Principles and Applications of NMR spectroscopy
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So we will now look at the last topic of this course which is a technique which is used in drug discovery process this is known as saturation transfer difference NMR spectroscopy or in other short it is called as TD-NMR spectroscopy. So where is where is this spectroscopy method used?

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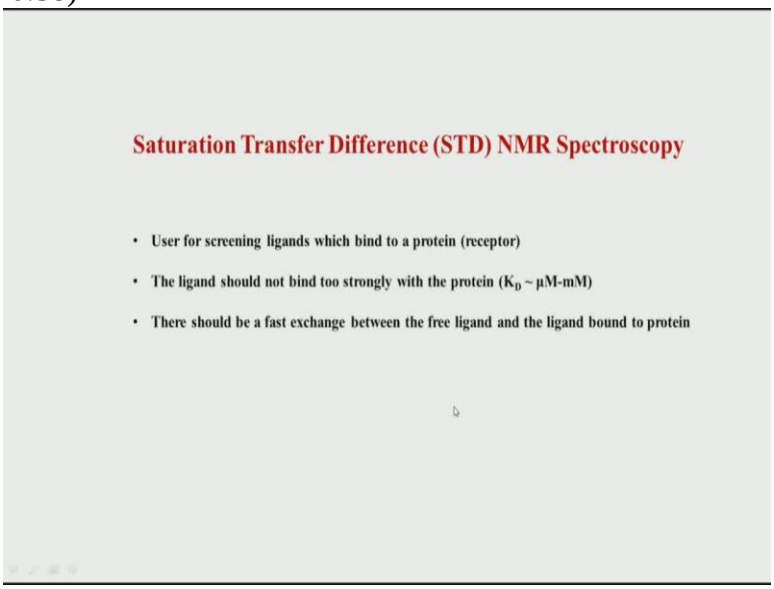

So this is shown here, typically we use it for screening ligands. So this is again remember let us say we have a target protein or a receptor which we have isolated and we are trying to find out which which molecule will bind to this protein and so we are screening a large number of molecules from the libraries.

So let us say we have library of thousand com.s or even a million com.s so the idea the goal is to find out out of this million com.s how many of them bind to the protein. So that is one reason why we would need to do this experiment that STD-NMR spectra experiment. Second the only criteria I mean we saw in the previous lecture we looked at the DOSY, so DOSY can also be used for this screening process, but the problem with DOSY is that yes it should be having a atleast a sufficiently strong binding.

Whereas here the ligand actually should not bind strongly, this is the criteria we will see why it so but this is typically a method used when the dissociation constant remember in ligand protein interactions we always measure the affinity of binding we characterize affinity of binding using this con method this number known as dissociation constant. So the dissociation constant should be for STD-NMR should be in this range should be micromolar to millimolar.

So you see this is a weak binding, if it is a strong binding, typically the Kd value will be in the nanomolar regime. But in if it is a very weak binding it will be in tens of millimolar or hundred millimolar. So our goal is to look at a medium binding medium to weak binding and the molecule should be in this range.

So we are not trying to screen here for those ligands which binds strongly we are trying to figure out which are the molecules have atleast weak binding to the protein. So what is the consequence of having a weak binding coefficient, the dissociation of constant? The consequence is that the molecule now exists as a fast exchange between the ligand and the protein. That means a molecule that is a ligand which we are trying to screen will not be tightly bond, it will be having an exchange rapidly between the solution the bulk free form and in the ligand bound form, and this is exactly what we want to exploit in STD-NMR to figure out which of this thousands of ligands which we are trying to look at which of them bind by exploiting this fast exchange.

