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Lecture – 47
Protein Ligand Interaction

Welcome back. So, today in this lecture-21, I will start talking about Protein Ligand Interactions. So, again these are these two topic two part lecture. In the first part, I will mostly focus on the thermodynamic aspects and experiments that can be used to determine the protein ligand binding constants. And in the second part, I will continue discussing protein ligand interactions, and I will mostly focus on the kinetics, and how you can measure the kinetics of protein ligand interactions.

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So, in this lecture, the topics that will be covered are basic thermodynamics and kinetics. So, I will give you an overview of the thermodynamics and kinematics of protein ligand interactions. I will also talk about the basic experimental setup, so that is something that is used for all the experiments that I will discuss. Then the techniques to study interactions, I will discuss three techniques in this lecture, and some more techniques in the next lecture.

And finally, the practical aspects of measuring interactions, because each technique has its own advantage and disadvantages. So, I will also try to discuss the important issues of

how you can choose, which technique should be used in which condition. So, the bottom line is that there is not single technique that can be used to measure binding constant for all types of interactions.

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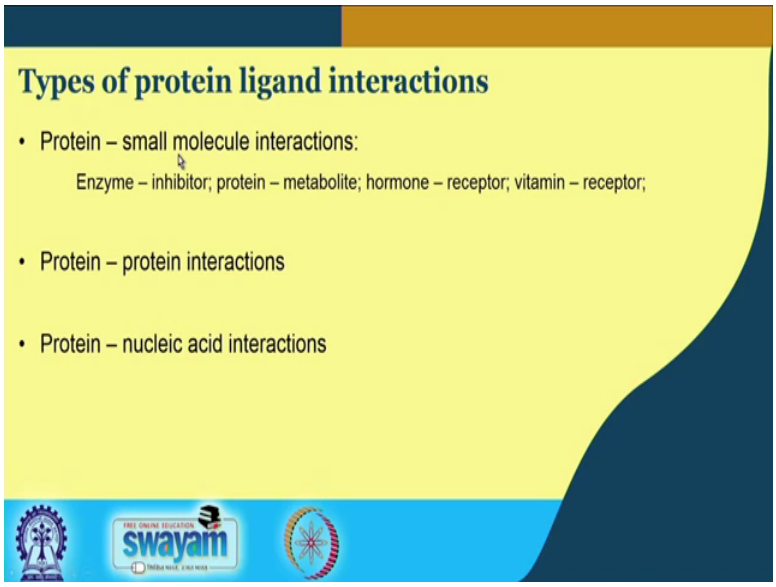

So, protein ligand interactions can be broadly classified into these three parts. One is where proteins bind to small molecules. The other one is one protein is in interacting with another protein, so these are macro molecular interactions. And another macro molecular interaction is where proteins can interact with nucleic acids, so nucleic acids such as DNA or RNA.

Protein-small molecule interactions there are several examples. So, if you have an enzyme, which binds to an inhibitor that is a protein-small molecule interactions; protein interacting with metabolites; receptors interacting with hormones or receptors interacting with vitamin. Protein-protein interactions are again same in all different source of biological systems, especially in the signaling events were proteins can one protein can interact another protein.

And protein-nucleic acid interactions are again seen in transcription and DNA replication. So, for example, in case of gene transcription, there are transcription factors which bind DNA promoter's sites. So, if you want to interact study the interaction of transcription factor with a DNA promoter site, then that will be an example of protein nucleic acid interactions. Then there are also RNA binding proteins which bind and

stabilize messenger RNA or the types of RNA. So, those will be again examples of protein nucleic acid interactions.

So, an important point to clarify here is the depending on the type of interactions, you will choose the experimental method that can give you the best information about that type of interactions. So, there are some experiments one experiment that I will talk to you about today is that can be used specifically for protein nucleic acid interactions, it is a very versatile experiment, but it cannot be used for protein-small molecule interactions. So, every technique has its limitations and also strengths. And we have to know about those, so that you can make out judicious judiciously choose the type of experiment, you want to use.

