NMR spectroscopy for Structural Biology
Prof. Ashutosh Kumar and Prof. Ramkrishna Hosur
Department of Chemistry
Indian Institute of Technology - Bombay

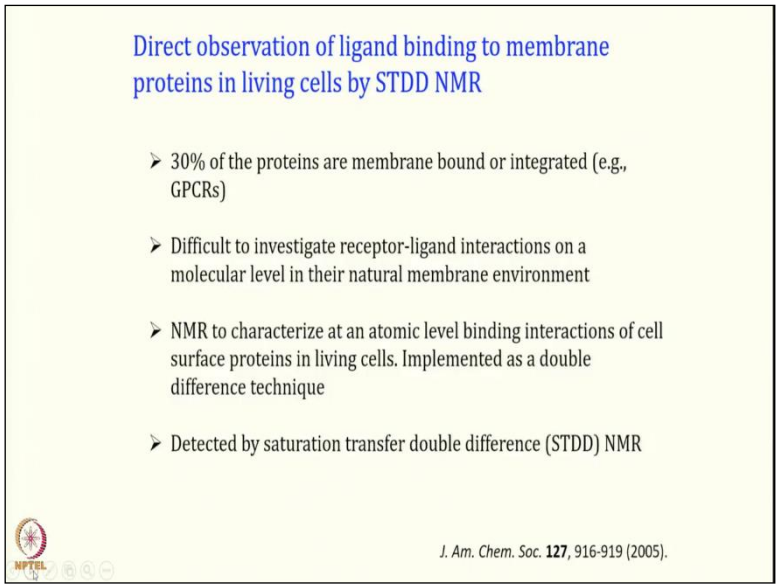
Lecture: 43
NMR Analysis of Ligand Specific Parameters in a Protein-Ligand Interaction -II

Good morning. So, we were discussing protein-ligand and protein-protein interactions using NMR spectroscopy. So, in the last class we started to understand what are the NMR parameters that can be used for understanding the protein-protein or protein-ligand interaction. We started with simple analysis of linewidth, the line shape, the intensity, and the resonance frequency, which can gives the idea about the protein-ligand interaction.

So, then we went ahead and we dissected the protein-protein protein-ligand interaction into two part where we are detecting first on ligand and then we can go and understand the detecting on proteins. So, on the ligand-detected experiment, I started with STD-NMR saturation transfer difference, where basically you can find it out binders; it is a high throughput NMR techniques for phishing out the binders of a particular protein.

And then I discuss the linewidth, how the ligand can have a sharper line, upon binding the linewidth can increase. So, that itself can tell about which are the binders, if they are binding to a protein or not. Then we discuss what STD NMR can be used for. So, it is a high throughput techniques. So, we are going to discuss little more detail how STD can use for finding it out the binders and quantitative parameters of the binding.

(Refer Slide Time: 01:55)



So, here we are directly observing the ligand, that is binding to membrane protein in a living cell, that is what we are going to do is advanced version of STD NMR, called STDD saturation transfer double difference. So, double difference, we are going to discuss now. To make you more familiar in STD-NMR what we are doing. So, here is a binder, here is a protein, they are binding, binder like a small molecule ligand has its own resonance, protein will have its own resonance.

We are going to saturate the signal exclusively from protein, if the small molecule is binding, the effect of that saturation will be transferred to the ligand. When we take a difference spectrum; when it is not saturated or saturated far away and saturated on protein, when we take a difference, the epitopes that binds to the protein will be illuminated. That is the STD NMR saturation transfer difference.

Here we are going to now discuss the saturation transfer double difference. So, what are the double difference and how you can use for understanding the intact cell protein. So, as a biologist you know, all protein cannot be purified and purified to high concentration. There are various intrinsic problem with protein purification and stability.

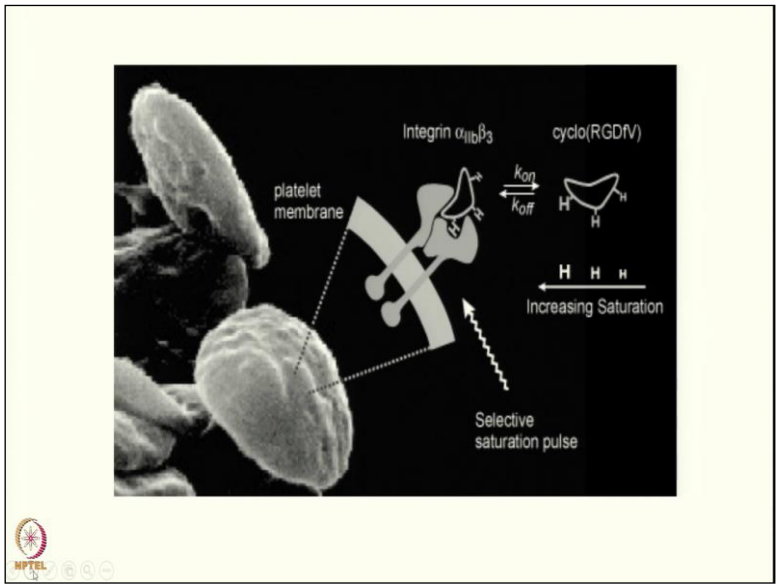
Now still there are some proteins which cannot be taken in solution, but they can be taken in an in a membranic environment, but again the reconstitution and all those are going to be really really tough. But those are main target like one of the example is GPCR, G-Protein Coupled Receptor; they are the major target for drugs because they are receptors for various molecules.

So, about 30% of protein are membrane protein, membrane bound protein or integral membrane protein, one of them is GPCR and these are important drug targets.

So, if you cannot understand their binder, then we are losing out lots of things. So, can NMR come up with a technique where we need not to purify protein, but still you can investigate what is the binder. How we can investigate? That was again offered by Meyer group and that was published about 15-17 years ago in JACS. This technique called STDD Saturation Transfer Double Difference.

