Lecture 29
Surface Plasmon Resonance- Principles and Assays-I

Welcome to MOOC codes, on applications of Interactomics, using Genomics and Proteomics technologies. Today's lecture will be delivered; by Dr.Uma SinhaDatta 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 immune diagnostics and RTPCR. She's an expert in surface Plasmon resonance technology, especially GE's biacore, certified trainer. And in Cell analyzer, G's 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 is fear words and how they may occur technology platform words, but also a brief overview of applications possible and how to process the data, analyze data and interpret in meaningful manner. So, let's have Dr.Uma Sinha Datta lecture today. So you all had a, chance to look at the Via Core, we talked about some of the SPR technologies, how it actually determines the interaction based on the surface Plasmon resonance technology, right. You know, what's the association phase? What's the dissociation phase? Regeneration is required right, to run the cycles. So, for that you know I would like to talk about, a little bit on the asset development part , you know why, when you are ready to do your or startup with your Via Core experiments, what are the things that you would like to, you know take care, you know, when you have to interact ins 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 lag in, 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's start, so this is the basic asset development, before I do that, I just, I would like to spend a minute, to talk about the organization, that I have worked for,

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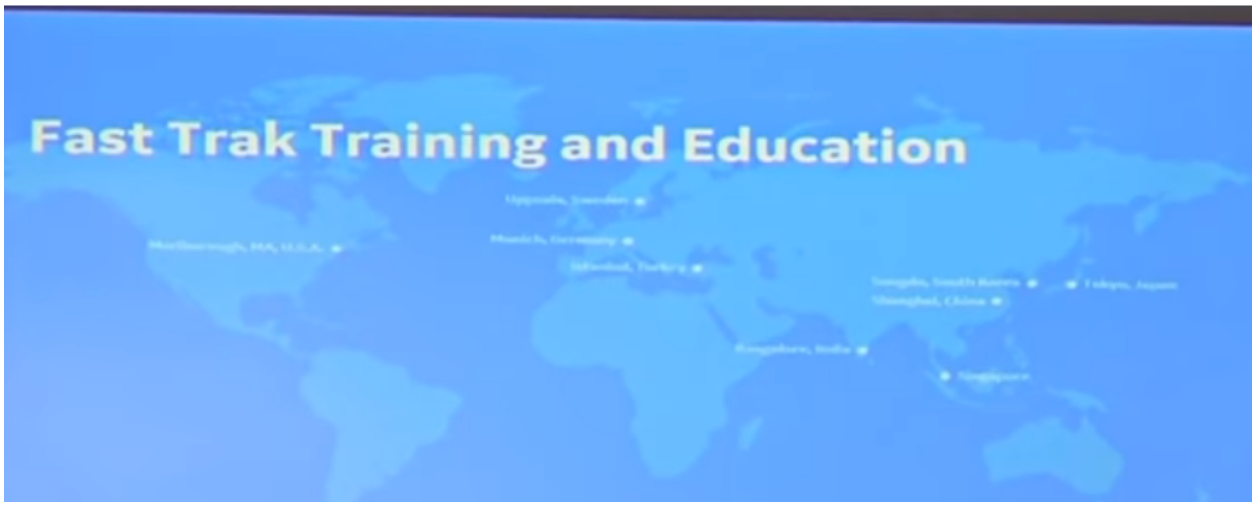

So I'm part of GE healthcare and I work for a small group called, 'Fast Track'. Fast track is actually, group which offers services to the customers, there are two parts to it, first is the process development; the other is their training and education. And I am currently the global fast track train, Training Manager. So I take care of all the trainings that happen globally. And fast track like I said it's a global organization is, located strategically over the world and you know, we do all kinds of process development and training. So, coming to the objectives of the lecture, so like I said, we will talk about, on the SI development part, what you would do, when you first would like to set up a Via Core, I say?
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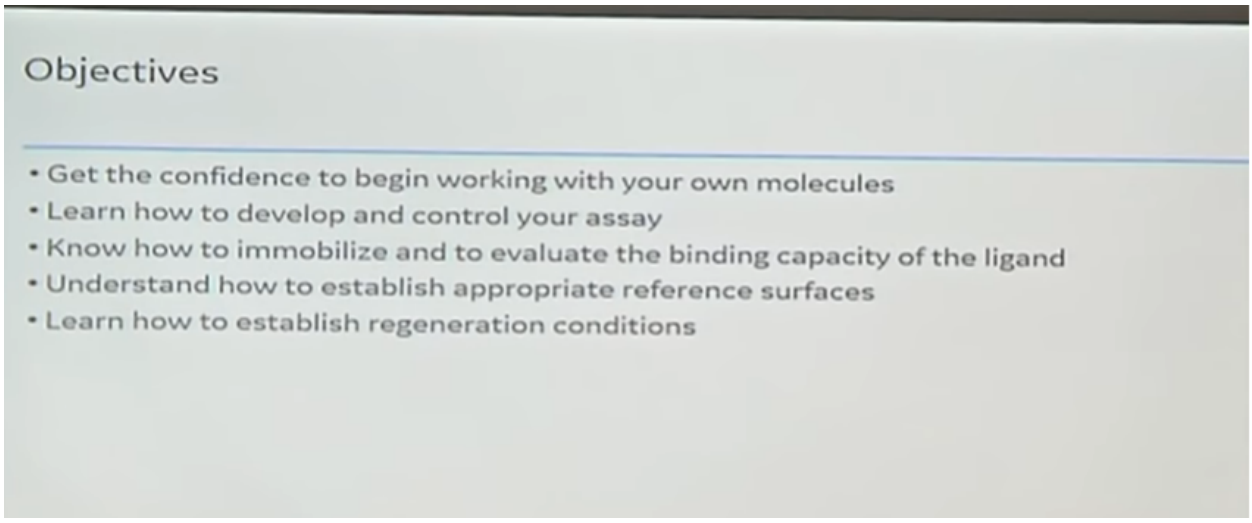

You know, what are the optimizations that you would like to do, what you know things like, which one -first of all how are the you know as, first I say how does it should look like, then which one can be alike in? What can be your analyte? What are the different reference surfaces that you can do? You 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 over, so well, right?