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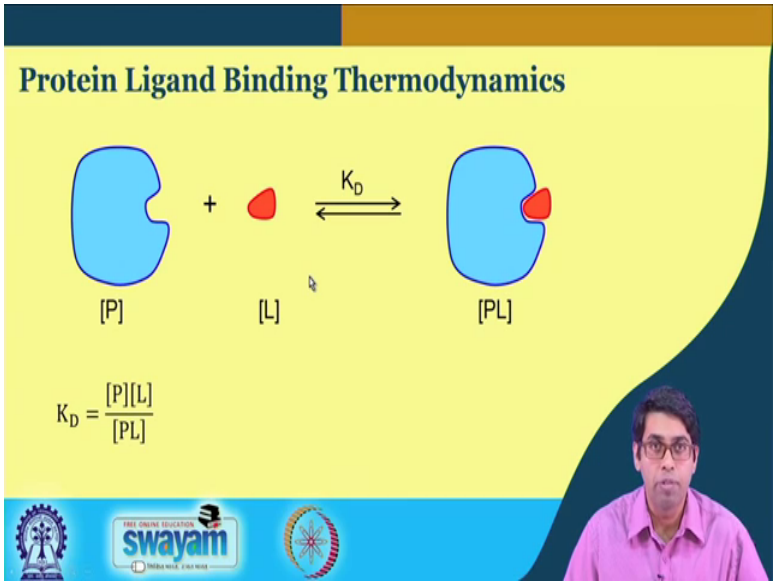

So, here is a brief overview of protein ligand binding. So, this is a (Refer Time: 04:01) of a protein, and this a ligand. So, if you just small molecule, it will look small like this. If it is another protein or a DNA and RNA, then it will be much bigger almost to the same size of this protein or it can be even bigger than that, because sometimes the DNA molecules that are used are much bigger than the proteins that we are looking at.

So, this free protein binds to the free ligand, and what you get is the protein ligand complex. The equilibrium constant of this type of interaction, this is the simplest interaction that I have shown here. It can it can be a complicated, where one protein is bound by two ligands or in case of a DNA, one DNA can be bound by two or even more

number of proteins. So, those things can complicate this equation, but this is the simplest form.

The equilibrium constant is a thermodynamic parameter, and you will notice that thermodynamic parameters are written as in capital letters in upper case. So, K D or dissociation constant also gives you the equilibrium constant of this reaction. And it is given by the product of free protein concentration, multiplied by free ligand concentration divided by the concentration of the protein ligand complex.

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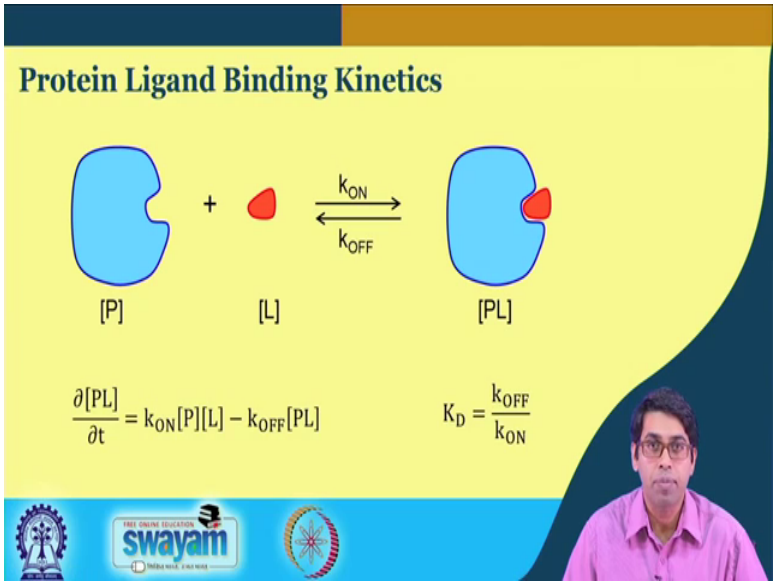

For the same reaction, we can also get kinetic information. So, here again notice that the rate constant kinetic rate constants are denoted in lower case letters. So, k ON is the forward reaction rate constant. So, this protein and ligand bind to give you PL or the protein ligand complex, and this forward rate is given by k ON. And the reverse rate were this complex dissociates into the free ligand and the free protein is given by k OFF.

So, at any given point of time, the rate of formation of the protein ligand complex is given by the summation of the forward reaction rate and the reverse reaction rate. So, the forward reaction rate is given by this rate constant multiplied by the free ligand concentration multiplied by the free protein concentration which is shown here. So, k ON times P, which is the free protein concentration times L which is the free ligand concentration. And you subtract the reverse reaction rate which is k OFF multiplied by

the concentration of this protein ligand complex, so minus k OFF times PL. So, this is the rate of the formation of this complex.

At equilibrium what happens is this left hand side becomes 0. So, just by doing a simple rearrangement of this equation, what you can see is that k OFF divided by k ON becomes P times L divide by PL, which is exactly equals to K D, we saw in the previous slide. So, K D is given as the OFF rate divided by the ON rate. So, this equation links the thermodynamic parameter to the kinetic parameters.

One thing that I should point out here, and I will again discuss this in the next lecture is that the rate constants this k ON and k OFF have different units. So, k ON is depends on the concentration of these two, whereas k OFF depends only on the concentration of this. So, the units of these two parameters are different, I will I will elaborate this in the more in the next lecture.