So, now we are not going to isolate protein, we are taking them in their natural membrane environment and try to understand how they are binding. So, NMR has to characterize at an atomic level the binding interaction of a cell surface protein in a living cell and that is implemented as this double difference technique, so, how we are going to do.

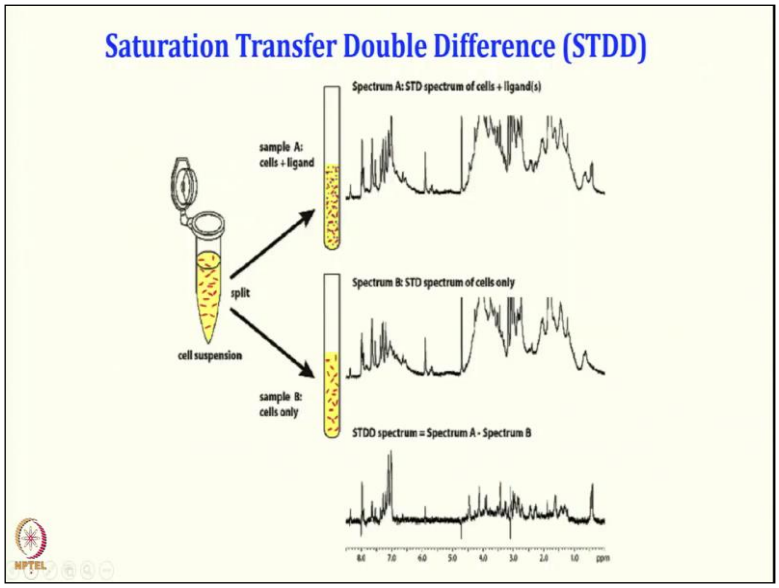
(Refer Slide Time: 05:08)



Here is our whole cell right and here is a receptor and in this receptor some binders are there. So, they are going to bind here. So, binding reaction is k_{on} versus k_{off} right. So, these are the binders here is a free ligand and it is binding. Now we are going to selectively saturate the receptor. So, receptor concentration of course will be very less. So, we have to choose a region typically methyl region where these receptors only gives the signal and we can even increase this saturation duration by increasing the duration of this pulse or strength of the pulse.

And when they bind, the effect of binding can be illuminated by saturating this receptor pulse. So, if receptor signal are saturated, it will transfer to the binder which is small molecules and that we are going to elucidate it in the STDD-NMR.

(Refer Slide Time: 06:15)



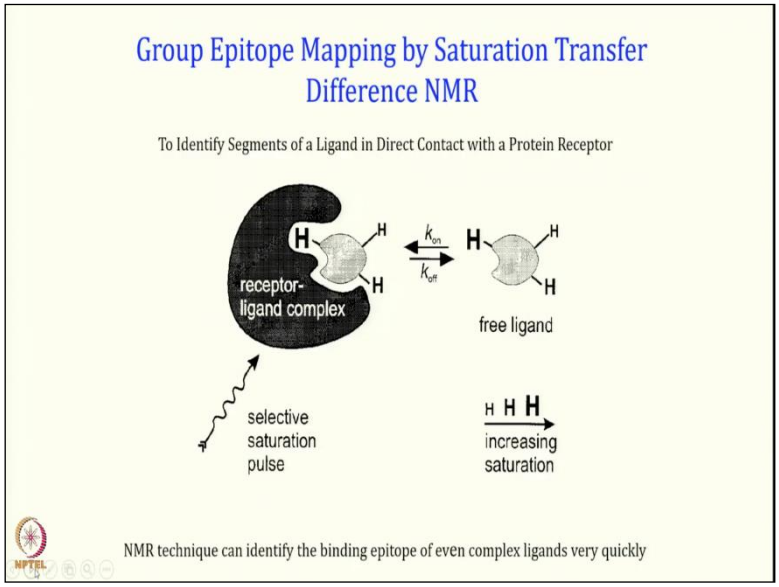
So, a typical experiment is done like this; you have a cell suspension, we have taken an eppednorf. Sample A will contain cells and ligand, and in Sample B we will have only cell. So, this is the Spectrum we are recording the spectrum of A where we have a protein signal like A or cell signal and ligand signal. So, cell and ligand is here, a spectrum B where we are doing STD of cell only.

And then we are going to take a difference STDD. So, here we have saturated the cell, look at the effect of those cells saturation on ligand, here just to be saturated the cell and then we took the difference of these two. So, now if you look at there are some things getting illuminated. If that is getting illuminated it says that there is a binder which is which has bound and the sharp signal that is coming are from those binders, the ligand that are bound.

So, this is the saturation transfer difference. To emphasize again, we have taken a cell suspension, splitted into two, in one we have added the ligand, in another we have not added ligand. In the first sample, we did STD-NMR like saturated the cell signal or protein signal and then without saturation, we recorded the STD-NMR for first one. Similarly we recorded the STD-NMR for the second one, we took the difference of those two.

So, a spectrum first minus Spectrum two, the illuminated signals are coming from this double difference STDD and that is telling that ok, here are the ligands that are binding. So, even without purifying protein taking from cell suspension, we can find it out the ligand that can bind to particular receptor right.

