Interactomics: Protein Arrays and Label-Free Biosensors.
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Lecture-6.
Basics of SPR: Experimental Design.

Welcome to MOOC NPTEL course on intractomics. In our last lecture, we learned about various kinds of commercially available surface chemistries to perform different bio-molecular interaction studies however there several other parameters including ligand purity, optimum immobilization levels, immobilization buffer, injection strategies, appropriate reference surfaces and analyte regeneration etc., which are involved in aptly designing a surface plasmon resonance based assay.

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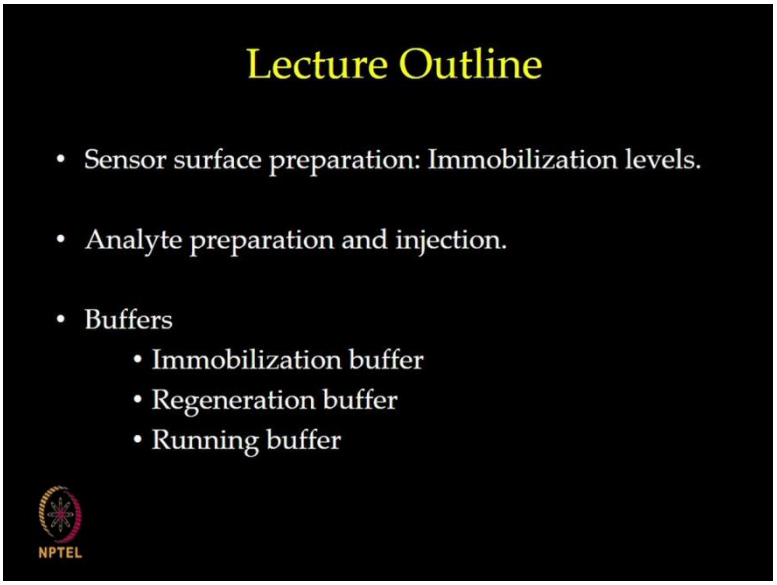

In today's lecture, Dr. Srinivas from GE healthcare will talk to us and highlight, the importance of some of these parameters including the sensor surface preparation, immobilization levels, analyte preparation and injection. Welcome back, Dr. Srinivas, today we are going to talk, SPR assays and different criterion which one need to take care of during these experiments.

So, experimental design is very crucial for the SPR experiments. Buffers can be a key to a success in SPR based experiment, which is a very crucial factor while designing this kind of experiment. Therefore, Dr. Srinivas will also elaborate on immobilization buffers, regeneration buffers, running buffers and buffer scouting.

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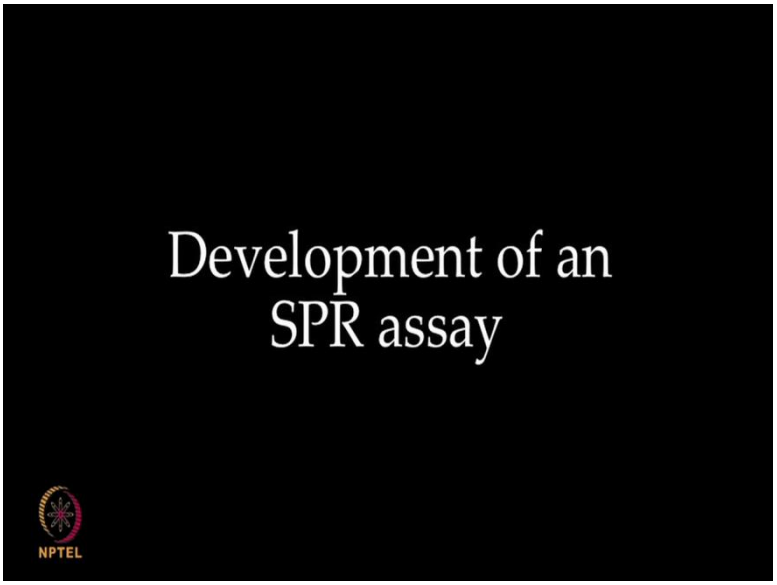

In this session, we will understand how we can develop an assay biacore or a surface plasmon assay and the objectives of this particular studies, how we will understand to work with a molecule? How we will develop an assay, control an assay, we will understand about the reference surfaces, we will also understand about the regeneration of the surfaces and all that. And coming to the different varieties of assays that we have, one of the very simple way of assay would be a direct binding assay.

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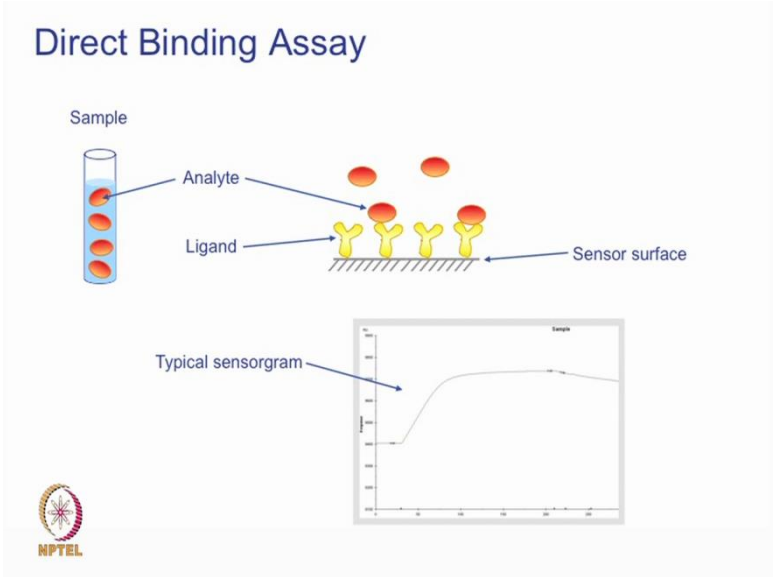

