Enzyme Science and Technology
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Module - VII
Enzyme Substrate Interactions
Lecture - 34
Enzyme-Substrate Interactions (Part-IV: SPR)

Hello everyone, this is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering IIT, Guwahati. And what we were discussing? We were discussing about different properties of the enzyme, in the course, enzyme science and technology. And in this context, in this current module, we are discussing about the different techniques and as well as the approach to to understand and as well as to calculate the enzyme-substrate interactions.

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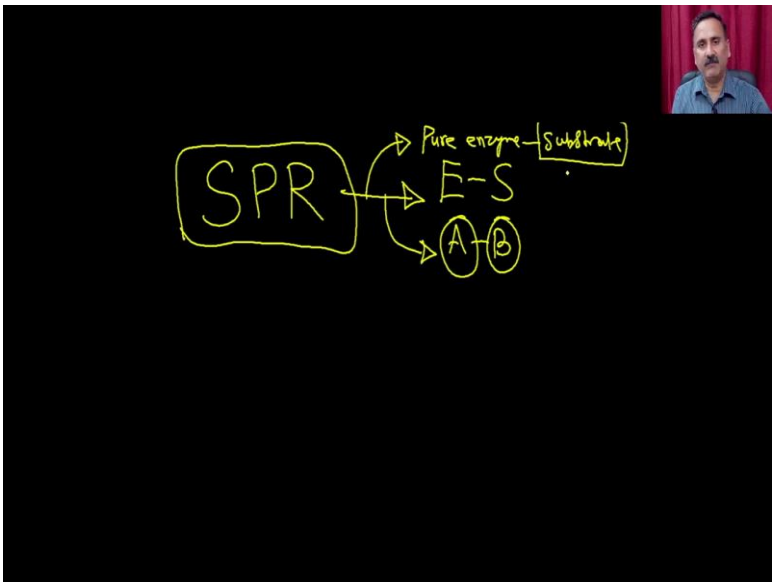

But what we are going to discuss today is actually the one technique which is called as the SPR or the Surface Plasmon Resonance. And SPR is a very, very robust technique to measure the enzyme-substrate interaction or it can actually be able to use any of the two molecules which you know that they are interacting with each other and you can actually be able to use the SPR.

For SPR, the system utilizes the pure enzyme, right? It utilizes the pure enzyme and pure-substrate, ok. So, you cannot use the multiple-substrate in a single reaction and you can actually be able to measure any kind of interaction. So, it actually works with the pure enzyme and substrate combinations.

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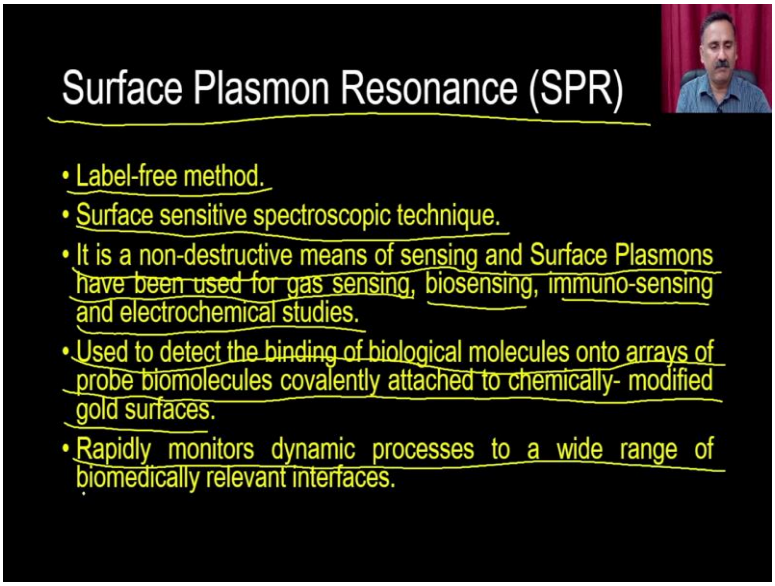

So, now what is the SPR? So, SPR or the plasmon Surface Plasmon Resonance is a label free method. It surface sensitive spectroscopic technique. It is a non destructive mean of sensing and surface plasmon's have been used for gas sensing, biosensing, immuno sensing and the electrochemical studies. It is used to detect the binding of biological molecule onto a array of probe of the biologically biomolecule covalently attached to the chemically modified gold surface.

And rapidly monitoring dynamic process to a wide range of biomedically relevant interfaces. We will discuss all this when we are going to discuss about, in detail, about the principle of SPR and how the SPR is can be used to detect the interaction between the enzyme substrate interactions and then only you can be able to follow some of these terms.

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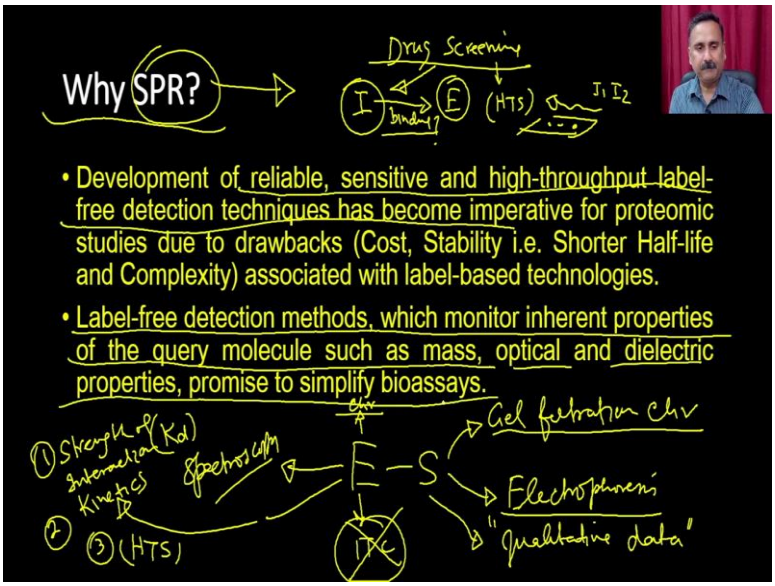

But the question is why we need an SPR, ok? We need an SPR because of the many reason. One is we actually have to develop a reliable sensitive and high throughput label free detection technique. So so far what we have discussed?