(Refer Slide Time: 08:21)



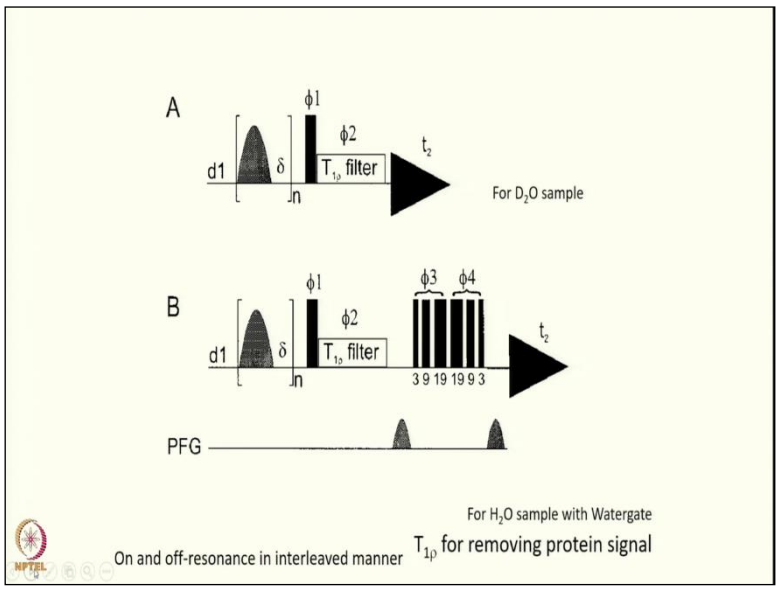
So, that is that is the STDD NMR. Now I will briefly discuss to you group epitope mapping by saturation transfer difference. So, group epitope, like I was talking about the epitopes that binds. So, basically what we are going to do is identify a segment of ligand that directly interact with the protein or receptor. So, the same concept little bit trick we have to do, same concept of the STD.

Suppose this is my ligand and this is the receptor. Now receptor is a bigger molecule, ligand is a smaller molecule. We are again saturating on the receptor molecules and we are looking at the effect of that saturation on these small molecules. So, receptor and protein are in exchange k_{on} and k_{off} right. So, here is a free ligand and here is a receptor-ligand. So, receptor protein complexes. So, we saturated essentially the receptor, now in this experiment, we are going to increase the saturation time.

So, the closest one will be first illuminated. Once we increase the saturation time, now the second one, which is closure here, the third one. So, this is the third, this is second, and this is the first. So, now not only we find it out which is actually interacting but also find it out how far they are from the receptor and that is called actually the group epitope mapping. So, by

increasing this saturation time recording the various sets of STD NMR we can find it out how where they are positioned in the receptor binding site.

(Refer Slide Time: 10:11)



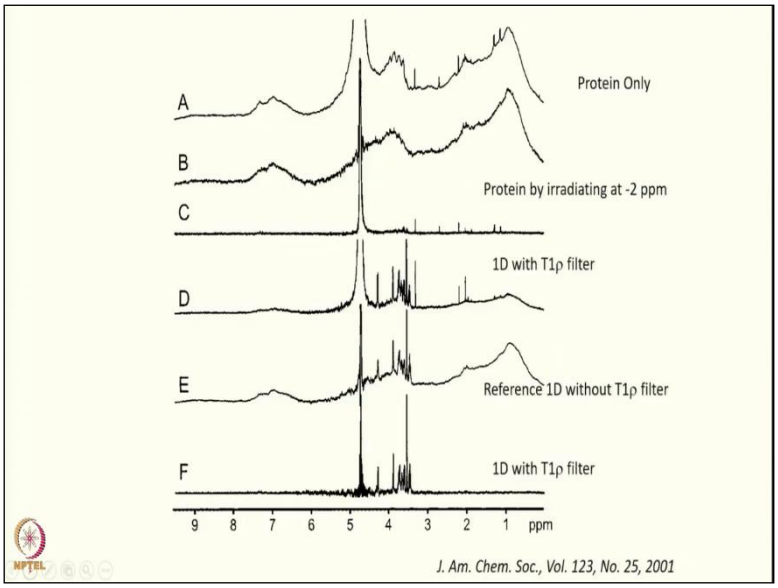
So, typically these are the pulse sequence. So, to have a simple explanation of these, here you have a D_2O sample, you can have pulse of saturation. So, this is relaxation delay D1. Typically you start with this; wait for some time for magnetization to come in equilibrium; then we using a pulse terrain, we are selectively saturating the protein signal and then we are exciting it, like this is a 90 degree excitation pulse, then we are using a T1-ρ filter and finally we are detecting at T2.

So, during this, like all the signal coming from the in other interactions will be rephased and essentially we are seeing this saturation effect for D_2O. If you are doing in water, we need to the kill the water signal. So, this is done by one of the water suppression technique, that we have discussed earlier. So, and remaining will be suppressed by the gradient. So, use of this selective water saturation pulse with gradient, we can kill the water and rest remains as it is like STD saturating on protein signal detecting on the ligand signal.

And we have to do on-resonance and off-resonance in interleaved manner. So, what is on-resonance, like when we are saturating the protein signal exclusively, off-resonance when we are not saturating protein signals. So, our irradiation is somewhere far right 20 ppm. So, the T1-ρ, here we have added again to remind you it is for filtering out or removing the protein signal that might come in this.

So, T1 filter ensures that we are only detecting the ligand and effect of the saturation on the ligand that is what actually it ensures. So, this is typical pulse sequence that we have for D_2O sample and for water, cleaning is important. So, we kill water using this 3-9-19 pulse right.

(Refer Slide Time: 12:14)



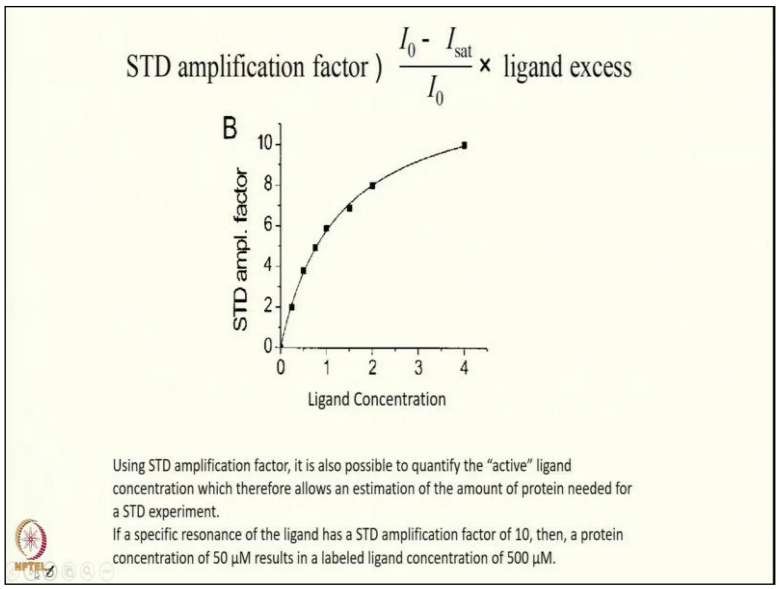
So, here like for an example, here we have only protein, you can see really broad signal. So, we irradiated the protein at -2 ppm, that only protein can have. Now if you add this T1 filter, you can see now the lines becomes really sharper. So, you only we are getting few peaks and with T1-ρ filter, you can see the lines are really coming out.