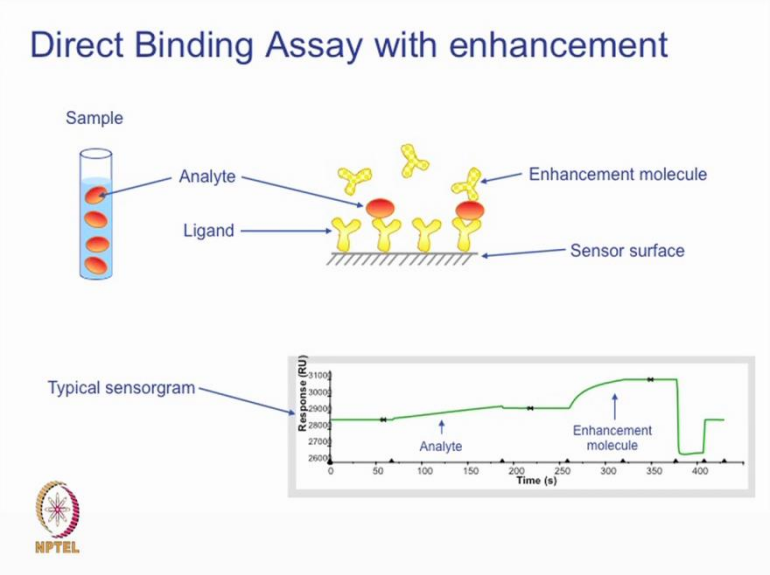

In a direct binding assay, you have the ligand that is immobilized on the sensor chip and an analyte that is present in your other sample is passed on the surface and the interaction is recorded and you can see on the screen the interaction shows this way a very simple binding and the regeneration, whereas sometimes binding generally does not go a very noticed, the reason is the responses from the analyte are very very little. And that particular stage what we can do is, we can pass an enhancement molecule, enhancement binds to the analyte and actually, shoots up the responses, so that it can be covered or captured by the system in a prominent way.

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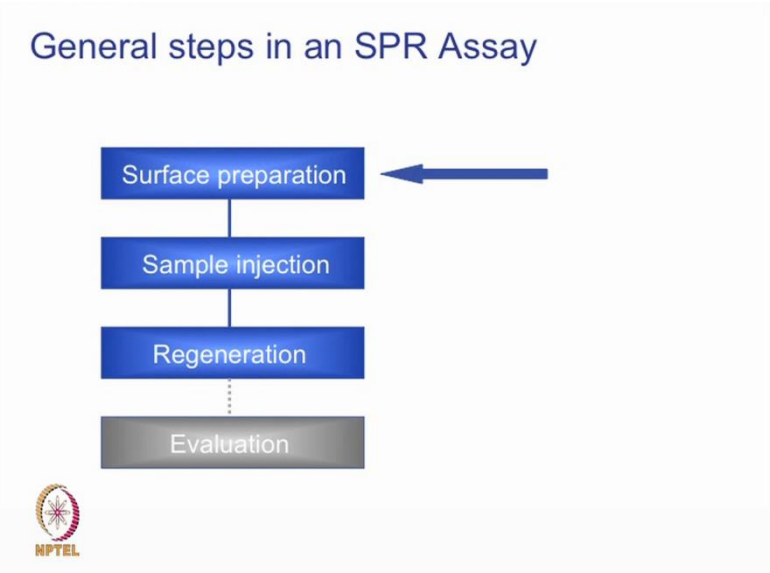

The way the experiment is designed will actually identify the molecules, whether they are binding to one particular side or they are binding to two different sides on the surface of the sensor. The various steps that are involved in a biacore assay are surface preparation, sample injection, regeneration and evaluation. So, we will start with the surface preparation. surface preparation is nothing but immobilization and the immobilization generally is by two ways done by two ways, one is a covalent linking and other is a capture method.

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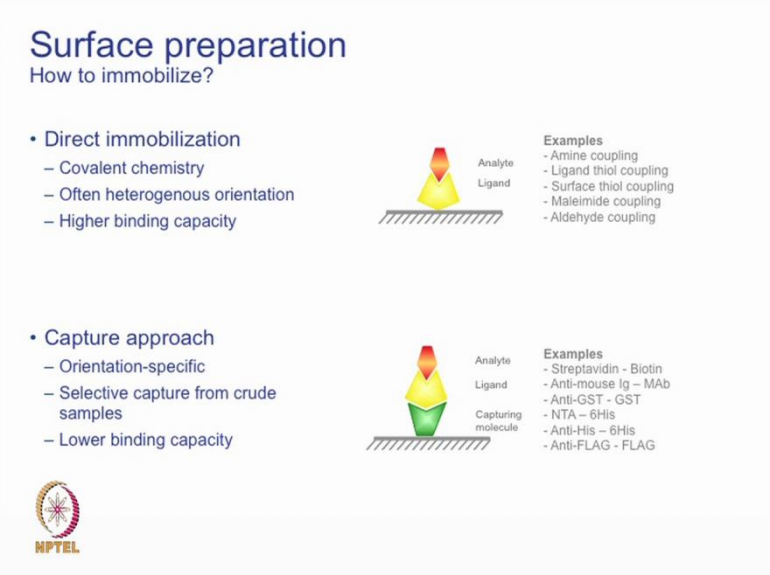