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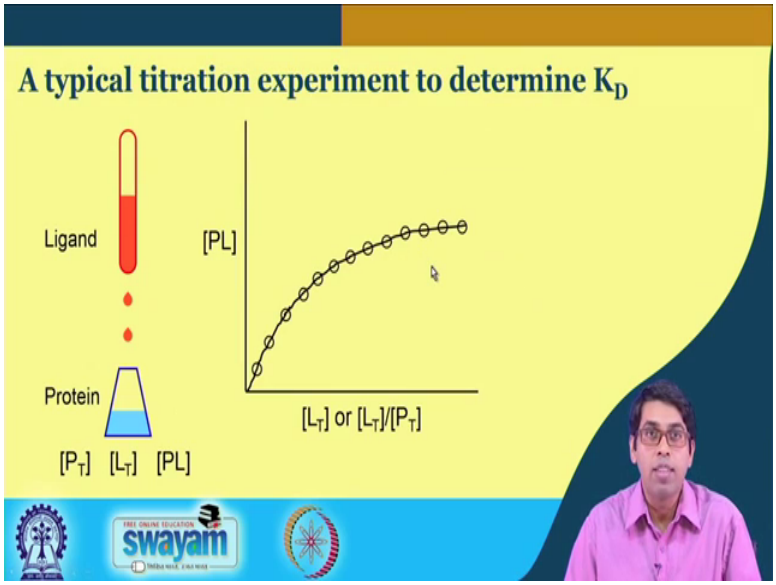

So, here is a typical set up of titration of a protein with its ligand. So, all experiments in essence will look something like this. The details will change, but overall what you will see is basically a titration experiment. So, here what we have is protein and we have protein and we know the concentration of this protein, so the concentration of this protein is given by P total ok.

And what we have up here is burette or a syringe or some device, where we have a ligand. So, we also know the concentration of this ligand, but this is the stock solution. So, this concentration is not going to be very useful in the titration experiment, we have to recalculate the concentration of the ligand that goes in here. So, at the first point, there is only protein, no ligand. So, protein ligand concentration is 0, you add may be one drop of ligand.

Now, you calculate the total concentration of the ligand here. So, whatever concentration was there, it gets diluted here, but you can easily calculate the concentration of the ligand, and that is your L total. You have some amount of ligand, and you have protein, so the binding event will happen, and you will get some protein ligand complex. So, this is the concentration of the protein ligand complex. So, you keep on doing this, you keep on adding the ligand which is titrated in the ligand. And the concentration of the ligand will keep on increasing, and the concentration of your protein ligand complex will keep on increasing.

So, if we plot the protein ligand complex as a function of the total ligand complex that is present in this reaction mixture, then you will get a plot like this. Because, as you keep on adding more and more ligand as we are adding more and more ligand, the concentration of the protein ligand complex keeps on increasing. And after sometime, you will reach a saturation point which mean that even after adding more ligand, you will not get any increase in the concentration of the protein ligand complex. So, it means that we have saturated the protein by adding a lot of ligand.

So, this is this discrete points are obtained from each titration points. So, if we add this ligand; so how many times we have aerated, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13. So, if we have added this ligand 13 times, we will get these 13 points, and this is our experimental data. And from this, we have to calculate the binding constant K D. So, how do we do that?

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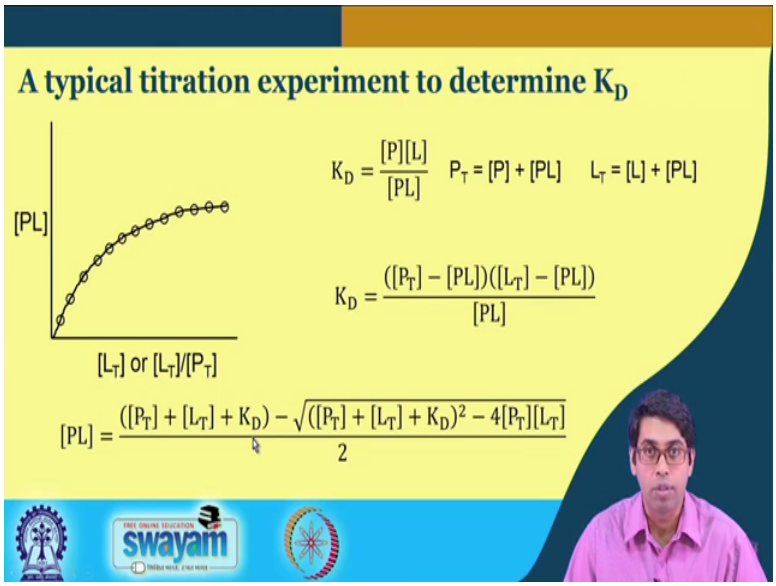

This is our data K D, we know is given by the free protein concentration multiplied by free ligand concentration divided by the protein ligand complex. Normally, the experiments are setup in such a matter that we can measure the formation of the complex. So, we know what is PL, we know the protein ligand complex concentration, but we do not know what is the concentration of our free protein and what is the concentration of our free ligand.