Therefore, if we are studying the complex system like this, here we probably need to put this T1-ρ filter that dephases the protein signal, just filters out the protein signal and we can get a sharp line which is shown here. So, protein here irradiating protein at -2, 1D with T1-ρ filter, you can see sharp signal and 1D with T1-ρ filter, you can really get a beautiful sharp spectrum coming from the interactions.

So, next is we find it out epitope. We also find it out the position of different epitope; how closer or how far they are. Why position is important, because now, as we discussed, your Medicinal Chemistry friend can come and start modifying it doing the molecular jugglery, just to increase the binders, increase the size of binding. So, that we can tune the binding property. So, that is helped by STD and STDD and group epitope mapping.

Now next is can we find it out, because that is what we are saying, can we find it out Kd of the binding.

(Refer Slide Time: 14:05)

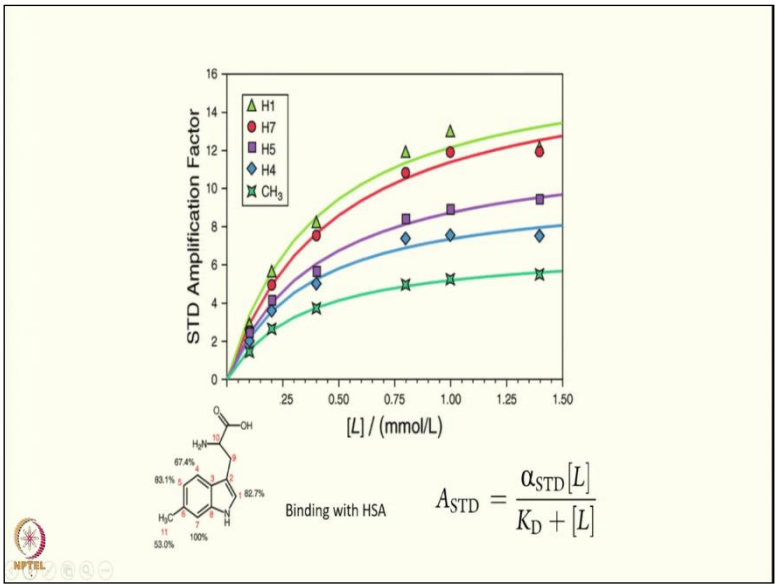


Yes, we can do, this is the simple experiment. So now, this is the simple experiment that we are going, we are increasing now ligand concentration and then STD amplification factor, how many times we can get more STD signal that can be detected. So, with concentration of ligand and STD amplification factor, we can find it out the Kd of this.

So, how STD amplification factor helps? So, we know the I_0 like without anything, then I_{sat} when we saturated, and we divide by I_0, multiply with the ligand excess, how many times ligand was excess. So, using these, we can fit this curve and that gives us the Kd, dissociation constant. So, STD amplification factor it is possible to quantify the active ligand concentration, which allow estimation of amount of protein needed for STD experiment.

And it is also a specific of the ligand which has STD amplification factor of 10 or so. So, using some of these techniques, we can probably get kind of the STD amplification Factor, how many times STD and this also opens an avenue for finding it out the binding strength.

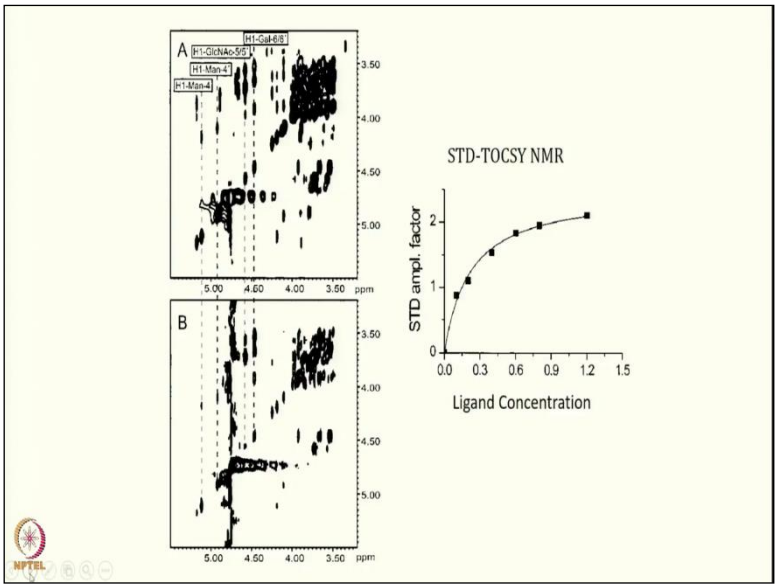
(Refer Slide Time: 15:26)



So, here one can find it out with STD amplification factor. So, how far or how close they are, what is the STD amplification factor, like this molecule was binding. So, we did various ligand concentrations of STD, ligand to protein ratio, we put it, how the signal is getting saturated of different atoms H1, H7, H5, H4, and methyls.

We plotted the signal intensity with respect to ligand concentration and we calculated the STD amplification Factor, where the binding to HAS, you can fit it, STD amplification Factor alpha times of the STD ligand concentration and one can determine the Kd. So, essentially for each of these moiety, using STD amplification Factor we can find it out, we can have an idea of Kd, how strongly or how weakly these are binding.