So, when we actually trey to go for an immobilization, the very important points that we need to consider are what to immobilize? How to immobilize? And what is appropriate level and what is a right sensor chip that is required and when we are immobilizing, we need to know a great amount of information about the protein that we are actually mobilizing or macromolecule that we are immobilizing, we need to understand the molecular weight of the interactant, the tags that are present on interacting the functional groups, the PI of the molecule, the purity of the molecule, the available amount and the required assay conditions and the binding activity, valency are some of the important points are which we need to consider when we are going for identifying a ligand.

Uhh Immobilization is done by direct method as we just discuss and that immobilization can be by covalent chemistry and there will be high binding capacity and the ways the various examples, of a direct immobilization are amine coupling, thyol coupling, aldehyde coupling, maleimide coupling are the methods that are available. Whereas when we look at the capture processes because most of the molecules are expressed these days with tags. And when you have tags on the molecule, they are different chips which will capture the tags, let say we have streptavidin chip that is available and biotin molecules like protein (mol), peptides, DNA, RNA all these molecules are actually captured by streptavidin chip.

Uhh most of the time we have antimouse antihuman capture kits are also available and these kits actually will help to capture mouse and human antigens and or antibodies and then we will understand the interaction with an antigen, also we have the antihis antibodies which will be able to bind to the His-Tagged proteins or a NTA chip which will capture histidine molecules. And a flag molecules are also captured when antiflag antibody and similar way.

The advantage of a capture approach is that capture approaches can be impure or you can have a crude mixture of a ligand when we actually go with, a direct binding method or a covalent coupling method.

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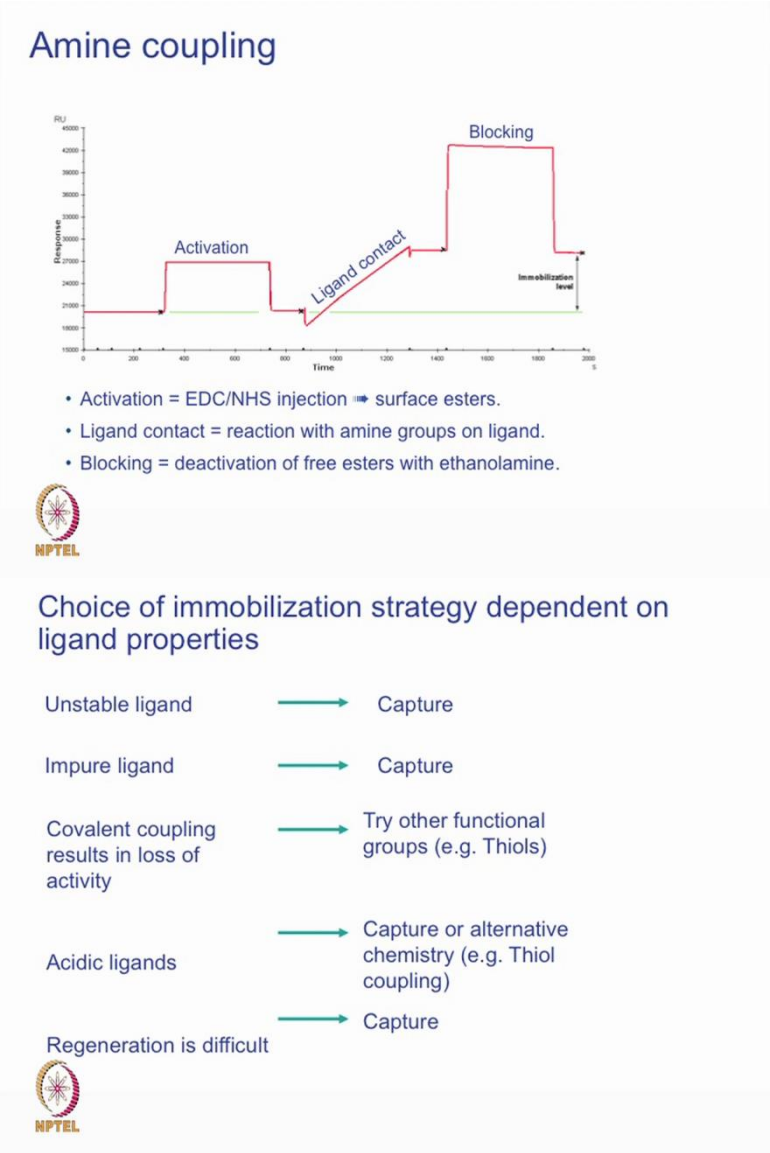

The various covalent coupling methods have been clearly distinguished in the literature, we will take one example a here, which is an amine coupling, which is a very common way of coupling proteins on the sensor surface. And here, the activation of the surface is generally done by chemicals EDC and NHS and they will activate the surface esters and the molecules from the amine groups are actually covalent coupled on the surface of the chip and the extra groups that are activated are blocked by ethanolamine.

The choice of the immobilization strategies generally depend upon the kind of molecule we have. Let us say, we have an unstable ligand then we go for capture. Let us say, we have an impure ligand we go for capture if we have a covalent coupling when we do a covalent coupling and that covalent coupling will lead to loss of the activity which means that your analyte is not binding then we will not proceed for a covalent coupling but in that case, we will identify capture process.