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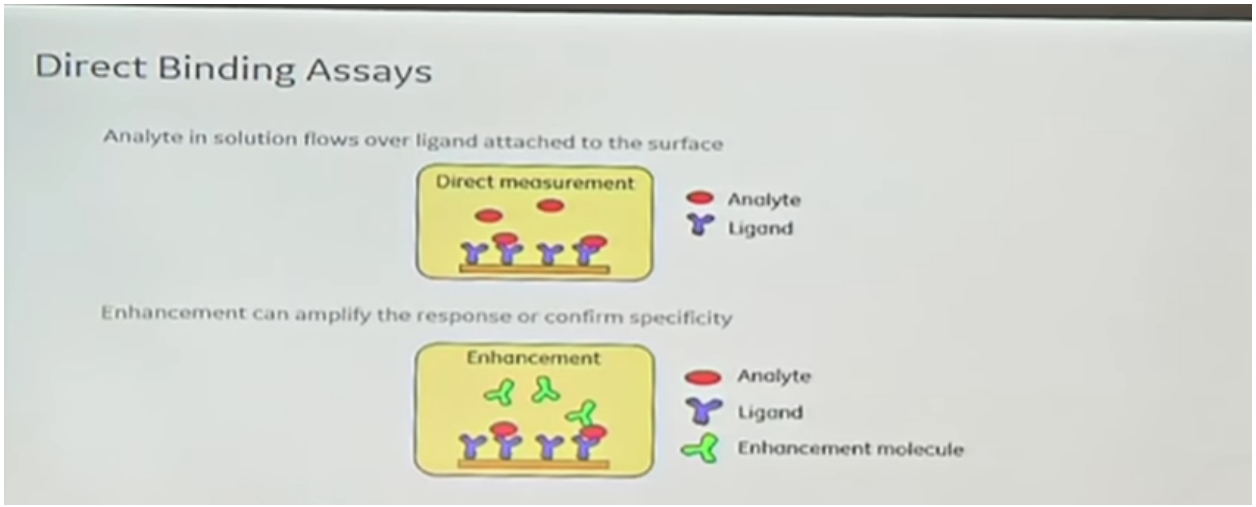

So, the first, so let's look at, some of the Assays format. The first one is the direct binding as a format, which is very simple, you know, you're you're interacting is actually immobilized on the surface, like you can see here. So, we talked about in Via Core, that you have a Ligand and a la analyte. Right? Whatever goes on the surface is called the, 'Ligand'. And whatever is flown on, the on the floor 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's, nothing if we call it an Enhancement or it is also similar to the capture method, right. What people are talking about? So the first the capturing molecule is immobilized, then you actually bind my your Ligand and then your actual analyte comes in binds right. So this is, we you can also call this an enhancement molecule, sometimes if

you're, if you're 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 sensor grams would look something like this.

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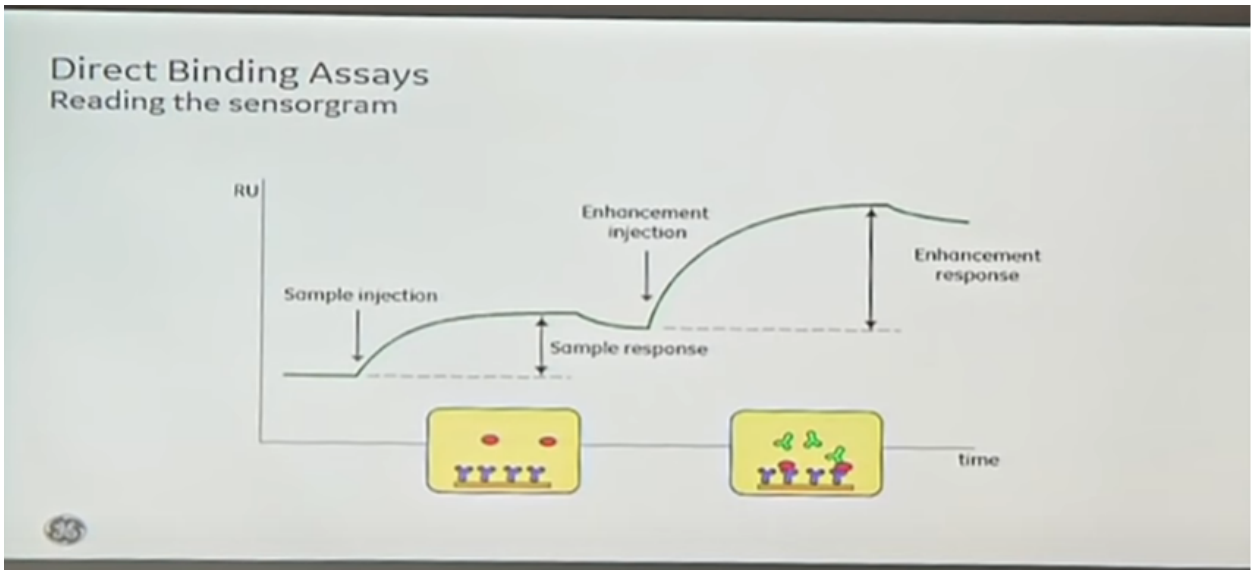

In, in this type you, this is where your baseline is, this is your association phase and this is your dissociation. And if you're looking at and after that you can actually do regeneration for this type only. But, if you're looking at this enhancement, what you do is actually first, this is where your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

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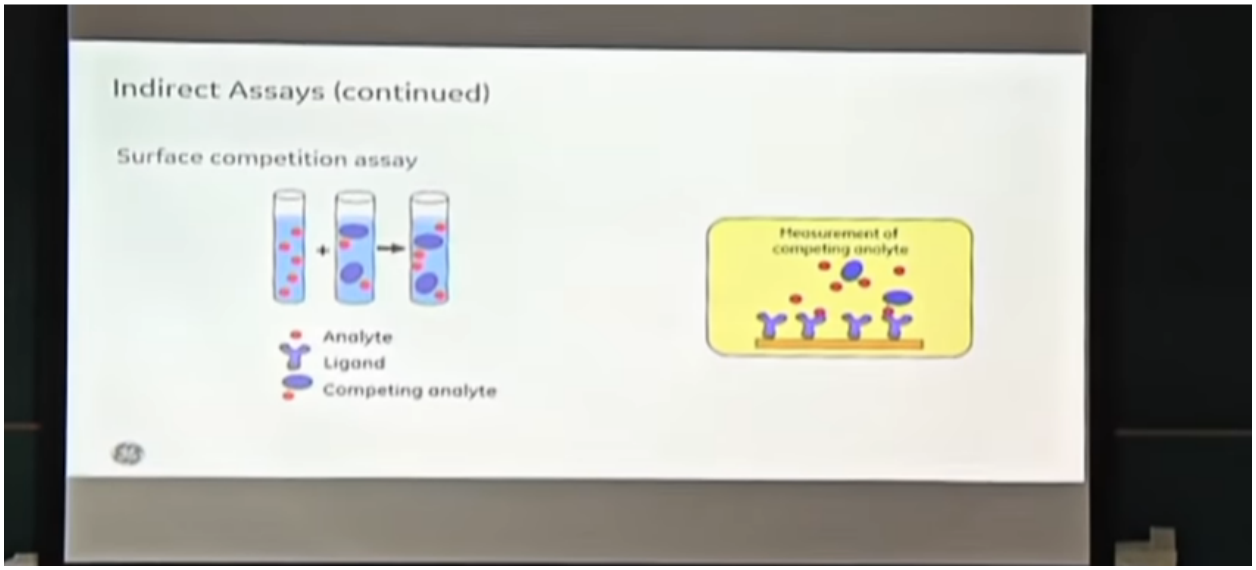

There are certain indirect bindings that you can do, where we use it, we utilize the competition. So the binding, doesn't really happen on the surface, you're having the binding happening on the, in the solution. Right? So this is called the, 'Solution Competition'. What you do is here, you mix the analyte and the detecting molecule in a particular portion, the analyte is kept constant, we're in this increasing concentration of detecting molecules. Okay? And you mix them, so with an increasing concentration of detecting molecule, what will happen is? You, you will with increasing concentration to Somali 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's actually a reverse, you know, with increasing, usually in the direct binding with the increasing concentration of analyte you see, increasing response unit, here you are seeing, just the opposite. Right? This is another one, it's a similar format. So here, you're actually having a competition, in the cells, in the term that you are, you're 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? Nowhere, when you, 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 it's a half high molecular weight analogue, where you link it with and the key is the high molecular weight analog, when you attach it, the size of this should be construe a larger than your analyte. You mix them, here they actually, in earlier case the binding was happening in solution, but here of there is no binding. When you put this mixture on, on your surface, where you're you know, Ligand's are bound , you see the binding only because of your competing analyte. Okay? When you these analytes are so small and negligible, you do not get to see your binding. So here, too with increasing concentrations of your analyte, you actually see less and less of binding. So it's again a reverse plot.

