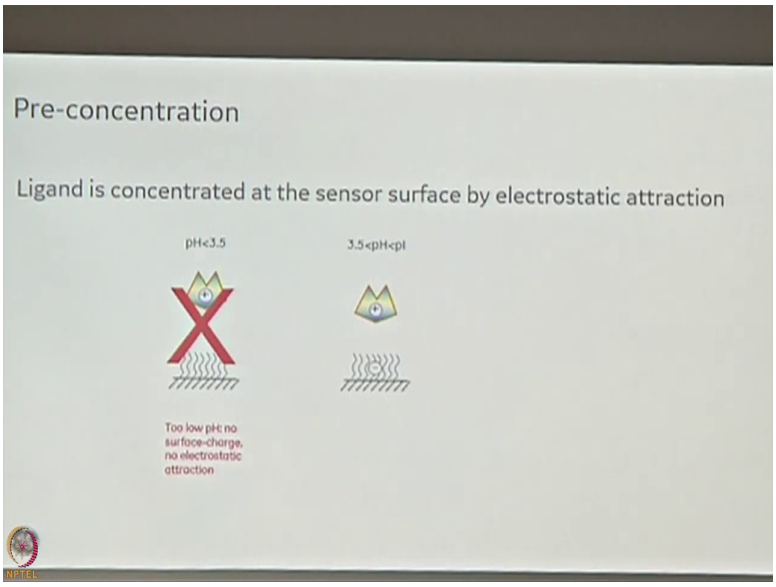

So, I am talking about a scenario where I would like to immobilize my ligand and I would like to put my ligand or the protein in a certain buffer where it is in a particular charge, positive or negative let us decide that, right. So, if I put my protein in a pH 3 0.5 say most likely if the protein of course, pI is higher than that it will be positively charged. But the surface actually loses its charge, it has absolutely no charge. So, in that case you know there is no attraction between the protein and the surface.

And why are we talking about the attraction between the protein and the surface? Because we talked about covalent linkage, right. But the covalent linkage to happen the protein has to come close enough to your surface, so that you know covalent bond can be formed, ok. So, this scenario where your pH is very low typically it is not a good scenario to immobilize your ligand. You do not see immobilization. So, it is too low for immobilization.

Now, at a pH higher than 3 0.5 you know your surface actually attains a net negative charge, ok. And if your pI is higher than that pH in that particular pH and you are able to keep the protein positive you know then your attraction happens, ok. And this is the ideal scenario where your you know covalent linking can happen very ideally. Again, if your pH is extreme higher than your pI you know then there is no attraction because both of them becomes negative, right.

So, that is what we call it as pre-concentration also. So, when we are doing an ligand immobilization and sometimes we see that we are not attaining the amount of RU that we are expecting. This is something that is extremely important apart from the chip quality and EDC quality and things like that. So, this is also something very important to keep. And this is the same thing that we talked about.

Now, you know not all the time you may have information about the correct pI, right, so we have a tool called scouting pH scouting which actually let tells you which buffer would be most convenient or ideal to use it for immobilization buffer, ok. For immobilization purpose for that particular protein, so you can take a little bit of your protein before you do your actual immobilization and run into this pH scouting experiment, ok.

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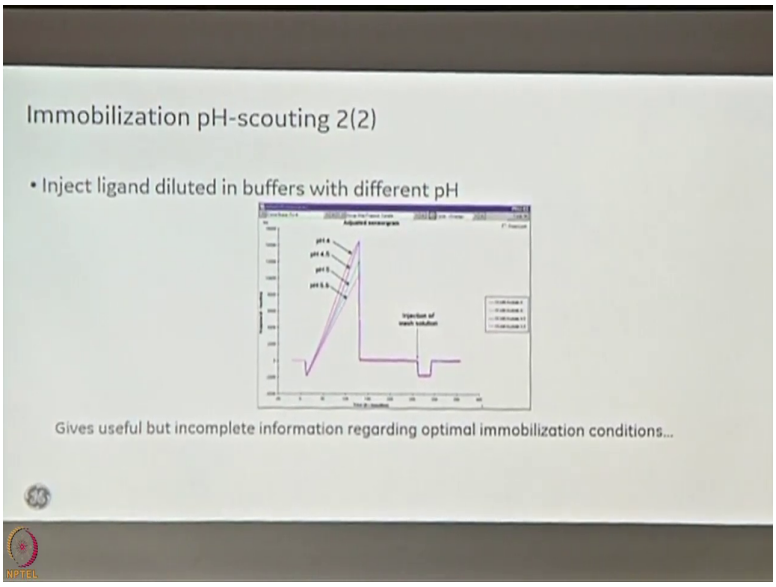