Sometime some of the ligands, the PI is very very close to acidic range like two or three and those kind of molecules, we will still prefer to go for a capture process. Sometime some molecules, the regeneration is very difficult in that also we go for a capture process. So, this way we distinguish between what molecules can be captured and what molecules can be covalently coupled. And a very important point during an immobilization design of an immobilization of a molecule or a development of an assay, most of the times we need to know PI or the isoelectric point of a protein.

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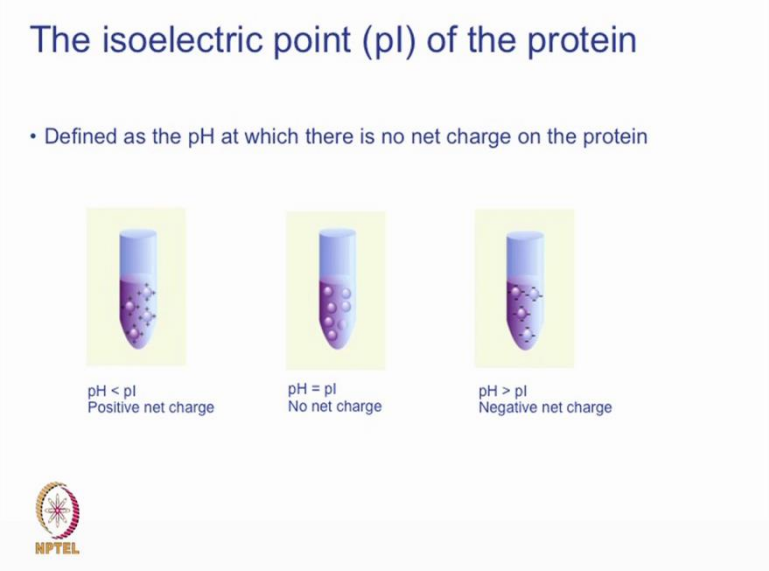

Uhh the PI isoelectric point is no net charge on the protein and or you can always say that you have equal amount of positive negative charges that are present on the protein. So, for immobilization strategy, it is always better that we go below the PI and at least one or two units below the PI where the amine groups are perfectly exposed or any of those functional groups are perfectly exposed and will be available for a covalent coupling.

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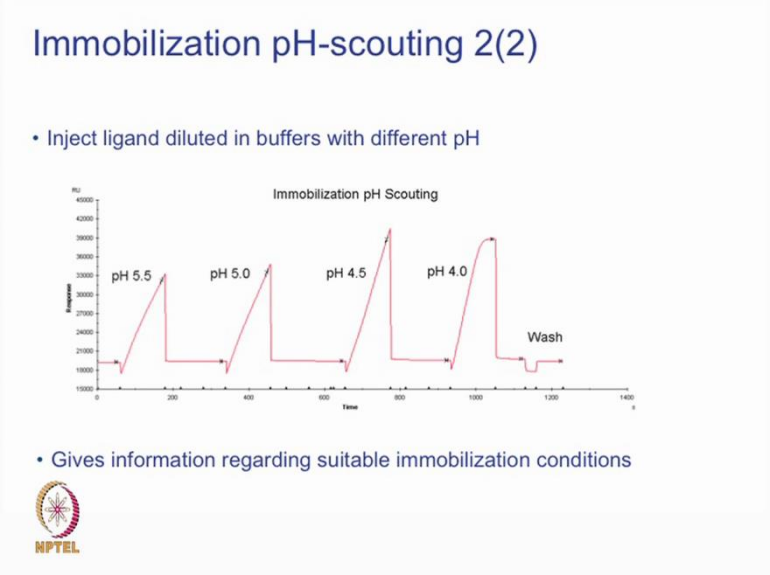

In this process, if we sometimes are not aware of a PI because the molecule is a new molecule and it is just recombinently expressed and we do not know the molecular isoelectric point, in that case we will go for an immobilization pH scouting, in which we will dissolve in protein at different pH then we tend to pass on the surface of the chip and see what is the responses that are coming up from the molecule. And wherever the molecular in pH is good enough of the immobilization, we go ahead and do immobilization at that particular range.

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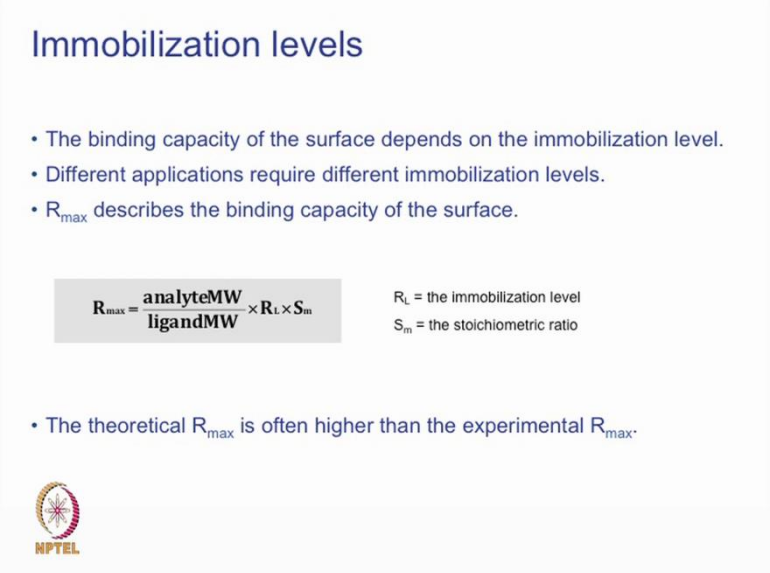

Most of the cases proteins are generally immobilized at 10 milimolar sodium acetate buffer pH 4.5, but this is a general assumption, go for the immobilization level can be done by two

ways; one is either you go for a control immobilization process which is called Aim for immobilization or you aim for an immobilization level based on the calculated Rmax stoichiometry, binding level and the number of binding sides the molecule has all that is taken into consideration and the level of immobilization is determined.