(Refer Slide Time: 16:40)

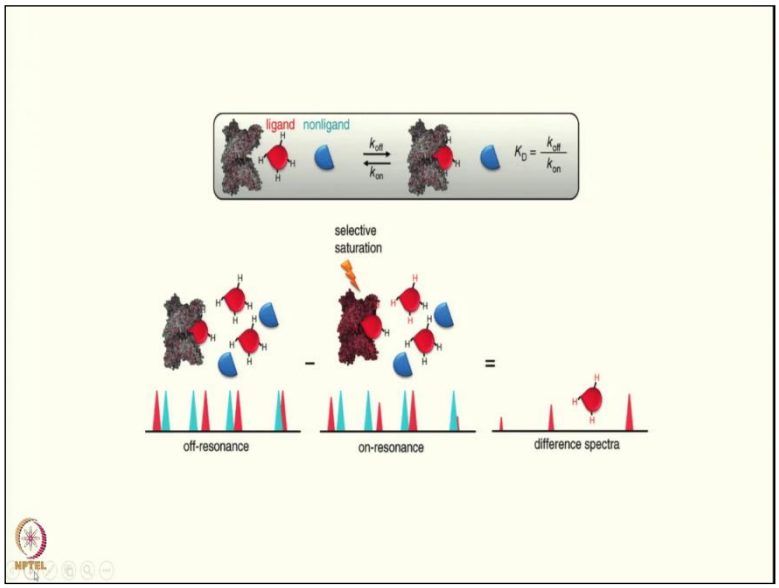


So, this simple experiment helps us in getting the signal. Now can we also go advanced version of the STD? What I mean, STD was just 1D experiment where we are recording 1D. Can we combine this with a 2D, we can get even more information. So, yes STD can be combined with site COSY or even TOCSY right like we can get even in 2D fashion.

So, that even our ligand becomes bigger you can get some information. So, here is one example where we have combined STD with a TOCSY. Now, you can see here different peaks you can identify in TOCSY region and since we have done STD. So, you have to record two experiment TOCSY without irradiation, TOCSY with irradiation or TOCSY on-resonance, and TOCSY off-resonance and take these spectrums, you plot with a various ligand concentration calculate the STD amplification factor.

And one can see for various epitopes, the STD amplification factor can be different and you can again the map the group epitope very nicely and also possibly you can get some idea about the Kd of this.

(Refer Slide Time: 18:05)

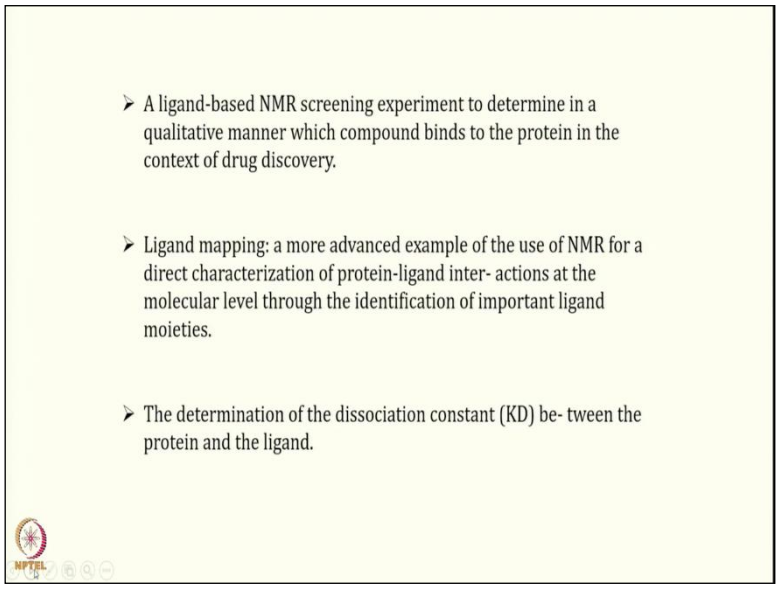


So, that is the extension of STD NMR saturation transport difference NMR. Now just to again revise you little bit, here is my receptor molecule, here is ligand molecule that binds, this is non-ligand which is not binding, they are in equilibrium k_{off}, k_{on}; binding-non-binding. So, binder binds and non-binder does not bind. So, we have:

$$K_d = \frac{k_{on}}{k_{off}}$$

And we have selectively saturated the protein in the STD fashion, we took the difference where there is no saturation, here there is a saturation. So, some peaks have lower intensity. So, this is no saturation called off-resonance and saturation called on-resonance; we took the difference here is a difference Spectrum. So, now we are getting the signal from the epitopes that bind. So, that is the STD NMR.

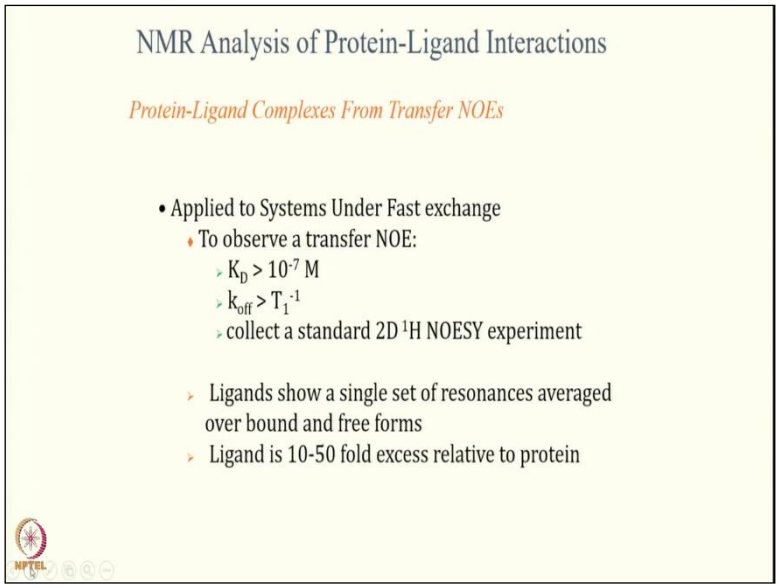
(Refer Slide Time: 19:08)



So, now to sum up, a ligand-based NMRs experiment screening to determine in a quantitative manner, which compound binds to protein in the context of drug Discovery. We can map the ligand a more advanced example to use the NMR for direct characterization of protein-ligand interactions at the molecular level through the identification of important ligand moiety. One can find it out and one can determine the dissociation constant between the protein and ligand,

where we are taking the ligand at a various ratio, we are plotting the STD amplification factor by fitting it, and one can find it out the Kd, dissociation constant between the protein and ligand. That is what we can achieve using is various STD experiment.

