Introduction to Complex Biological Systems
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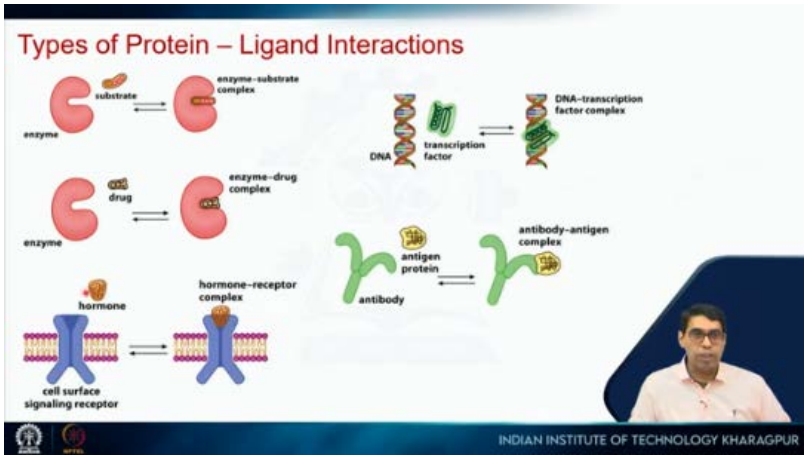
Lecture 58
Protein - ligand interactions

Welcome to the last week of lectures. So this is Lecture 58. Today I am going to discuss protein-ligand interactions. So we will see some methods where I will talk about protein-ligand interactions.

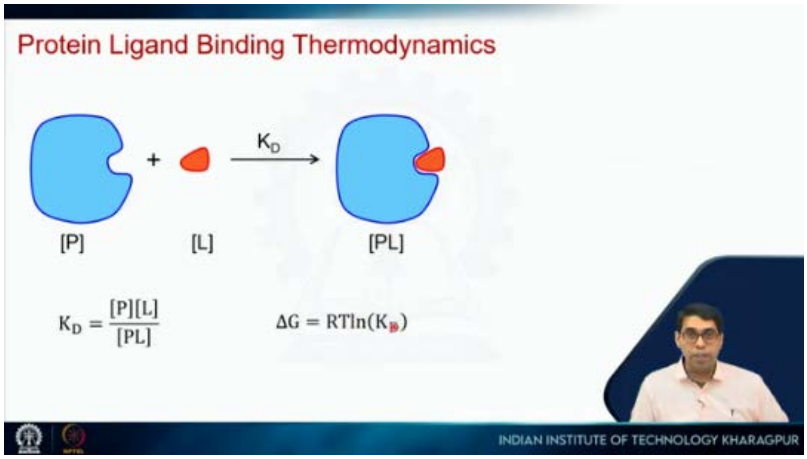
So you have already seen this. I have already discussed the types of protein-ligand interactions. So we can have enzyme-substrate interactions, we can have enzyme-drug molecule interactions, so this will be an inhibitor. We can have DNA transcription factors, so this is a protein, this is DNA, protein-nucleic acid interaction. We can have protein-protein interactions, where this is a protein, which is an antigen, and this is the antibody, which is also another protein. We can also have another type of protein-protein interaction, where this is a protein or a peptide, which is a hormone, and that binds to this membrane-bound protein, which is the receptor.



So, all of these interactions can be studied using the techniques that I am going to discuss today. So this is again a recapitulation of what we have seen. These are proteins, this is a ligand, and you get a protein-ligand complex. We can measure how tightly this ligand is bound to this protein using a number called the dissociation constant. So K_D stands for the dissociation constant, and it is given by the concentration of the free protein multiplied by

the concentration of the free ligand divided by the concentration of this protein-ligand complex. So you can see that this is concentration, this is concentration.

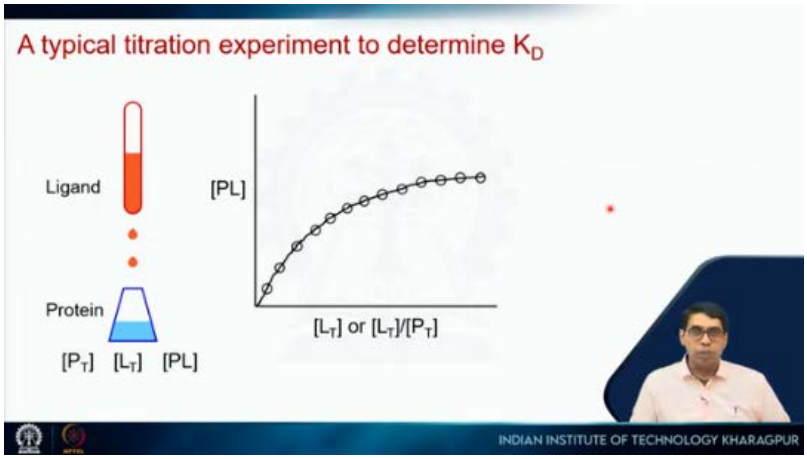
So, concentration squared divided by concentration. So, its unit will be in concentration. The free energy of binding is related to this K_D by this value. So, R is the universal gas constant, T is the temperature at which you are measuring the dissociation constant, and this is the natural log of K_D. So, that gives you the ΔG.



So, pictorially, we can see it like this: You have the free energy of the protein, which is somewhere here. In this, we are plotting all the different conformations or molecules, and here we have the free energy along the y-axis. So, the free energy of the Free protein is this. The free energy of the ligand by itself is this, and when you mix both, the free energy of the total system, where both protein and ligand are free, is this. But once the ligand binds to the protein, the free energy decreases to this. Since there is a decrease in free energy, the binding will be spontaneous. So, if the free energy does not decrease, then the binding will not be spontaneous. Here, we are talking about spontaneous reactions. The typical experimental setup to determine K_D will look something like this: where we have a protein in a flask and a ligand in a burette, syringe, or some device. So, dropwise, we will add the ligand to the protein, and somehow we will measure the concentration of the protein-ligand complex that is formed. You can also reverse this. The ligand can be here, and the protein can be here, so you add the protein to the ligand and measure it.

Now, when you do that at every point, it will be called a titration point. So, we are doing a titration. So, at every titration point, you measure the formation of the protein-ligand

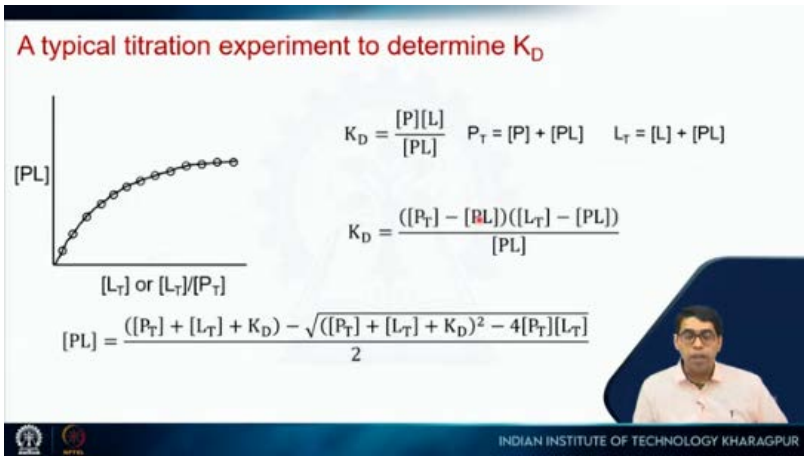
complex. So, this complex is plotted here, and this is the amount of ligand you have added or the ratio of the ligand to the protein. So, this will increase. So, as it increases, you will get more and more bound protein-ligand complexes. So, more and more protein will bind to the ligand. So, the concentration of the complex will increase. Eventually, it will reach some saturation where, even after adding ligand, no more protein will bind, and you will achieve this if all the protein is bound to the ligand. So, we want to determine this protein-ligand concentration; however, this is not something that we know. What we know is the total ligand concentration that you have added to your experiment, the total protein concentration that is there in the burette to start with, or the flask to start with.



So, these two you know, but now, if we look at the equation of K_D, it depends on free protein, free ligand, and the protein-ligand complex. But we can say that the total protein concentration will be the summation of free protein plus bound protein because the protein is present in one of the two states: either it is free or bound, so the total concentration will be $[P_T]$. Similarly, the ligand is either free or bound, so the total concentration will be $[L_T]$. and this is something that we know, and this is something that we know. So, we can replace in this equation the free protein by $[P_T] - [PL]$. So, that is what we have done here.

