NMR Spectroscopy for Chemists and Biologists
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Lecture 59
Protein-Ligand Interaction- II

So, welcome to today's lecture. We were discussing important topic of protein ligand interaction and how you can use NMR spectroscopy to understand this. So, in the previous class we looked at a very simple technique that is based on polarization transfer can be used for quick determination of ligand which is binding or which is not binding to protein and that can be used for a screening of ligands and essentially this is high through put technique for the pharma industry to find what is the binding epitope, whether this ligand binds to protein or not where actually on ligand site binds to protein.

That is a very simple technique but I just said let us take it to the next level and we find out how we can use this technique for finding it out not only soluble protein, also integral membrane protein or a protein on the self surface because you know the membrane proteins are one third of the total proteins and they have very very important functions like they are involved in signal transductions we discussed briefly this in last class.

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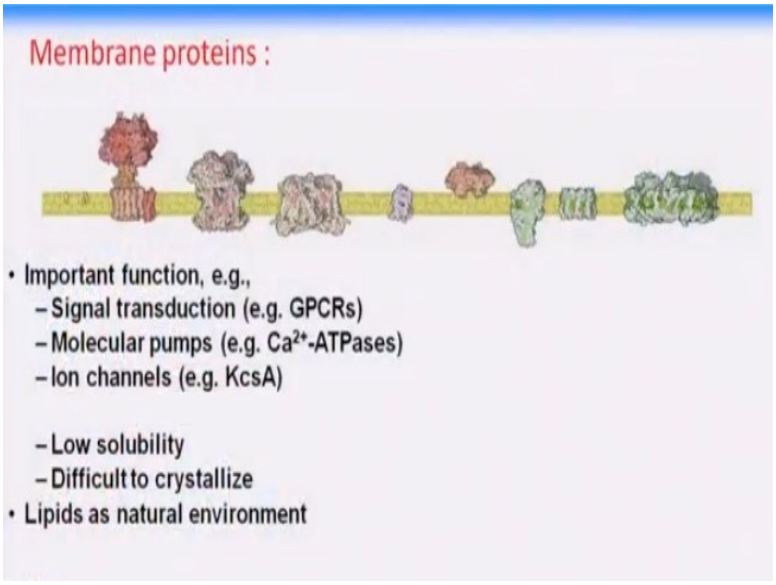

Like GPCR so G protein coupled receptors and the GPCRs are one of the primary target for drugs. So many drugs that are discovered or designed just for GPCR targeting so, GPCRs are very important. Then pumps, like molecular pumps like calcium pump, potassium pump, and

all those or Ion channels these are all membrane proteins and they are also very very important for many pharma industry but they are difficult they are difficult for solubilizing and therefore in a typical conventional way the protein ligand interaction becomes difficult.

Because they are membrane-embedded so their natural environment is around lipid so they cannot be solubilized and they cannot be crystallized therefore crystallography is also difficult to find protein-ligand interaction in this case and this is primarily because lipid is in their natural environment.

Now one can crystallize, one can solubilize but you need to have lipids for these molecules so that becomes a project in itself. Here for pharma industry, we need to find the target not getting into the structures.

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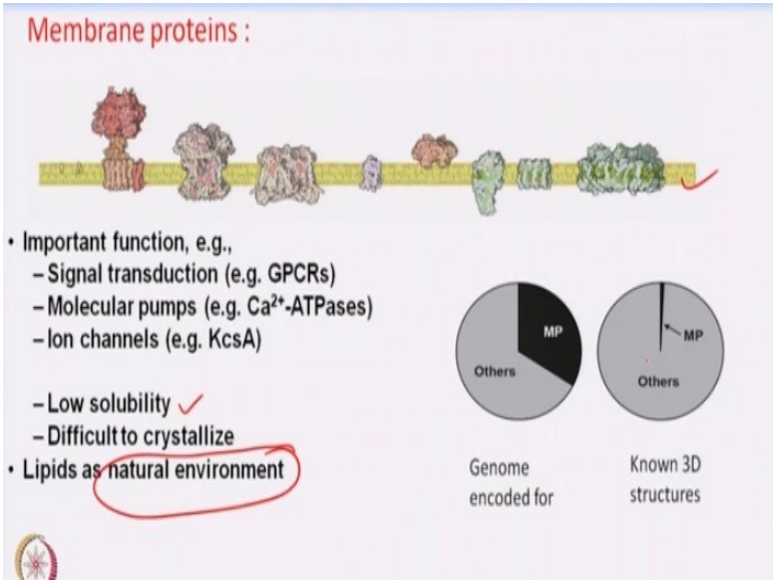

So, if you look at actually genome encodes one like I would say almost 30 percent of proteins but structural known are very less. So, membrane proteins although it is important target for pharma industry but structurally very very limited knowledge we have compared to other soluble proteins therefore, suppose you want to quickly get into the drug determination and you do not want to get into the structure solving process it takes years to solve a structures, you can use these techniques that we were discussing quickly to identify a binding ligand. So how we do that?

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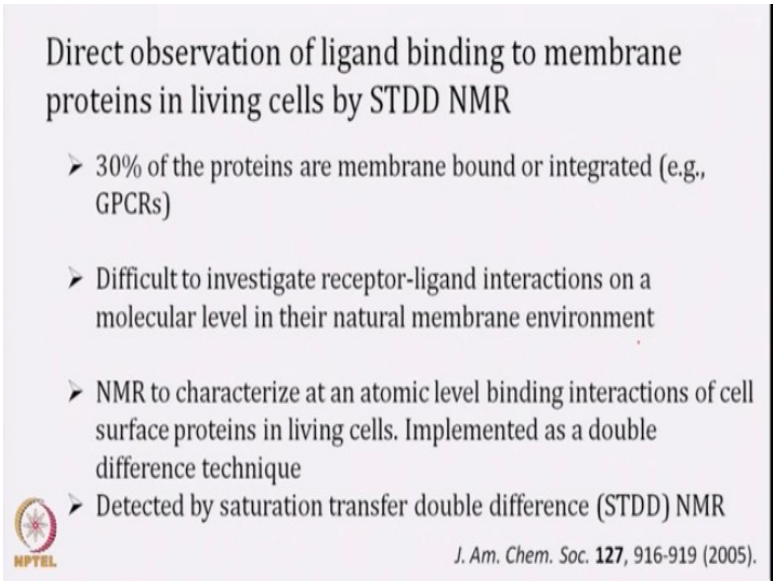

