Interactomics: Basics and Applications

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Lecture – 32
Basics of SPR: Experimental Design

So, we have been discussing the basics of SPR, surface chemistries and now about the experimental design. In the last lecture we have learnt about various kinds of commercially available surface chemistries to perform different type of bio-molecular interaction studies. Apart from this sensor chip surface there are several other parameters including like impurity, optimum immobilization levels, buffer injection strategies, appropriate reference surface, analyte regeneration which are also involved in aptly designing a surface Plasmon resonance based experiment.

In today's lecture Dr. Srinivas from G. E Healthcare will talk to us and highlight the importance of few of these parameters including sensor surface preparation, immobilization levels, analyte preparation and injection. Experimental design is very crucial for the SPR assay and buffer plays a key role in designing of these experiments. Therefore, Dr. Srinivas will also elaborate on immobilization buffer, regeneration buffer, running buffers and buffer scouting. Some of these are very crucial parameter in the experimental designing of SPR base experiments. So, let us have this lecture by Dr. Srinivas.

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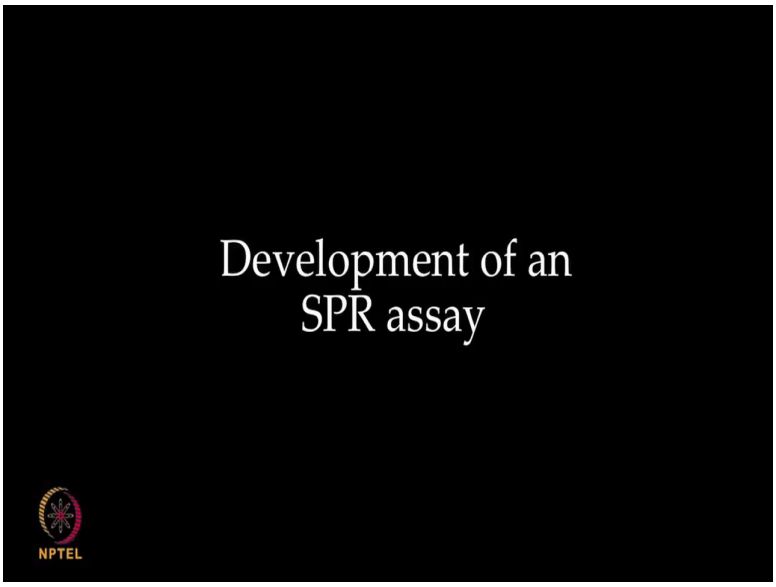

In this session, we will understand how we can develop an assay Biacore or a surface Plasmon assay.

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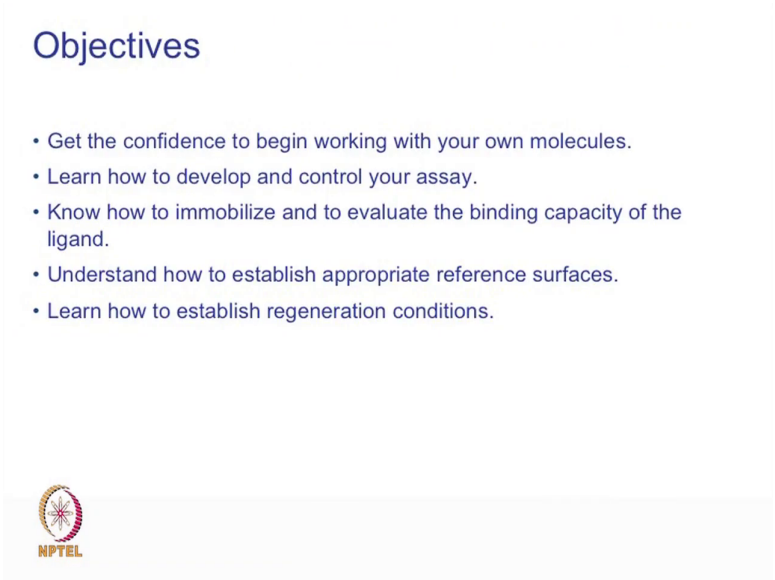

And, the objectives of this particular study is how we will understand, how to work with the molecule, how we will develop an assay control an assay we will understand about the reference surfaces, we will also understand about the regeneration of the surfaces and all that.

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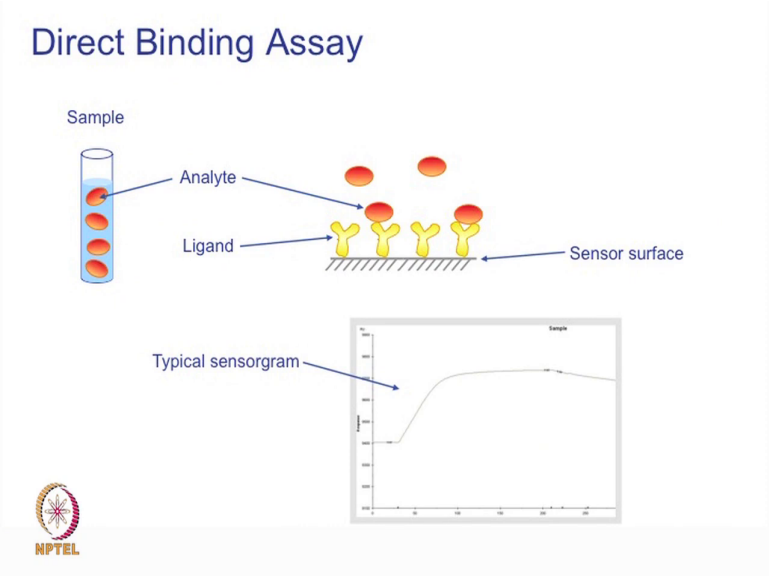