But, what we do know as experimental control is the total concentration of the protein, because that is what we started with. And the total concentration of the ligand, it turns out that total concentration of protein is the summation of the free protein plus the protein ligand complex, these two concentrations. Similarly, the total ligand concentration is the summation of the free ligand plus the protein ligand complex.

So, if we substitute this P with P total minus P L, and then this L with L total minus P L, we get something like this. So, now K D is given by known parameters, so we know P L, we know L total, and we know P total. So, we can calculate what is our K D? Since, the experimental data is obtained in this fashion, where we get the protein ligand complex concentration as a function of the total ligand concentration, it is easier.

If we can solve this equation and get the protein ligand concentration as a function of total protein, total ligand and the dissociation constant. So, this quadratic equation you can see this is a quadratic equation, because you will get P L square. And if you solve

that quadratic equation, this is one of the solutions, and this is one of the physical solutions that you get. So, in this case, what you have to do is you have to simply fit this data to this equation, you know P total, you know L total for each titration point, and you can solve for the dissociation constant.

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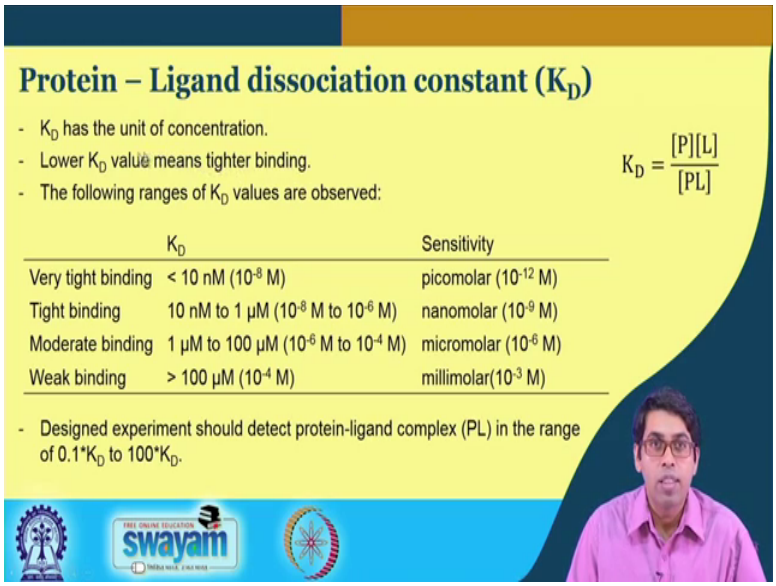

So, if you notice here, you will see that the dissociation constant has a unit of concentration, because these two have units of concentration, and you divide it by another unit of concentration, so you are left with one unit of concentration. So, K D has the same unit of concentration, and it turns out that lower K D value means tighter binding.

So, if a K D value is one nanomolar, and if another system has a K D of one micromolar, the one nanomolar K D value means tighter binding ok. So, the range of K D values that are observed in different biological systems can be divided into this four groups. So, I have tried to summarize this into a table.

So, you can divide the K D values that we see in different biological systems into this four different groups; one is very tight binding, then tight binding, moderate binding, and weak binding. And the typical ranges for each of these are given in the second column very tight binding is when the binding is less than 10 nanomolar. Tight binding is when it is between 10 nanomolar to 1 micromolar. Moderate binding is when it is between 1

micromolar to 100 micromolar. And if it is more than 100 micromolar, we call it weaker binding, in brackets these numbers are given in terms of molar ok.

So, it turns out that a particular experiment cannot be used for this wide range of binding constants that are observed in nature. So, some experiments are very good in this range for very tightened tight binding. We will see one example, so EMSA it is very good in this range, but it not that good for moderate or weak binding. And then there are other experiments which are very good for weaker binding or even moderate binding, but they cannot be used for tight binding systems.

So, again you have to have some idea about the range in which your protein ligand binding constant will appear, and you have to choose the experiment accordingly. It turns out that the experiment that we are going to use, its sensitivity has to be lower than the binding constant that you are going to measure. So, if you are going to measure a binding constant in the nanomolar range the experiment that you will use should have a sensitivity in the picomolar range. Similarly, if your measuring weaker binding constant, then much lower sensitivity will suffice. So, the sensitivity should be lower than the binding constant, so that you can cover a range of protein ligand concentrations to get the constant.