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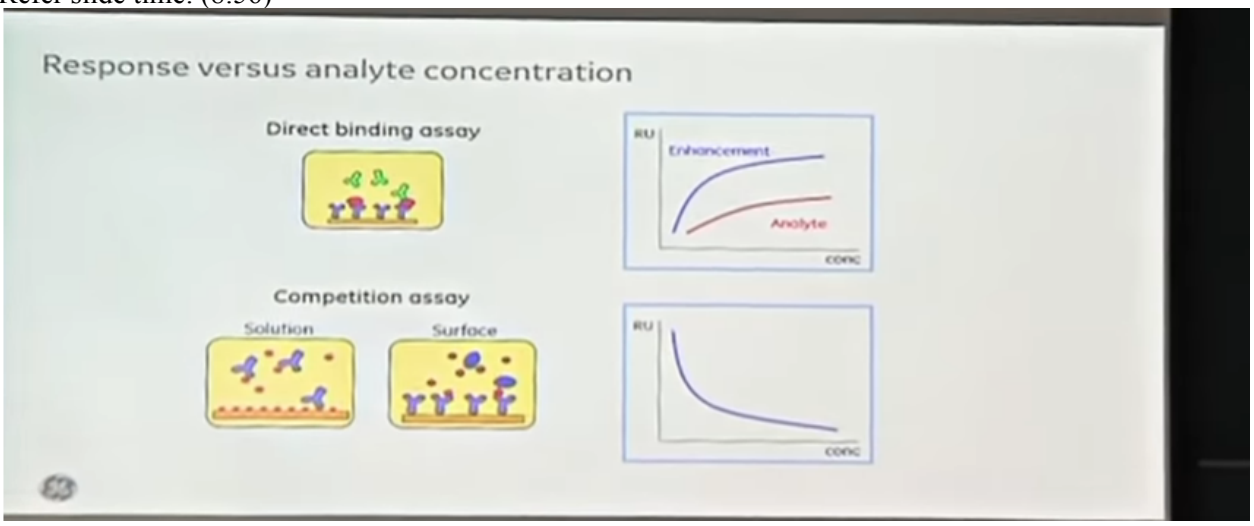

So if you, summarize a response versus analyte concentration in direct binding, you see increase scenario with analyte, whereas in indirect, you actually see a decrease in binding.

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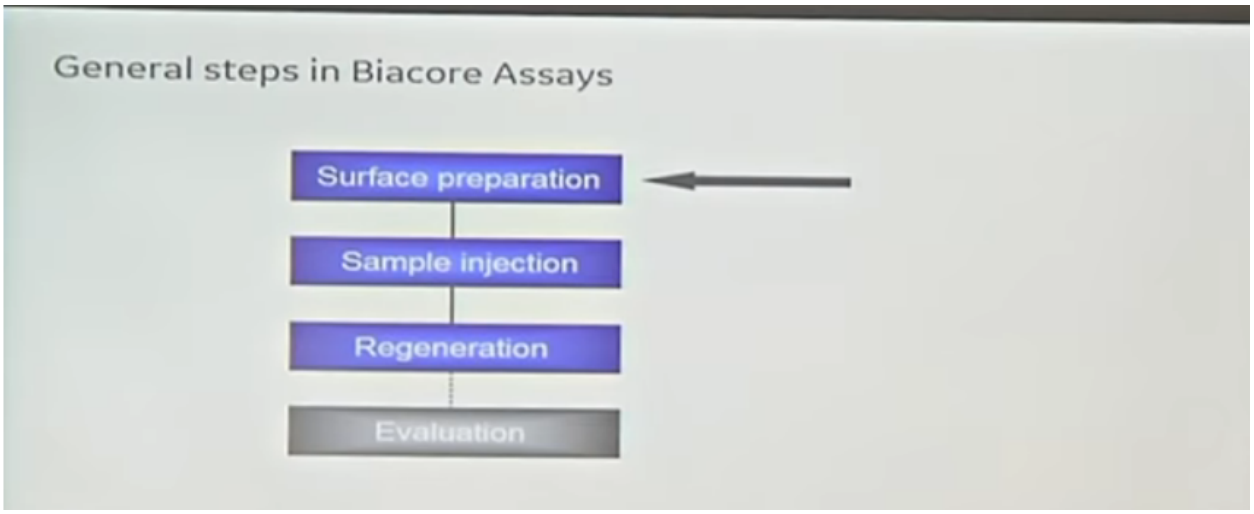

so, these are basically two types of that's, that's right, so you, you probably will not get the rate constants there, you can only do, only certain applications like concentration determination, yes. That's a very good point, yes. So you, so these are different various formats that are offered, but then again, it is limited to what applications you are using to yeah.Okay? Coming to the general steps of vehicle assay, I think I don't need to explain that, you are all now quite, comfortable with this you understand this, but we'll talk about now in detail on the surface preparation, by you know, so surface preparation. So, when you have, set up to interact to Mars. Right? You, you would like to use it in Via Core to see and bind it. Right? So you need to first understand, why you would like to, which fun you would like to choose as a Ligand? Which one you would like to choose it, as a analyte, things like that. Okay?

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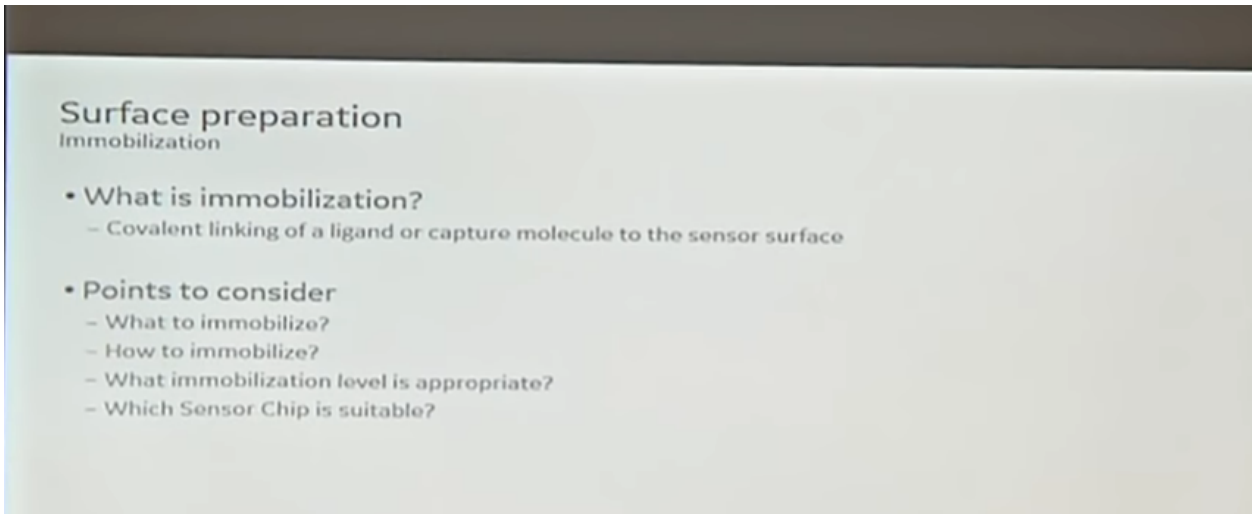