Take a small amount and then mix it with various buffers, typically the buffer that we supply is from pH 5.5 to pH 4, ok. Mix them in all different same concentrations, but different buffers and then one them. And then try to compare and see what is the peak like. Remember here there is no immobilization happening it is just the pre concentration, only the attraction that is happening and then you see the increase in IO out here, right.

So, in this case that you can see that pH 5.5 is the lowest and the highest is pH 4. So, what does it what do you think it means? Does that mean that pH 5 4 is the best for immobilization? So, most people think you know that you know increasing the more lower the pH, we go we can attract the protein more and eventually we can get the best and well there is some amount of truth in that, but not always you know.

So, if you see out here in pH 5 0.5 which is actually kind of like the highest pH here you attain quite a large amount of RUs, ok. You go to around something like I cannot even see 10000 RUs, which is a extremely huge amount of RUs. You do not need that much amount of proteins to. You know if you are reaching around 4000 or 3000 its more than enough to you know do any of your applications that we are talking about.

Now, so we actually did not get enough information from there. So, we took the same thing and we ran it through the whole immobilization process, right.

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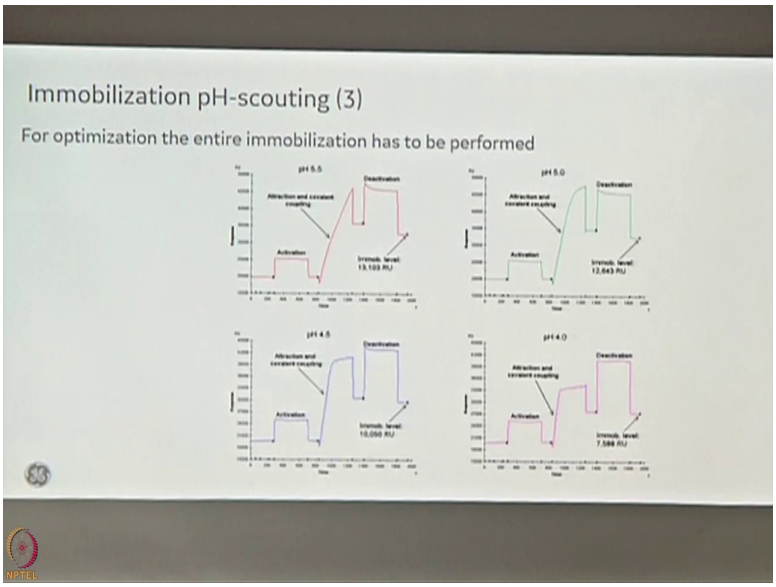

And you see in pH 5.5 you get to around 13000 RUs, right and pH 5 you get to around close or maybe similar, I do not think there is I mean 13.5 and 12.6 I think I would say it is very close to each other.

4.5 there is a reduction, it is much less and then again 4, it is even less. So, whereas, our pre-concentration earlier showed you know with 4.5 and 4 it was still increasing, but here actually you are actually attending a much lesser RU than in considered to pH 5 and pH 5.5.

The logic out here is actually, so when you are actually attracting so much in pH 5.5 increasing, lowering the pH even further and attracting does not really help for covalent linkage. So, that is where you start getting your steric hindrance and where your you know immobilizing your covalent linkage is not again you know happening in an ideal scenario.

So, the key thing like she said is to have your proteins in an comfortable environment close to where your proteins will be comfortable, not stress them out in you know in harsher condition. If required, if you do not see any rise like for example, in these cases if you do not see any rise in pH 5.5 and 5 and suddenly you see a pH rise in 4.5 that is where you go into a low you know lower pH like 4.4, 4.5 or 4. Otherwise, stay close to you know more comfortable environment does it make sense, ok.

Now, I think somebody was asking about the immobilization level. So, this is, so how do we find out the immobilization level and in Biacore term and how much should we immobilize actually when we are doing a Biacore assay?