Once the level of determination is done, we will go ahead and ask the machine to immobilize at that particular level. Sometimes we can always go with time and flow mode in which you will just ask the machine to flow your molecule in that particular pH for some amount of time at a particular flow rate and whatever the immobilization happens at the end is the immobilization level.

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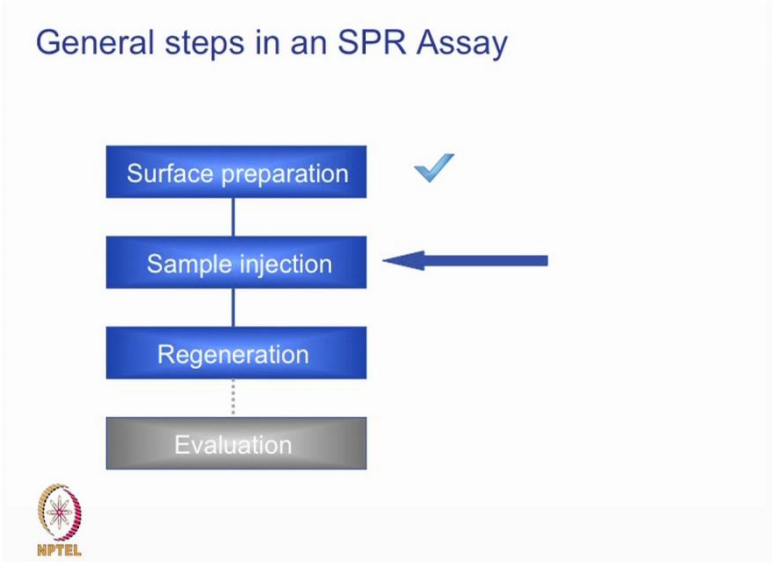

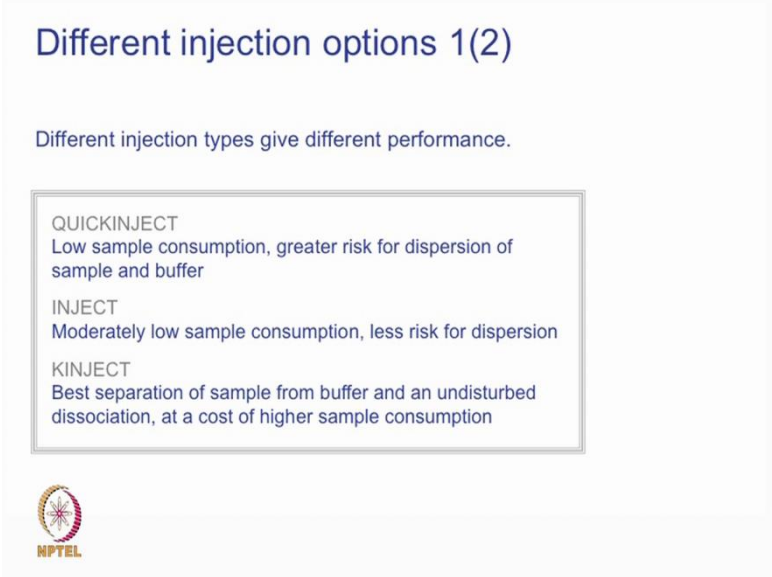

So, coming to the different steps in our assays. So, the next important step developing an assay is a sample injection. In a sample injection mode, you inject your analyte and the way the molecules are injected depending upon the way the molecular interaction process is happening. If you are screening then there is a very slow way of injecting your molecule, slow means the flow rate when you arte injecting the kinetics, the kinetics are injected with a certain flow rate.

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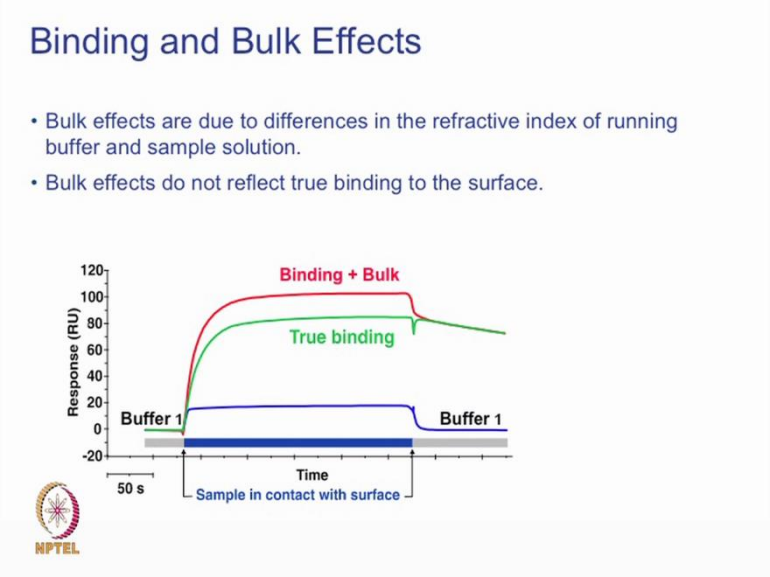

So, the sample injection process actually depends upon the flow rate and the different modes of injection are generally called as quick inject, kinject and inject and these modes depending upon the mode of interaction or the type of study that you are doing let it be screening, kinetics, thermodynamics, the mode changes and based on the mode the injection process changes and the interaction is recorded at that particular flow and contact time.