That we will go to this technique which is called STDD. We will discuss today. So we are going to do direct observation of ligand binding to membrane protein in living cell by something called STDD NMR (**S**aturation **T**ransfer **D**ouble **D**ifference). So how we will do that? That is what we are going to today discuss. So now you understand why it is difficult because membrane proteins are not soluble there the lipids are in their natural environment so we cannot look at STTD NMR but these are important targets, so therefore, we need to understand this and we can implement this binding by saturation transfer double difference

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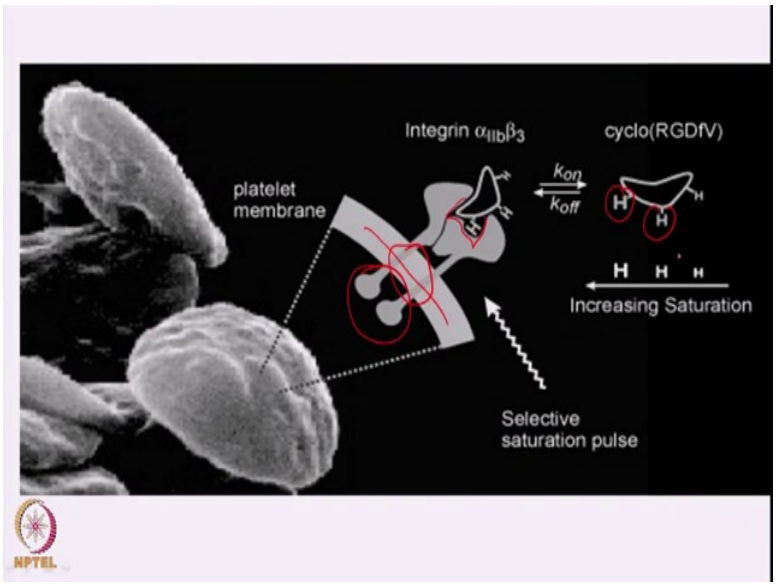

So here is just schematics, suppose this is my cell surface and here is one receptor and this is the environment for this protein receptor so some part inside the cell and some in the

membrane and some is extracellular domain. Now, this extracellular domain suppose we have a binding pocket where my molecule is binding so there is the binding site here and in this site the molecule RGDCV some molecule which binds.

Now this protein has protons. This ligand has protons, they are binding and going of so micro molar binding is there, K_{on} and K_{off} is happening. So now here what we are going to do now is same concept we are going to use it. Select a saturation of protons on the protein site here receptor site so we are selectively saturating the signal on the protein or the receptor site and looking at the effect of that saturation on the ligand. So suppose you increase the saturation effect what will happen the ligand, which is the ligand proton which is quiet close will be more perturb and which is little far will be less perturb and so and so far.

Now if we keep increasing the saturation the effect will be transferred in a distance so this will be like what will be nearer to the binding site will be more perturb than this and than this. If you will increase this, this will be perturb, this will be perturb, but this will be less perturb if you further increase all of them will be perturb and that is what you can find it out the protons or the epitopes which is closer to the binding site which is relatively far to the binding site.

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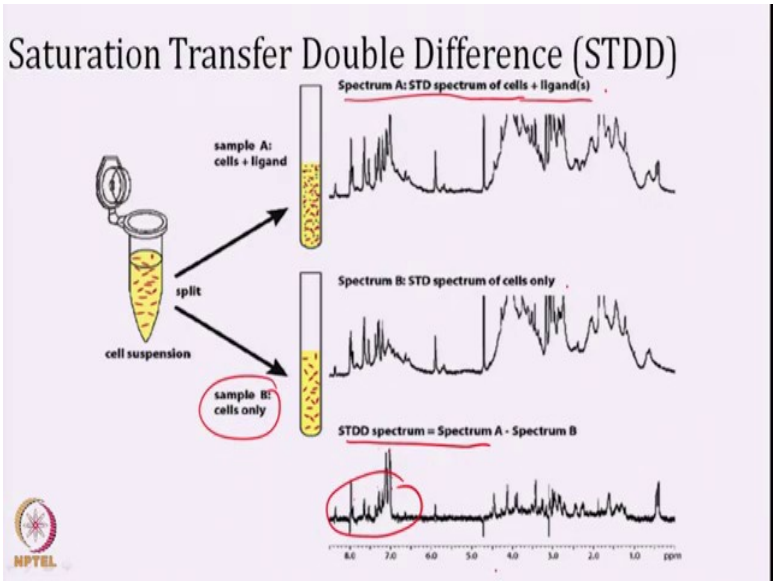