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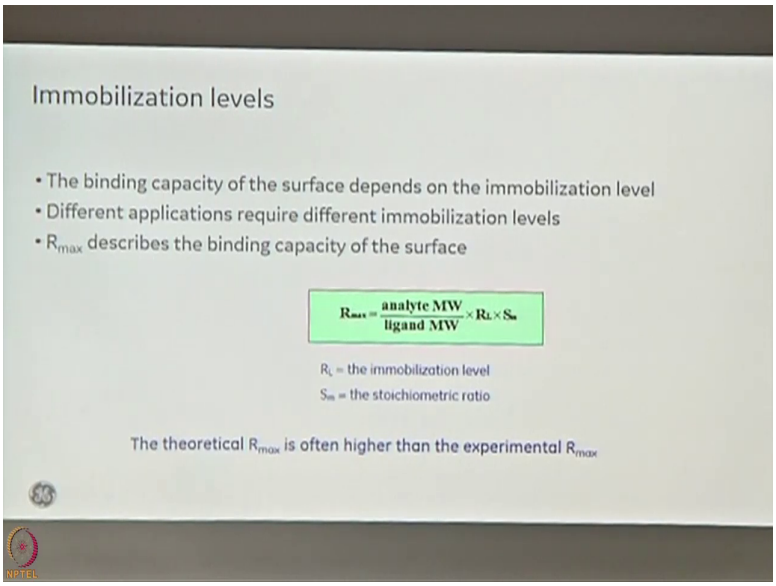

So, we have this formula which we call it as it is kind of like a what do you say [FL] in Biacore. R max is equal to molecular weight of analyte by molecular weight of ligand multiplied by R L into the stoichiometry. So, R max, we have not talked about R max yet. R max is called the maximum binding capacity of your surface. Once you immobilize your surface the capacity of the surface to bind maximally your analyte that is your R max, ok.

R l is the ligand immobilized, ok. So, when you immobilize your ligand you get a particular RU, right. Say for example, you immobilize 500 RUs or 1000 RUs, right, so that is your R L and stoichiometry of your binding whether it is 1 is to 1 or if it is a bivalent it is 2, right. So, typically theoretical R max what we did find out from here is higher than your experimental R max. Usually, when you actually mobilize your ligand you tend to you know lose some activity you know and or your ligand to begin with may not have 100 percent activity.

But in some cases when you see much higher R max you know in experimentally as compared to theoretical immediately it should strike a bell that, a bell should ring in your mind then something is wrong either the stoichiometry that we considered was not right or there is some nonspecific activity or there is a aggregates happening, right.

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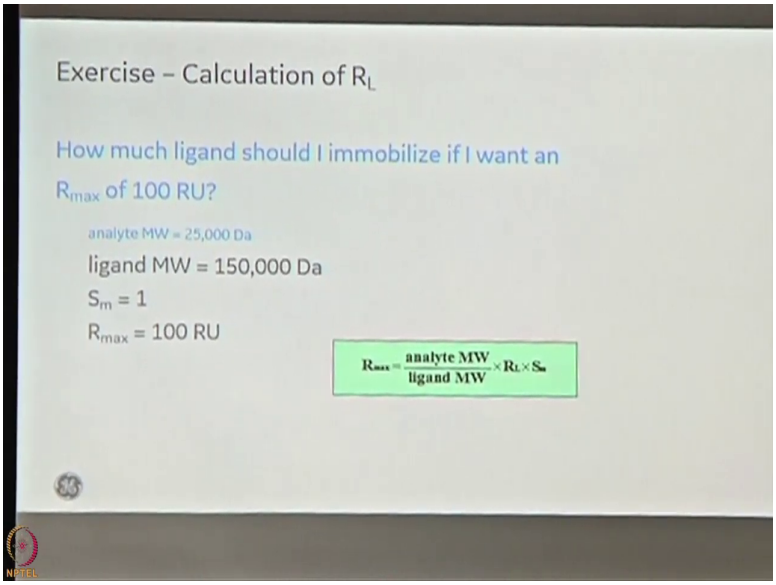


So, there is a small exercise, if you would like to do hopefully it will wake you up if you guys are sleeping. So, for a say for example, if we have a ligand that I would like to immobilize and I would like to work at an R max of 100, ok. And the molecular weight of your analyte and ligand are given. So, your ligand looks like a map like 150 kilo Dalton, the analyte is 25 kDa, stoichiometry is 1 and I would like to reach an R max of 500, right. So, how much R L, how much of ligand should I immobilize? Anybody?