Another important parameter when that to be considered when we are designing these experiments is about the bulk. Bulk is nothing but the difference in the refractive index of running buffer and the sample solution. So, this is the place where most of the people go bad in their assays and will not get proper a kinetic data. So, I would suggest that people have to equilibrate their analytes to the running buffer so that to get a good kinetic data. And another important point in a designing an experiment is the references, that there is always reference upstream your assay but the references have to be not giving much of response.

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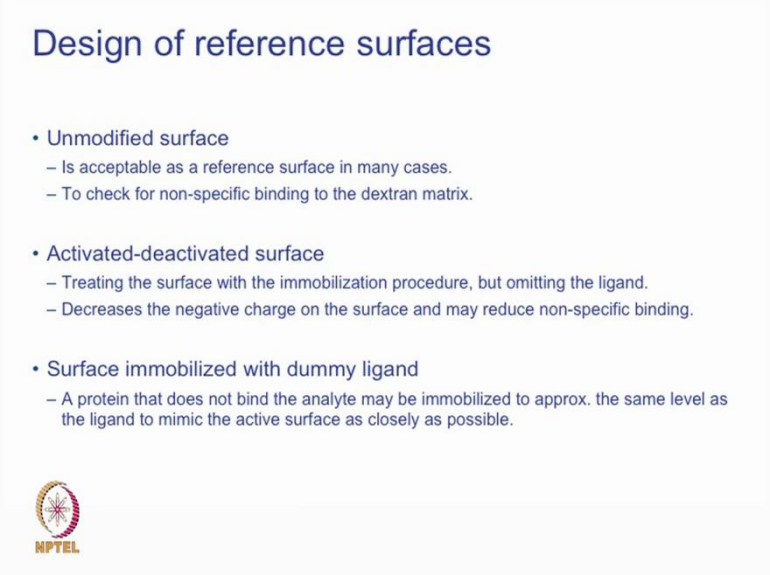

Non-specific response is something that is quite common in these kind of an assay widely followed a different reference surfaces are unmodified surface in which you take the reference surface as such or a modified surface in which you activate and block the reference surface. Or a third way of doing is an immobilization of a dummy ligand on the surface of the reference and use it to subtract the non-specific binding and the coming to the sample, sample or analyte is a major contributor for a bad data. So, it is very important that we purify them or characterize them before our experimentations.

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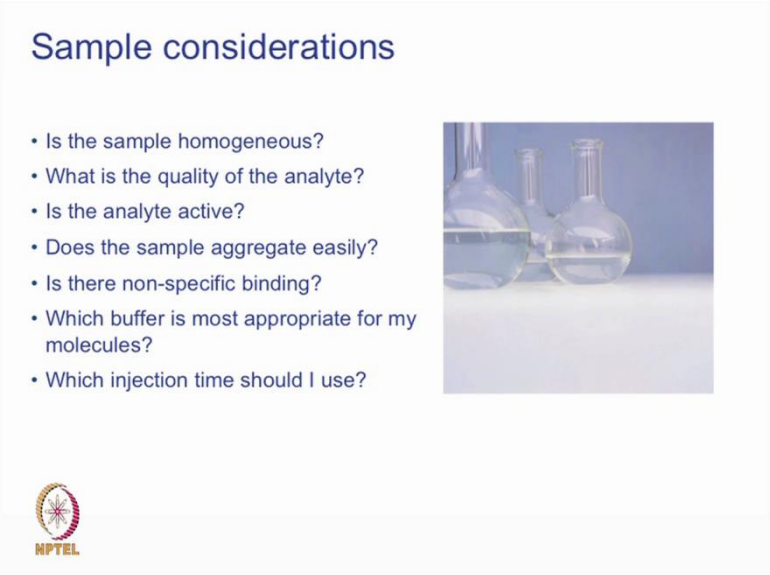

Homogeneous samples are good enough to be considered please mixture that your sample are highly homogeneous, good quality, active and they do not aggregate and they do not give us a

lot of non-specific binding and it is very important to understand the right buffer for a right sample and also once we have identified the good sample conditions then we should actually think about the association time and the time at which we need to setup our biacore experiments buffer or a running buffer or a sample buffer or a very important area that we need to understand little bit here.