So, again the typical titration setup will be set in such a way that you cover a range of almost 1000 fold. So, this 0.1 K D, so if our binding constant is say 10 nanomolar, you should be able to detect 1 nanomolar upto 100 K D, so 10 nanomolar times 100 is 1 micro molar. So, you will actually detect a range of 1 nanomolar to 1 miromolar in your experiment to determine a binding constant of 10 nanomolar.

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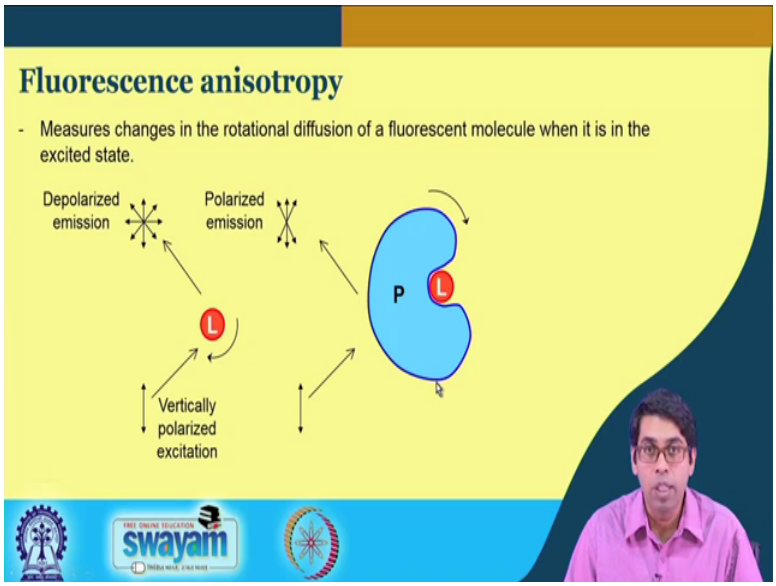

So, here is one example which is widely used in case of protein ligand interactions, and it is a fluorescent anisotropy. This experiment is used because of its ease of setup, but again you will have to know, whether your system can be used in setup like this. So, the basic principle of process anisotropy is very simple. In this case, what you do is your ligand has a fluorescent label.

So, if you excite your ligand with a vertically polarized light, once it gets excited, it will emit after sometime. But, while it before it does that, this ligand is assuming, it is not very big, it will have a rotational diffusion. And that will result in a emission of a depolarized light, because by the time this emission happens, all these molecules have rotated in different directions. So, the emission ends up to be depolarized. So, you excite with the vertically polarized light, and you get a depolarized emission.

Now, if you titrate in your protein, so if you titrate in a protein, you will get a protein ligand complex. For the complex, this is the ligand, and the protein is let say much bigger than the ligand. So, the overall size of the complex is much bigger compared to the size of your free ligand. So, in this case, the rotational diffusion becomes much slower, because bigger molecules diffuse slowly ok. So, the rotational diffusion will become slower.

Now, if we again shine the ligand or excite the ligand with a vertically polarized excitation by the time the emission happens, there is not much rotational diffusion that

has occurred because of the bigger size of the complex. So, the emitted light is somewhat still polarized, it is not completely depolarized like the free ligand. So, as we keep on adding more and more protein, we will get more and more complex, and the emission spectrum that you will get will become more and more polarized.

So, the different between this and this will give you a measure of how much protein ligand complex is formed at each titration point. So, again one point, I would like to clarify here is that in this case, you are titrating in the protein and not the ligand. Because, in the basic experiment, when I the basic setup of I said that you have protein, and you add ligand. But, in this case since your observing the ligand, you are titrating in the protein.

So, you will see that depending on the technique, what you titrate in keeps on changing. So, the basic idea is whatever you are observing the concentration of that species remains constant, and what you do not observe you titrate that in. So, in this case, we are observing the ligand which means you can detect the free ligand, and the bound ligand. So, the concentration of this, we will maintain as constant. And the protein is something that we are not going to see, so we will keep on titrating the protein.

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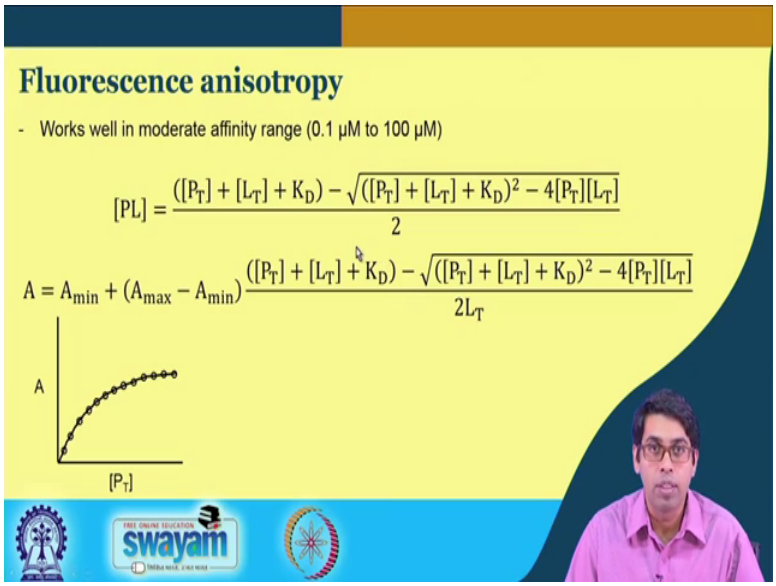