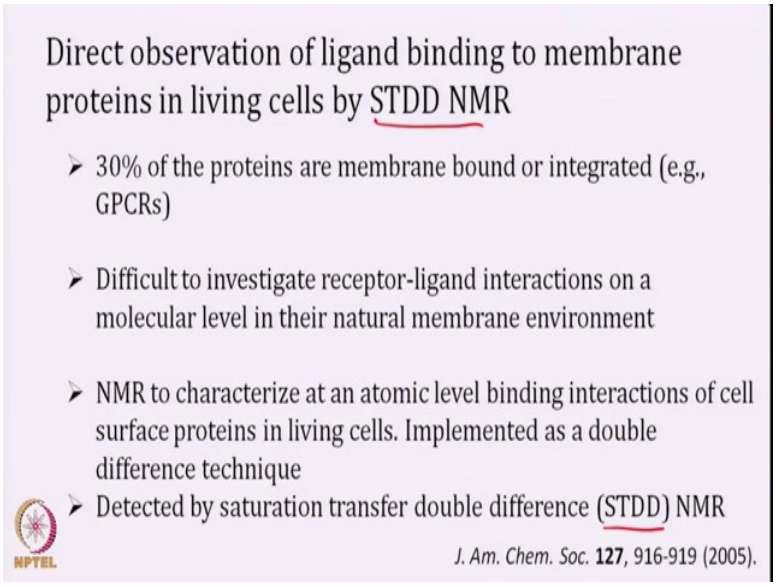

Ok, so experiment is simple. In saturation transfer double-difference you do not have to do much. You just take a cell suspension. Ok, just I forgot to tell you. This method was again developed by the Myer Group in Germany and that was published in JACS some 14 years back. So what we are going to do here in this experiment. We are taking a cell suspension. We are splitting it into two.

So this is an eppendroff where we have cell suspension and this cell suspension has our like membrane-embedded protein or protein expressed on the cell surface.

So here you can see, this is solution, these are from cell suspension so all these integral cells are there. We are splitting into two. Sample that has only cell, sample that has ligand and as well as cell. Now here same thing we are doing. Spectrum of STDD spectrum of cell and ligand. We are doing STDD that means so let me explain again. Here STDD we are going to do on both. We have total cell suspension. We divided into two. One where only cells are there, another where cell plus ligand are there. We are going to do two STDD experiment, one with only cell suspension another with cell with ligand.

So here in this STDD experiment, we perturb somewhere on -1 or so. Look at the STDD difference. Then here again we perturb at -1 ppm or so and that effect will be visualized on the ligand. Since there this is a point heterogenous mixture so we do not know because the ligand is not very clean. So, therefore, ligand has signal which is here as well and cell suspension has all the signals coming from the proteins. So if you take a difference of this STDD where cell plus ligand was there and STDD where only cell was there. Take the

difference that is called saturation transfer double difference Spectrum A minus Spectrum B. You get few of the peaks that are actually shown in this spectrum.

So if there is no binder ligand, you should get a sensory zero signal because there is no saturation transfer. But if you are getting these peaks that means there is a binder and these binder peaks has like this. So now you can record only ligand signal and you can find it out which protons in the ligand are actually interacting with this receptor. Just by this simple experiment, one can find in whole cell that whether there is a binder or not. So just again to summarize, this is a little complex technique.

What we are doing. Our target is a ligand for the receptor which is on the cell surface. For this, we are doing an experiment called saturation transfer double difference. We are taking the whole cell suspension. We are dividing that into two. One only with cell, one cell plus ligand. We are going to do two STDD one for sample number A, one for sample number B. Now this two STDD are subtracted. If you are getting the signal in the difference, double difference spectrum we identify that this suspension has ligand which actually is binding to the receptor.

Now, we can record the ligand spectrum and we can find it out the atoms that are involved in the interaction and that is how you can find it out in STDD that what is the binding mode.

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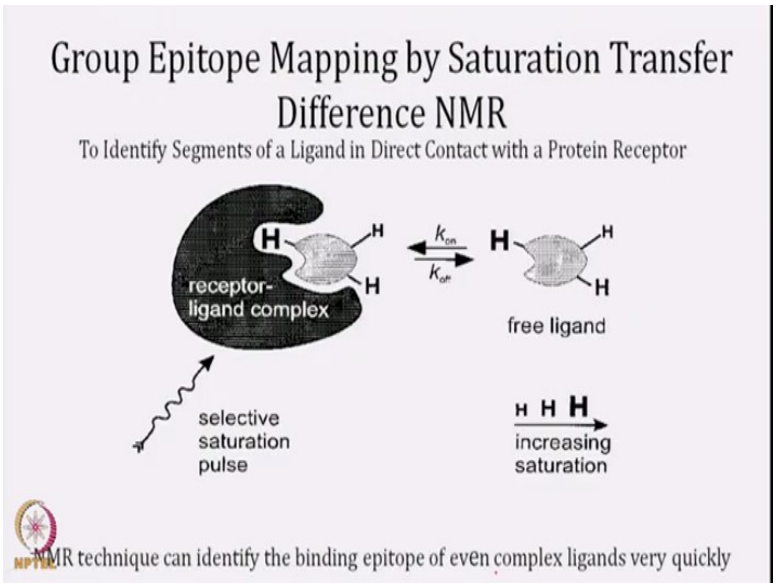

Now let us go further and this is called, this experiment is called Group epitope mapping by saturation transfer difference NMR. What we want to determine? The segment of a ligand in direct contact with a protein or receptor. So suppose protein is big anyhow and ligand is also

big. So all the moiety in the ligand is not interacting with the protein. Some moiety is interacting. And that is what we have to find it out that which moiety is in proximity of this. So essentially briefly I discuss it but let us go in detail

So here is our receptor ligand complex. Receptor is big, ligand is small and ligand has many protons. We are selectively saturating on protein looking at the effect of that saturation on the ligand. The only condition is that they are in micromolar binding range. So k_{on} and k_{off} is there, so this is free again. Now, we are doing several experiments. We are increasing this saturation strength. So if at low saturation only direct contact protons will be shown in STDD. If you increase saturation, this will be shown then further increase, this will be shown. So on increasing saturation, you can find it out how the protons are getting affected by this transfer. This can identify the binding epitope even for the complex ligand very quickly.

So let me repeat it again. Here is my receptor. This is my ligand. So, suppose these two are my moiety of the ligand. So this binds and this does not bind and if I increase saturation first protons from here will show STDD effect but if you keep increasing slowly from protons from here will also show STDD effect. So we know that this is closer and this is relatively far and that is what group epitope mapping by STDD NMR.