And, coming to the different varieties of assays that we have one of the very simple way of assay would be a direct binding assay. In a direct binding assay you have the ligand that is immobilized on the sensor chip and an analyte that is present in your or a sample is passed on the surface and the interaction is recorded and so, you can see on the screen the interaction shows this way, a very simple binding and a regeneration.

Whereas, sometimes binding a generally does not go very notice the reason is the responses from the analyte are very very little at 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. 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.

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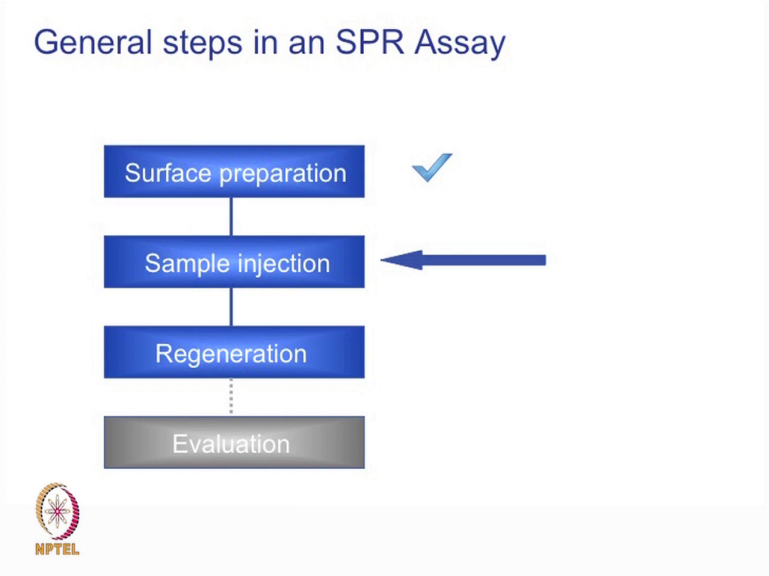

The various steps that are involved in a Biacore assay are surface preparation, sample injection, regeneration and evaluation. So, we will start with the surface preparation.

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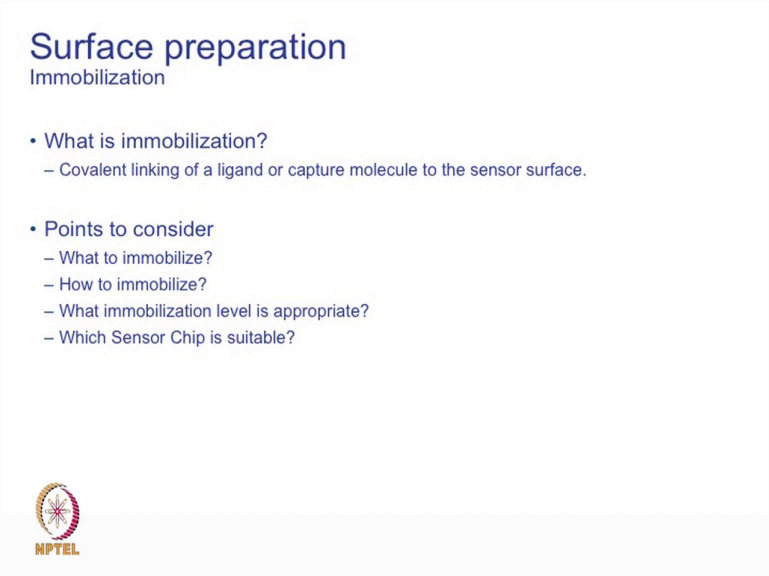

Surface preparation is nothing, but immobilization and the immobilization generally is by two ways done by two ways – one is a covalent linking and other is a capture method. So, when we actually try to go for an immobilization the very important points that we need to consider are what to immobilize, how to immobilize and what is appropriate level and what is a right sensor chip that is required.