We have said that the enzyme when it is interacting with the substrate, this measurement or this interaction can be mapped by the gel filtration chromatography, it can be used by the gel filtration chromatography, it can be mapped by the electrophoresis and it can be measured by the spectroscopy, right?

It can be measured by the ITC and it also can be measured by the other kind of chromatography techniques, right? Majority of these techniques except ITC are actually giving you a qualitative data, right? Its not going to give say tell you. So, its actually giving you the qualitative data, its actually going to tell you that, ok.

This enzyme this substrate is interacting with each other and that you can be able to know by the gel filtration chromatography, electrophoresis, spectroscopy and ion exchange chromatography, hydrophobic interaction chromatography.

But it will not tell you about many things, it will not tell you about the strength of the interactions, right? So, it will not tell you the strength of interactions number 1, right? This means it will not going to give you very precise the Kd values, it will not going to

tell you the kinetics and most important is majority of these techniques cannot be used for screening the multiple ligands.

For example, if you have if you want to do or if you want to use the SPR for the drug screening, right? You can actually be able to use the SPR for drug screening because you can actually be able to say whether the inhibitor is binding to the enzyme or not, right? Whether it is inhibiting or not, that is already you have done it, ok? So, whether the inhibitor is binding to the enzyme or not.

So, first would be that criteria only to choose the inhibitor which you for which you want to do further test like whether it is inhibiting the enzyme or not, whether that inhibition is leading to the death of the parasite or not and so, on, right? So, that is secondary, right? First you have to know whether it is binding to the enzyme or not, right? So, that binding experiment can be done in a HTS mode or the high throughput screening mode and that is how you can be able to use the SPR only the technique.

You cannot do that for ITC, you cannot do that for spectroscopy because all these are actually going to be very time consuming and laborious. And on the other hand, if you do so, it is actually going to be cost effective also, right? Because in the SPR, you would only require a you know chip and on this chip, you are actually going to have the enzyme and then you actually can flow the multiple types of inhibitors. So, in one batch, you can inhibitor 1, then inhibitor 2 and so on, right?

So, label free technique check-in method which monitors inherent properties of the query molecules such as mass, optical and dielectric properties promises to the simplify bioassays.

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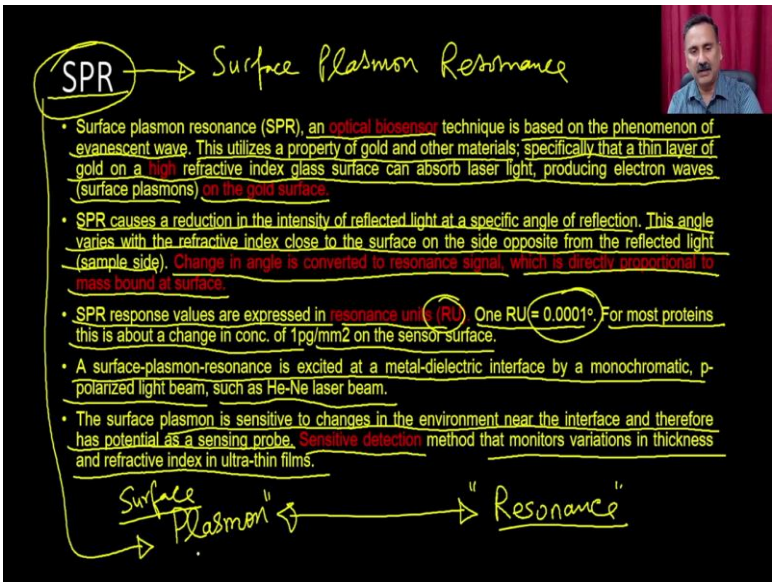

Then SPR is a optical biosensor, right? So, its technique is based on the phenomena of the evanescent wave and this utilizes a property of gold and other metals, other materials specifically that a thin layer of gold on a high refractive index glass surface can absorb the laser light producing the electron waves or the surface plasmons onto the gold surface.

SPR causes a reduction in the intensity of reflected light at a specific angle of reflection. This angle varies with the refractive index close to the surface on the side opposite from the reflected light, that is the sample side. Change in the angle is converted into the resonance signal which is directly proportional to the mass bound at the surface.

SPR responses values are expressed in the resonance unit or the RU, right? And one RU is equivalent to the 0.0001 degree change in the angle. For most protein, this is about a change in concentration of 1 picogram per millimeter cube square on the sensor's surface. Surface plasmon resonance is excited at a metal dielectric interference by a monochromatic plane polarized light beam such as the helium neon laser beams.

The surface plasmon is sensitive to the change in the environment near the interface and therefore, has a potential at a sensing probe. Sensitivity detection method that monitor variation in thickness and the refractive index in the ultra-thin films. So, what you see here is that SPR is actually going to rely on two things. One, it is actually going to work on the plasmon, right?

So, as the as the name suggests, right? What is the SPR? It is called surface, plasmon resonance, ok? So, it has two terms which we have to understand and then only we can be able to understand this particular technique, right? So, we have two terms. One is called resonance, the other one is called as the surface plasmon, right? And surface plasmon and these are the two technique, two terms what we are going to discuss in the second next slide.