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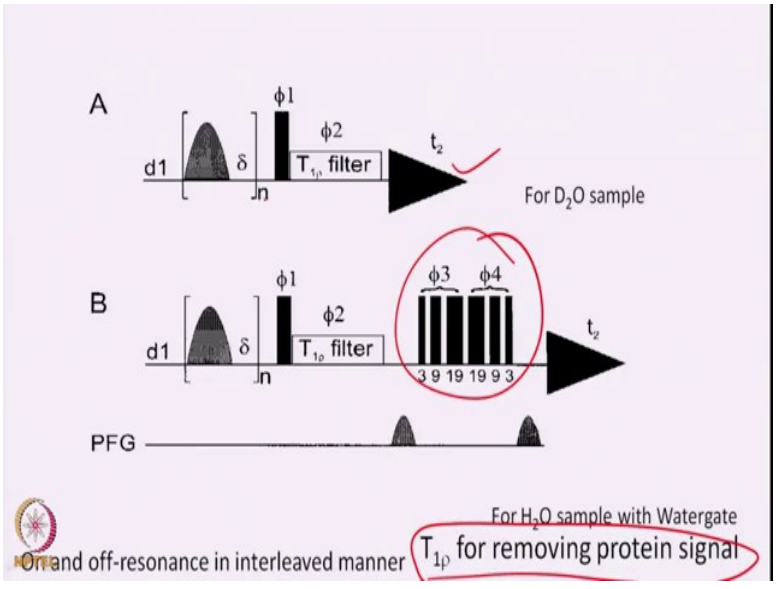

So essentially this is the pulse sequence. You do basically in D_2O so that you remove water background. You can do even in a H_2O but then you need to add something called water suppression peak. So, this is water gate and then you need to have a $T_1\rho$ filter just to remove the protein signal. So, experiment is very very simple. Here is a saturation, then you have a

90˚pulse and then $T_1\rho$ filter for removing the protein signal then you detect it. Now, you have to increase this saturation time. So if you increase the number of *n*, the saturation time will increase and that will be shown essentially on the protons of the ligands. Closer one will be affected first then following.

So you have to do two experiments on resonance and off-resonance where you saturate and where you do not saturate and that you can do in interleaved manner and then you can subtract it to save the time. So this interleaved will save the time. So you saturate it, you get a STD signal, you, if you keep on increasing saturation more and more peak, will be affected and that will tell you the group binding epitope. Ok. So one example.

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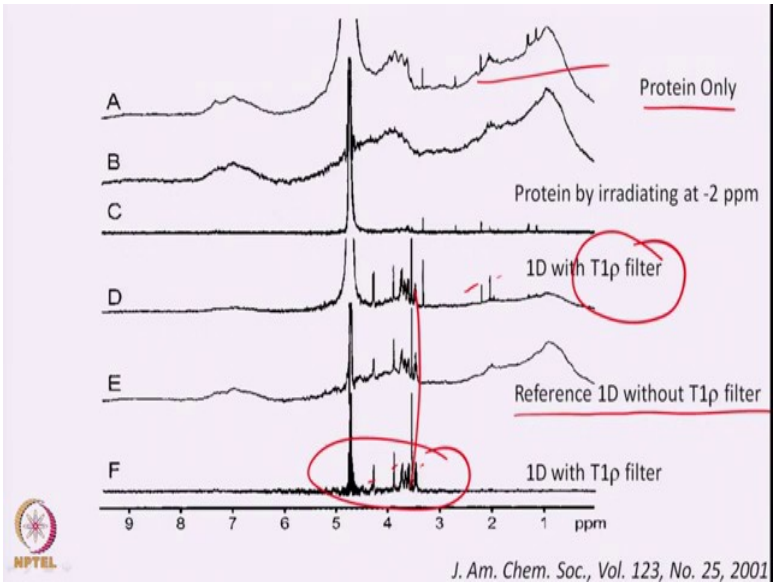

Here if we take a protein, because protein is huge. So if you take just 1 D of the protein we are getting the broad signal. Now suppose, you take a protein and irradiate at -2 ppm. So -2 ppm is essentially some protein signal that comes something from too much shielded that will be off field shifted we so essentially if you take if you see this all the protein signal will be gone that means -2 is the right ppm for saturation. Now you do 1D with $T_1\rho$ filter. So as I discussed $T_1\rho$ filter you can put I do not want to go in detail but $T_1\rho$ filter essentially can remove all the protein signal. So here mostly we are getting now the signal coming from the ligand. You can see the sharp line as we discussed previously.

Sharp line is line from signal, broad line is from complex and protein, so sharp line coming from the ligand. Now you do reference spectrum 1D without the $T_1\rho$ filter you get a broad line, 1D which are $T_1\rho$ filter you get a sharp line. So if you do STDD you get these peaks and

these peaks will tell that this particular ligand is interacting. Now you can increase the saturation time and you can see the effect on these different protons and that will tell you which are nearer or farther.

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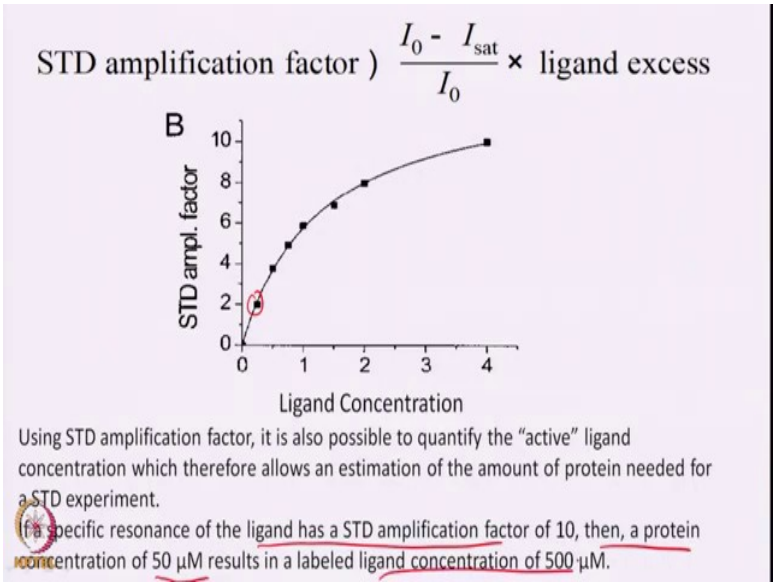

So if you do that saturation time, you can plot it the effect of that as a either ligand concentration or saturation time concentration. So by doing that one can find essentially an STDD amplification factor. So what is STDD amplification factor

$$\frac{I_0 - I_{sat}}{I_0} \times ligand\,excess$$

I_0 means non-saturated and here is I_{sat} saturated. So, now STDD amplification factor can be fitted into this and that can give you binding strength of this particular protein to the ligand.