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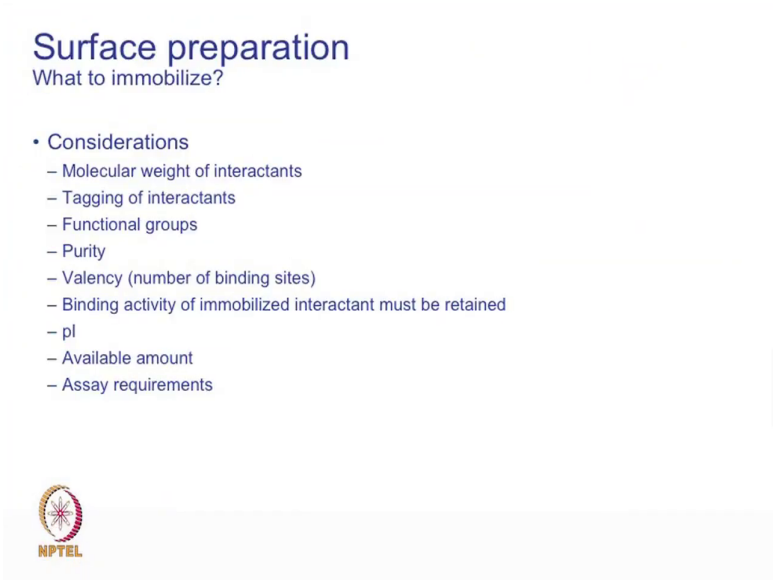

And, when we are immobilizing we need to know a great amount of information about the protein that we are actually immobilizing or a macromolecule that we are immobilizing. We need to understand the molecular weight of the interactant, the tags that are present on interactant, the functional groups, 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.

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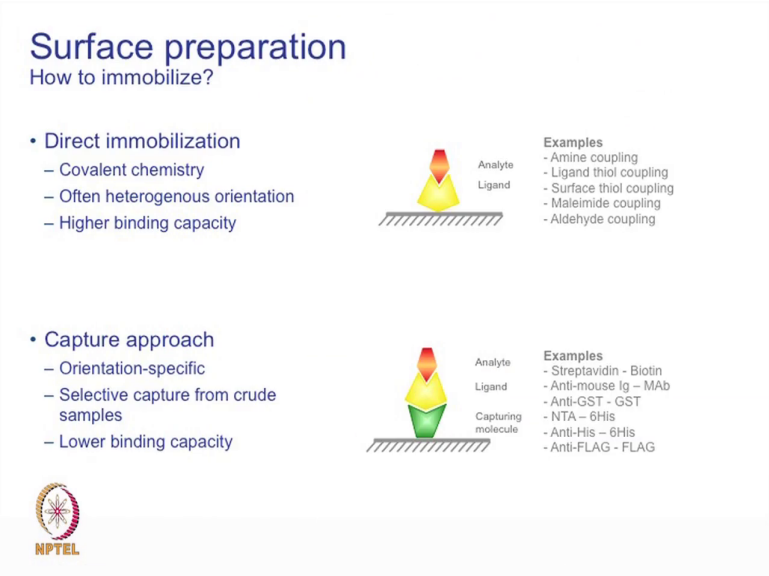

Immobilization is done by direct method as we just discussed and that immobilization can be by covalent chemistry and there will be high binding capacity. And, the ways the very examples of a direct immobilization are amine coupling, thiol coupling, aldehyde coupling, maleimide coupling are the methods that are available.

Whereas, when we look at the capture processes because most of the molecules are explicit these days with tags and when you have tags on the molecule. They are different chips which will capture the tags let us say we have a streptavidin chip that is available and biotin molecules like protein peptides, DNA, RNA all these molecules are actually captured by a streptavidin chip. Most of the time we have anti-mouse, anti-human capture kits are also available and these kits actually will help to capture mouse and human antigens and or anti antibodies and then we will understand the interaction with an antigen.

Also we have the anti-his 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 anti-FLAG 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. The various covalent coupling methods have been clearly distinguished in the literature. We will take one example here which is an amine coupling which is a very common way of coupling proteins on the sensor surface.

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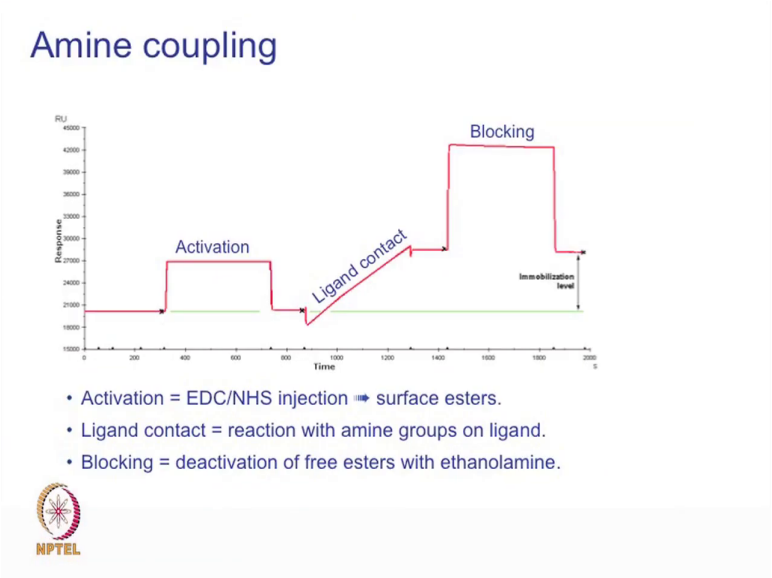

And, here the activation of the surface is generally done by chemicals EDC and NHS and they will activate the surface esters and the molecules from the amine groups are actually covalent

coupled on the surface of the chip. And, the extra groups that are activated are blocked by ethanol amine.