(Refer Slide Time: 20:02)

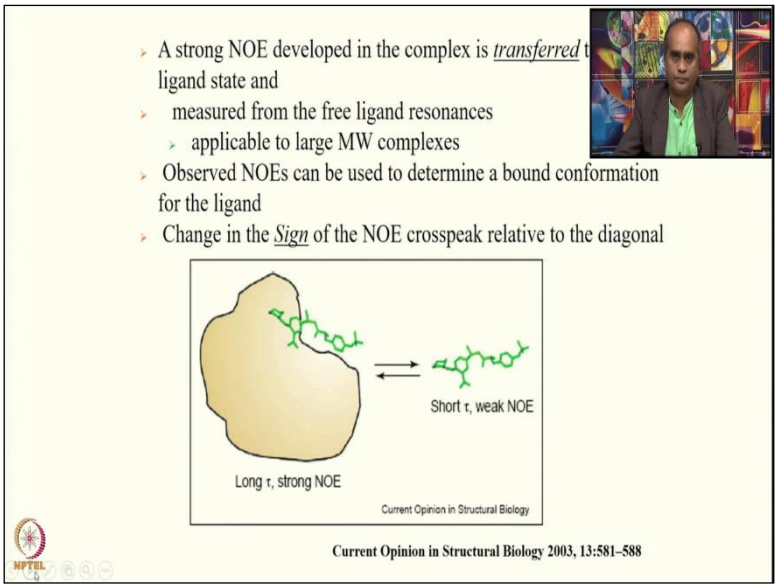


The next technique that can be used is called a transfer-NOE, protein-ligand complexes that is called transfer-NOE. Here again, we are mostly detecting on the ligand side. So, two techniques already I explained you, the looking at the linewidth which is determined by the relaxation property, then next we talked about STD and it is it is a variations STDD, group epitope mapping and how we can use for Kd determination.

Now the third techniques is transfer-NOE. So, this is applied to a system, where it is in fast exchange right. So, the ligand and protein is in fast exchange, Kd is typically 10^{-7} M and k_{off} rate is like a the $T1^{-1}$. Here we are going to observe the NOE, the NOE between the ligand and protein that's a transfer-NOE. So, we have to collect standard 2D NOESY experiment like we can do 2D NOESY experiment where ligand is added to the protein molecule.

And if there is a transfer of signal coming from the protein, that is transferred from ligand, we can detect it. So, ligand show a single set of resonances averaged over bound and free state. Here also, ligand should be more, like excess relative to protein, we record a series of NOESY experiment and we can find it out.

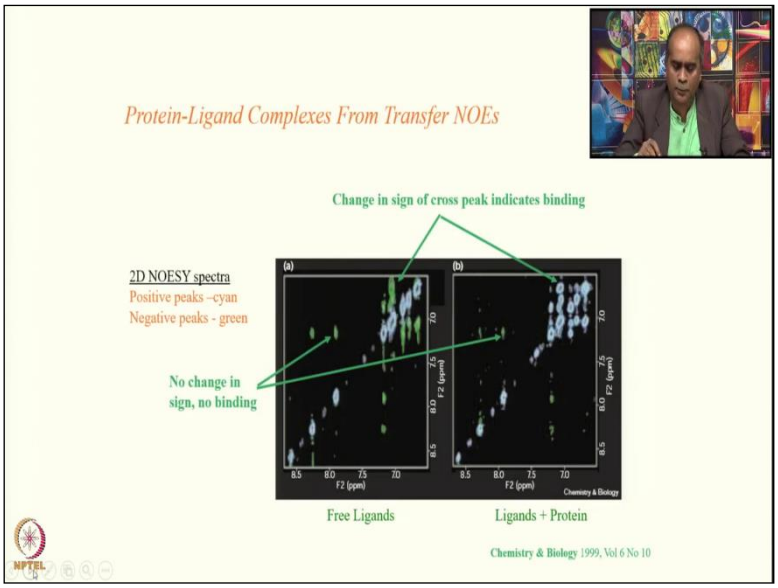
(Refer Slide Time: 21:35)



So, a strong NOE developed in a complex is transferred to free ligand state and one can measure the free ligand resonances, it is even applicable to a molecular weight which is large kiloDalton, several kiloDalton. Observed NOEs can be used to determine the bound conformation of ligand. If suppose, this is ligand, this is protein, and we are getting the NOE from the ligand to protein, we know the distance right.