So using STDD amplification factor it is also possible to quantify the active ligand concentration which is therefore allows an estimation of the amount of protein needed in the STDD experiment. Right, so we are just increasing the ligand excess. So here say protein is 2µM ligand is 200µM so do we need that much. So just to find that we keep on increasing the ligand concentration and find out what is the optimum ligand you are going to use for this particular protein target and now you see this is giving an important aspect.

Suppose we are targeting one protein now how much like pharma industry can also take this in account that how much protein you need to saturate this receptor or to bind completely with a receptor. So that helps in determining dose of the pharma industry. So this ligand

concentration you can just get by simply doing STDD NMR. So after sometime it becomes saturated and you do not need any more ligand for that.

So in a specific case, a ligand has an STDD amplification factor of 10 then protein concentration is 50 µM and ligand concentration should be 500 µM so that is what it gives you the idea of saturation.

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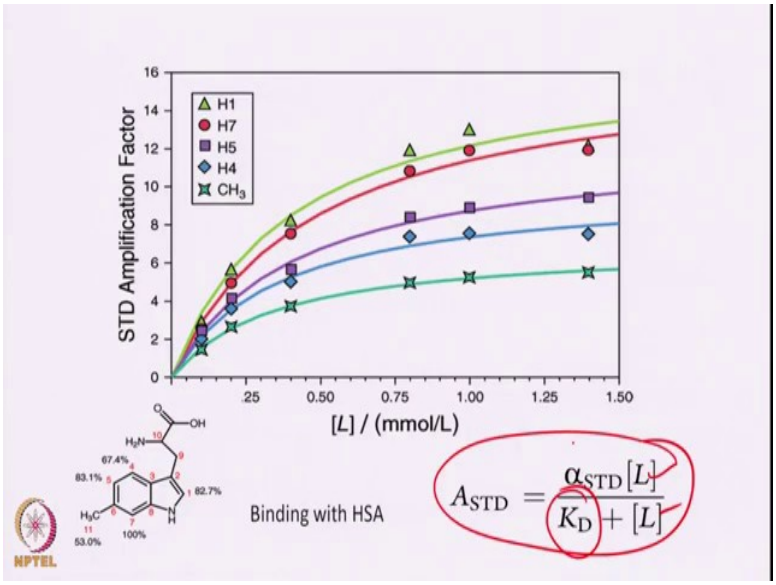

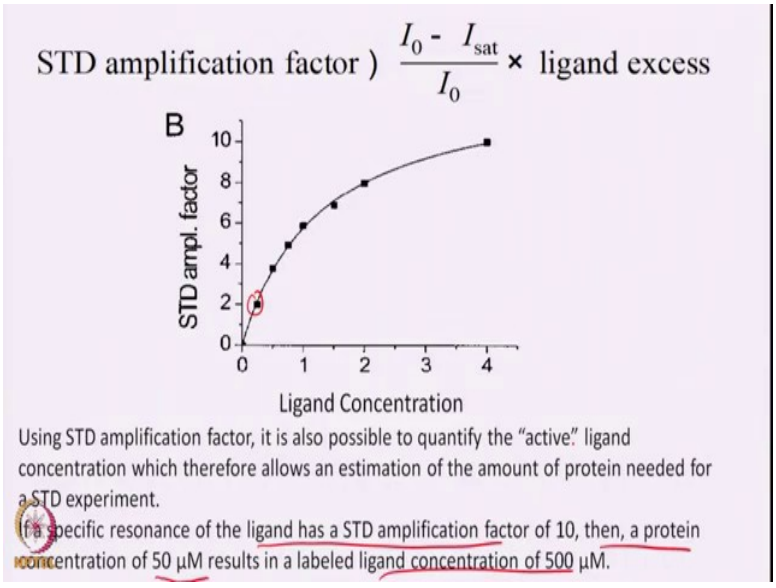

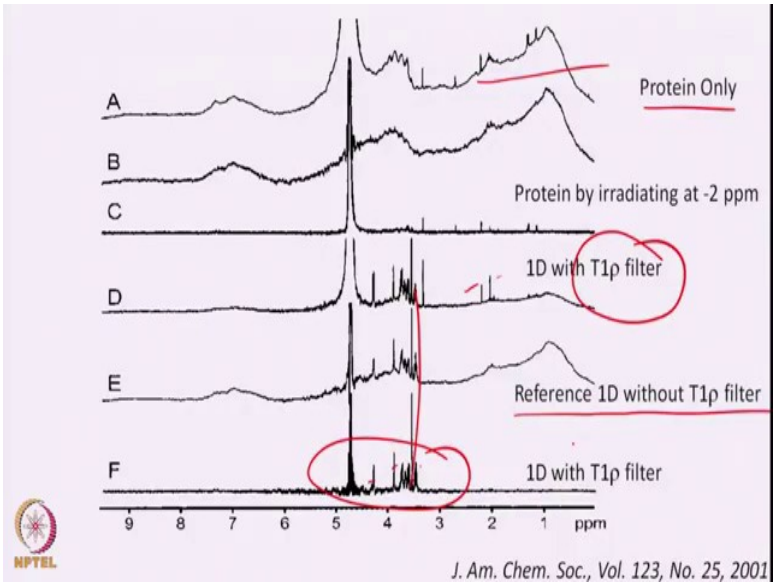

As we discussed STDD amplification factor, you can find it out essentially which proton is near and which proton is far and you can find it out here like H1, H7, H5, H4 they have a different saturation range in a ligand 2 concentration manner and that can be used to find it out essentially the K_D. This is the ligand concentration and this is the amplification pattern for STDD. So with STDD one can find it out the binding epitope we can find it out the group epitope that we just looked at here, group epitome mapping, the position of the binding atoms and we can find it out what is the saturation by STDD amplification factor, what is the minimum concentration required for the saturation of the receptor then we can find out the K_D ok.