So, first of all what is immobilization? We all know right. How do we stick it to the surface? We covalently linked it right. it is not just any attraction it's, it's covalent linkage. So, once you immobilize, that is you know immobilize for good, you cannot strip it out. Okay? So, once you immobilize something on the surface, you can't strip it off. 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're 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? Okay? 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 Ligand's have to be mobilized very, very low, whereas if you are doing a concentration analysis, you need to have a high Ligand concentration. So this knowledge is very, very important. Okay? And which sensor chip is suitable for your Assay. We talked about various sets of censorship, like no cm 5, cm 3, c1, when you have a lot of nonspecific, hydrophobic 4 HPA, so all these things, NTA for nickel tagged, if you have something like that. Okay? What do immobilize? You 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 interact ants and one of them is large and one of them is small, which one would you like it to go as aligned. The small one absolutely, because you can use the larger one, as an analyte. So, you get a user you know, higher response you know, but having said that, with the Via Core 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 100 by the sensitivity, was not as high, with t200, tagging of the interact ants, if you have a tag, you would obviously like to use a capture method to put it in there as aligned. Right? functional groups, the few things functional routes and binding activity after 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, you know, then there's 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 an impure Ligand, the more junk you immobilize the data good 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's 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 doings binding it's fine to have a slightly impure analyte. Valances, the more number of valances where do you think it should go, it should it, you know, the so like an antibody and an analyte. Or so you, you used anti a beta 2 micro globulin today. Right? And beta 2 micro globulin as analyte, which one did it go on the Ligand, the anti, the valiancy was 2, you put that on the surface. Right? So, but if you had done the opposite and okay. so the question is, if you have an antibody on the surface and have an analyte on the flowing, on the surface, it is actually a 1 is to 1 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, 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, P I of the protein, PI of the protein, I would like to slightly stop by out here and we will explain it, in slightly greater detail one, in upcoming slides.

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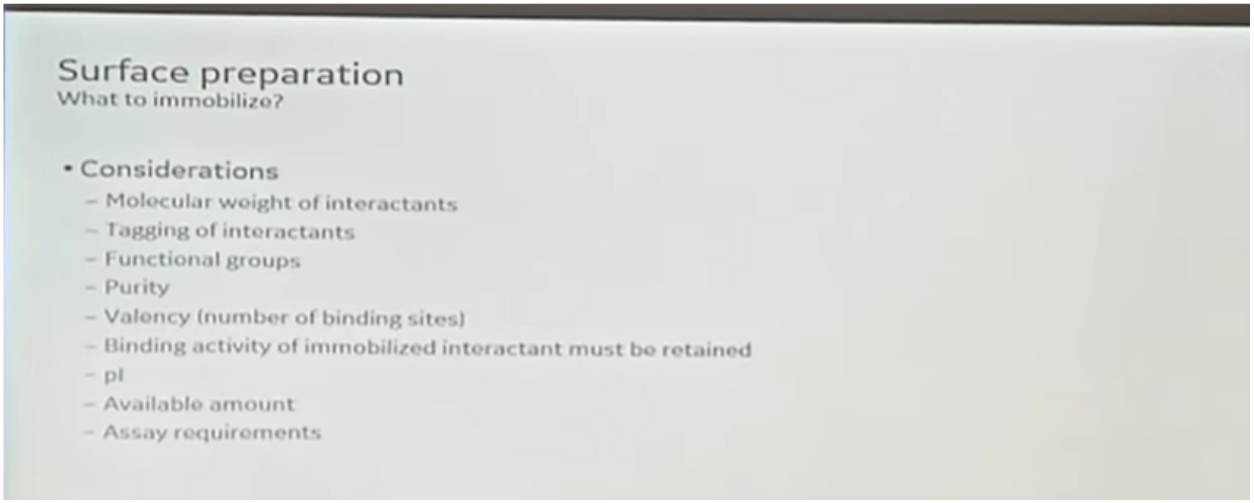

Because, that you will see is an extremely important, when you're immobilizing your Ligand. Okay? Amount of available, obviously if your amount is very, available is very little. then you know, you would rather use it as a Ligand, because when you are using it as analyte, you have to you know run various number of cycles, leading to more number of you know, requirement of analyte. And then, an Assay requirement 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 this slide you, you know, a surface can be prepared in two ways, one you directly immobilize the Ligand, right? The in the other one, you actually capture the, capture your Ligand.

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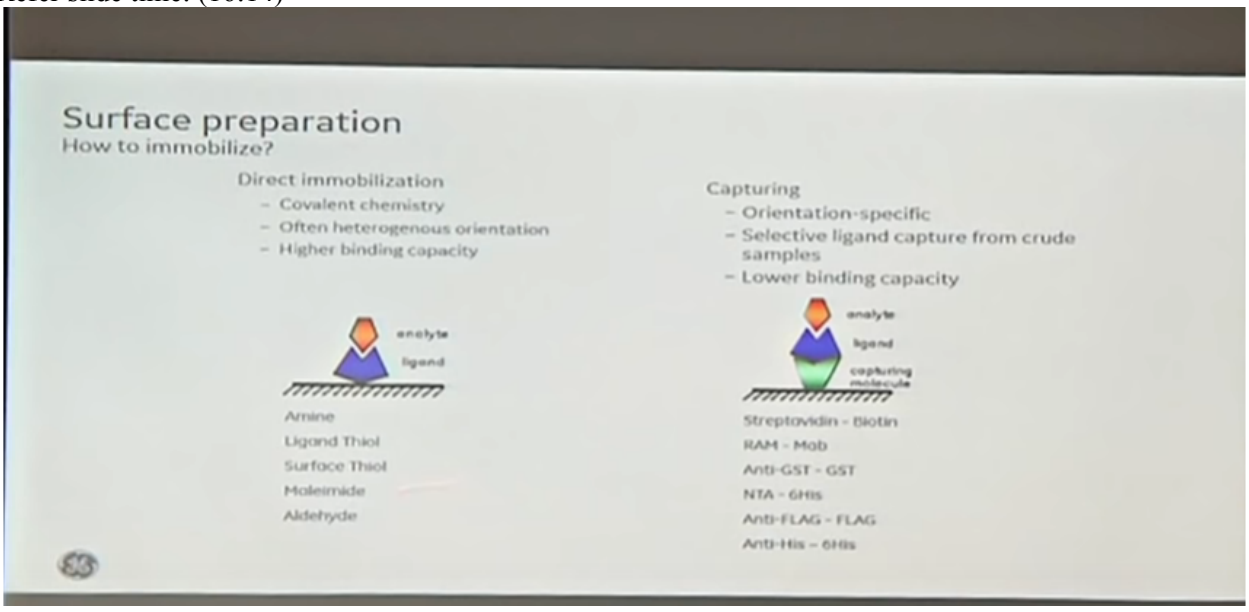

So you're, in capture you are actually, capturing molecule is actually immobilized directly on the surface. Now, the difference would be is that, you know in this case you; you lose the directionality of your Ligand like we were discussing. So, it is actually immobilized at random using, you know, any of the free

amine groups or the tile groups, on the surface. Whereas if you are using a capture molecule, you maintain a directionality of your, Ligand opinion.