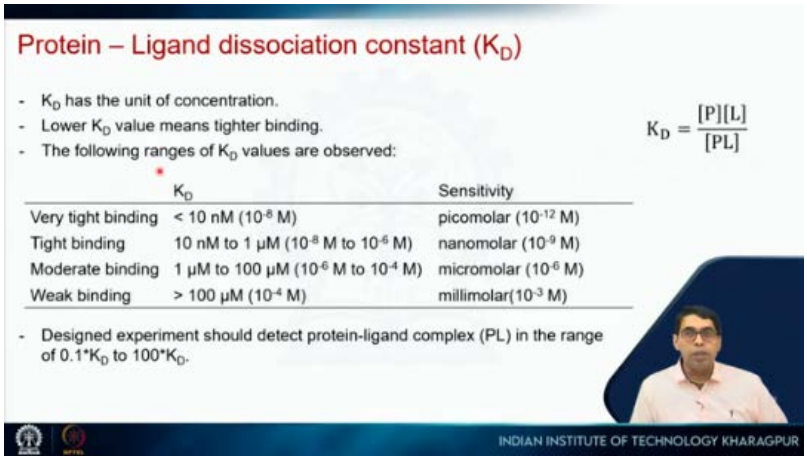
So, we have replaced free $\frac{[P]}{[P_T]} - [PL]$, and we have replaced free $\frac{[L]}{[L_T]} - [PL]$. So, $[L_T] - [PL]$, and in the denominator, we have $[PL]$. So, in this equation, you have P Total that you know, $[L_T]$ that you know, but you do not know what $[PL]$ is, and you do not know what K_D is. Now, if we multiply the terms and collect them together, we will get a quadratic equation for $[PL]$. So, you can actually see that $[PL]$ $times$ $[PL]$, you will get a $[PL]^2$. So,

if you collect all that and then solve the quadratic equation, you will get a solution like this: where at this position, you will get a plus and minus sign. Now, if it is plus, then it means that the protein-ligand concentration. This will be the summation of total protein plus total ligand plus K_D divided by 2 plus something. So, which is physically not possible. So, your protein ligand concentration cannot exceed the total protein or the total ligand concentration. So, you can throw out that solution. So, this will be minus and that is what is shown here.



So, $[PL]$ is given by this. So, now you can see this is your experimental data. You know $[L_T]$, you know $[P_T]$. So these two and you are getting a measure of $[PL]$ from something. So, we will see that indirectly we can measure it from fluorescence or heat released or things like that.

So, you will get a plot like this. So, for each of these plots they will obey this equation. So, we can do a numerical fitting where we can figure out what K_D is. So, typically that is what is done that this type of data will be numerically fitted to this equation where $[PL]$ is on the y axis and this $[P_T]$ and $[L_T]$, is something that you know $[P_T]$ will be most probably constant $[L_T]$, is something that you are increasing for each titration point and then you fit for K_D and you will get the K_D value. Here are some of the parameters that you can keep track of when you are setting up or designing an experiment.



So, remember that K_D has the unit of concentration. Lower K_D value means tighter binding. So, if K_D is nanomolar, it is tighter binding. If K_D is micromolar, it is a weaker binding. If K_D is millimolar, it is a very weak binding.

So, the following ranges of K_D values are observed. For very tight binding, you get nanomolar or less than nanomolar. Tight binding will be 10 nanomolar to 1 micromolar. Moderate is 1 micromolar to 100 micromolar and weak will be more than 100 micromolar.

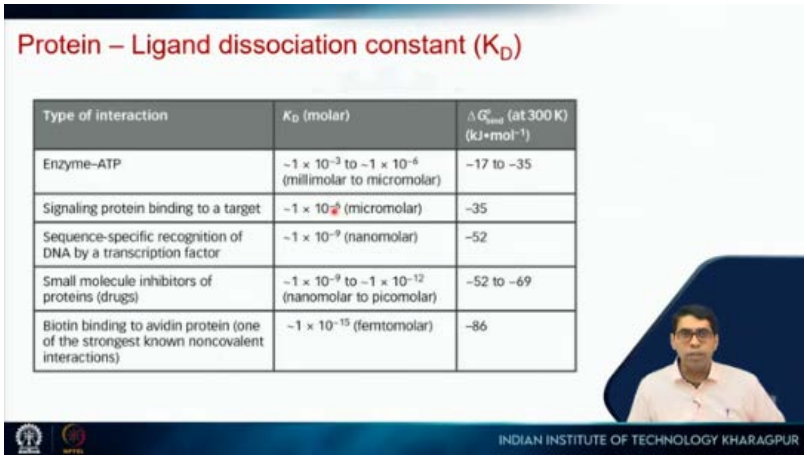
So, it goes into the millimolar range. Now, to determine K_D values in this range your experiment should be sensitive in this particular range. So, to get something that is less than 10 nanomolar your experiment should be sensitive in the picomolar range. Picomolar means that it should be able to determine the concentration of the free ligand or the free protein or the ligand-bound complex, whatever it is determining, and that should go down in the picomolar range. So, to get this tight binding, which is 10 nanomolar to 1 micromolar, it should go down to the nanomolar range.

So, 1 nanomolar or even less. So, roughly whatever the K_D value you are measuring, your sensitivity should be, to a good degree, at least 10-fold less. So, a 10-fold less amount you should be able to detect with good reliability. Now, the experiment that you will design will use or detect the protein-ligand complex in the range of 0.1 K_D. So that is why you have to go 10-fold less, up to 100 K_D.

So there will be almost a 1000-fold range of titration that you will do. You will have to determine the protein-ligand complex in this particular range. So your experiment should

be sensitive not only in the lower range but also in the higher range. Again, these are some examples of protein-ligand complexes where you see that enzyme binding to its substrate will be quite weak; it will be in the micromolar range.

Signaling protein binding to a target, so hormone or something else binding to a receptor, will be in the micromolar range. DNA transcription factor, because transcription factors have to bind to these promoter sites with high specificity and affinity.

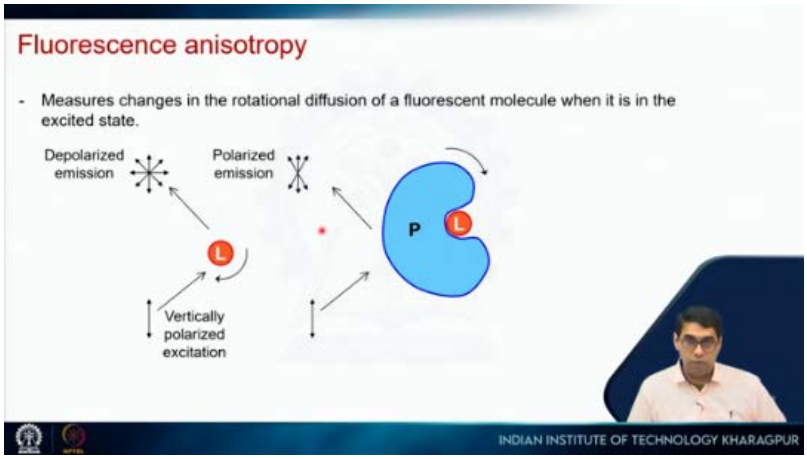


So in that case, the binding constant is much tighter. So we get nanomolar or even it can go down to the picomolar range. Inhibitors of proteins or enzymes, ideally we want them to be in the nanomolar range; if you get something in picomolar, that is even better and biotin binding to avidin is one of the strongest known non-covalent interactions, which is in femtomolar. Now let us look at some experiments. The first one that we are going to talk about is fluorescence anisotropy. We have discussed a little bit about fluorescence in Lecture 56. Here, let us see: we have a ligand, so we want to look at a protein-ligand interaction, and let us say our ligand is a small molecule. This is a ligand, and it fluoresces; maybe it has its own fluorescence or you have attached some fluorescent tag to this ligand. Now, what we do is we excite this with vertically polarized light. So, the light is vertically polarized like this and then it will emit this light. So, it will absorb and then emit, and that is what we are going to detect.