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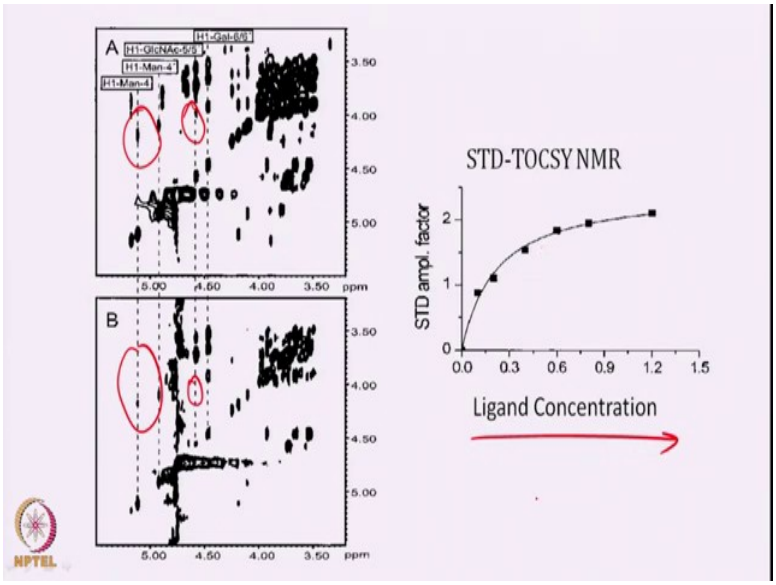

This STDD is a simple module that can be added even to a 2D. So suppose we can add actually you can add STDD in TOCSY so you can record one STDD TOCSY without saturation one STDD TOCSY with saturation and you can find it out here just for an example you can see these peaks are vanishing here you know that these peaks or even the peaks from here one can find it out that like this peak if you look at in the reference and this STDD are disappeared so not only 1D you can extend this to 2D but the time required will be more. In the 2D manner you can find out even the site like many other atoms in more resolve way which is actually involved in the binding and similarly you can do in STDD TOCSY manner you increase the concentration of the ligand record several STDD TOCSY and you can find it out K_D of this binding protein ligand interaction, K_D you can find it out also group epitope mapping you can find it out just like fitting this curve.

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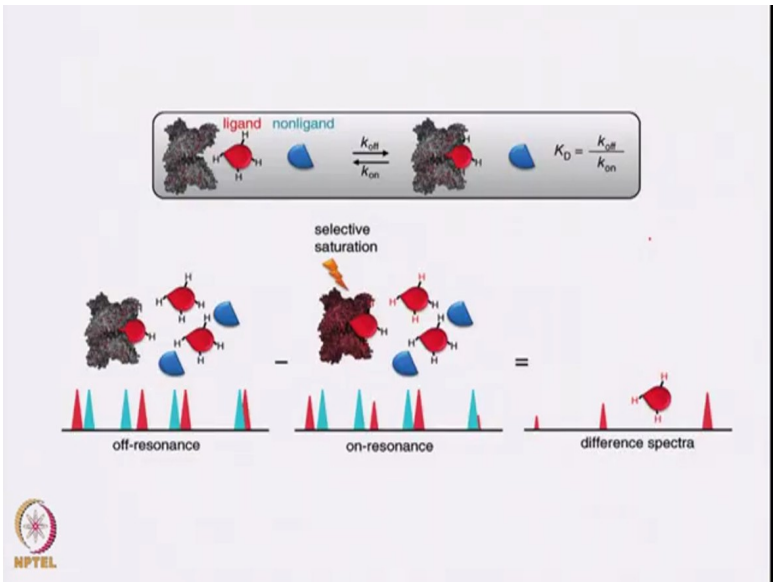

Ok, so essentially STDD is a powerful tool so what we learn, the protein ligand interaction if this ligand is interacting this is non ligand there is a k_{on} and k_{off} this ligand binds to protein

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

So now what we are doing off-resonance all the peaks will appear from all the ligands now if you saturate it the effect of the saturation is transferred to the binders and you can see some of the peaks have different intensity and you take this spectrum you can find it out the difference spectrum and that is given by these protons of the binder.

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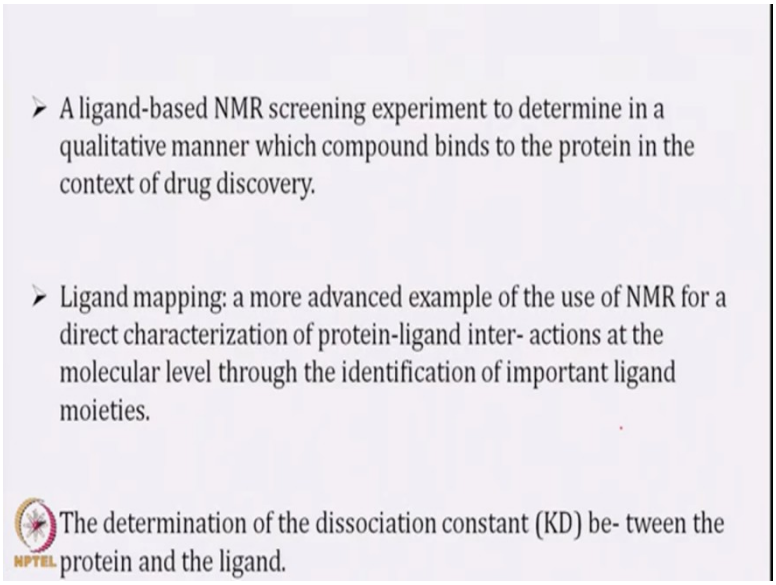

That is what we looked at. So to summarize this is a ligand-based NMR screening experiment to determine in a qualitative manner which compound binds to a protein in context of drug discovery. It is quite rapid and you can use that as a throughput technique for finding it out binders to a protein. So ligand mapping you can do a more advanced example we just showed use of NMR for direct characterization of protein ligand interactions at the molecular level and identification of important ligand moiety.