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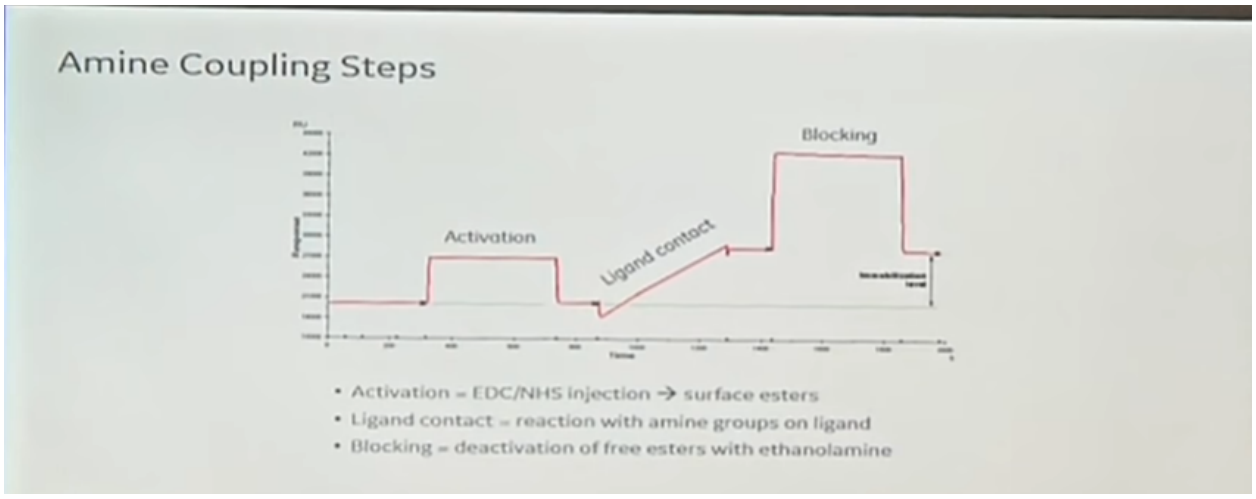

If you, if you're say requires so. This one that you're looking at is actually the step of your immobilization. So, how do we mobilize is like you know, first we activate our surface, using EDC /NHS and don't ask me, what's the full form, because I really cannot remember ever, the EDC NHS it what it does it actually activates your surface, into a the carboxyl groups, into a reactive ester group. Okay? And that's where you're seeing the EDCNHS is being pushed, after that you know, at this point your surface is actually activated and then, you push your Ligand. Okay? 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. The 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. Okay? choice of in immobilization strategy, it will depend on your Ligand again, amine coupling is very widely used, you know, in, 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 are available in most proteins. Right?

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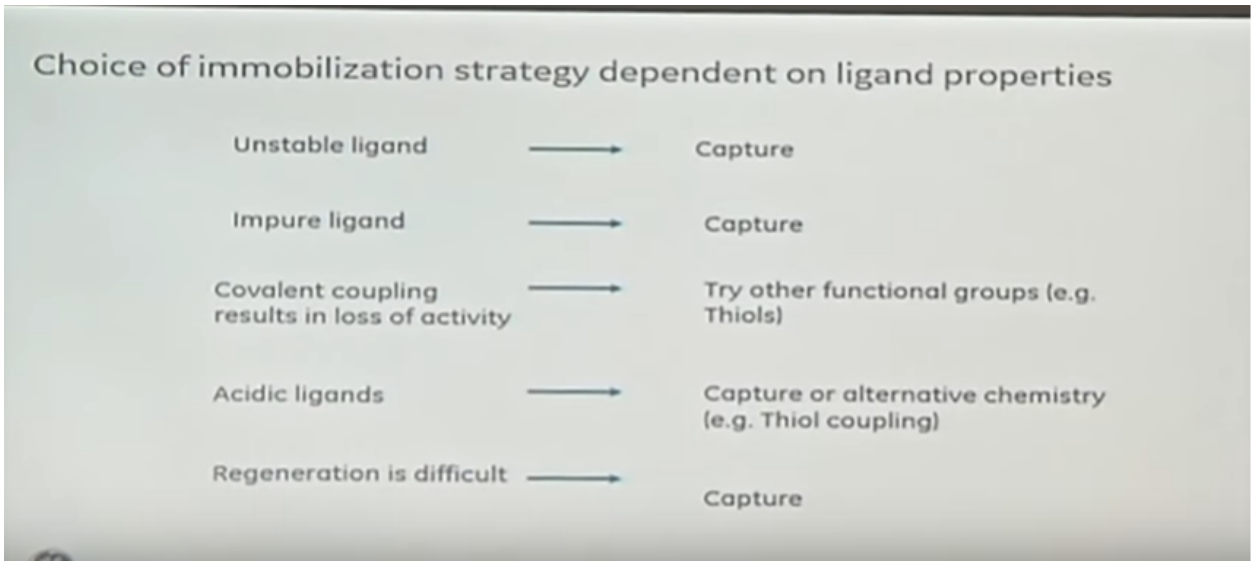

So, if your Ligand is actually unstable, then you would actually use a capture method. Right? If you, if you're, if you're using a covalent chemistry to immobilize and you're like and you know, loses his activity, then you would rather use a capture method. If it's an impure Ligand, like remember you 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 proper you know, results in loss of activity, say for example, you have done I mean, then you and it loses the activity, then try and use a chiral coupling or an aldehyde coupling, for acidic ligands typically, you know capture, chemistry is, 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. Okay?