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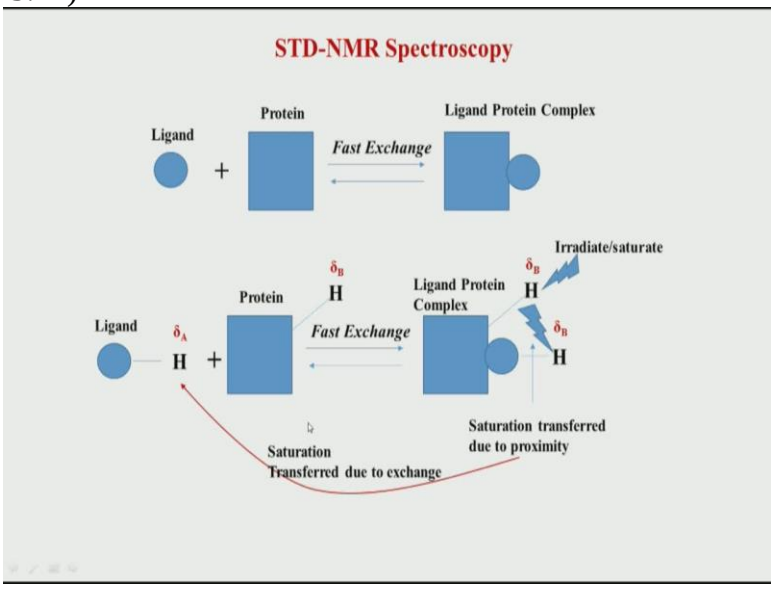

So let us see how this works, so this is just a schematic drawing so imagine that this is a ligand and this is the protein and this ligand binds to the protein so it forms a complex, but now this is a fast exchange process which means this one dissociates readily and goes back to this form associates comes back to this and so on so forth. So this process keeps happening a large number of times per second, so we use a word fast exchange. So this way of course there will be a rate constant for this which we saw in the previous classes that you can use second method of chemical exchange to determine this constant, you can also determine the binding affinity using the methods we saw in the previous classes.

So how this is useful for STD-NMR? So this happens like this. So suppose that ligand has a proton which is now having a chemical shift value delta A stop and suppose there is a protein a proton which is on the protein part of the protein and suppose its chemical shift is delta B. Now what will happen in the ligand this will be something like this. So let us assume that the chemical shift has not changed much between the bound and the free form I mean bound and the free form.

So now what we do in STD-NMR is that first we irradiate or saturate this proton signal. How do you do that? Remember this is typically what we do by applying a weak RF irradiation. So if you recall we did we discussed this when we talked about solvent separation. In a solvent separation we remember if you remember we said we do a selective frequency RF irradiation, means we look at the solvent proton and we irradiate at that solvent proton continuously for let us say few seconds and that completely saturates equates the population of the two levels and completely makes the signal go to zero.

So we do the same thing here, we identify a particular peak of a ligand or a protein and that peak is now selected for irradiation or what is called saturation. So when we do that continuously this is completely saturated this means there is no signal on can be observed for this particular hydrogen atom. So what how is that useful? What happens is that because of this distance suppose this distance is close to this or even if it is not close to it, it is close to some hydrogen on the protein and that protein is close to the hydrogen on this and that hydrogen is close to this.

So basically, if you have a collection of spins which are close to each other, then this irradiation of this hydrogen affects the irradiation of this hydrogen because of the proximity. You see this is

a saturation so whatever we have done to this gets transferred to this hydrogen because of its proximity. So this is similar to again if you recollect we discussed this in the study state noe concept in the NOSY experiment. We discussed that if you irradiate one hydrogen atom selectively then you will see an enhancement or a decrease in the signal for some other which is close in space within five to six armstrongs.

Here similar situation but is this not be close in space because there is what is called spin diffusion, now that also is a terminology and concept we discussed in NOSY we say that spin diffusion happens when there is no direct transfer but indirect transfer of magnetization polarization from one hydrogen to next neighboring to the next and so on it is a kind of a relay and that relay process we also use our spin diffusion. So because of spin diffusion the magnetic polarization of this hydrogen if I destroy by irradiation the destruction is communicated to all the protons in this whole system.

So one of the protons in this system is the hydrogen of the ligand which is now bound to this complex to this protein. So this is also now a part of this system, so that means any hydrogen which saturates, the saturation is communicated throughout it is propagated its something like in one part of the city something happens it completely propagates to the remaining part of the city. For example this routinely you can observed in that if you there is a traffic jam in one part of the city, slowly it transverse in the whole city many nearby areas get also traffic the traffic gets jam.