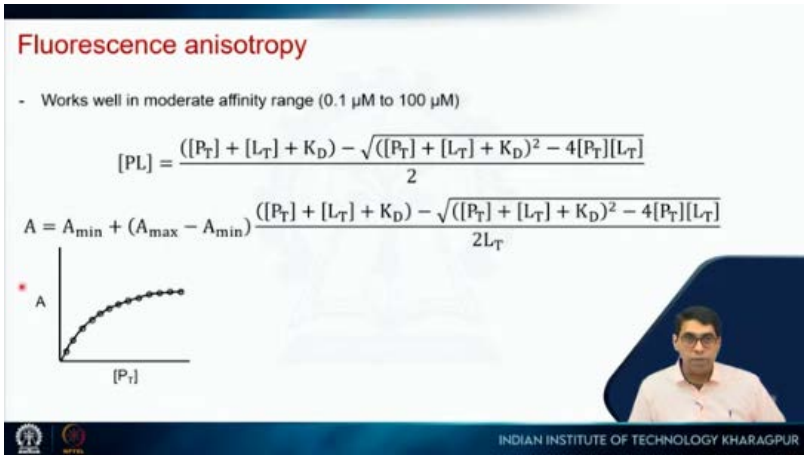
However, before emission, this ligand, since it is small, will tumble, but since it is small, it will tumble faster. So, it will tumble in all different directions, and that will depolarize this light. So, whatever the light that was polarized because of this rotational or tumbling

motion of the ligand, what you will detect in the emission is depolarized light. So, you can measure how much it is depolarized. Now, let us say we add protein.

So, we are doing a titration now; we add a little bit of protein. So, some of the ligands will bind to the protein. Since the protein is a bigger molecule and the ligand is bound to the bigger molecule, this whole complex will tumble at a rate much slower than this. Now, since this tumbling is slower, the depolarization that happens will be smaller.

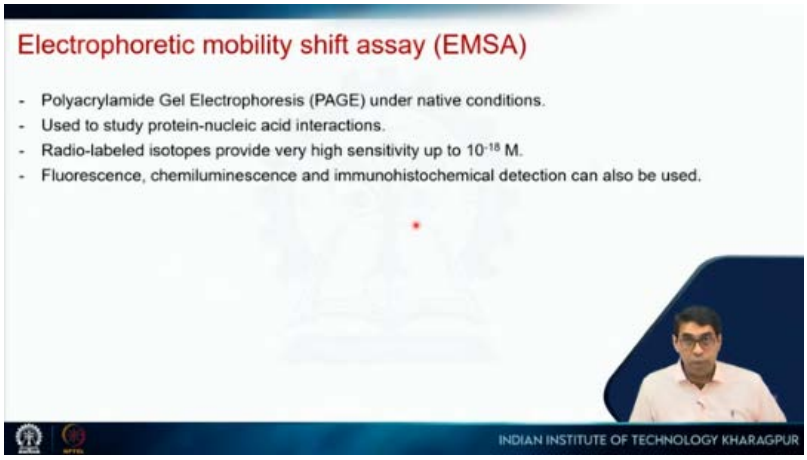


So, here we get depolarized emission, and here we get somewhat polarized emission. This degree of polarization or depolarization will depend on how much ligand is bound to the protein. So, as you precede with your titration, as more and more ligands binds, you will get increasingly polarized light compared to this.

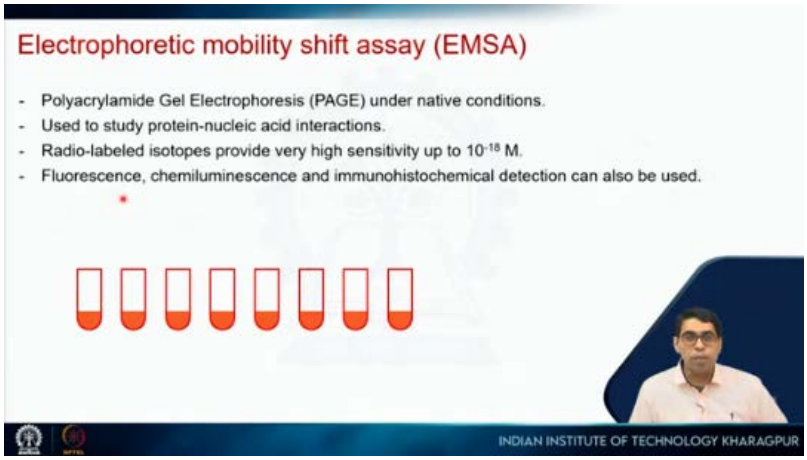


We can measure the rate or the amount of change between this depolarized and this polarized light and estimate how much ligand is bound to the protein. Once we do that, we

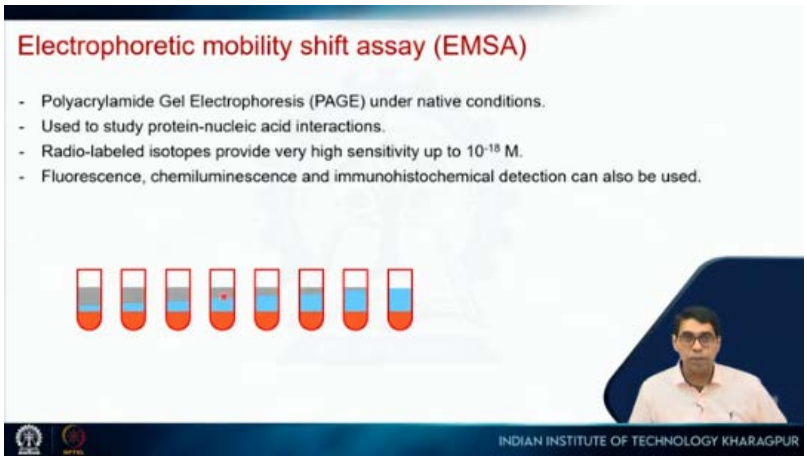
can measure it, so this is something that we are measuring, and we can then also normalize it. So, there will be a maximum value and a minimum value. So, we can normalize that using this, and then you can see that this is the basic equation we have derived. So, the same equation comes back. So, you fit to this, and you know$[L_T]$, you know $[P_T]$, and you are also measuring all these A values so you fit forK_D, and you can get your dissociation constant. It works well. This type of experiment works very well for moderate affinity ranges, which are 0.1 micromolar to 100 micromolar. There is another very popular and widely used experiment called electrophoretic mobility shift assay. We have already seen polyacrylamide gel electrophoresis, but we saw it under denaturing conditions. Here we will use the same method, the same gel electrophoresis, but under native conditions and typically, this experiment is used to study protein-nucleic acid interactions. As I mentioned a few minutes back, protein-nucleic acid interactions are very tight. So, EMSA is something that can actually detect much tighter interactions. If we are using radio-labeled isotopes, then it provides very high sensitivity, up to 10^{-18}molar. So, you can measure picomolar or femtomolar binding constants using this type of experiment.



So instead of radiolabel, fluorescence, chemiluminescence, or immunohistochemical detection can also be used, but they will have lower sensitivity compared to this. So using fluorescence, you can go up to picomolar sensitivity, which is good for most applications. This is the typical setup of the experiment. Here, we are going to do a titration experiment, but instead of having one flask, we will have a series or range of tubes. These are small tubes, 1.5ml sample tubes and what you have is the DNA. This orange color is the DNA.

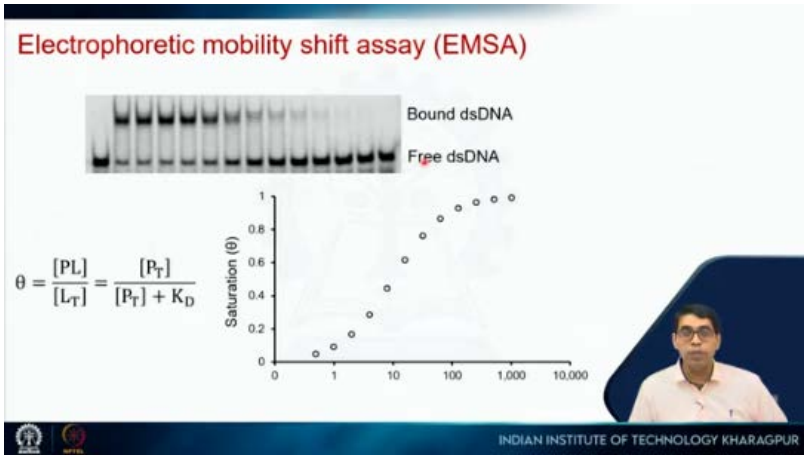


So you add maybe 5 microliters or 2 microliters of your DNA. The same amount of DNA is added to all the tubes and this DNA is either radio-labeled or labeled with a fluorescence tag. Then you add varying concentrations of your protein. So, there will be one tube where you will have no protein, and then you add some more protein, and then some more protein so you keep increasing the amount of protein, but you have to keep the total volume the same. So, you make it up with a buffer. This gray is the buffer. Now, the total volume is the same. The DNA gets diluted to the exact same amount; the protein gets diluted, so you will have less protein here and more protein here. So, that is what you have and that is your titration. Then, there will be one tube where you will have only DNA and a buffer made up to the same volume. Then, you take these samples, load them onto a polyacrylamide gel under native conditions, so you will not add any SDS or other denaturant, and you will run it.