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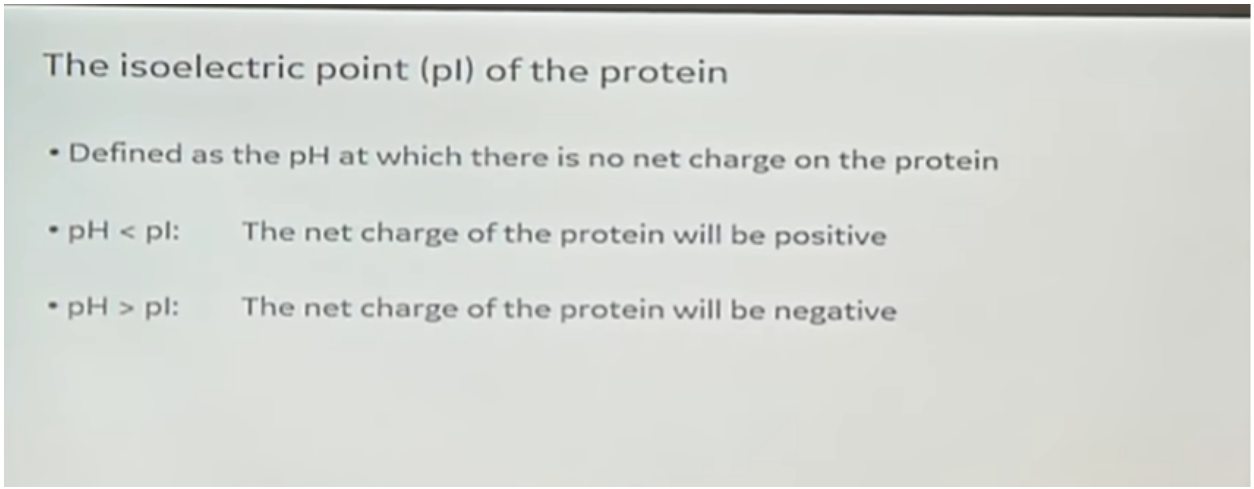

Coming to the PI point, which is extremely important here when you are immobilizing your Ligand. and I think, I don't need to reiterate this you know, we all know, what's the you know, what happens when your, when your protein is put in a pH, less than your PI, your protein is actually positively charged. Right? And if it is more than your PI your protein is actually negatively, charged so when we use this, it's the same thing, in a more schematic thing. Now, the pKa of the surface thecae, the chip. The pKa of the surface is actually close to 3.5. Okay? so if you are going less than ph 3.5, so I'm 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's, let's decide that. Right?

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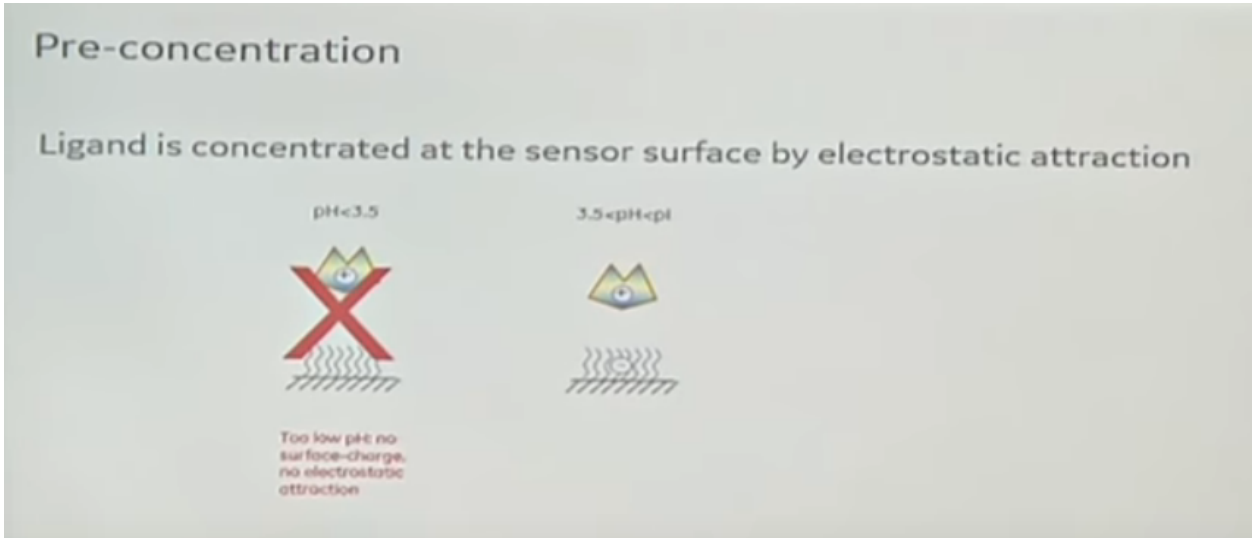

So if I put my protein in a pH 3.5, say most likely if the protein, of course PI is higher than that, it will be positively charged. But, the surface actually loses its charge. Okay? It has absolutely no charge, so in that case you know, there is no attraction between the protein and the surface. And 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 your covalent bond, can be formed. Okay? So, this scenario where your pH is very low, typically it's not a good scenario to immobilize your Ligand, you do not, see immobilization. So, it's too low for immobilization. Now, at a pH higher than 3.5, you know your, surface actually attains a net negative charge. Okay? And if your PI is, higher than that pH and in that particular page, able to keep the protein positive, you know, then your attraction happens. Okay? and this is the ideal scenario, where you're you know covalent linking, can happen very ideally. Again if your pH is extreme, a higher than your PI ,you know, then there is no attraction, because both of them becomes negative. So, that's what we call it as free concentration also, so when we are doing an Ligand, immobilization and sometimes we see that, we are not attaining the amount of REO 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. 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 led tells you ,which buffer, would be most convenient or ideal to use it for, immobilization buffer. Okay?

For immobilization purpose, for that particular protein, so you can take a little bit of your protein, before you do your actual immobilization.

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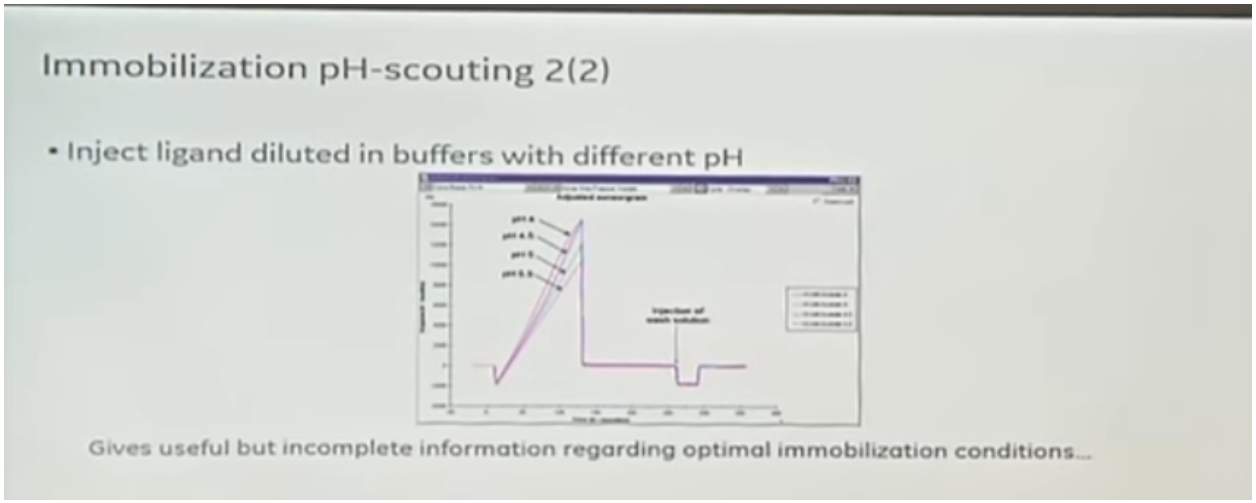

And run into this, pH scouting experiment. Okay? Take a small amount and then mix it with various buffers, typically the buffer that we supplies from ph 5.5 to pH 4. Okay? Mix them in all, different con, same concentrations, but different buffers and then bond them. And then try to compare and see what 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 outing. 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? That does that mean that, pH Phi, 4 is the best for immobilized. 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 5.5, which is actually kind of like the highest ph here, you attend quite a, large amount of our use. Okay? you go to around, something like I can't even see ten thousand dollars, which is a extremely huge amount of our use, you don't need that much amount of proteins, to you know, if you are reaching around four thousand or three thousand, it's 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, same thing and we ran it through, the whole immobilization process. Right?