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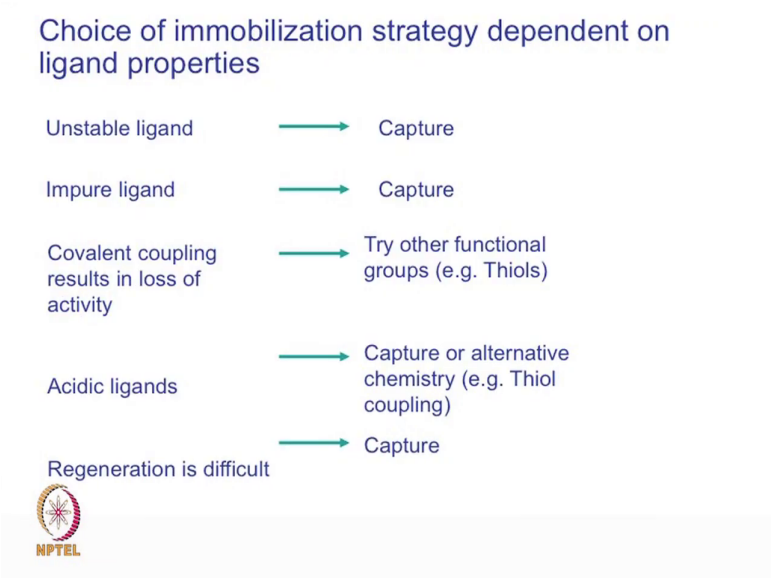

The choice of the immobilization strategies generally depend upon the kind of molecule we have let us say we have an unstable ligand then we go for capture. Let us say we have an impure ligand we go for a capture if we have a covalent coupling even do when we do a covalent coupling and that covalent 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 we in that case we will identify a capture process.

Sometimes some of the ligands the pI is very very close to acidic range like 2 or 3 and those kind of molecules we will still prefer to go for a capture process. Sometimes some molecules the regeneration is very difficult, in that case 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 the pI or the isoelectric point of a protein.

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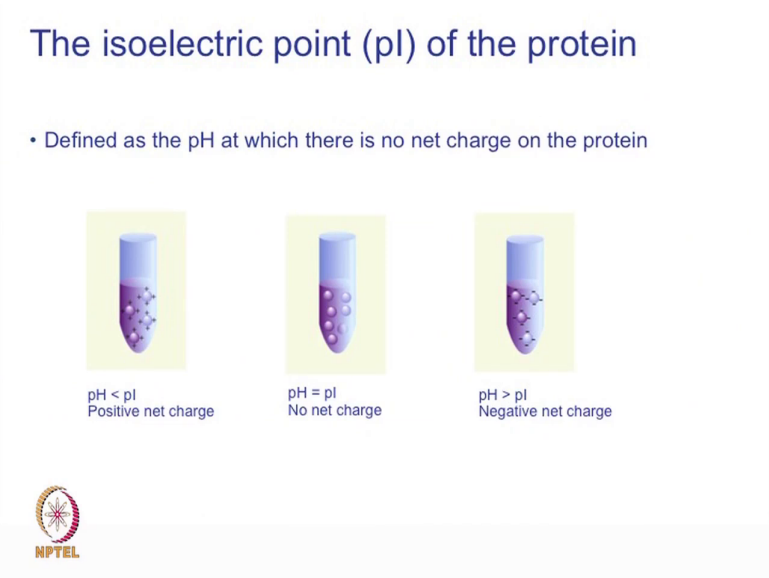

The pI isoelectric point is no net charge on the protein and or you can always say that you have equal amount of positive negative charges that are present on the protein. So, for a immobilization strategy it is always better that we go below the pI and at least 1 or 2 units below the pI where the amine 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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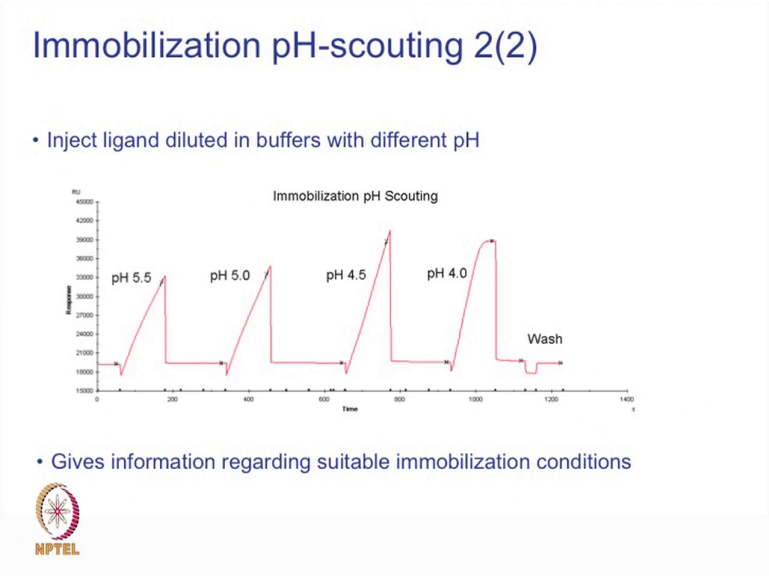

In this process if we sometimes are not aware of a pI because the molecule is a new molecule and it is just recombinantly expressed and we do not know the molecular isoelectric point in that case we will go for an immobilization pH-scouting in which we will dissolve the protein at different pH and then 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 pH is good enough for the immobilization we go ahead and do immobilization at that particular range. Most of the cases proteins are generally immobilized at 10 millimolar sodium estate buffer pH 4.5, but this is a general assumption.