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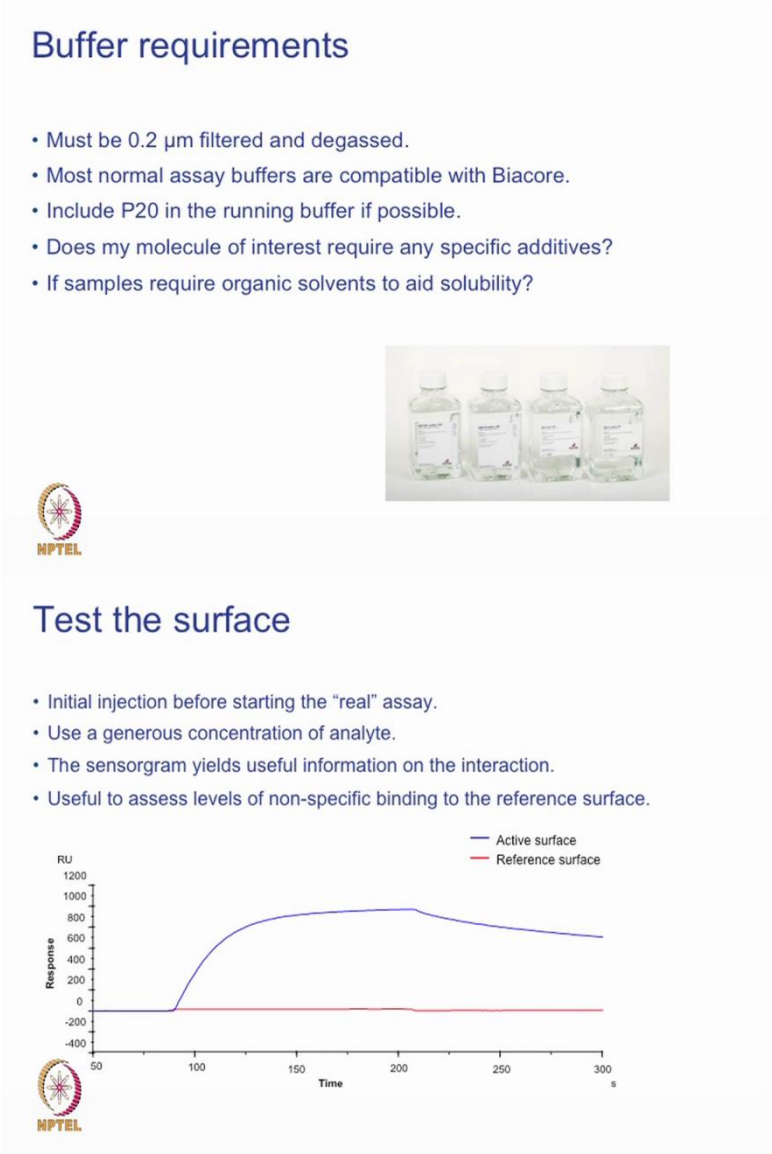

A buffer have to be filtered thoroughly degassed and as many of the machines have a degasser and filter that is available in the system but it is obvious that we can also filter them and degassed them prior to experimentation. All buffers are compatible for SPR systems and it is very important to add sometimes a detergent to make sure that there is a very little amount of non-specific binding.

Most of the molecules of the detergents that are used for into running buffer to prevent nonspecific binding is a polysorpy 20, twin, SDS all these molecules are added into buffers and buffers are prepared. And also sometime some of the additives are especially required for your pure running buffer sometimes likes metal ions, some kind of other activating molecules, activators all these need to be added into your running buffer or a sample buffer to maintain the conditions for your experimentation. And it is very important sometimes when we filter and degas, we need to check for the proper filters, which are compatible with water and organic solvents.

And once the experimentation conditions are set, the analytes are prepared, the ligand is immobilized then the it is very good way to start by injecting a small amount of your sample to see what is the binding responses that you are getting and to what time you should continue your binding and when to dissociate and all that. The process is called test of a surface you can just test the surface to understand the interaction and also understand the regeneration condition and level of non-specific binding that is coming up from these interactions.

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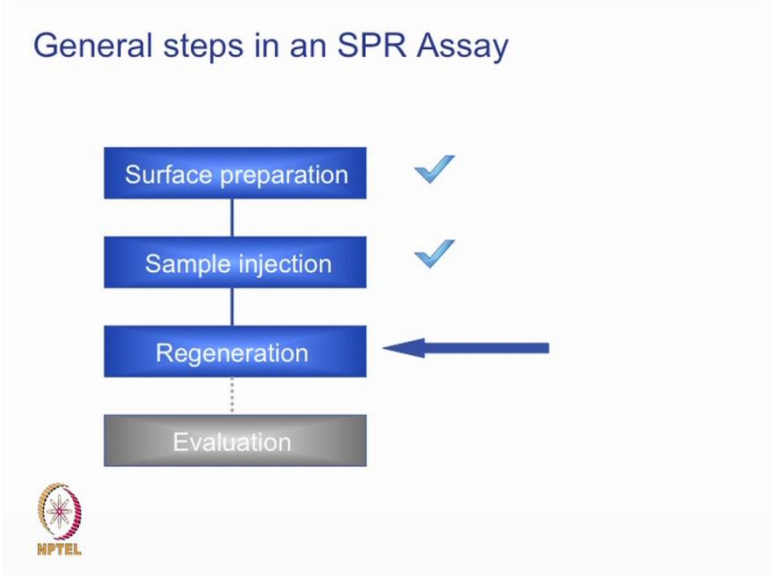