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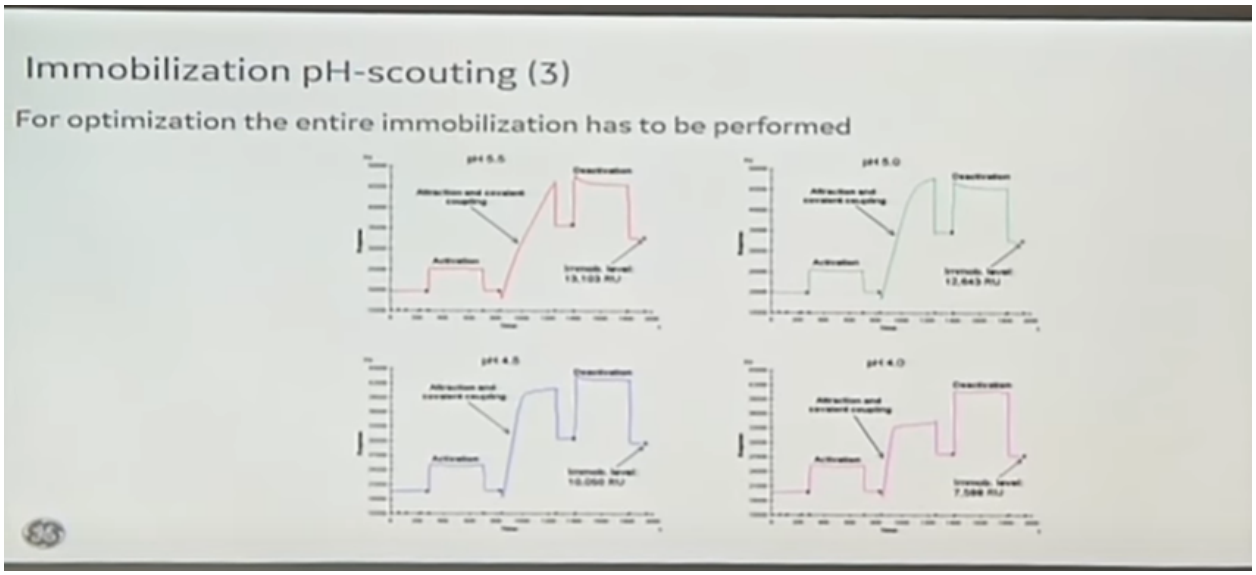

And you've seen ph 5.5, you get you get to around 13,000 are use. Right? and ph five, you get to around close or maybe similar, I don't think there's, I mean, thirteen point five and twelve point six, I think I would say, it's very close to each other. Four point five there's a reduction, it's much less and then again 4, it's even less. so where is our pre concentration earlier, should you know, with four point five and four it was still increasing, but here actually, you are actually attending a much, lesser are you than in considered to ph v and ph5.5. The logic out here is actually, so when you are actually attracting so much in ph 5.5, increasing the pH, lowering the pH even further and attracting, doesn't really help for covalent linkage. So that's where you start getting your steric hindrance and way or you know, immobilize, it 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 uncomfortable environment , close to where your p, proteins will be comfortable not stress them out in you know, in harsher condition, if required, if you do not see any rice, like for example, in these cases, if you do not see any rise in pH five point at five and five and suddenly you see, a pH rise in four point five, that's where you go into a lower, you know lower pH like four point four, four point five or four. Otherwise stay close to you know, more comfortable environment, does it make sense. Okay? Now, I think somebody was asking about the immobilization levels, so this is, so how do we find out the immobilization level? And in India K anta and how much, should we mobilize actually, when we are doing a Via Core. I say,

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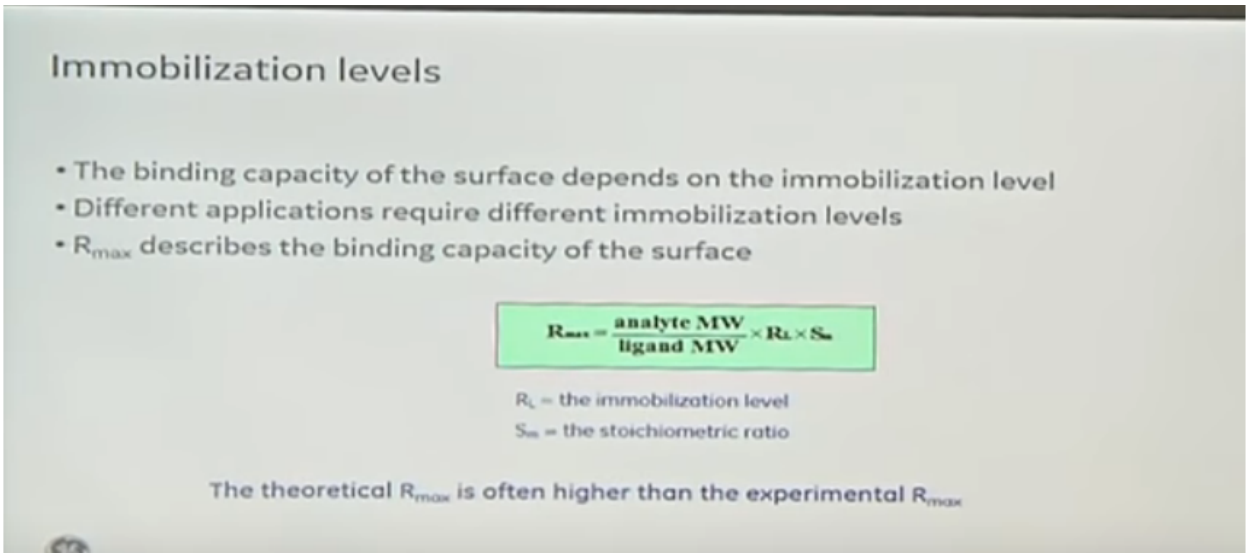

So we have this formula, which we call it as, it's kind of like a, what do you say? Ved Vakya in Via Core, our max is equal to molecular weight of analyte, by molecular weight of Ligand, multiplied by RL, into the stoichiometry. So R max we haven't talked about R max yet, R max is called the maximum binding capacity of your surface. Once you immobilize your surface, the capacity of the surface to bind maximally your analyte, that's your R max. Okay? RL is the Ligand impum, immobilized. Okay? So, when you immobilize your Ligand, you get a particular RL. Right? Say for example, you immobilize 500 R use or thousand R used. Right? So, that's your RL, I was toy come a tree of your binding, whether it's one is to one or if it's a by 1 it's two. 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 or your Ligand to begin with may not have 100% 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 and something is wrong, either the stoichiometry, that we considered was not right or there is some nonspecific activity or there is aggregates happening. Right?