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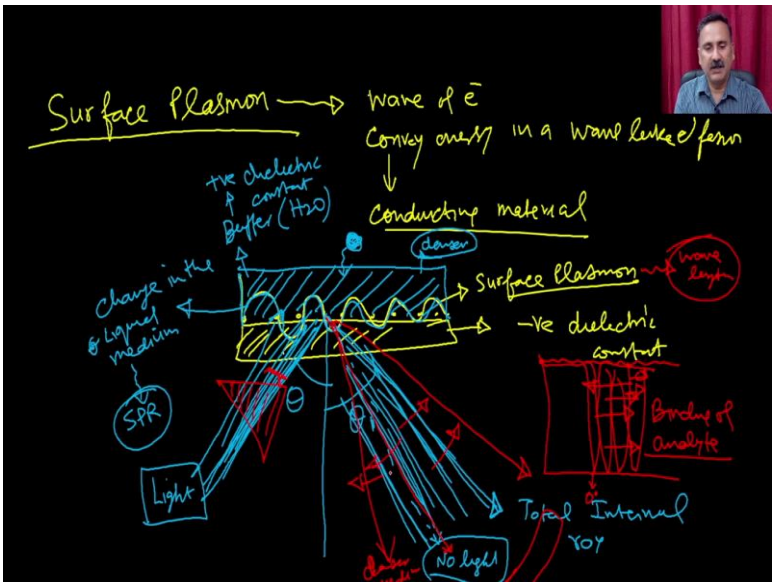

So, what is mean by the surface plasmon? Ok. So, surface plasmons are actually the wave of electron which are going to conduct or which are going to convey the energy from in a wavy pattern, ok? Or in a wave like fashion, in a wave like fashion, ok? Its means, and this will happen only with the only with the conductive material, right?

For example, conductive material. So, for example, if I take a glass, if I take, for example, if I take the gold, right? So, if I take a gold thin film, right? Ok. So, this is a gold, right? What will happen is that, the gold is actually going, it is a very conductive. So, it actually has the very low dielectric constant.

So, it is actually going to have the negative dielectric constant, right? And because of that, the top surface of the gold will actually going to have the electrons and these electrons are actually going to pass the energy from one electron to another electron, another electron to first say third electron and that is how it is actually going to form a wave like pattern, ok?

And these wave like pattern are actually going to be called as, because it is happening on the surface, it is going to be called as the surface plasmon. But it does not happen when, until you are actually going to have the top surface, any kind of modification in the top surface, ok?

So, if you are actually going to have the two gold particle, one to another one, then there will be no surface plasmon which is going to be formed. How it is actually going to form is that, you are actually going to have the. So, you are going to have the water molecule, ok? So, if you keep the water molecule in front of this particular, in front of this particular metal surface.

So, what will happen is that the gold, the water or buffer for example, right? So, buffer is nothing but the water, its actually going to have a positive dielectric constant, right? And because of this positive dielectric constant, it is actually going to you know, cause the production of these plasmons, ok?

So, when the plasmons are formed, they are actually going to travel throughout the surface of this metal surface. And these plasmons are very sensitive for, in the change in the for the change in the liquid media, ok? And that is what is actually going to exploit in the technique which is called as SPR or the surface plasmon resonance.

So, these surface plasmon resonance, they are moving from one end to another end, right? And to detect or to measure the value of the surface plasmon, what you are going to do is, you are actually going to illuminate these particular, you are actually going to illuminate this with a light, ok?

So, what you are going to do is you are going to illuminate this gold film with the help of a light and you are actually going to follow a particular wavelength or particular angle at which it is actually going to transmit directly or it is actually going to show you a total internal reflection because it is going to be get reflected. So, at particular wave particular angle, it is actually going to be get completely reflected and that is how it is actually going to show you a angle of theta 1 and that is the high intensity what you are going to get.

So, this total internal rate what you are going to collect, ok? Now, what will happen is that if you are actually making a modification in this external environment, right for

example, if you are introducing a ligand molecule, you are actually changing this particular surface, right? And because you are changing this particular surface, it is actually going to, if you make the media denser, right?

If you make the media denser, right, the this particular ray which is. So, this is actually going to give you multiple rays, right? But at a particular angle, right, there will be a complete absorption of this particular light and that is how at that particular wave, that particular angle, you will actually going to see no light, ok or you will not going to see any of the reflected light.

So, if I show you like how it is actually going to give you is if you are illuminating the sample with multiple rays like this, you are actually going to accept multiple rays like this, right? But in between, there will be a ray, there will be an angle at which you are actually going to see a reflected ray, right? So, reflected ray from the sample which is not going to show you any color.

So, although I am showing it with the red light, but there will be no light. This means, if I monitor this into a wavelength. So, what will happen is that, if there is a no change in the refractive index, the intensity will remain like this. So, it is actually going to remain as a straight line. But if there will be any change of the external media because of which there is a change in the plasmon wave also, then it is actually going to show me a dip.

So, this dip is nothing but the loss of light because at this particular angle, this is particular angle, there is a absorption of light and because of that, there is a loss of light, ok? And this actually is very very specific for the binding of the analyte, ok? Analyte to the molecule which is present on the surface.

So, plasmons are actually having, and how it happens that surface plasmons are actually moving a with a particular wavelength, right? And you are eliminating this with a particular wavelength. So, you are actually putting a prism, right? Or you are putting a prism here. And with all the, with the help of the prism, you are actually selecting a particular lambda light through which you are eliminating this particular complex.

And as a result, when you are changing this lambda at a particular lambda, what will happen is that the light is actually going to be completely absorbed by the complex which is present on the cell surface. And as a result, you are actually going to see a

absence of light. Otherwise, you will actually going to see the total internal reflection and it is because of that, the complete light is actually going to be get reflected.