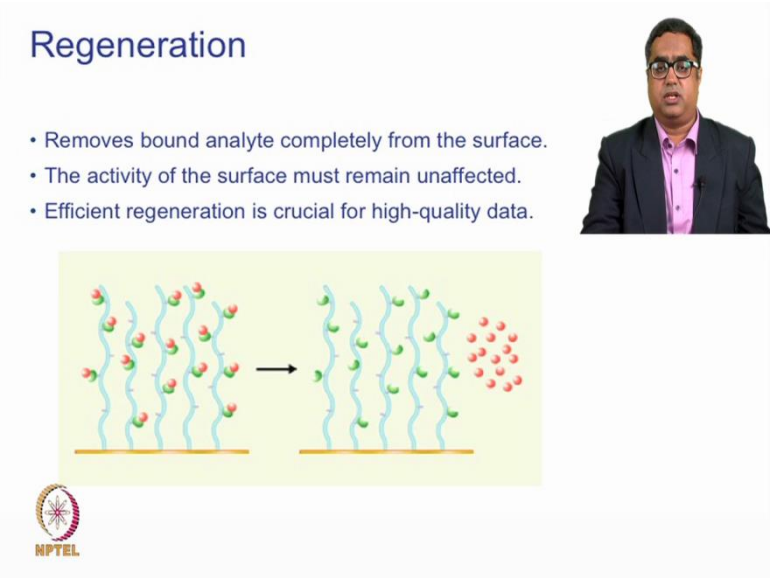

Another part of the assay is regeneration and in regeneration it is very important that we look at what are the different regeneration conditions and what are the ways of doing a regeneration. Regeneration is nothing but regenerating the surface back or the immobilized protein and the analyte interaction is been prevented by some kind of a solution, so that the surface is regained and again useful for another interaction.

So, that process can be studied and there are different ways to study that regeneration conditions, develop the regeneration condition require for a particular interaction. Regeneration as I just said removes the bound analyte completely from the surface, the activity of the surface must remain unaffected after regeneration and an efficient generation is crucial for high quality data.

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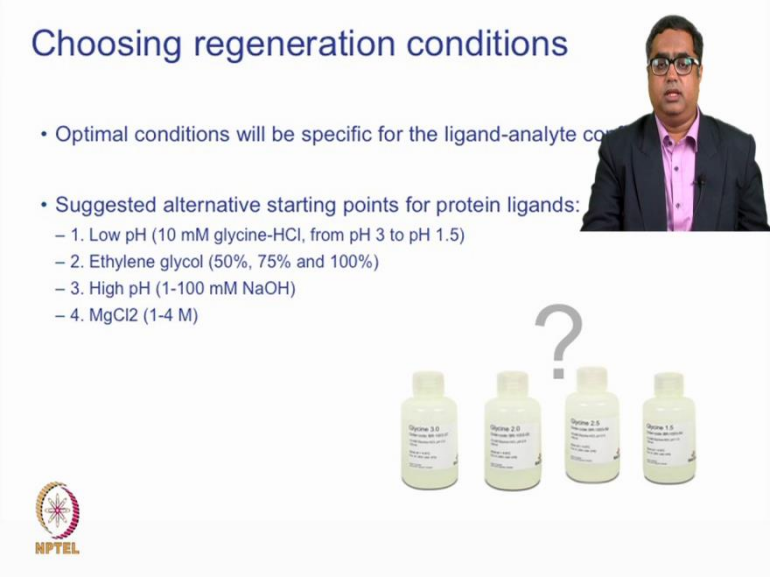

And regeneration conditions we need to test the regeneration surface, we can do a regeneration scouting before start of an assay or develop an assay and then identify the right or the appropriate regeneration conditions. And some of the very natural or a regular usage of regeneration conditions are low pH, ethylene glycol, high pH and magnesium chloride.

So, once we identify the proper regeneration solution, it is very important for us to identify, once we use this regeneration condition are we getting our reference surface on our active surface back after injecting this reference. In this particular session, we have gone for identifying what is an appropriate ligand or conditions for immobilization of a ligand? What are the different ways we can make an analyte sample, purify characterize the sample, conditions that are required for, biacore assay. And finally we went through what is bulk, what is non-specific binding and also, we identified or studied about a different reference surfaces and also, we went through a great detail about regeneration conditions.

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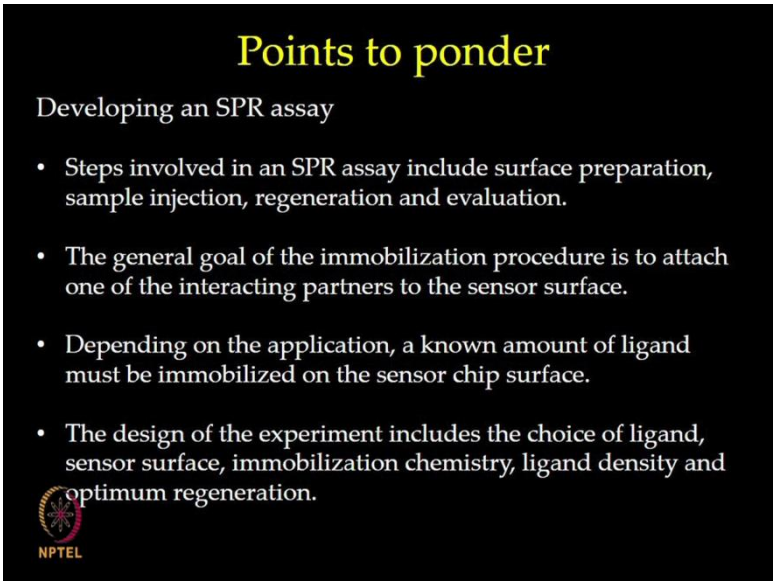

Thank you very much Dr. Srinivas for very nice lecture. It was very useful discussion on SPR surface chemistry and experimental design which we have discussed in last two lectures. Thank you very much, thank you.

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