Then you detect it. If it is a fluorescent tag, you use an instrument that can detect fluorescence, or if it is a radio label, you detect that. So, you will get an image like this. Again, you see that this is the first lane or the first sample where you had only DNA. That is what you see, this is the free DNA. That is how you can actually tell which is your free and which is your bound. Nothing here, you see something here.

This is your free DNA and so here, it is done in the reverse order. In this case, the amount of protein was less, and the protein concentration is increasing in this direction. So, as protein concentration increases, you see that this free DNA is decreasing. The band is becoming smaller and smaller, and there is a new band that shows up.



So, this is the bound DNA. So, the DNA is bound to the protein molecule and it shows up here. So, this is the DNA bound protein complex and since it is bigger it runs slowly. There is a native page where DNA is a negatively charged molecule. So, it will run towards the positive end.

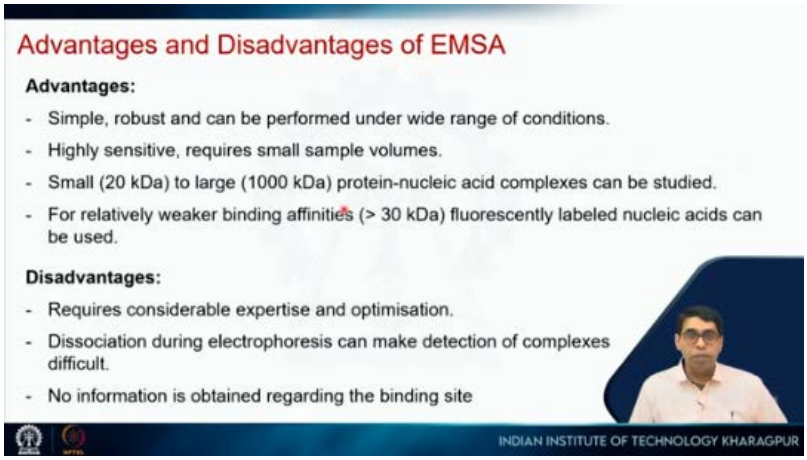
All the free DNA, they will run at the same speed. However, when the DNA is bound to the protein, the size increases so it will run slower. This is the mobility shift. That is why it is called electrophoretic mobility shift assay.

Since the DNA is binding to this protein, there is a shift in its mobility. It runs slower and you can detect the bound DNA. If you get a gel which is good like this, you can quantify this, or it is much better to quantify the free DNA. So, typically that is what is done. You quantify the free DNA in each lane and you divide it by the intensity in this lane.

So, you measure the intensity in each of these lanes and divide it by this. That is normalization, and that tells you the saturation. So, if this was 0, then this divided by this will be 0, meaning that 100% is bound. If this divided by this gives you 0.5, it means that 0.5 of the DNA is bound. So, that gives you the saturation.

So, whatever you get, subtract it from 1 and you will get the saturation, how much of the DNA is bound to the protein, and that can be expressed in terms of this. So, this is the total protein concentration, and this is the dissociation constant and you can see that it does not depend on the DNA concentration. It only depends on how much protein you have added at each titration point. You can plot this saturation as a function of protein concentration, and it will look something like this. Here you see that it looks like a sigmoid curve because we have plotted this on a log scale. So, you should be aware of that. This is not a sigmoid curve. So, there is no allostery or co-operativity in this. It is just because we have plotted it in the log scale. It looks like this. Now we can fit this equation. We have theθ, we have our total protein concentration for each of these titration points. So, we can fit them and we can get the K_D value.

So, roughly you can see that when my protein concentration equalsK_D, θ becomes 0.5. If I go to 0.5 like this and if I go to this, whatever protein concentration is here, that will be my K_D. So here the K_D will be somewhere in the range of 10. So if this is nanomolar, then this will be maybe 15 or 20nanomolar binding constants. So, what are the advantages and disadvantages of this experiment? EMSA, or electrophoretic mobility shift assay, is a very simple, robust experiment and can be performed under a wide range of conditions. It is very sensitive; it requires a very small amount of protein, so you do not have to purify large quantities of protein or use very costly ligands, since you are using a very small amount, it is relatively cheaper and you can repeat your experiments multiple times to get good statistics.

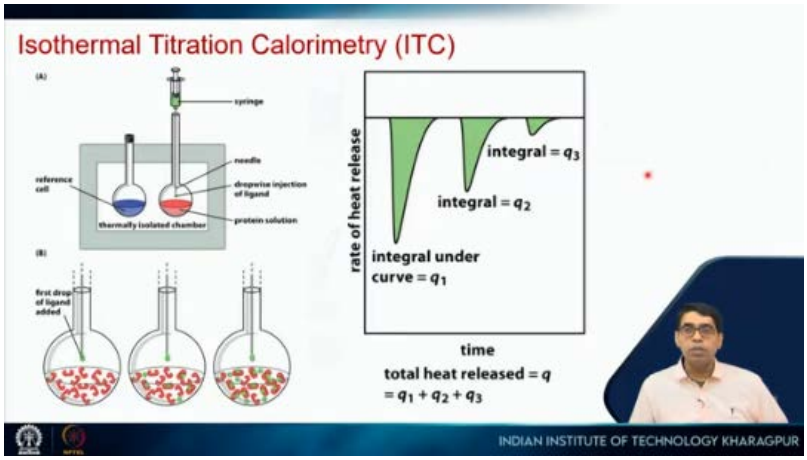


It is applicable to small to large protein-nucleic acid complexes, and for relatively weaker binding affinities, fluorescently labeled nucleic acids can be used. So, if your affinity is in the micromolar range or nanomolar range, you can use fluorescently labeled nucleic acids. Disadvantage: it requires considerable expertise and optimization but this is true for any experiment. So, you have to do these experiments several times to get a good handle on it, and you have to optimize it according to your sample.

There is, of course, that time that is taken. However, most biochemistry labs run gels. So, running a gel is something that students normally know. This is something that they can easily do or optimize. Dissociation during electrophoresis can make detection of complexes difficult.

So, in many cases you will not be able to see the complexes well because of dissociation; the complex, the band that is formed by the complex, becomes diffused. So, you cannot quantify it, but then the free ligand or the free DNA band will be quite narrow and crisp which you can easily quantify and normally people use that for measuring the K_D value. No information is obtained regarding the binding site so you can tell whether your DNA is binding to the protein or not and you can measure how tightly it is binding.

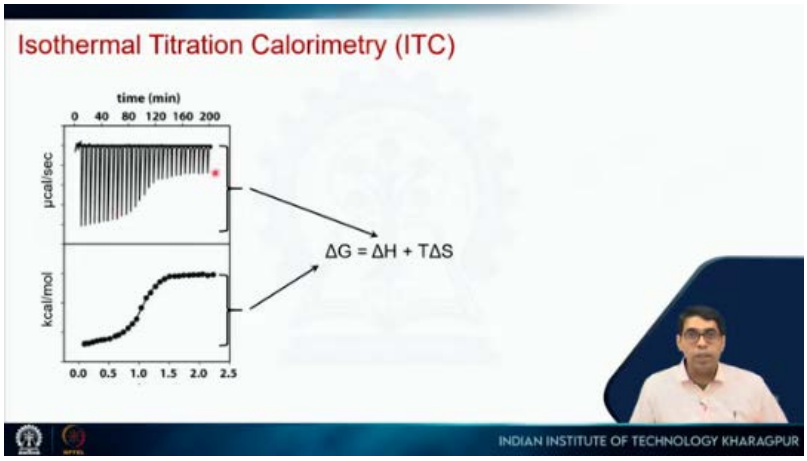
But you cannot tell where it is binding, what is the binding site and you will also not know the kinetics from this particular experiment, the way we have set it up. So another very useful and very popular experiment that is used to determine K_D is isothermal titration calorimetry. The instrument looks something like this. So it has this thermally isolated chamber and there are two vessels.