This moiety is binding or that moiety so in a group epitome mapping one can find it out the portion of ligand. We can also determine the dissociation constant K_D between the protein and the ligand. Ok, so now little bit more than STDD, there is another method which can be used for protein ligand interaction.

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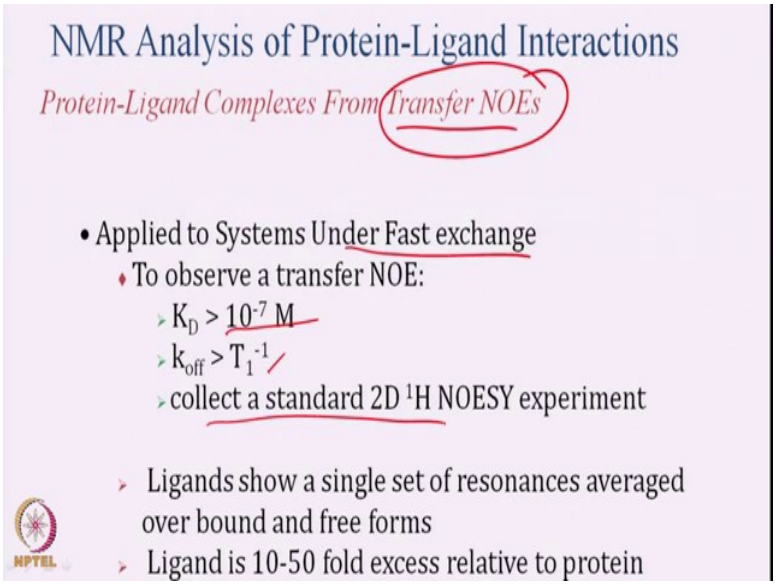

This is called transfer NOE, transfer NOE, you know nuclear overhauser effect. Now transfer NOE mean we are transferring the NOE from ligands to protein to find it out whether this ligand is interacting with protein or not. So this transfer NOE method is also used and this is again saturation transfer based method so for ligand protein interaction. So only thing it has to be in fast exchange and K_D should be in quiet strong like 0.5 or greater than 1 μM and then k_{off} also should be smaller. One can collect the 2D NOESY experiment.

So essentially what we have to show. Ligand shows a single resonance like if the protein and ligand is interacting and they are exchanging very fast shows only single set of peaks right and average of over bound and free from and ligand is generally in excess to protein. That is what is transfer NOE. Quiet a strong binding that should be in micromolar range and one record NOESY and then ligands should show only one set of peaks because they are in quite a bit of exchange and ligand should be excess of protein.

So if you do that the strong NOE will develop. Now this is binding right so NOE is distant dependent phenomenon if it is binding and all the time it is spending here so then NOE will develop from the complex and that is transferred to the free ligand state and that one can measured from the free ligand resonance and that can also be applicable to the large molecular weight right. So absorb NOE can be used to find it out bound confirmation of elements.

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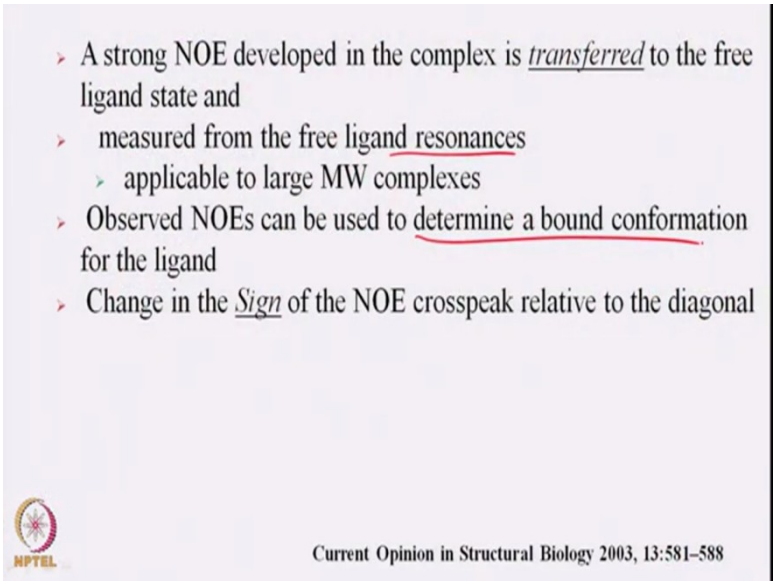

So what we are saying now my ligand is binding to protein and it is exchanging so sometimes expands in bind form and sometime in free form. Now if there is complex formation the NOE will develop between in the complex and that will be shown in the spectrum, NOESY spectrum. So all the NOE that coming from complex you can absorb it and find it out the proton that are in the close complex of the protein molecule.

So one can find it out now by what is the binding epitope in transfer NOE manner. So now when the protein will interact with the ligand now ligand NOE sign will change because now it becomes bigger molecule, so therefore with NOE cross peak relative to like sign will change and that is what one can find out.