So, it is like you are actually going to have a beam of light and within that beam of light, there will be an absence of light. That absence of light will happens only when the wavelength of the surface plasmon is actually going to be have the same order as the wavelength of the light what you are through illuminating.

So, when there will be a binding, the angle is actually going to be on this side, which means, when there will be binding, the angle is actually going to move on this side, ok? This means you will see a binding, right? You will actually going to see another binding. You will be going to see another binding; you are going to see another binding.

So, it will be keep shifting as the molecules are binding because if they bind one molecule, you will see one shift. If they molecule if they bind another ligand, you will you going to see the binding, ok. So, this anyway, I am going to show you in the next slide, but how it happens actually.

And so, this is what it is actually going to happen, right? So, when if it is going through denser media, it is actually going to deflect in this direction. If it is going through in a lighter media, it will go into this direction, ok. So, that is how you are actually going to see a change, ok. So, it will, if it goes in this direction, it is actually going to say binding. If it goes in this direction, it is actually going to say you the dissociation.

So, this is what is the basic principle of SPR, where you are actually going to take a thin film of, you know, the gold, right? And on this thin film, you are actually going to have the buffer. And because you are actually going to have the buffer or the water, the gold film is actually going to show you a surface plasmons, ok. And these surface plasmons can be excited with the beam of light.

And because of that, the beam of light is going to be get reflected and it can be collected by a detector what is present here, right? So, you can actually be able to place a detector here and that actually can collect the illuminations. And at a particular angle, what will happen is that the lambda of this light is actually going to match with the, the frequency with the wavelength of the surface plasmons and that is how it is actually going to show you a complete absorption.

And when it is actually going to show you the complete absorption, there will be a loss of light or there will be no light. And that you can be able to measure with the help of the detector. So, you can be able to know whether the light is deflecting in this direction or whether the light is deflecting in this direction. And that is how you can be able to say whether there will be a binding or there will be a dissociation. Now, how you are going to perform the surface plasmons resonance spectroscopy?

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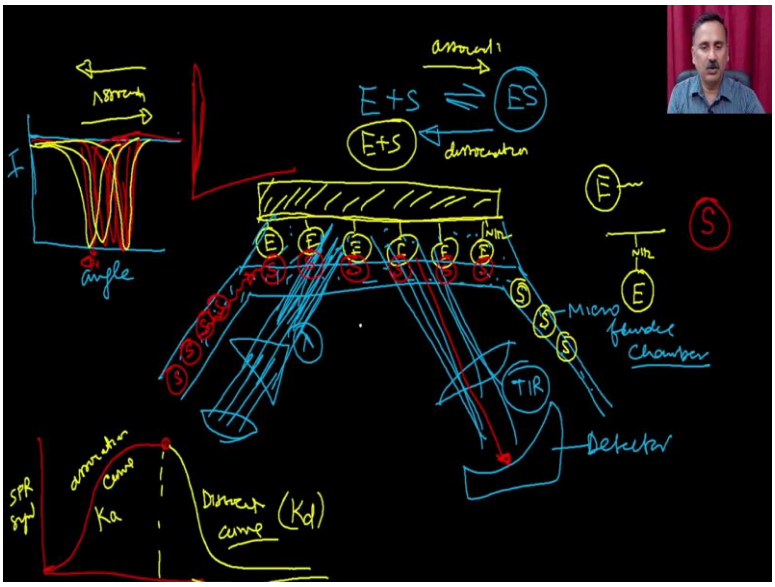

So, what you require is you require a very simple system, right? So, what you require is you require a glass, a metal sheet, ok. And on which you require the glass, ok? So, this is actually going to be a glass, ok? And then on this metal surface, what you are going to do is you are going to, suppose I have I want to; I want to choose an enzyme, ok? So, this metal surface is going to be functionalized. So, suppose this has the amino group, right? So, its going to have the amino group.

So, if this metal surface has amino group, I can use this and I can couple the enzyme for which I want to measure the or I want to calculate the dissociation. So, what will happen is that the enzyme is actually going to couple to the surface on this side, right with the help of any kind of conjugating material, what is present, right? And then what I am going to do is I am going to have the system. So, I can actually have the micro channels, ok?

So, I can have the micro channel like this, ok? And in this micro channel, I can actually be able to flow the liquid, right? So, this actually can go like this. It actually will cover the metal surface also, right? So, I can actually be able to have the metal surface. So, which I can be able to flow the liquids, right?

And below to this, I can have the light source. So, I can have a light source here. And we can have the prism. So, that I can be able to collect. I can be able to. So, I can actually be able to eliminate the sample with the help of this particular wavelength, ok? And then I can also be able to collect the wavelengths like this. And this side also, I can actually be able to. So, this side and then I can have a detector.

So, this is the detector what we have, right? And this is the microfluidic chamber. So, this is the microfluidic chamber, ok? And I have only conjugated the enzyme. I can conjugate any of the pair. I can conjugate the antibody or antigen or any kind of pairs. And what I will do is I will first collect the spectra, right? So, I am going to see some. So, if I show you like this, ok?

So, if I have done that, I can collect the spectra and see how the binding is happening or not. So, what will I am going to see is I am going to see the intensity versus angle, right? So, I can actually be able to see a no change in the change in the intensity because all the lights are actually going to be captured.

So, if I am if I am sending the light with lambda, it is also being collected by the detector because it is going to show me the total internal reflection, ok? So, it is because of that, the all the lights are going to be covered. Now, what I am going to do is I am going to flow the analyte. So, suppose I have a substrate molecule. So, I am going to flow the substrate molecule, right? So, what happened is that as soon as the substrate molecule will flow.