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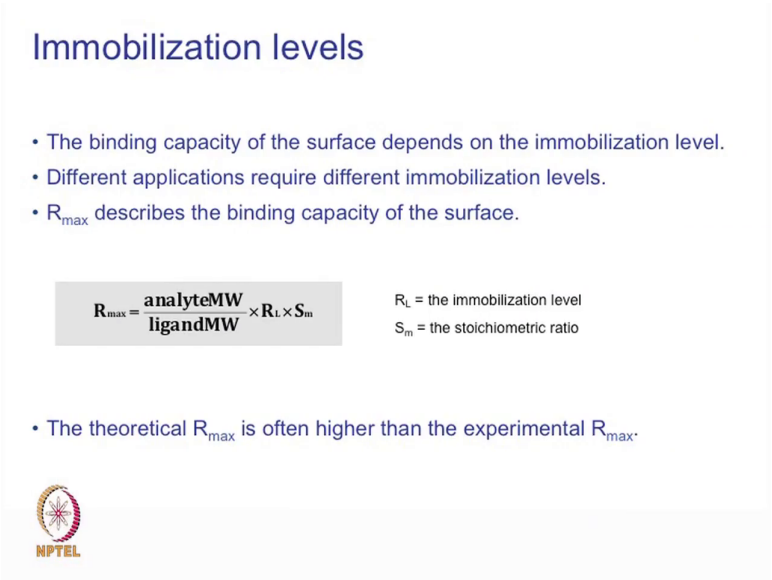

Go for the immobilization level can be done by two ways one is either you go for a controlled immobilization process which is called aim for immobilization. Or you aim for an immobilization level based on the calculated R max, stoichiometry, binding level and the number of binding sites the molecule 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.

So, coming to the different steps in our assays the next important step for 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 are injecting kinetics the kinetics are injected with a certain flow rate.

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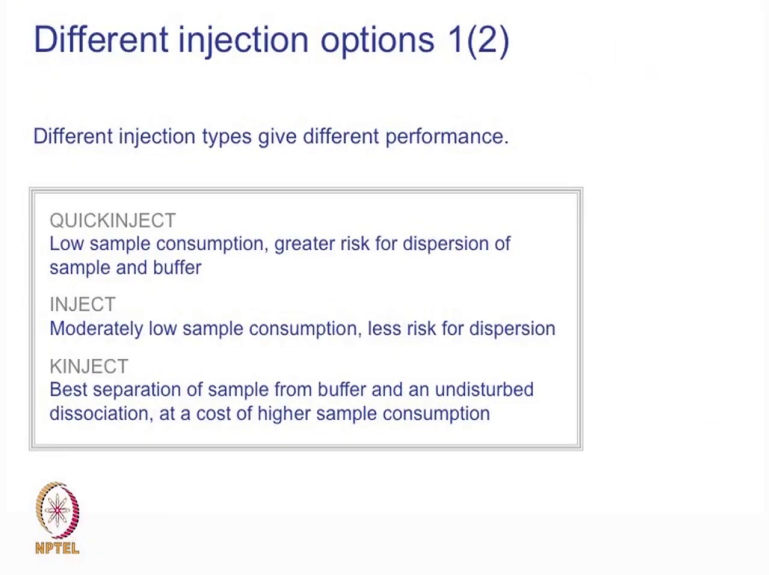

So, the sample injection process actually depends upon the flow rate. And, the different modes of injection are generally called as quickinject, kinject and inject and these modes depending upon the mode of interaction or the type of study that you are doing let it be screening, kinetics, thermodynamics,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

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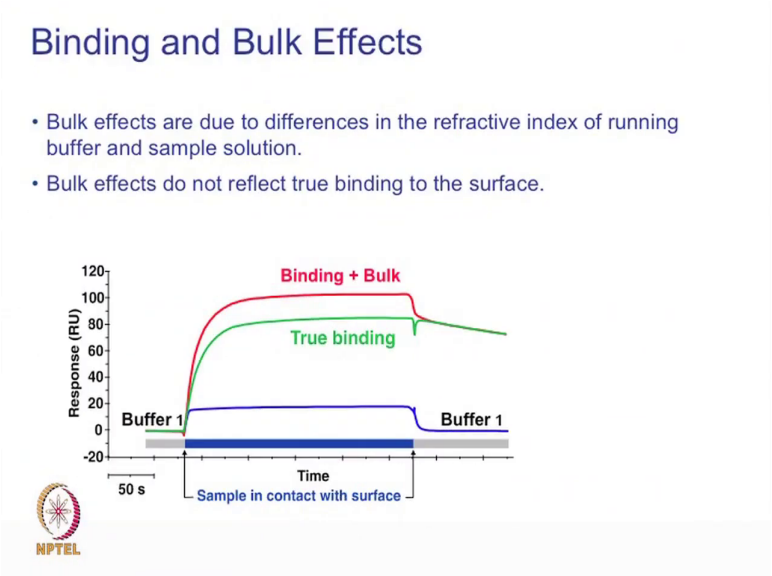

Another important parameter when to be considered when we are designing these experiments is about the bulk. Bulk is nothing, but the difference in the refractive index of running buffer and the sample solution. So, this is a place where most of the people go bad in their assays and will not get a proper 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.