So, at the end of your titration, again you get a curve like this. So, this is the absorbance as a function of the total protein concentration, because we have titrated in the protein. This is the equation that we derive, and this will be the equation, where you will fit your

data. These are just the minimum and maximum absorbance that you will see, and they are they are for normalization, but the basic equation is exactly the same. And you fit your data to this equation to determine the dissociation constant.

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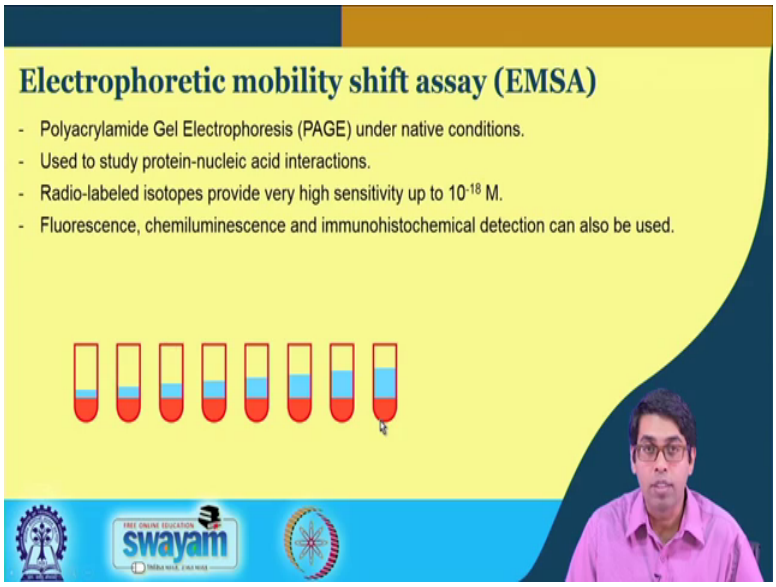

Another experiment that is widely used, and is very popular, and it is highly sensitive is electrophoretic mobility shift assay. So, we have discussed polyacrylamide gel electrophoresis in great details in previous lecture. So, here we are going to use the same page gel, but this gel will be run under native conditions. So, no SDS which means that the protein will not be denatured and it is essential, because you want to detect the formation of the protein ligand complex. If the protein is denatured, then no complex formation will take place.

These type of experiments are very useful to study protein-nucleic acid interactions. So, it can be DNA or RNA. And again in this case, the nucleic acid if we which is the ligand is the one that will be labeled. So, we can label them with radio isotopes, so phosphorous 32 is most popular choice and it has the highest sensitivity, it can detect upto 10 to the minus 18 molar.

If the binding is not very tight, then fluorescence, chemiluminescence or even immunohistochemical detections can be used. So, again the basic setup of the experiment is something like this. You have these different tubes, where we have the DNA, the labeled DNA same concentration of labeled DNA in each of these tube. And what we do

is we add the protein in different amounts. So, we are adding the protein in increasing concentration, you have to make sure that the total volume is constant, otherwise the concentration of the DNA will change. So, the total volume should be constant, but the concentration of the protein that you add keeps on increasing.

And what we do is if you again have an idea about the binding constant, let us say the binding constant is 1 nanomolar, then your DNA concentration should be much less than that. So, your DNA concentration should be around 0.01 nanomolar or 10 picomolar. The protein concentration will vary from 0.1 nanomolar to 100 nanomolar in this different reactions that you have setup here.