So, you have multiple substrate molecule, right? It will go and bind the enzyme, ok? So, as soon as it goes and bind the enzyme, there will be a change in the absorption pattern, right? And because of that, you are actually going to see a light or the absence of light. So, you are going to see a light which is going to be absent in this particular light being, ok? And as a result, it is actually going to show you a dip, ok? And then again it will come back, ok?

Now, when you. So, this is, suppose this is at lambda 1, ok? Now, when the second molecule of substrate its actually going to bind, it will actually going to show me a curve like this, ok? And I can plot this, I can plot this in a another curve. So, I can plot this in another curve. So, where I can actually be able to show how the things are happening. So, it is actually going to show me like this. Going to show like all that angle is increasing, right?

And then it will be going to reach at plateau. So, it is actually going to be keep increasing, keep increasing, and then ultimately it is going to keep showing me the same angle. This means, now the system got saturated. Now, once the system got saturated, you are actually going to see that all the enzyme molecules are actually having the substrate.

Now, what you are going to do is you are simply going to flow only the buffer, ok? So, there will be no, there's no flow of, substrates, right? So, if when you are going to flow only the substrate, then what will happen is that, it is actually going to have the dissociation because the enzyme is making a interaction with substrate, right? And it is actually going to form the enzyme substrate complex.

So, it will be keep making the enzyme substrate complex until you are supplying the substrate, right? When you do not supply the substrate, it is actually going to have the backward reactions, right? It is actually going to have the breakdown of this enzyme substrate complex. And as a result, what will happen is that it is actually going to show you, substrate which is going to be removed, right? So, substrate molecules are actually going to be come out from this enzyme substrate complexes.

So, it is actually going to show you this, ok? So, this is actually a association, right? And this is actually the dissociation, right? So, when there will be a dissociation, this peak is actually. So, earlier when there was a association, when it was association, the peak was moving from this direction to this direction and you are actually going to see a change in the angle on a higher side.

When there will be a dissociation, right? So, peak will actually going to move in the backward direction. This means, it is with going to show you like this, then it is actually going to show you like this and then it is actually going to show you the original peak, ok? And it is actually going to show you like this, ok? And it is going to become flat, ok?

This means, this is going to be your association kinetics, right? So, this is going to be association curve and this is going to be dissociation curve, right? And this is actually going to give you information about the association kinetics association constant and this been actually going to give you the dissociation constant.

And by looking at that time. So, this is actually going to be in the time versus signal, right? So, this is going to be called as SPR signal, right? And time versus you can be able to calculate the kinetics, how long it takes for getting into the saturation state, how long it takes for the breakdown of this particular enzyme substrate complexes and so on. Now, if you want to perform this, as I say, you know, these are the thing, the multiple component what you require, right?

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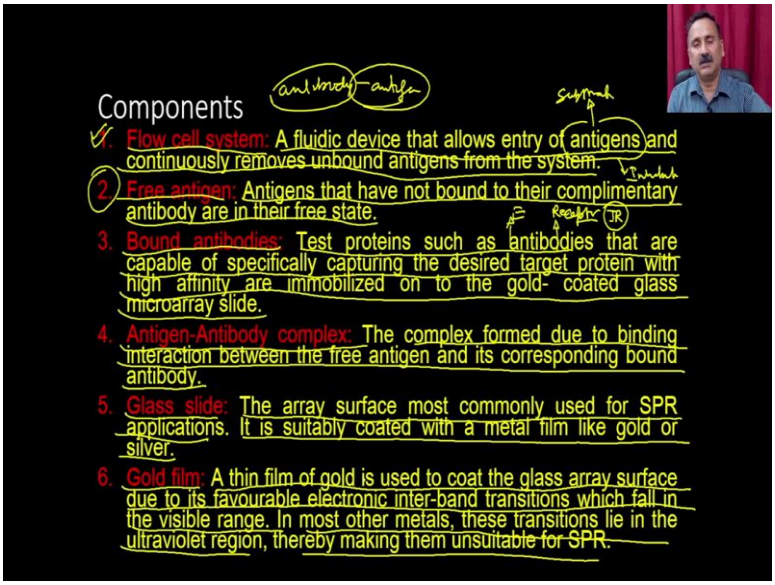

So, there are multiple component what you require, right? You require a flow cell. So, in a flow cell system, a fluidic device that allows the entry of antigen and continuously remove the unbound antigen from the system. Although it is saying the antigen, it could be anything, it could be substrate, it could be inhibitor and so on, ok?

Then we can also have the free antigens. So, you are actually going to supply the antigen, although we have taken an example of the antibody and antigen. It can be antigen or enzyme substrate complex or enzyme inhibitor complexes. So, then you have to have antigen that have not been bound to their complementary antibody that are in their free state.

Then you also require the bound antibodies. So, the test proteins such as antibody or the enzyme or the receptor, you can use anything, right? You can use the receptor, for example, you can use the insulin receptor and you want to measure how the insulin receptor is interacting with the insulin or not. That are capable of specializing capture the desired target protein with the highly affinity are immobilized onto the gold coated glass microarray slides.

Then we also require the antigen antibody complex and complex formed due to the binding interaction between the free antigen and its corresponding bound antibody. Then we have the glass slide, the array surface most commonly used for SPR applications. It is suitably coated with a metal film like the gold or the silver. Then you require the gold film.

A thin layer film of gold is used to coat the glass array surface due to its favorable electronic interbanded intransitions which falls in the visible range. In most other metals, these transition lies in the ultraviolet region thereby making them unsuitable for the SPR. So, why we use the gold? Because gold is only giving the transitions in the visible range whereas, for other metals, it is actually giving the transition in the ultraviolet range and that is not suitable for the SPR.