These will normally be metallic vessels. It can be gold or it can be some other alloy which is very inert so most of the samples can be easily used with them and they have to be, of course, conducting. There is a reference cell and then there is the reaction cell. Both are in the same isolated chamber, and both have exactly the same volume. So, in the reference cell, you fill it with your buffer. If your protein is in, let's say, a PBS buffer, you will add the same PBS buffer here, and in this case, you will add the protein solution. Then there is this syringe which will have the ligand. So, this can be a small molecule; this can be another protein; this can be DNA, anything. Now, you will add your ligand to this protein dropwise. The moment you add it, some of this ligand will bind, and this binding can result in a change in heat, so it can release heat or it can absorb heat, and this chamber, what it does is it will try to maintain the temperature, so it will measure that change in heat, and that change in heat is plotted here, so, as you inject, as the first injection happens, There is some release in heat, so you will get a peak like this, and then the instrument will go back to its equilibrium. It will go back to this, and then, after some time, another injection happens, so you will get another peak, and then it will go back. Then you add another injection, there will be another peak, and it will go back. You can see that progressively, this peak will get smaller and smaller and smaller. The area under the peak gives you the heat that is evolved or absorbed at that particular injection. Now, why is it getting smaller and smaller? Because, the first time when you are injecting the ligand, all the protein molecules are free. So, you will get the maximum amount of binding in the first injection.

In the second injection, there are quite a few protein molecules which are bound, so the amount of protein molecules that are free is less, so when you do the second injection where

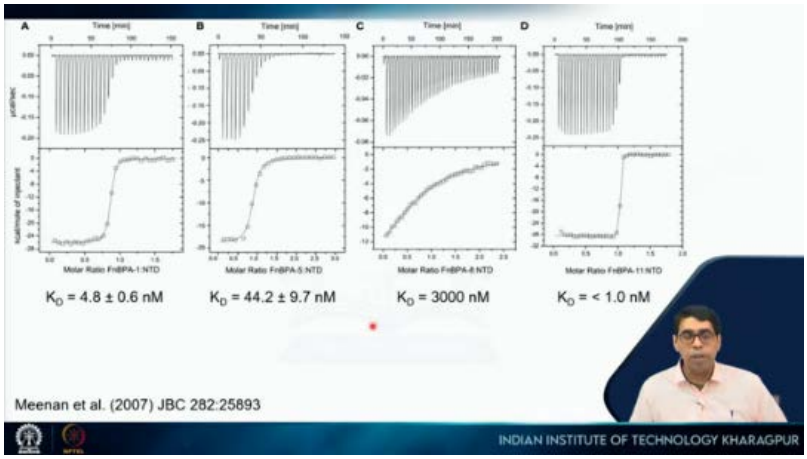
you are injecting the same amount of ligand, you will see a lesser amount of proteins that are getting bound. The heat will be less, and finally, you will reach saturation. Only three are shown here, but typically we do around 20 injections. By the time you go beyond 15, you will see that this heat is very low, so it has reached almost saturation. So, a typical ITC data will look something like this. So, you can see that the first injection gives you a very big peak, and then slowly it becomes smaller and smaller, and then finally it flattens out. You can still see that there is a peak here. Even though it has flattened out, the same amount of peak is there. The reason for this is the dilution effect of your ligand. So, your ligand is very concentrated. You are adding, let us say, 2 micromolar, which is going into 200 microliters of the protein sample. So, there is a 100-fold dilution, and that dilution will result in some heat change, and that is called the heat of dilution.



Typically, what you should do is you should do another experiment where you inject the ligand into the buffer, not the protein, you will see peaks of this height, but there will be no curve like this. It will be flat like this. So that heat of dilution, you can do the same number of injections and subtract that heat of dilution from this, and that will remove the heat of dilution. Only the heat that you will get will come from the binding. Then, you can measure the heat or integrate this area under each peak and plot it here. So, this is the protein-ligand ratio; this is the heat per mole that is released for each injection, and your data will look something like this. Then we can fit it to a standard equation, which will look very similar to what I have shown you before, and you can get K_D from this. So, it turns out that once you get the K_D, you know the ΔG, the difference in heat from here to here or, in other words, the heat that you get from the first injection that gives you a measure

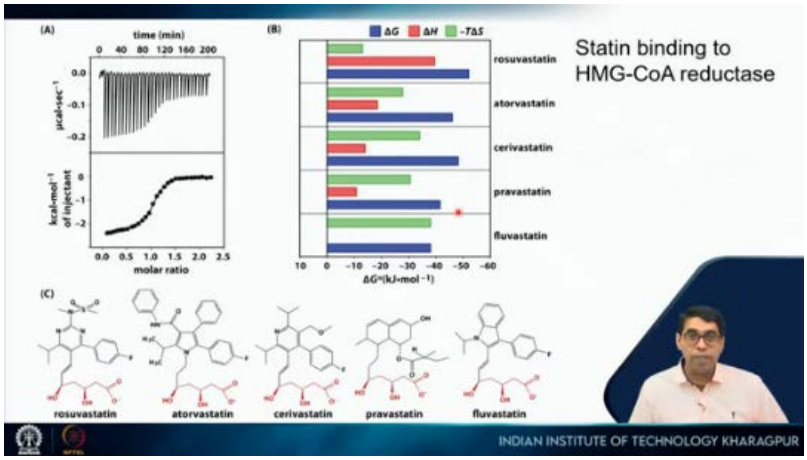
of ΔH, the heat that you get from the first injection. Of course, you have to subtract the heat of dilution that will give you the ΔH. So, if you know ΔG, which comes from K_D, and if you know ΔH, you can also measure ΔS. So, ITC not only gives you the K_D or the dissociation constant, it also gives you the thermodynamic parameters, which are the enthalpy and the entropy. So, these are some typical ITC curves for different protein-ligand interactions.

So, you can go through this paper to see the actual conditions and actual binding assays. If you see something like this, this tells you that the binding is very weak. On the other hand, if you see something like this, you see everything is binding, everything is binding and then suddenly it reaches saturation. So, when you see something like this, then it is, then the binding is very tight. So, weak, very weak, very tight and then these are intermediate. So, you can see that this is some binding and this is some binding. So, this is still sharper than this.



So, you can just by looking at it, you can tell that this will be the tightest binding, then it will be this, then it will be this and this will be the weakest binding and once you do the actual fitting and measure the K_D value, you will see that is indeed the case. K_D is less than 1 nanomolar so it is very tight binding here. In this case, it is around 5 nanomolar.

This is 40nanomolar and this is 3000. So, it is quite weak. So, it is going to the micromolar range. As I said, you can not only get the K_D value but you can also get the ΔH and ΔS, so enthalpy and entropy values also. So some of the statins, these are different statins which bind to *HMG COA* reductase, so that is an enzyme.

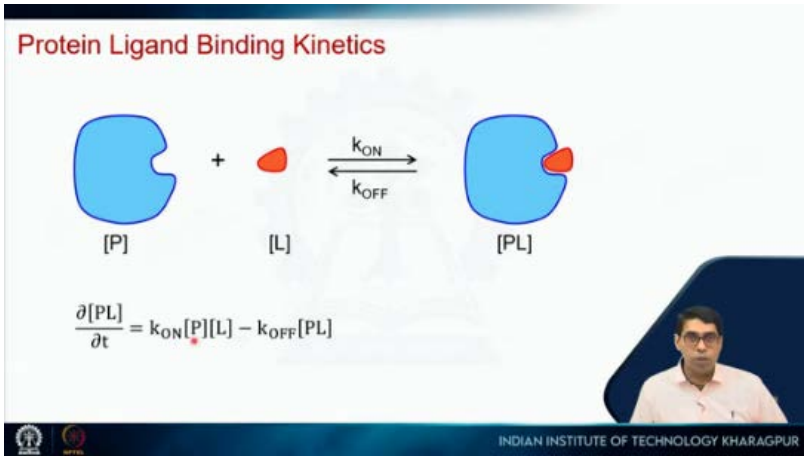


These statins binding to this enzyme have been characterized or studied by ITC and the ΔG shown in blue, ΔH shown in red and ΔS or $T\Delta S$ shown in green, they are all shown here. So, you can see that if you just look at the blue, you can see the blue is more or less similar. So, this is tightest binding, this is weakest binding, but they are in the similar range. However, if you see the ΔH and ΔS, they vary a lot.