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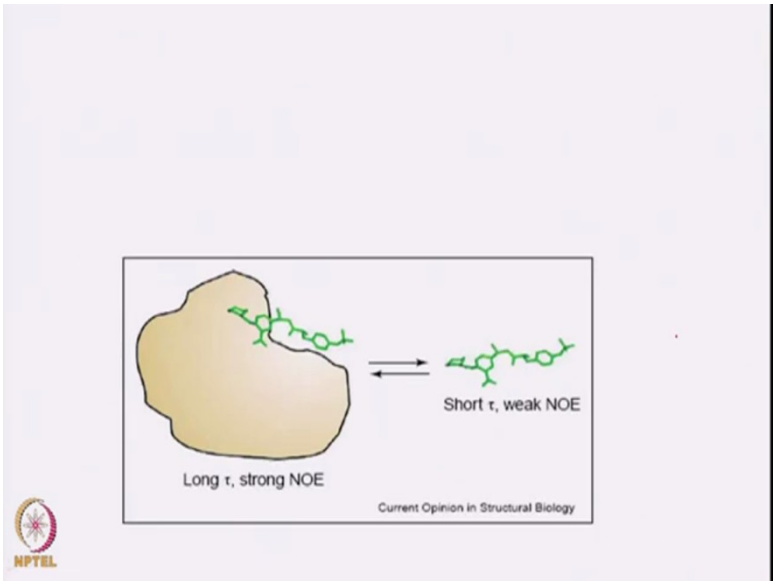

So essentially this is the case. Protein has a long τ, strong NOE, ligand has a short τ, weak NOE and if it forms the complex like here the NOE pattern of the complex will change and that is what you are going to see here.

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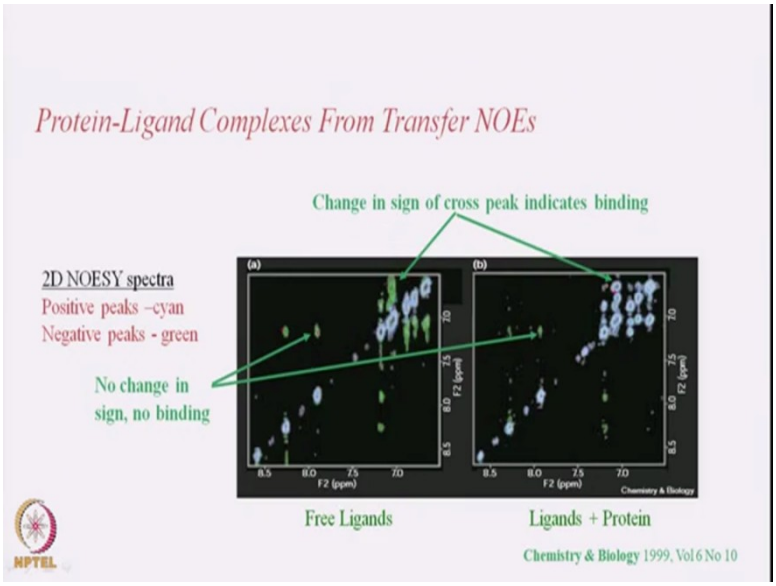

So here are all those negative NOE ok, so negative NOE are green this is coming from the complex and these you can find it out. Here if you look at this was positive NOE upon complex formation the sign has changed. So this means these are the protons that essentially are binding. Some of them they are not changing so that means those are not binding ok so that is what you see and that one can find it out whether it is binding or not binding.

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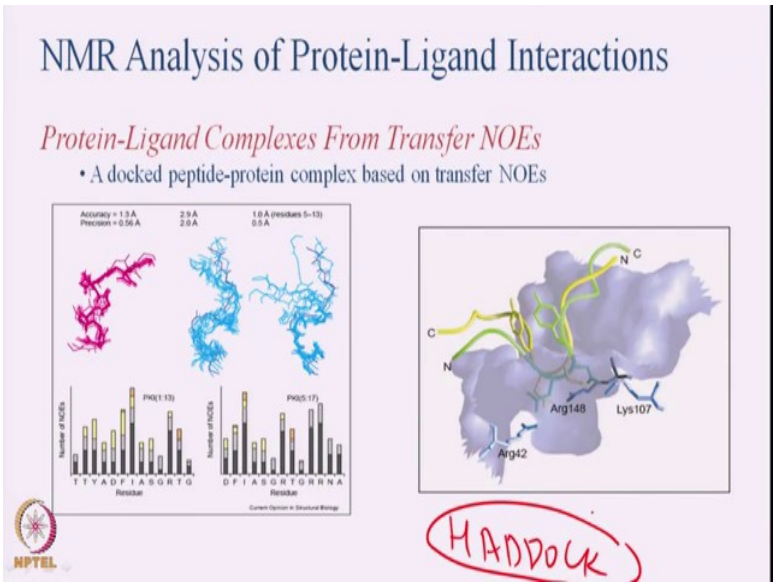

So one can find the complex ligand and one can use this information in a residue-specific manner and you can find where my ligand is binding to the protein by doing by getting information from the transfer NOE and you can use this information to create a dock model something like HADDOCK. HADDOCK is software which can be used for getting this complex model. So you can use those information to create a complex model of binding from protein to ligand.

So to sum up what we have here so STDD, I showed you so STDD can give you the binding strength like K_D also the group binding epitope. You can do that by quickly but STDD is not only method against polarized transfer-based method like ligand protein interaction can be also used by a transfer NOE. In transfer NOE what we are going to do is looking at the NOESY spectrum of ligand if it is in exchange with protein in the complex form.

So because of this interaction, it gets a negative NOE sign and those negative NOE can be identified and those are the protons of the ligand that are interacting with the protein. You can use those information to find it out which proton are interacting with the protein. One can use this information and can create a dock model using HADDOCK and can have a complex protein ligand complex structure.

So these are very very easy simple techniques that can be used in a structural biology as well as in pharma industry to find it out protein ligand interaction. Thank you very much. I hope these two lectures on protein ligand interaction will be useful for you in future for drug designing, drug discovery and so on. Thank you and looking forward to see you in the last class.