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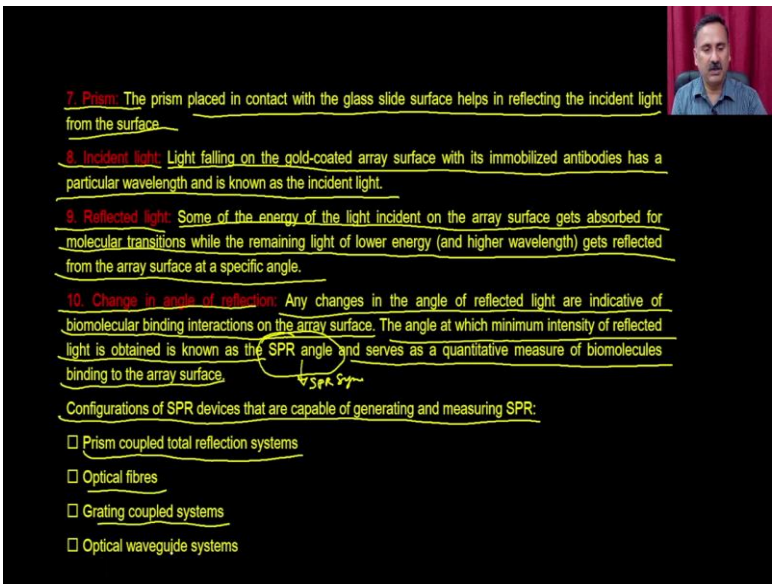

Then we also require a prism. So, prism plays in the contact with the glass slide surface helps in reflecting the incident light from the surface. Then you also require the light

source like the incident light. So, light falling on the gold coated array surface with its immobilized antibody has a particular wavelength and it is known as the incident light. Then you also require the reflected light.

So, some of the energy of the light incident on the array surface get absorbed for the molecular transition by the remaining light of lower energy get reflected from the surface at the specific angle. Then you also require the change in the angle of reflections. So, any change in the angle of reflections are indicative of the biomolecular binding interaction on the array surface.

The angle at which the minimum intensity of the reflected light is obtained its known as the SPR angle and this is the angle at which you are actually going to see the SPR signal. Right and serves as a quantitative measure of the biological binding to the array surface. So, configuration of the SPR devices that are capable of generating and measuring the SPR is the prism coupled total reflection optical fibres grating couple system and the optical wavelength systems.

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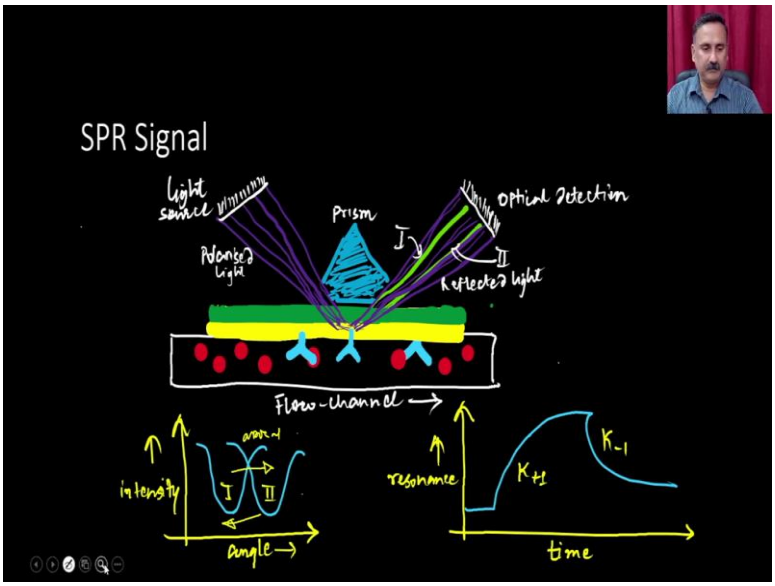

And this is what I have explained already how the SPR signal is actually going to be measured. Right so, you are actually going to see a deflection in this direction when there will be association and you are going to see a moment in this direction when there will be a dissociation. And this is what it is going to show. This is actually the association kinetics and this is actually the dissociation kinetics.

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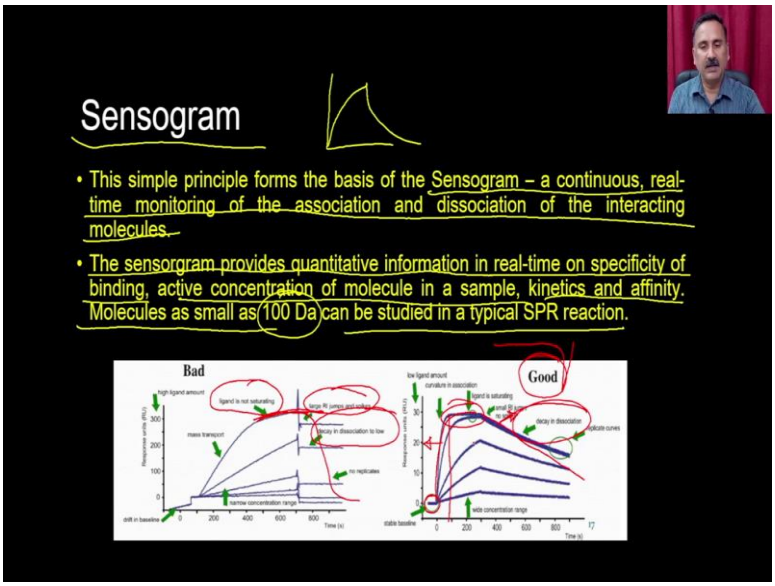

And this is what is called as the sensogram. So, what you have seen this pattern right this pattern what you have seen is actually being called as the sensogram. And just like as you remember and recall when we were looking at the pattern of the protein getting elute from the chromatographic column it is called as chromatogram sensually. So, same way when you are actually going to have the you know the SPR signal plotted across the time, it is actually going to be called as sensograms.