So, distance is kind of measured here and that distance fix the orientation of the ligand in the binding pocket and here the sign of NOE cross peak will be opposite and you can get looking at that this is coming from the transfer NOE. So, here we have a ligand which is small molecule, protein big molecule. So, we have a long τ, strong NOE;

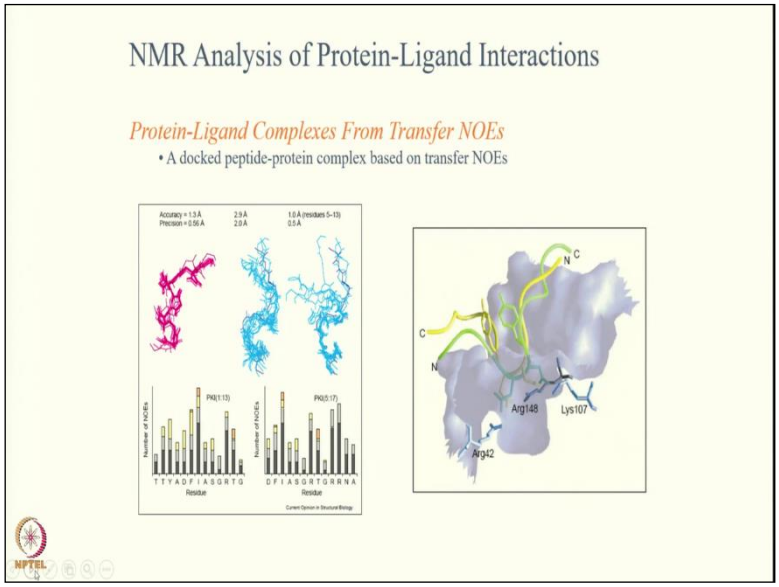
(Refer Slide Time: 22:40)



And when it is in free, it will have short τ, weak NOE. Now when we record the spectrum of protein-ligand complex, one can see that these peaks which are coming here, it is of negative sign and when the positives, like when there is a colour change positive peak is cyan, negative peak in green. So, the cyan change of the cross peak indicate the binder and if they are not binding they will not bind.

So, one can see here for these guys the peak sign is changing. So, here a protein-ligand is happening. So, some of these you can see these are not changing. So, if the sign of the peak has changed that means it is binder, they are coming from the transfer NOE experiment.

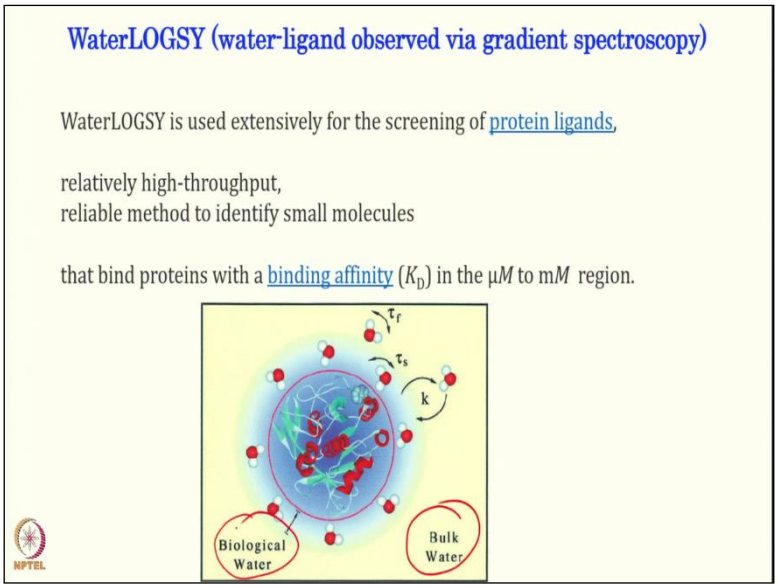
(Refer Slide Time: 23:28)



So, now since we find it out what are the binders, you can use this for getting a protein-ligand complex. So, protein-ligand complex can be determined by transfer-NOE. Since now we have established the NOE connection between protein and ligand, you can fix the orientation of this ligand in the protein complexes and using few of the experiment, we can docked it and create a dock model which will tell that how these proteins are binding.

So, one can illuminate some of the residue that are in contact with the ligand. That is how you can create a protein-ligand complex using transfer-NOE experiment right.

(Refer Slide Time: 24:06)

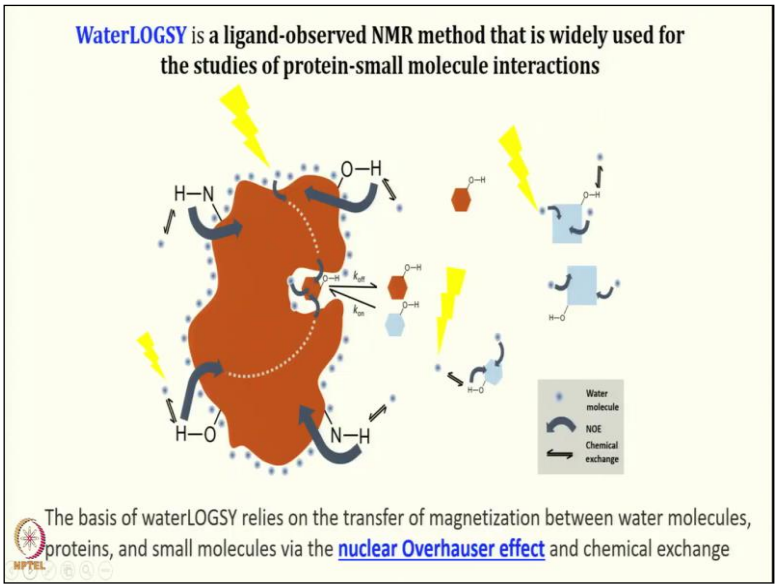


So, next experiment that I will be discussing is a water-LOGSY experiment. Water-LOGSY as name suggest it is a water-ligand observed via gradient spectroscopy. So, here the concept is like a there is a protein and there is a ligand, but in between there is a lots of water, what kind of water, some bulk water, some biological water. Biological water which is in vicinity of the protein molecule. So, water-LOGSY is used extensively for screening against the protein-ligands. It is again a high throughput techniques and it is a reliable method for identifying a small molecule.

The requirement is that the binding affinity should be from micromolar to millimolar. So, what we are going to do here. So, assumption is there is a ligand which is binding to protein and in between there are lots of water, and those are in exchanging. So, what are exchanging, bulk water bound water is exchanging and then protein when it is binding that is also exchanging with the water present here.

So, exchange of water that's what the name is, water ligand exchange that is happening and we are observing using gradient spectroscopy in water-LOGSY experiment.