So, you see ΔH, so this one for binding of this molecule to the enzyme, and it is mostly driven by enthalpy. You see, red is the biggest. On the other hand, for fluvastatin, there is no enthalpy; it is completely driven by entropy. So binding of this molecule, which is this. to this enzyme, this is completely driven by entropy, whereas for this molecule it is driven more by enthalpy than by entropy, and then these 3 molecules are in between. So, this is something that is very interesting, and you can sort of correlate whether enthalpy will contribute more or entropy will contribute more by looking at the polarity of this molecule. So, you can see that this molecule is more polar and it has more enthalpy contribution, whereas this molecule is less polar and it has much less enthalpy contribution.

So not only protein ligand interaction thermodynamics, we are also interested in kinetics. So how do you measure kinetics? Again, this is a ligand binding to a protein. The rate at which it binds will be given by the ON rate k_{ON}, and the rate at which it dissociates will be given by k_{OFF} or the off rate and we can look at the rate of the formation of this protein ligand complex like this. So, the rate of formation is given by the forward reaction. So, the ON rate multiplied by the ligand concentration multiplied by the protein concentration which is shown here and this complex, the concentration is decreased, or the complex is

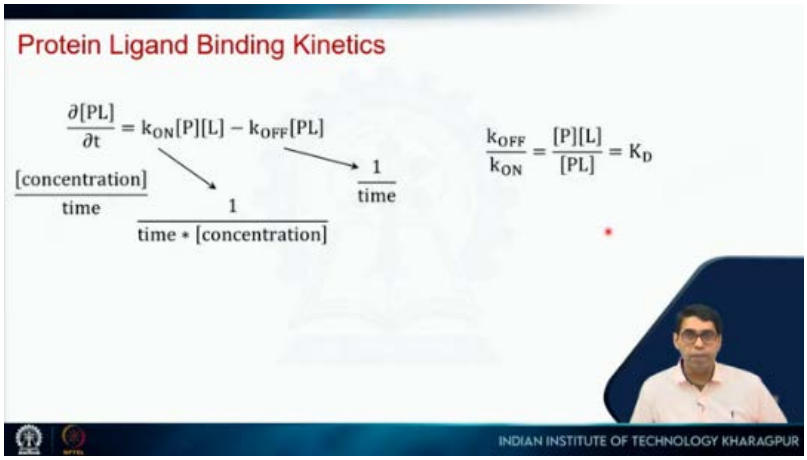
destroyed by this reverse reaction. So, it will be the OFF rate multiplied by the concentration of the protein-ligand complex. The OFF rate multiplied by the protein-ligand complex, and since it results in reduction, there is a negative sign here. So, this minus this gives you the rate of formation of the protein-ligand complex. Now, at equilibrium, what happens is that the protein-ligand complex concentration will not change with time, which means that $\partial[PL]/\partial t$ will be equal to 0.



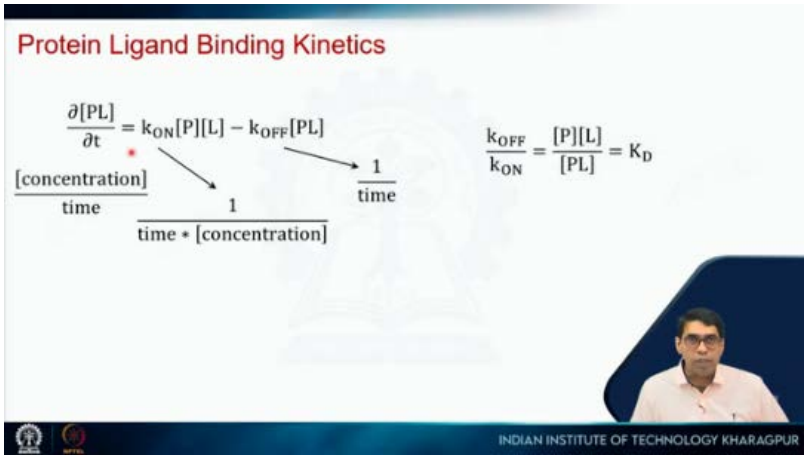
So, if you set this left-hand side equal to 0 and if you take the ratio, you will get that k_{OFF} by k_{ON}will be equal to $[P][L]/[PL]$, which is nothing but the K_D. So, under equilibrium, the dissociation constant equals the ratio of the OFF rate and the ON rate. Now, if we look at the dimension or the units of these two rate constants, on the left-hand side, it is $\partial[PL]/\partial t$.

So, it is concentration over time. k_{ON} will be 1 by times concentration. So, time to the power minus 1, concentration to the power minus 1. So, that you can easily figure out by doing a dimensional analysis. So, this is concentration over time, this is concentration, this is concentration.

So, this will be 1 divided by time times concentration. Similarly, k_{OFF} will be only 1 divided by time so time inverse and then, if you put these dimensions here, you will get the same thing back, the same equation back. What is the typical experiment to determine the ON and OFF rates for a protein-ligand interaction?

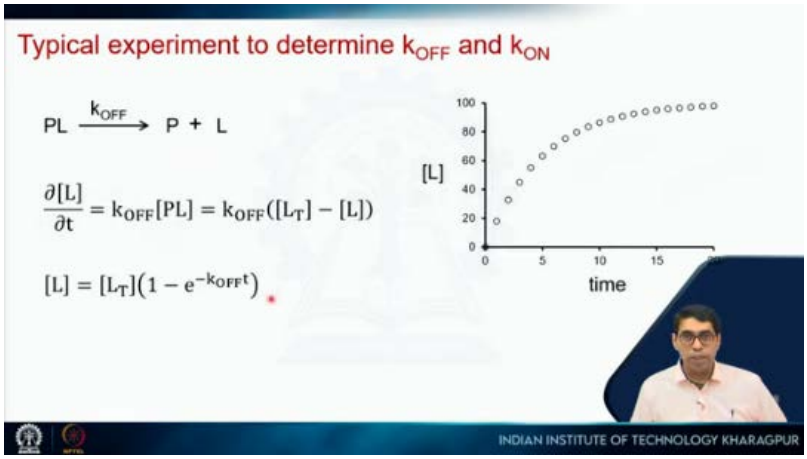


This is our guiding equation. So, we have the ligand. Let me just go back and show you this. So, protein-ligand concentration is this. You see that the protein-ligand complex is destroyed by this OFF rate. This is the reverse reaction. The protein-ligand complex is getting destroyed by the OFF rate, and you are getting protein plus ligand.

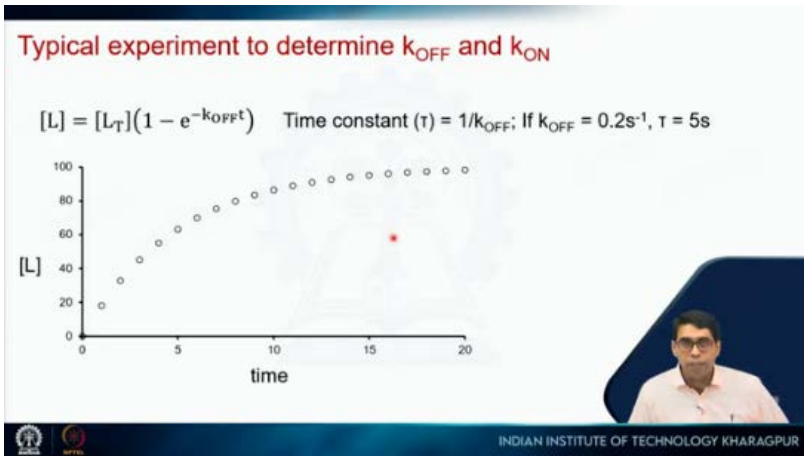


So, if we focus on this, then we can say that the rate of ligand formation is nothing but the OFF rate multiplied by the concentration of the protein-ligand complex, which is this. Now, we can replace $[PL]$ by this $[L_T]$ minus L, because $[L_T]$ will be the free ligand plus the protein-ligand complex. So, we can solve for this equation and get something like this, where the free ligand concentration depends on the total ligand concentration times 1 minus $e^{-k_{OFF}t}$. So, this is a first-order reaction, and this is what you get. So, you can simply see that when t equals 0, $e^0 = 1$.