So, sensogram is a continuous real time monitoring of the association and dissociation of the interactive molecules. The sensograms provides the quantitative information in real time on the specific of binding active concentration of the molecule in the sample kinetics and the affinity.

Molecules as small as 100 the iron can be studied in a typical SPR reaction. And you see this is a these are the two different types of SPR, sensograms what we are showing and this is a bad quality sensogram whereas, this is a good quality senseogram. One of the major reason why this is a bad quality sensogram is that the ligand is not saturating. It is actually not showing you a plateau right it at should show you a plateau and then it should show you a down right.

And its actually going to show you a lot of spikes ok. So, its also have a spikes it actually have a decay in dissociation and so on. And because of that this is a bad quality sensogram, it is not actually going to give you the accurate values for the Kd values and

other kinds of parameters. Apart from that this is a good quality sensogram because what you see here is that it is actually having a very stable baseline right.

So, baseline should be stable right because initially you should have a straight line and then it actually should show you a dip right. And it is actually going to show you a saturation. So, for example, it scores high and then there is saturation. So, this is a saturation phase and then its having a decay right. So, decay is a linear right. So, its actually going to show you a decay in dissociation also.

And that is why this is a good quality sensogram. And this sensogram has two phases. One is if you like right line here right this is a association phase and this is the dissociation phase and both of these phases can be used to calculate the different types of parameters.

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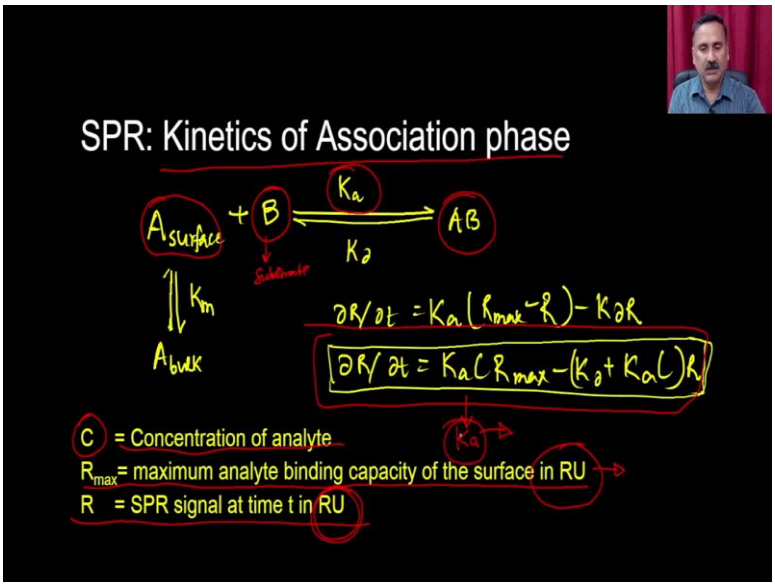

So, kinetics of the association phase. So, you can have the for example, A right. It can be antibody, it can be enzyme, it can be anything. And then we have the B which we are actually going to say substrate right. And its actually going to get associated. So, its going to have the association kinetics and that is how it is actually going to give you AB complex.

And you can be able to use this particular equation to calculate the different types of parameters where C is the concentration of the analyte. R max is the maximum analyte

binding capacity of the surface in the RU. So, this is the maximum capacity of the ligands what are being bound onto the gold surface. And then this R is the SPR signal at a time t in the in the resonance unit.

And that all these three you can actually be able to put into this equation and that is how it is actually going to give you the values for the association kinetics and association constant and other things.

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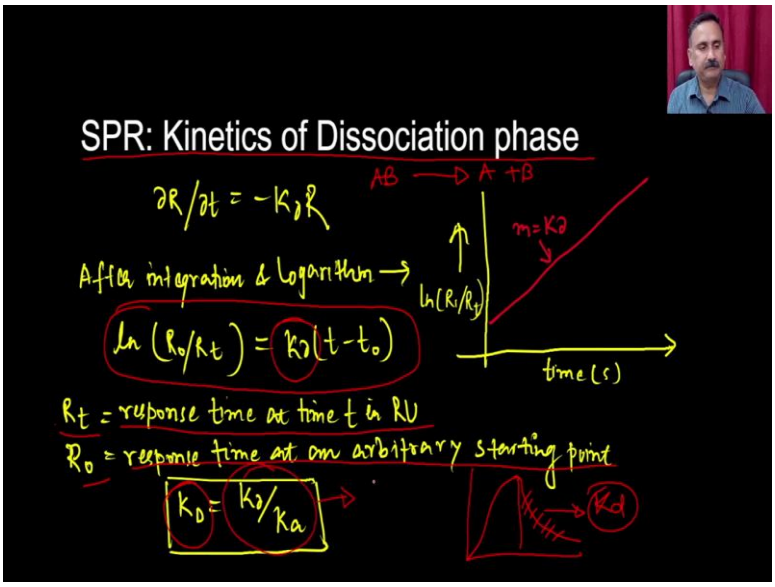

And then we have the kinetics of dissociation phase. So, kinetics of dissociation phase where you are actually going to monitor the AB giving rise to A plus B. And if you recall from the pattern or the sensogram it is actually like this right. And then it goes like this. So, this is the phase which you are going to use for calculating the dissociation kinetics or the equilibrium kinetics.