Similar process because it is propagating, so same thing happens here the saturation of a signal hydrogen atom if a protein is propagated or communicated such that hydrogen atom of a ligand also now gets saturated. Now if you see carefully this hydrogen is now in exchange with this hydrogen now, because remember we discussed this is known as fast exchange. So in a fast exchange scenario this hydrogen is exchanging with this hydrogen rapidly.

So therefore this hydrogen is next in turn further transferred or communicated to this hydrogen here, because this is in fast exchange. So if you recollect the exchange lectures on the exchange part chemical exchange we saw that when there is a fast exchange all the NMR parameters acquire an average value and that average value is a population weighted value. That means whatever I change for the first state B affects the state A because they are in constant exchange with each other.

So that means whatever I do to this hydrogen, if I saturate or destroy or reduce the magnetization of this, that is now transferred to this and this is also reduced in intensity. So what is the advantage of this? The advantage of this is that now I can looking at this form of the signal this is a free ligand, so looking at the NMR spectrum of this ligand by saturating this hydrogen of a protein I can figure out whether this ligand is indeed binding to it or not because if it was not binding to this ligand to this protein this ligand if it was not binding to this protein, if I saturate the protein nothing should happen to this ligand because it is no way interacting with the protein.

But if it is interacting with the protein, then if I saturate the protein signal it gets communicated to the bound form of the ligand and because a bound is in exchange with the free form the free gets affected. Now the question is why do we need a fast exchange? What if it was a slow exchange? That means suppose a ligand is binding tightly, if the ligand is binding tightly to this complex, then what will happen? That you will transfer the saturation but the bound form will be saturated now and the bound form is not able will not be able to communicate to the free ligand because it is in slow exchange means it is always sitting here it is not exchanging very rapidly with this.

So whatever I do to the bound form will remain in the bound form information and that information cannot be conveyed or communicated to the free form because there is no exchange. So if there is a tight complex then I have to monitor the bound form only, but remember the bound form can be a very huge sample signal I am sorry protein, and because of that the signal intensity is very weak. That is what happens in proteins and protein when you go to large molecular weight protein your intensity of the signal is goes bad and further this if it is bound, many of the ligands you cannot study.

So the STD-NMR is therefore a very nice technique which can be used for weak exchanging means fast exchanging weak binding ligands which exchange rapidly and remember ligands are small molecules, so there signals are always stronger, much stronger compared to what you get from a protein, because proteins are large molecules more complex so signals are not easily identifiable. So therefore this exp technique of STD-NMR what you do is you take the protein ligand in large excess means large amount compare to protein, so protein is very less and then you irradiate.

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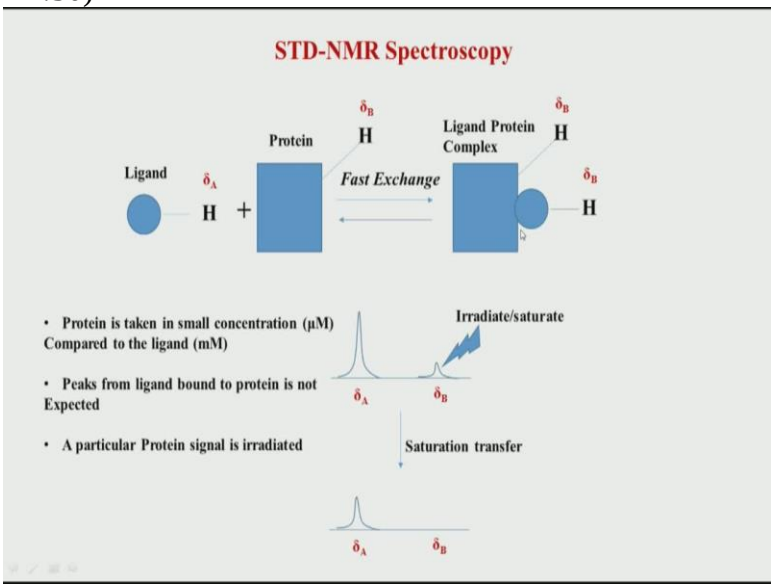