(Refer Slide Time: 25:34)

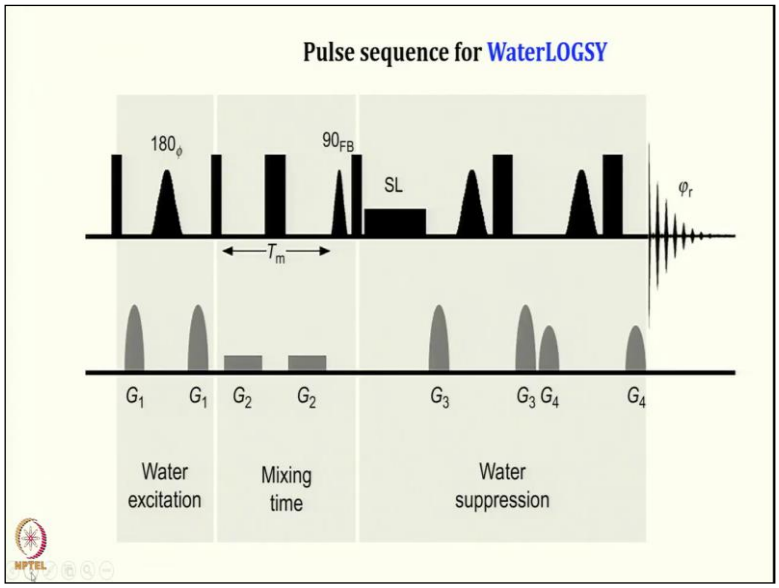


So, actually it is ligand observed and like we are observing the ligand. Its NMR method that observe the ligand, it is widely used for study protein-small molecule interactions. Here in this case we are irradiating now water. So, this irradiated water, there is a like a nuclear overhauser effect and chemical exchange that is happening with the bound water, the protein and the ligand.

So, here transfer of magnetisation between water molecule with protein and small molecules happens by a nuclear Overhouser effect, because they are in close proximity. So, if they are in exchange and they are in close proximity, when we irradiate on water molecule the irradiation can be transferred to protein molecule and that eventually can be transferred to a ligand molecule that is what essentially the concept.

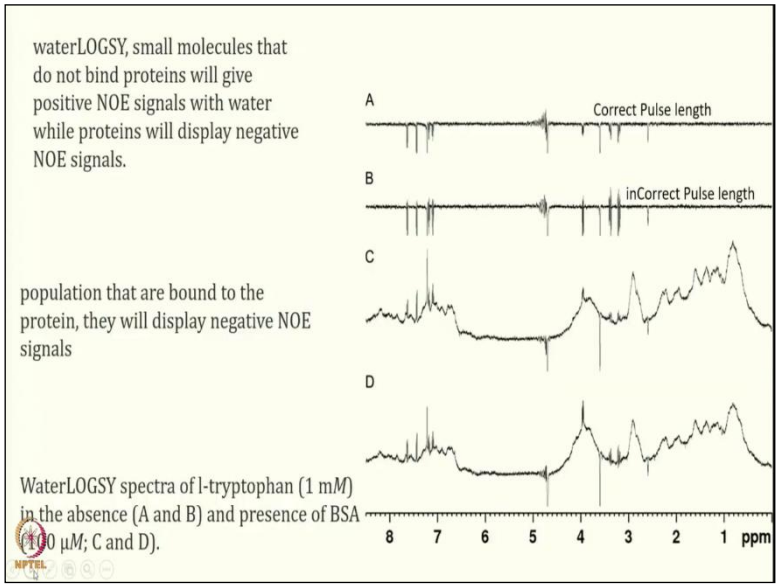
Irradiate water, gets transferred to protein, gets transferred to ligand, if they are binding and that is how you essentially understand using water-LOGSY experiment.

(Refer Slide Time: 26:48)



So, pulse sequence is essentially simple. So, here you excite water and then mix so that the magnetization of irradiation is transferred and finally you suppress water, look at the signal that is coming from the ligand molecule and effect we are seeing on the ligand molecule.

(Refer Slide Time: 27:08)



Typically, we look at the small molecule, few things you have to take care that pulse length is correct. So, if you have a correct pulse length, you are basically getting to get a good line shape. If you start with incorrect pulse length, you see the distortion phase. So, now you can see the sharp line, you can assume that sharp line is coming from the ligand molecule. Traditionally it is plotted negative, when there is no water LOGSY signal when there is no transfer.

So, that does not mean that this is giving negative peak, it is plotted like that and now we do water-LOGSY here. Now you can see that in presence of the protein molecule, you see few of

these peaks. So, in case of BSA, you see the C and D all right. So, these are basically with correct and incorrect pulse length again you can see it. So, essentially you have to compare these two and you can see now the peaks are coming here and these peaks are coming because the transfer happened.

So, water molecule in water-LOGSY, small molecules that do not bind to protein molecule does not give a positive NOE signal with water, while protein will display the negative NOE because that is how it is plotted. And population that are bound to protein they will display the negative NOE signal. So, that is what we are seeing. So, now the experiment was done with one molar of tryptophan in presence of BSA, 1 mM of ligand signal, 100 µM of protein signal, experiment was done.

And NOE was observed on the ligand and this says that they are interacting and that is how you can find using water-LOGSY. So, here I will end it with water-LOGSY. So, these three four techniques that I discussed, where ligand observed, the linewidth detection, the effect of T2, the STD-NMR and some variation of STD-NMR, STDD. Then we looked at the transfer NOE and finally water-LOGSY.

So, these are the four commonly used techniques that are there when we observe ligand. Now the next class onwards I am going to discuss the protein-detected experiment, which can be used for understanding the protein-ligand protein-protein interactions. So, here I sum up and I close. Here I look forward to you with lots of questions if you have any doubt do not hesitate to write to us.

We will be happy to answer all of your question for protein-ligand interaction where we are detecting on the ligand molecule. Thank you very much, see you in the next class.