So, $1 - 1 = 0$. So, there is no free ligand. At time t equals 0, you have everything as bound. When t is a very large number, then e to the power minus large number becomes 0, and $[L]$ becomes $[L_T]$. So, all the ligand is free, which means that this has completely dissociated and remember that we have set it up as an irreversible reaction, meaning there is no rebinding of the protein and the ligand. So, we can plot this as a function of time. If I plot this as a function of time, it will look something like this. So, if this is my experiment, and if I can measure the free ligand concentration as a function of time, I will get this and if I can fit it, I can extract my k_{OFF} value.

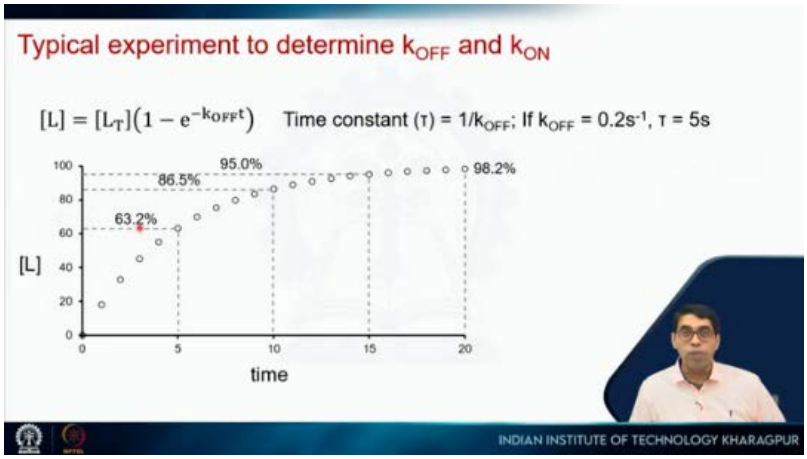


So, I can determine what k_{OFF} is. Now, these are some of the typical set of parameters that you should be aware of. So, let us say you are aware of the k_{OFF} value, and you have some idea that the k_{OFF} is something like, let us say, 0.2 per second. So, the inverse of that is called the time constant. So, if k_{OFF} is 0.2, the time constant will be 5 seconds.



So, the question is: how long should you run your experiment, and how often should you measure? So, these are the two parameters that you typically have to set in an experiment. So, if I let us say run the experiment for a time which equals this τ which is 5 seconds, you will see that your ligand will have dissociated, or the free ligand formation will be only 63%. So, if you are fitting to this curve, the k_{OFF} value that you will get will not be very good.

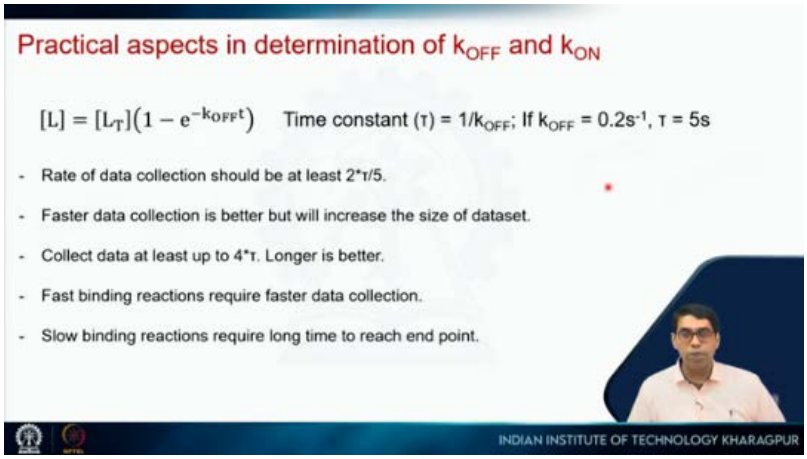
If you wait for 2 times this time constant, that is 10 seconds, this will be 86%. This is somewhat okay. If you go for 3, if you go for 4, here it is almost 98%, more than 98%. So, typically, you should wait for 4 time constants. So, if you think that your k_{OFF} rate is 0.2 per second, then you have to run this experiment for 20 seconds. So, that you can get a good curve like this, and you can get a good fitting and then, how often should you collect?



The rate of data collection should be at least 2 times τ by 5. So, what is τ? τ is 5 seconds. So, $\tau/5$ is 1 second multiplied by 2. So you should collect your data every 2 seconds or faster. So, faster data collection is better, but if it is of course, it increases the size of your data set, but normally that is not a big concern. So, it will be limited by the way you are doing the experiment. You should collect the data at least up to 4τ. So, in this case you should collect your data for up to 20 seconds. So, collect data every 2 seconds up to 20 seconds.

So, you will have 10 data points and you can fit it to get a very good estimate of this k_{OFF}. Fast binding reactions require faster data collection. So, this will completely depend on the instrumentation whether you can collect data at a faster rate. Slow binding reactions will

require a long time to reach end points, so we have to collect data at a slower rate, but you will have to collect data for a much longer period of time. So, it will be something where stability of the experiment will be very important.

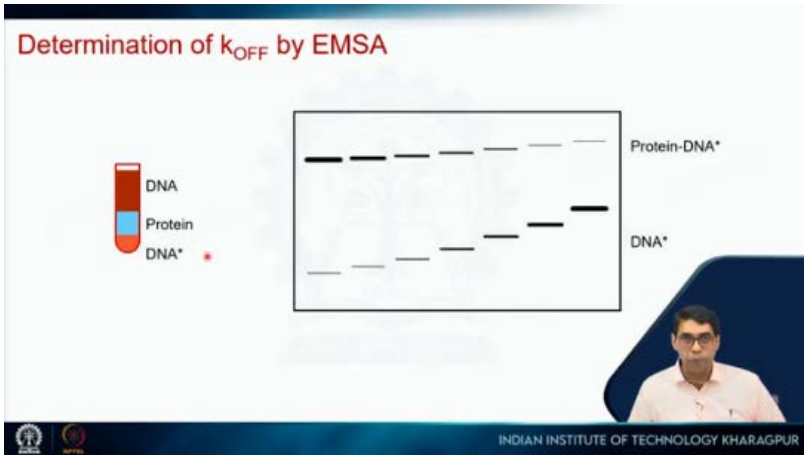


We just saw that EMSA can be used to determine the K_D value. If we set it up properly, we can also use EMSA to determine the OFF rate. So let us say you have a DNA which binds to protein, the DNA will also dissociate. So you want to determine how fast the DNA is coming off. Typically it will be a very slow process because the OFF rate will be very less because protein DNA interactions are typically quite tight.

So let us say you are studying something like that. So, this is my DNA which is fluorescently labeled or radio labeled, I add my protein. So, I will get a protein DNA complex. So, you give some time for this complex to be formed and reach equilibrium. Then what you do is you add the same DNA without the label.

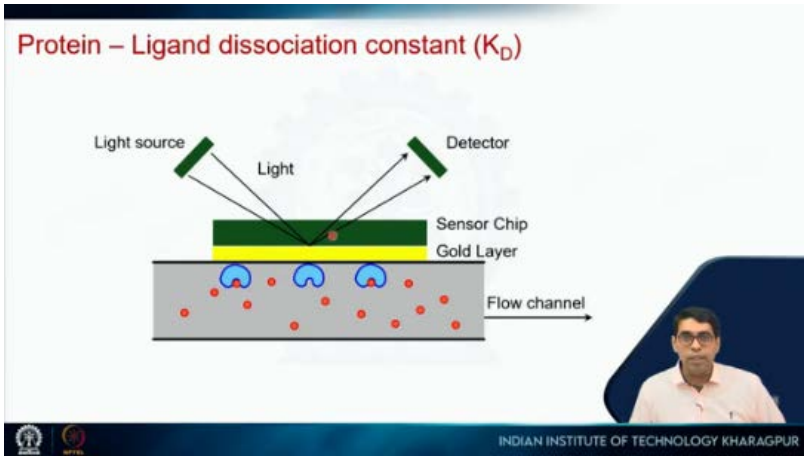
So, if this is fluorescently labeled, there is no fluorescent label. If this is radio labeled, there is no radio label here. Now, what will happen is that this DNA will compete with this DNA to bind the protein. So, since we have added more of this DNA, it is more in concentration or volume, whichever way you want to see it; this DNA is more, so it will outcompete this DNA. So, as you watch over time, you will see that less and less of complex will be there because there is dissociation, so you have this in one tube. So let us say you wait for 5 seconds, you add it here. You wait for another 5 seconds, then you add it to the next lane. You wait for another 5 seconds, and then you add it to the third lane, but in this case, you will keep doing that while your gel is running. So this is something that you have to do for

a kinetics experiment so you have to be very careful. So you are adding your sample while the gel is running. So of course they will run differently because I am running them at a later point. So by the time I add this this is already moved. So that's why we see a pattern like this. But since we are adding them at different time points initially there will be more complex as we go here. There will be less and less complex because more of this protein DNA complex is being replaced as we go from here to here, we will see less and less complex because more and more of this labeled DNA protein complex will be replaced by unlabeled DNA protein complex and you will see more and more of the free DNA.