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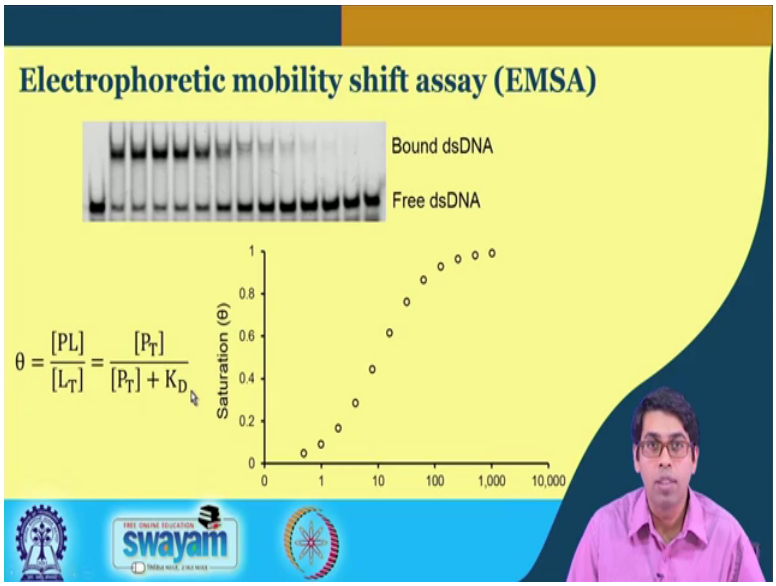

So, once we have that you load them in different lanes, again this you remember this is the native page, you may get very similar to what we have discussed before. Except that you leave out the SDS, and the first lane has the free DNA, and these are the lanes where you have loaded the reactions. So, here the protein concentration was the smallest, then you have more proteins, more protein, and more protein. So, these bands are for the free DNA. And these bands are for the DNA bound to the protein. As we increase the protein concentration the free DNA, band becomes thinner and thinner, and the complex the band that corresponds to the protein DNA complex becomes thicker and thicker.

So, typically in this type of experiment, we do not measure the intensity of this bands, but we measure the intensity of the DNA band. So, here the duty of this experiment is

that it we can detect the free ligand and the bound ligand simultaneously. Normally, there is these bands become diffused, so this a very good example of a gel. But, you might see that this band becomes diffused due to dissociation of the protein DNA complex. So, it is much easier to quantify the intensity of the free DNA bands, because they are always remain the same.

And from that we get a titration curve like this. So, this titration curve in the y-axis, what we plot is the saturation. And saturation is given by the concentration of the protein ligand complex divided by the total ligand. And this is the equation to which we will fit this, we know the total protein concentrations, but we do not know K D, so you are going to determine K D. So, if you fit this to this equation, you can determine the K D value.

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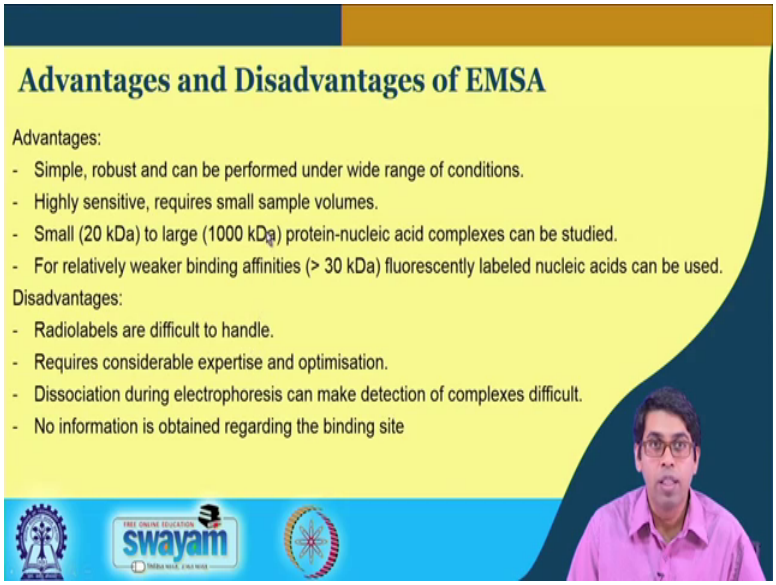

So, these are some of the advantages and disadvantages of EMSA. The advantages that it is very sensitive, and requires very small amount of samples, and it is highly robust which means that you can repeat this experiments and get reproducible results. It can also be used for wide range of protein DNA complex sizes. So, smaller complexes like 20 kilo Dalton's upto 1 mega Dalton size complexes can be studied by this method.

For relatively weaker binding, we can go to the alternative labeling methods, for example labeling the DNA with fluorescent labels. The disadvantages are that for high sensitivity, you have to work with radioactive samples which are always combustion and also difficult to handle. This type of experiments even though its running a gel, but it requires

a considerable amount of expertise and optimization; so, it is not that you can just run one gel, and you will get a very good data.

So, this experiments needs to be repeated several times to get very good and reproducible results. Dissociation during electrophoresis can make the detection of the complexes difficult, but we have a worth around for that where we detect the free ligand instead of the protein ligand complex. And it does not provide us any information about the binding site. So, this limitation is true for most experiments, because what you see is the protein ligand complex, but you do not know exactly where the protein is binding or where the ligand is binding.