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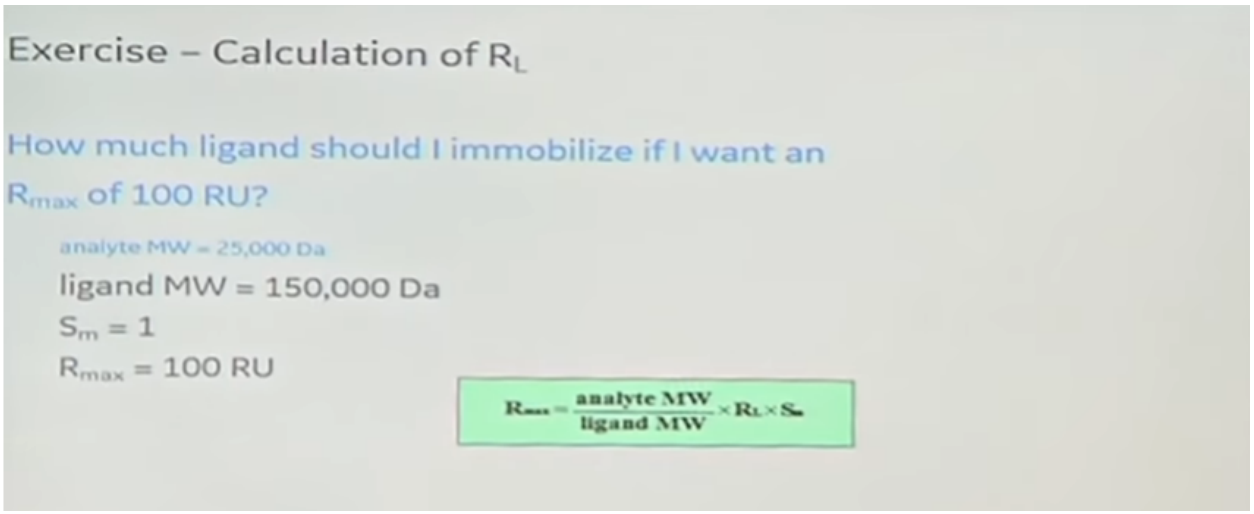

So there's a small exercise, if you would like to do hope it'll, wake you up, if you guys are sleeping. So, for a say for example, if we have a Ligand, that I would like to immobilize and I would like to work, at an R max of 100. Okay? and the molecular weight of your, analyte and Ligand are given, so your Ligand looks like a map, like 150 kilo Dalton, the analyte is 25KDA, show fame try is one and I would like to, reach in R max of 500. Right? So how much RL? How much of Ligand should i immobilize? Anybody, 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 it's 600 from your, theoretical I would go ahead and remove less 700 or 800 to compensate little bit and then, you know start your experiment. Okay? Is it okay? Right? Yeah, so, so if you're doing a kinetic analysis, you have to be, even 100our use is quite high sometimes, sometimes you have to go as, low as 50, 20 or you know ,T to 100 actually allows you to work as, as low as, five are use, R max. Okay? So you can still get a, very decent nice, no without noise, graph which we can perfectly, do an evaluation on and get a k, KD value of it. if it's 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, you find out the surface saturation, say for example, you know, if you're immobilizing a 150 KDA molecule, so your surface saturation comes at, close to around 10,000 to 15,000 R use. Okay? That's the surface saturation. Yeah, roughly. so and if it is say around 25KDA, then it is around 2000 or 2500, that's the surface saturation and when you're doing a kinetic, you should never be in a cell saturation mode you, you kno w, there is something called, mass transport emit a, 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. R use, resonance you, response unit. Yes, exactly, so like I said, right way it is related to the refractive index, so the change in refractive and the change in mass, will change the refractive index and that is related to your response unit, response unit. no analyte molecular at all, well it can allow a lot, I mean, and then it's not about T to 100 allowing it, it's the, the analyte and the ligands molecular weight, which will contribute to it yes, five R use ,of R max, yes. Okay?

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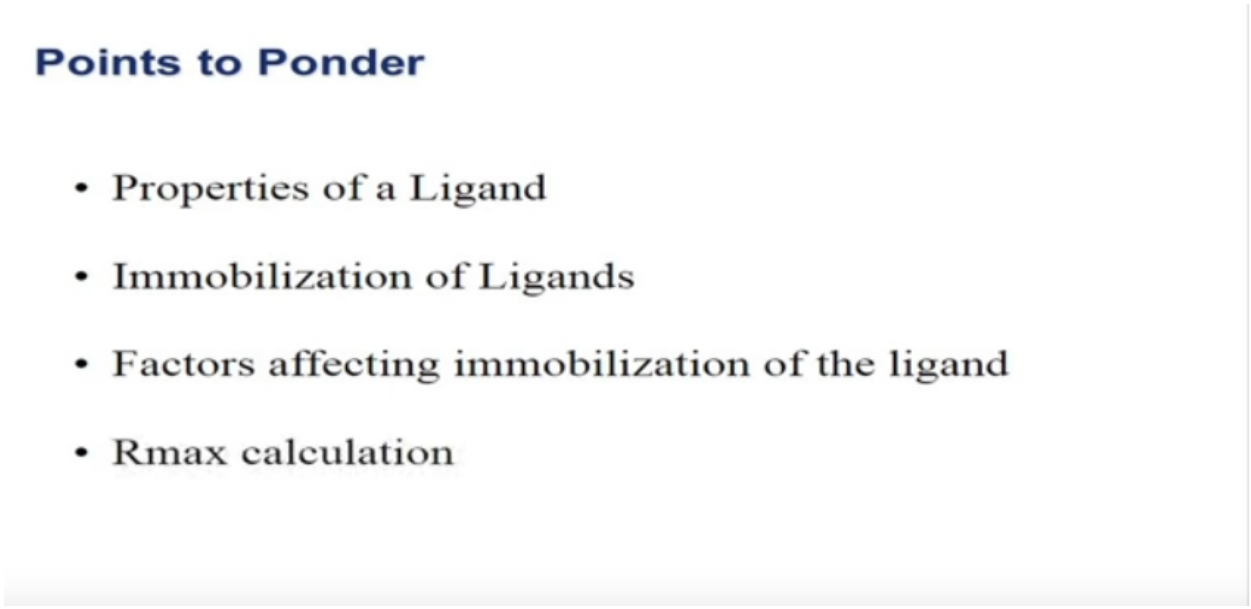

So, I'm sure this was very informative lecture, by Dr.Uma. You are convinced, that she is able to convey, the very hard ideas and principles involved, in this technology, in a very lucid manner. In the next class, Dr.Uma will continue to explain, some more detail of biacore technology. She'll also provide,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