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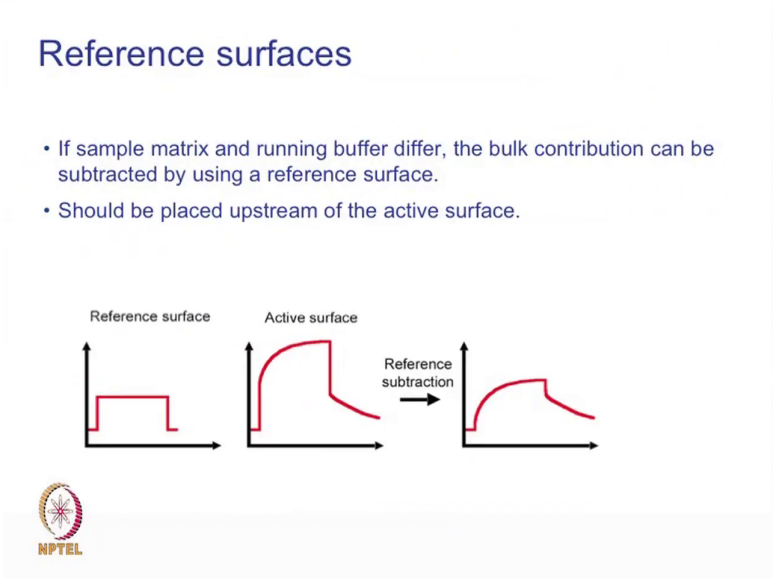

And, another important point in a designing and experiment is the references. There is always a reference upstream your essay, but the references have to be not giving much of a response. Nonspecific response is something that is quite common in these kind of an assay.

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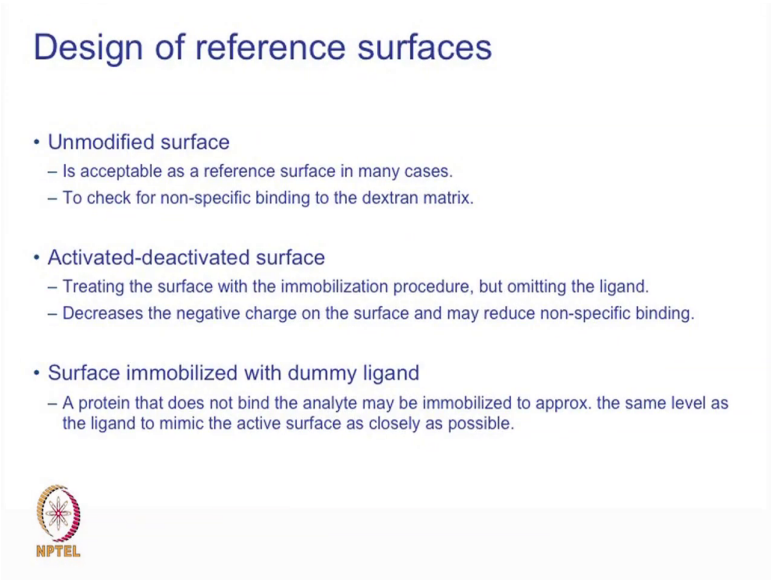

It is the widely followed a different reference surfaces are a non-modified surface in which you take the reference surface as such or a modified surface in which you activate and block the reference surface or a third way of doing is an immobilization of a dummy ligand on the surface of the reference and use it to subtract the non-specific binding. And, coming to the 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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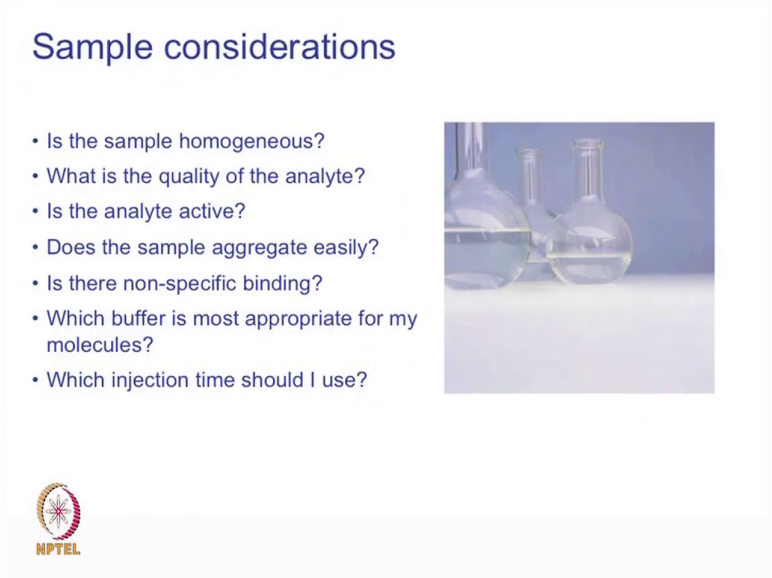

Homogeneous samples are good enough to be considered please make sure that your samples are highly homogeneous, good quality, active and they do not aggregate and they do not give us a lot of non-specific binding. And, it is very important to understand the right buffer for a right sample and also once we have identified the good sample conditions then we should actually think about the association time and the time at which we need to set up our Biacore experiments.