So we can quantify this or we can quantify this as a function of time and we can get the OFF rate. So this is my free ligand. I have it as a function of time. So I can go back to the previous equation and fit it to get my OFF rate. So, this is my free ligand, I can fit it to get my OFF rate.

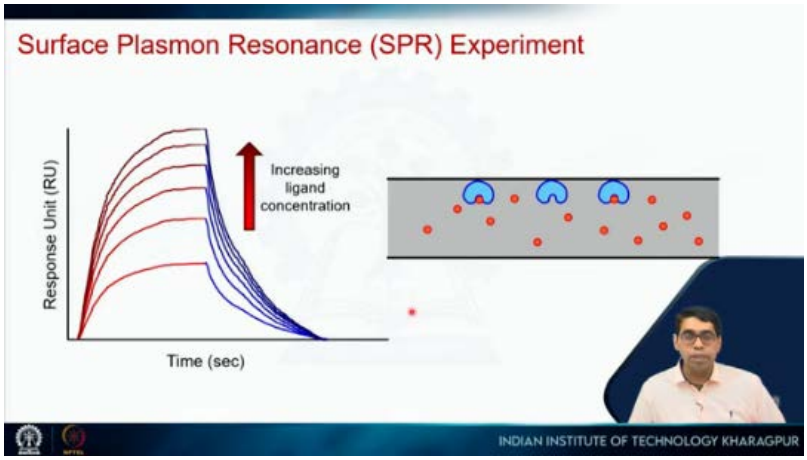
This is the last experiment that I will discuss which is called the surface plasmon resonance. It typically has this flow channel, there is a gold layer and then there is a sensor chip and what we do is we immobilize our protein here. Then we pass the ligand through this flow channel, so as you pass the ligand, some of it will bind and you reach an equilibrium. Then you stop passing the ligand and pass the buffer. In that case, what will happen is that the ligand will wash out and then you will see whatever changes happen. So what are the changes? We incident some light and that light is reflected.



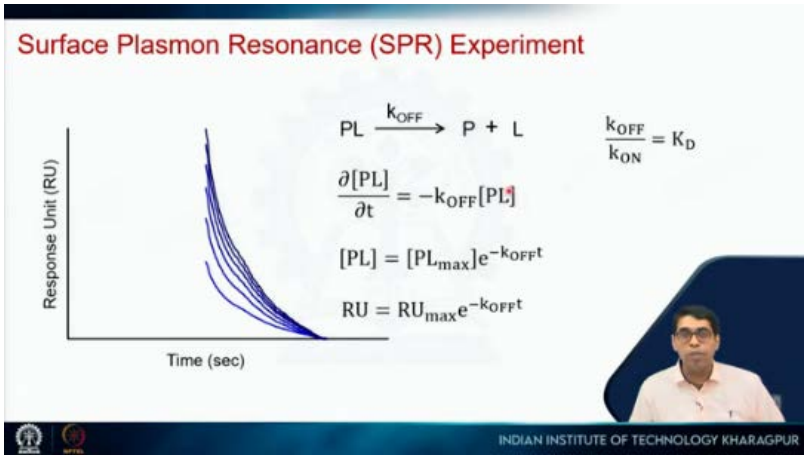
So, this is total internal reflection that happens and this reflection depends on the mass that is attached to this sensor chip. So, it changes something which is called an evanescent wave. So, we are not going into the details of the physics, but there is a change in the reflection of the light, which depends on the amount that is attached here. So, if the free protein is there, there will be some signal.

If a ligand is bound to it, then the total mass is changed, that signal is changed and that is something that we can detect and it is called RU, the response unit. So, it is some arbitrary unit that we refer to. So this is your response unit, you start your experiment, more and more ligands bind, we will see the response changes and then it will reach saturation.

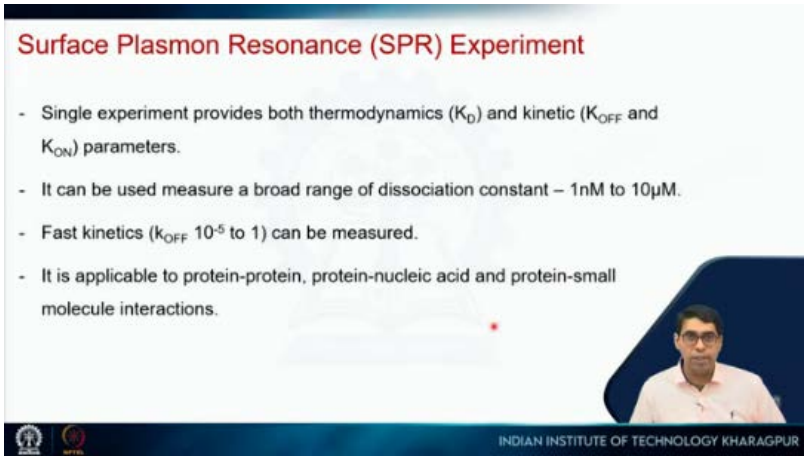
Then you stop passing the ligand and you pass the flowing buffer. As the buffer flows, you will see that more and more of this bound ligand are going out. So, your response will decrease and you will get a curve like this. This is because of association binding of the ligand to the protein, and this is because of dissociation, the release of the ligand from the protein. We can do these experiments by passing different concentrations of ligands. So if you add a lower concentration of ligand, your response will saturate to a lesser point. As you keep on increasing the ligand concentration, your saturation point will go up, and then, of course, when you start turning the buffer, you will get the dissociation. So we can just focus on the dissociation and we can just focus on the dissociation. We can fit it to the same equation that we have seen before and we can get an estimate of the OFF rate.



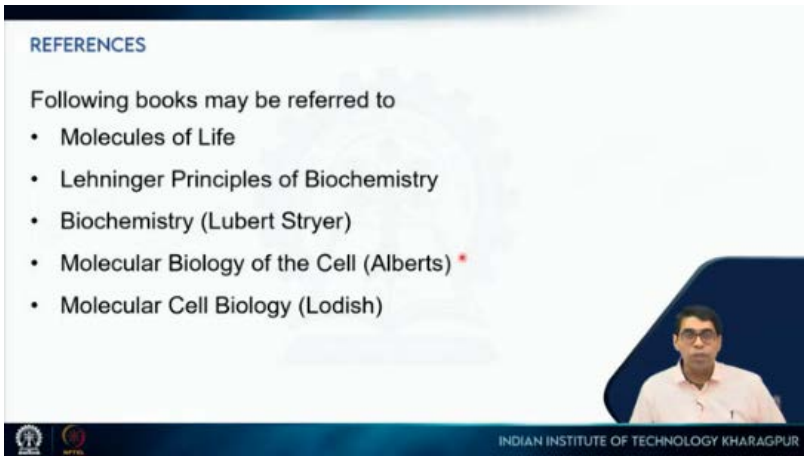
Since you have the off rate, you can also measure the K_D by doing experiments like this. So, you have this series of experiments, you have these different responses. You get this different response as a function of ligand concentration so from there you can get the K_D. Once you have the K_D, once you have the OFF rate, you can plug in the OFF rate, you can plug in the K_D, you can also get the ON rate. The instruments themselves have this fitting routine. So they can also fit the association part of the curve to get the ON rate. There are many ways you can get the ON and OFF rates and the K_D from SPR experiments. This is a single experiment which provides both thermodynamics, which is K_D, and kinetics, which is OFF and ON rate parameters. It can be used to measure a broad range of dissociation constants, starting from 1 nanomolar to 10 micromolar.



It can be used to measure very fast kinetics, from 10^{-5} per second to 1 per second. It is applicable to protein-protein interactions, protein-nucleic acid interactions, as well as protein-small molecule interactions. So, this is a very versatile technique, just like ITC.



So for today's lecture, you can follow some of these books, especially these first two books, 'Molecules of Life' and 'Lehninger Principles of Biochemistry'.



Thank you.