So this what we are going to see now, so how is does is manifest in the spectrum? So it happens like this, so you take the protein in a small concentration typically a few micromolar, remember our affinity is also in this order, and the ligand is taken in millimolar. So it is almost a thousand times more or hundred times more than the protein. So typically hundred times more concentration is what we choose. Then what you do is you record you know the spectrum of the protein you record a free spectrum means without the ligand and you choose a particular proton which you want to irradiate.

So that is what is shown here a particular protein signal is chosen for irradiation or is irradiated, okay. Now peaks from the ligand bound to the protein is expected, why? Because remember there is a difference of hundred fold so whatever ligand binds to the protein will be in the micromolar concentration assuming one is to one binding, okay so there is one molecule binding to one molecular protein there will be a thousand times or hundred times less concentration of the ligand in the bound form so we do not expect to see any of the bound form.

So our focus will be now only on the two states, state A is the free protein, free ligand sorry and state B is a protein signal, we are not looking at the bound form of the ligand. So you can see here suppose I have a hundred times more or here it is roughly 10 to 20 times more so this will be the scenario the protein signal will be very weak in intensity compared to the free ligand which is very high and in excess.

So next what I do? I irradiate this signal. So when I irradiate this signal of this protein as we discussed in the last slide this signal will go to zero, but not only that it will transfer its decrease in the intensity because of saturation through to this ligand which is bound and from there it gets transferred all the way to the free ligand because free ligand is also in exchange. So therefore, if you see the spectrum now will look something like this. After saturation as transferred the free (lig) the protein has gone to zero because we have completely saturated the protein.

But that signal bound the free part is also decreased, and why is the free part decrease? Because it is now exchanging with the bound form. So one thing you can see is that there is still approximity information if suppose the ligand has several peaks, suppose this is just an example but let us say our ligand has about five peaks then only that peak each peak is coming from one atom in let us say in the ligand. So only that peak which is closest to the this ligand will definitely be the most affected.

And that peak which is far away, the hydrogen which is far away from this binding site, so we use the word binding site, binding site is this side here that is the interface of the ligand and the protein. If that proton or hydrogen on the ligand is far away from this site, then obviously the saturation transfer is not communicated to far away protons as effectively as it is communicated to the protons at the interface. So we also get the information of which proton in the ligand is close to the interface based on how the intensity of that proton is affected by saturation transfer.

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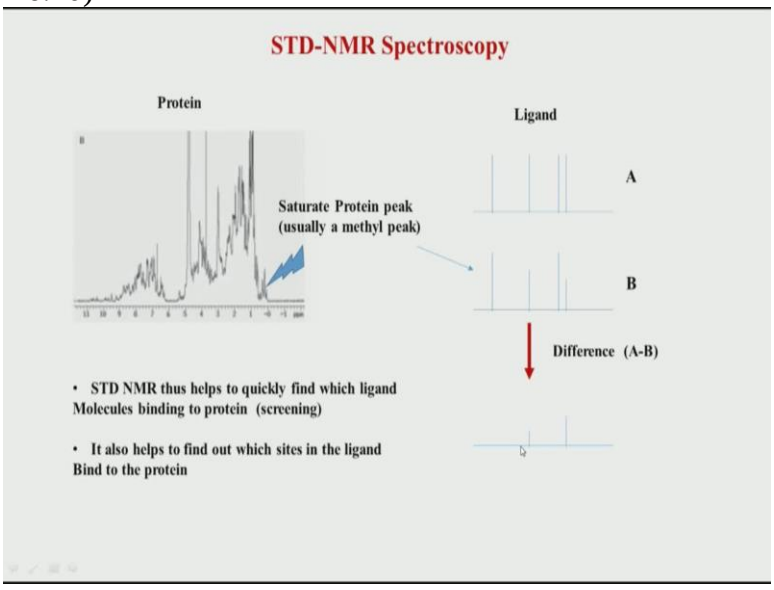