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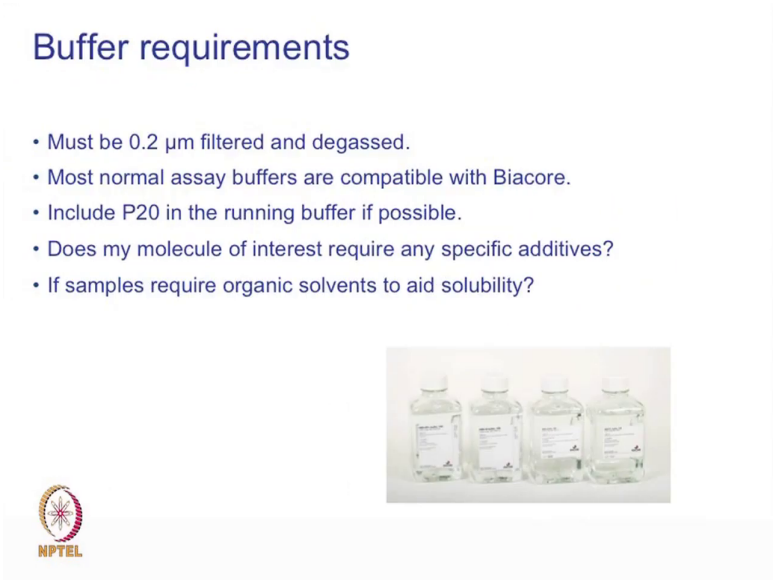

Buffer or a running buffer or a sample buffer or a very important area that we need to understand little bit here. Buffers have to be filtered thoroughly degassed and you know many of the machines have a degasser and a filter that is available in the system, but it is obvious that we can also filter them and degas 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 or the detergents that are used for into running buffer to prevent non-specific binding is a Polysorbate 20, Tween, SDS all these molecules are added into buffers and buffers are prepared.

And, also sometimes some of the additives are especially required for your running buffer sometimes like 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 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.

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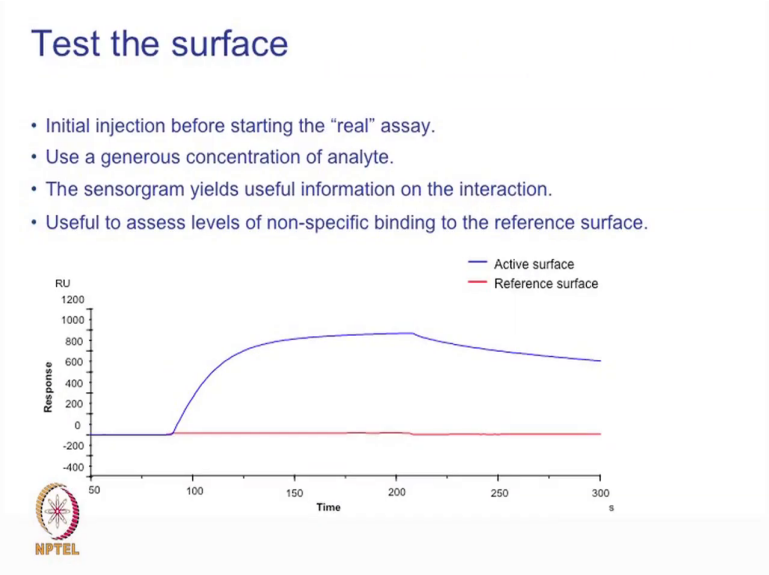

Then it is a very good way to start by injecting a small amount of your sample to see what is the binding responses that you are getting and to what time you should continue your binding and went to dissociate and all that. That process is called test of a surface you can just test the surface to understand the interaction and also understand the regeneration condition and level of non-specific binding that is coming up from these interactions.

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 being 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 they are different ways to study that regeneration conditions, develop the regeneration condition required for a particular interaction.