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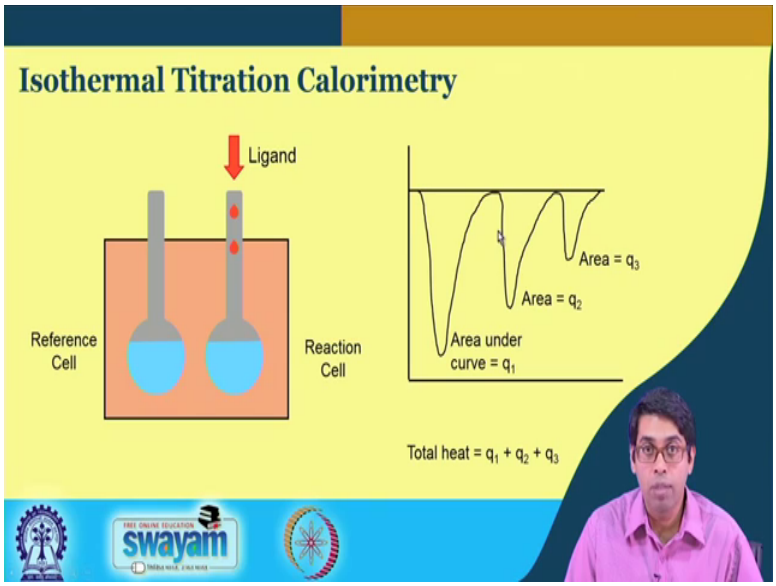

So, the last technique that I will talk about today is called isothermal titration calorimetry, again this is another titration experiment. But, here what we detect is the heat evolved, when the protein and the ligand bind. So, this a typical setup of this experiment, where you have two reaction vessels inside isothermal chamber, it means that the temperature of this chamber is kept constant. This is a reference cell, where you have your protein in its buffer and nothing happens, you do not do anything here.

And you have exactly similar chamber here, where you have the exact same protein, and the exact same buffer. But, in this case you keep on adding the ligand, and measure the heat change or the heat that is evolved or absorbed in each titration point, and that is

done by subtracting the change in heat between these two reaction vessels. So, this is the reference cell, and this the reaction cell.

So, after the first titration point, what you will see is signature peak like this. So, the area under this peak gives you the that is evolved, after the first titration. When you add another little amount of ligand that is the second titration point, you will get another small amount of heat. And as you keep on adding more and more ligand, you will see that the amount of heat keeps on decreasing, so that can be plotted something like this.

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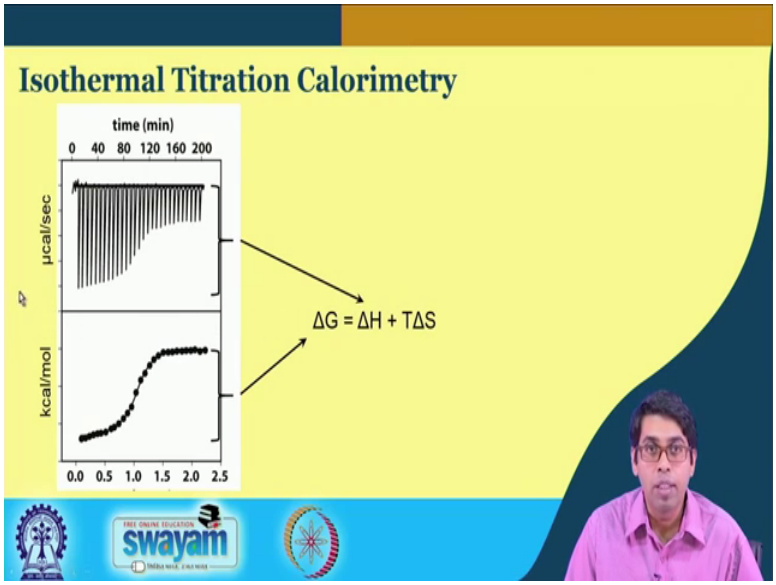

So, this is your this is a typical ITC experimental data. And if we integrate the area under each peak and plot them, you will get something like this. And there again standard equations. So, we can fit this to get the binding constant. Now, ITC provides you with additional information apart from just the K D or the binding or the dissociation constant. So, this is the standard equation that we know, it turns out that when you fit this, you get K D and K D is related to delta G.

So, from K D, you can get a measure of delta G. And the difference between these two is in kilocal's per mole right, so that difference gives you the delta H of the reaction. So, once we know delta G, and once we know delta H, we can measure the delta S. So, we can actually get the enthalpy and entropy of a reaction from isothermal titration calorimetry in addition to the dissociation constant.

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So, again these are the references that we will follow. And in the next class, I will talk about the kinetics of protein ligand interactions.

Thank you.