So this is what is shown here so we let us say we have a protein whose spectrum is complex and looks like this so typically you can remember this is a amide region, this is a alpha region, this is a beta, proton beta gamma proton and this portion is a methyl peaks here. So typically what we do is we choose a methyl peak for irradiation, the reason being that we assume that the methyl is far away from the ligand peaks and secondly the methyl peaks are a strong. They may be present in the hydrophobic or the interface.

And that will communicate or propagate the spin diffusion rapidly. So now that you saturate this peak, so this let us say our ligand spectrum is something like this. This is the free ligand, we will call it as A. So now once I saturate and I record another spectrum after saturation. So first is only the ligand, so I do not saturate anything here, it is the free form of the ligand. Then I saturate my protein and how is the saturation done? Remember again it is done by weak RF irradiation.

And typically the time is twos two three seconds, means we irradiate for a long time. And during that time seconds the molecules will exchange with the between ligand and the protein very rapidly. So they will they will exchange several times, several thousand times. And because of that the transfer whatever you are doing at the protein side is communicated to the ligand by exchange. So therefore if I record now a spectrum after saturation I will get something like this.

Remember I am again (())(17:59) any protein peaks here because they are at very low concentration, so that is why it is not shown here all I am going to expect to see only the free ligand. Because that is 100 times more compared to the protein. So when I saturate the protein after saturation the protein peak I will expect to see some decrease in the intensity of the ligand. Why is it happening? That is because this B, the bound form is in exchange with the free form.

So because of this exchange after saturation I am expecting a weak smaller decrease. All the peaks may not show the same decrease because these two hydrogens ofcourse each one is one hydrogen atom. Each hydrogen atom will have a different location in the binding compared to the binding site. So one which is closest to the binding site for example this peak will get maximally affected by spin diffusion. And which is far away from the protein binding site they will not see any affect.

So now I take a subtract, I subtract this and take a difference, so when I take a difference I will now get these two. Because these are the two have gone to 0. So what is the advantage of difference? My difference is telling me two things that if there was no binding I would expect complete cancellation why? Because this peak would be same as this peak, this peak would be same as this peak when there is no binding or if there is very weak binding there is no change in the spectrum whether I irradiate or not.

And therefore the signal will look the same. So when I subtract I will get 0. But if I do not get 0 means there is a binding going on, that is the information I get. Second information I will get is which of the atoms of the ligand are actually binding. So you can see now from this difference I can say that these two atoms are binding in the ligand and this is binding the most because its intensity has got subtracted the more most.

Otherwise this is binding less and other two are in fact very far away from the binding site. So by binding site information again all of them are binding equally because they are all part of the same molecule but relatively they are different because they are far away or nearby the binding interface. So I also get the information by STD-NMR, what is the residue atoms in the ligand which are actually close to the binding interface.

So therefore STD-NMR helps us to quickly find out which ligand molecules bind to protein. So this is useful for screening. So you can setup automated screening process where you pass each and every molecule through this process and we can quickly figure out which of the ligands. Because this is typically not more than a few seconds may be maximum 30 seconds experiment. So in half a minute, you can or a minute at the most you are able to figure out which molecule actually binds in your library of molecule which binds to the ligand sorry to the protein.

And not only that it helps me to find out binding it also finds helps me to find out which sites in the ligand bind to the protein. Because there will be a site specific differences in the saturation. So this brings us to the end of all this techniques.