So, here we can use actually the this particular equation and you can be able to calculate the K d values where R t is the response time at time t in the resonance units and R 0 is the response time at an arbitrary starting point. And the K d which is the dissociation constant is K d by K a and that actually is going to be calculated from the sensogram.

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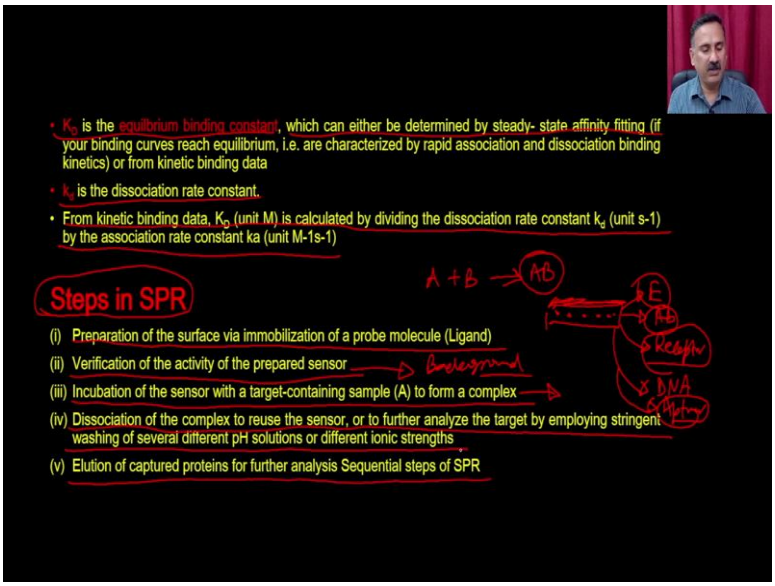

So, K d is the equilibrium binding constant which can either be determined by the steady state affinity fitting and K d is the dissociation rate constant from the binding data. K d can be calculated dividing the dissociation phase constant by the association rate constant. And the steps. So, steps in the SPR you are first going to prepare the surface by the immobilization of the probe.

So, first you are actually going to take the glass slide or the gold skated, gold plated glass slide right and you are actually going to immobilize the enzyme or the any kind of analyte what you or the probe molecules right. So, you can actually be able to use the enzyme you can actually be able to use the antibody you can actually be able to use the receptors and so on.

And anything, but you can actually be able to immobilize onto this surface you can use DNA for example, you can actually be able to use a tumber, you can use anything ideally you can use anything, but actually has the affinity for a particular ligand. Then verification of the activity or the prepared sensors. So, you actually should show the background signal right.

So, you can actually be able to see that it is whether it is showing me a low background or not, then incubation of the sensor with a target containing sample to form the complex. So, you can actually be able to do the incubations and that is how the A is actually going to react with B and its actually going to form the AB complex right.

So, this also you have to optimize you have to give the enough time so, that AB is going to be formed then dissociation of the complex to reuse the sensor or to further analyze the target by employing the stringent washing of the severe detergent different pH solution or different ionic strength then illusion of the captured protein for further analysis or the sequential steps of the SPR.

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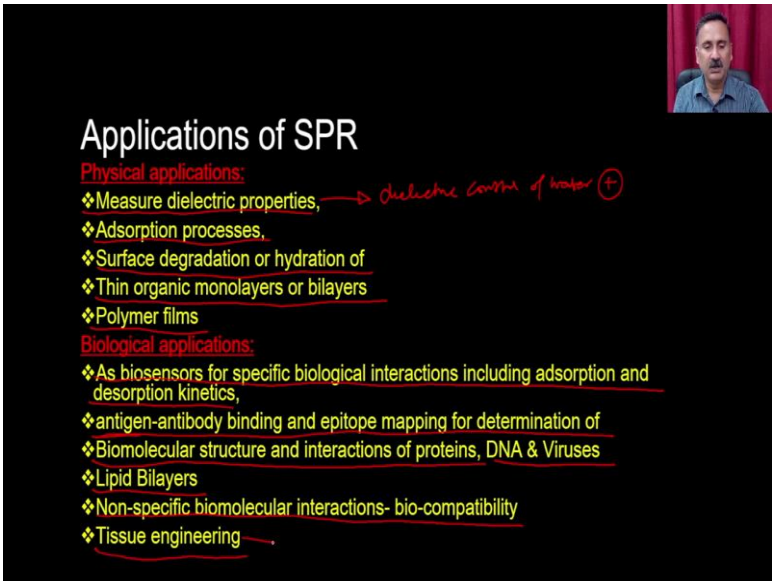

Now, what are the applications of SPR? So, you can actually be able to measure the dielectric properties remember that I said right you are actually going to have the surface plasma on because of the dielectric constant of the water right. So, its actually going to be in the positive side. So, if you are actually going to measure if you want to vary these you can also be able to measure the dielectric properties of the buffer actually.

Then you can also be able to use the adsorption processes, you can actually be able to use the surface degradation or the hydration, then you can also use the thin organic monolayer or bilayer and you can also be able to use the polymers films. What are the biological applications?

So, as biosensor for specifically biological interaction include adsorption and the desorption kinetics you can actually be able to use the antigen and antibody binding and epitope mapping for the determination of the biomolecular structure and interaction of the protein, DNA and viruses, you can use the lipid bilayers you can use the nonspecific

biomolecular interaction biocompatibility and you can also use this for the tissue engineering.

So, this is all about the surface plasma on resolence and how you can be able to use and what are the different aspects of the SPR. So, what we have discussed? We have discussed about the basic principle of SPR and how the surface plasmons are formed onto the gold films and how you can be able to do you know detect those surface plasmon with the help of the particular wavelength light and so on.

So, with this I would like to conclude my lecture here in our subsequent lecture we are going to discuss some more aspects related to enzyme.

Thank you.