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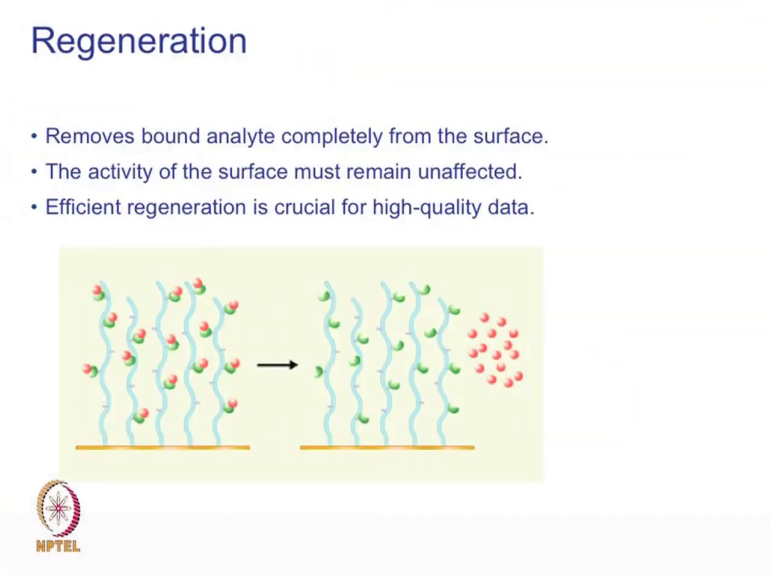

Regeneration as I just said removes the bound analyte completely from the surface. The activity of the surface must remain unaffected after regeneration and an efficient regeneration is crucial for a high quality data. And, regeneration conditions we need to test the regeneration surface, we can do a regeneration scouting before start of an assay or a develop and assay and then identify the right or the appropriate regeneration conditions.

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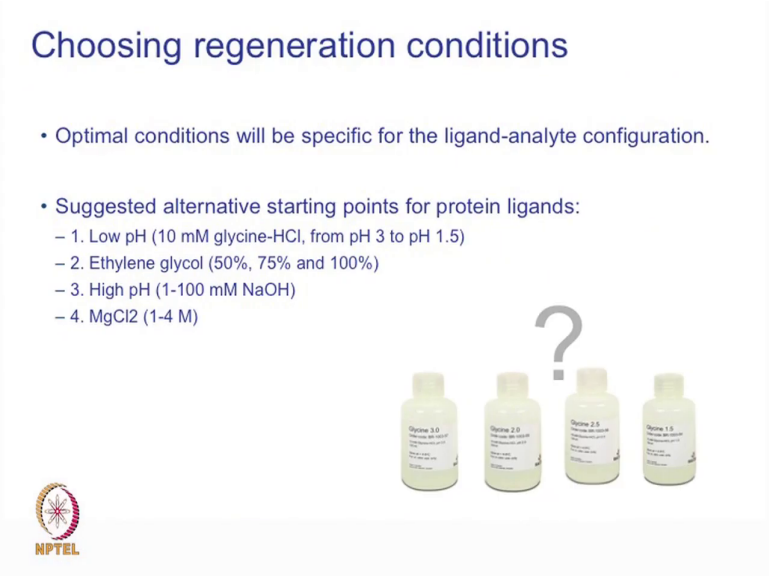

And, some of the very natural or regular usage of regeneration conditions are low pH, ethylene glycol, high pH and magnesium chloride. So, once we identify the proper regeneration solution it is very important for us to identify once we use this regeneration condition are we getting our reference surface 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 nonspecific binding and also we identified or studied about different reference surfaces and also we went through a great detail about regeneration conditions.

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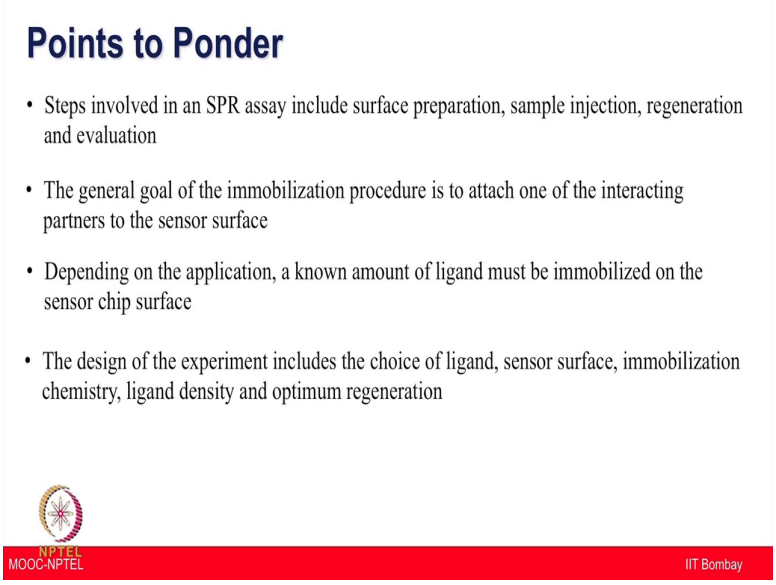

In today's lecture we discussed about the conditions that are required for proper immobilization of the ligand, different methods of analyte preparation and the conditions that are required to perform surface Plasmon resonance space assays. We also talked about the bulk and non-specific binding different reference surfaces and regeneration conditions that could be implemented for the surface stability which is essential for the high quality data.

I would like to reiterate that obtaining some binding information from the SPR experiment is not the only goal. We really want to make sure that you are obtaining very specific binding information and to really achieve that you have to look into many parameters. And, I hope some of the points discussed today, will help you in thinking about the right type of experimental design which is required for you to identify the very specific binding and the

conclusive data from your experiments. We will continue discussing about more of these points in the next lecture.

Thank you.