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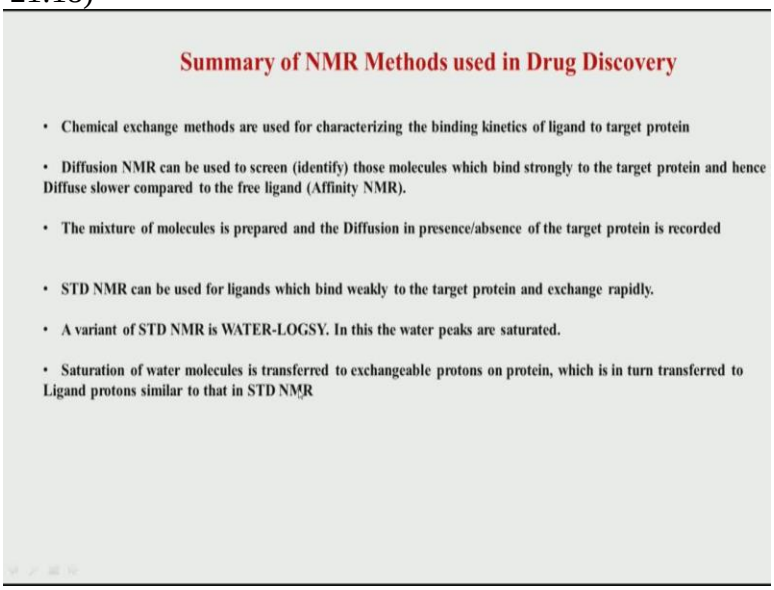

So this is a summary we have looked at different methods to be which can be used in drug discovery and we looked at this chemical exchange method which is very useful for characterizing the binding kinetics. It is also useful for finding by finding out estimating the dissociation constant or the binding affinity of the ligand to the protein. So these are all these techniques which are typically used in the drug discovery process.

And we looked at DOSY which is basically looking at diffusion process, translational diffusion keep remember. The translation diffusion can be a parameter which can be used for screening or identifying those molecules which bind strongly to the protein target protein. So that is what I said we used the word affinity NMR, this is being used in literature. The mixture of molecules so how do you do that? Again as we saw in the STD-NMR you prepare a mixture and then you can figure out which proteins which molecules are diffusing slowly in the presence or absence of the protein.

So in the absence in the presence of the protein if the molecule changes its diffusion it means it is binding to the protein. And we just saw the STD-NMR pros technique, this is also used for looking at ligand which bind weakly to the target protein and which exchange rapidly because of the transfer of saturation we can figure out which ligands are even with diffusion you can do that remember. In diffusion also if there is a weak binding, fast binding the change is also transferred

to the free ligand that is also can be done. And there is a another variant of STD-NMR which is Water Logsy.

In this what happens is you are now instead of saturating the protein peak remember in STD-NMR we had saturation on the protein peak, peak of a protein of the protein. But here we saturate the water and what is the advantage the advantage is remember water molecules are always hydrating, covering, surrounding a protein. So if I saturate the water the saturation of water is transferred to the exchangeable protons on the protein.

So remember we looked at hydrogen deuterium exchange and we saw that hydrogen exchange happens when the hydrogen is exposed or exchanging with the water water molecules. So therefore if I saturate my water peaks then it will also saturate my exchangeable proton peak because they are exchanging. Now that saturation of amide proton peak further will be communicated or transferred to the ligand protons which are binding to the ligand to the protein.

So this is again similar to STD-NMR that I am transferring a saturation from one position, one part of the system to another part of the system using spin diffusion. But here the one part of the system which is saturated is not the protein peak in Water Logsy but it is the water as such because water signals are very very clearly visible, we can saturate on water and that will communicated to the hydrogen amide protons and further communicated to the ligand and that is similar to what we seen in STD.

So those residues those atoms on the ligand which are reduced in the intensity, will be the one which are binding to the protein. So this brings us to the end of NMR course I hope you enjoyed this course we look through various basic aspects this is a course meant for basically going through very rudimentary NMR methods and we looked at many things in a qualitative manner it was not done in a very technically sound manner because of the mathematics involved.

The idea was to reduce the amount of mathematics needed to understand this subject so this was only basically mend to go through collectively how NMR works. How it can be used for small molecules, organic molecules how it can be applied to peptide and protein structure determination and how it can be used in the drug discovery process. So will I hope you enjoyed this course and I look forward to see you again in the next course.