Student: 600.

600, right. So, if you get 600, then typically I would since your theoretical R max is usually higher than your in experimental R max. We typically go ahead and if its 600 from your theoretical I would go ahead and immobilize 700 or 800 to compensate a little bit and then you know start your experiment, ok. Is it ok?

Student: How you decide R max?

Right. So, if you are doing a kinetic analysis you have to be event 100 RUs is quite high sometimes. Sometimes you have to go as low as 50, 20 or you know a T200 actually allows you to work as low as 5 RUs, R max, ok. So, you can still get a very decent nice no without noise graph which we can perfectly do an evaluation and get a kd value of it.

If it is a concentration or affinity determination, then you it has to be on a higher range. Like you know, when I say higher range it can be around say 3000 to 5000 ranging on that. So, you find out the surface saturation. Say for example, you know if you are immobilizing a 150 kDa molecule, so your surface saturation comes at close to around 10000 to 15000 RUs, that is the surface saturation. Roughly, So, and if it is say around 25 kDa then it is around 2000 or 2500 that is the surface saturation.

And when you are doing a kinetics you should never be in a saturation mode, you know there is something called mass transport limitation that happens which actually affects your concentration of your analyte. So, you would have to keep your ligand concentration extremely low. So, otherwise you get erroneous kinetic results.

Student: Ma'am, when (Refer Time: 31:12) when you said 600-700 what is the unit like?

RUs, resonance response unit.

Student: It means intensity.

Yes, exactly. So, like I said, right way it is related to the refractive index. So, the change in refractive, the change in mass will change the refractive index and that is related to your response unit response unit.

Student: The analyte molecular weight is not given. So, we have to prove also (Refer Time: 31:47)?

No, analyte molecular weight is there.

Student: There it is, ok, sorry.

Yeah, ok.

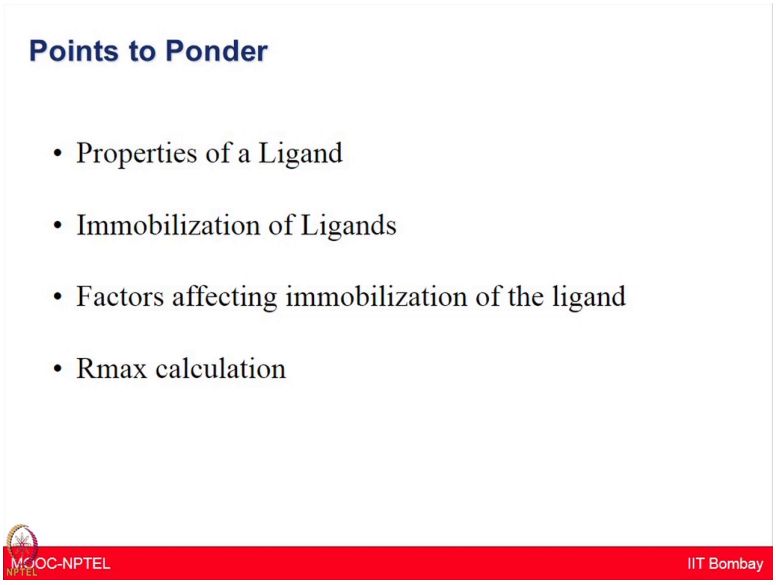
Student: What is the maximum R max that T200 allow?

Well, it can allow a lot. I mean and then it is not about T200 allowing it, it is the analyte and the ligand molecular weight which will contribute to it. Yes.

Student: What is this 5 you just said?

5 RUs of R max, yes.

(Refer Slide Time: 32:26)



So, I am sure this was very informative lecture by Dr. Uma. You are convinced that she is able to convey the very hard ideas and principals involved in this technology in a very lucid manner. In the next class, Doctor Uma will continue to explain some more detail of Biacore Technology. She will also provide a demonstration and working off how to do a SPR experiment on Biacore platform in the next class.

Thank you.