Multidimensional NMR Spectroscopy for Structural Studies of Biomolecules

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Lecture – 41
Understanding Protein ligand interaction by NMR: Diffusion ordered Spectroscopy (DOSY) – Part I

So, we will look at method for determining the interaction between protein and ligand.

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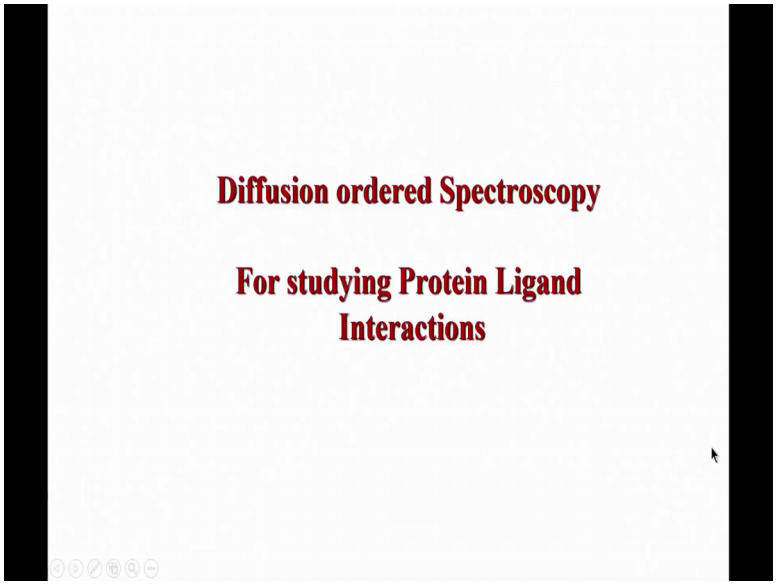

This is based on what is called as translational diffusion. So, this is a very popular method in NMR spectroscopy. And this is known as diffusion ordered Spectroscopy. So, we will see how that can be used for determining the interaction of a ligand small molecule with larger molecules such as proteins.

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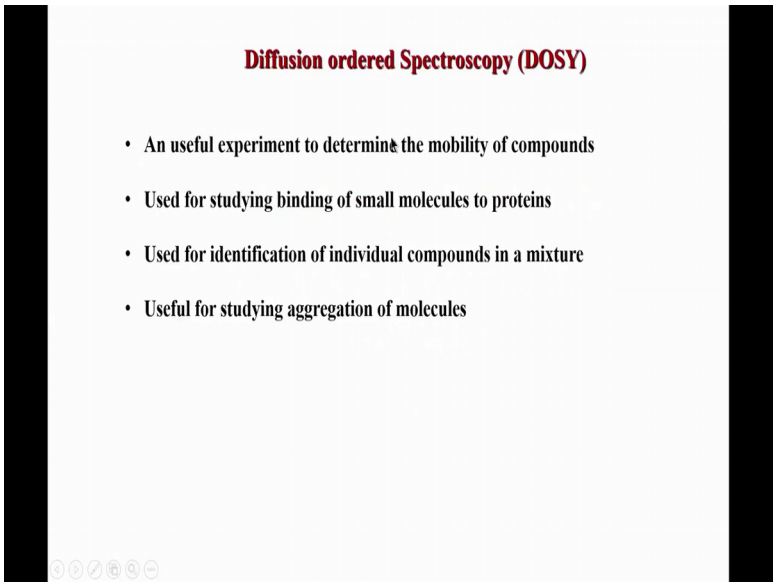

So, Diffusion ordered Spectroscopy is also known as DOSY this is acronym for the experiment. And this is a very useful experiment to determine the mobility of compounds. So, we are looking at now translational mobility the remember there are two types of mobility in molecules. One is known as the translational, mobility another is rotational mobility. Rotational mobility essentially is a rotational tumbling of the molecule which is characterized by the rotational correlation time. So, that is something called tau c which we have seen in earlier lectures when NOESY especially when we discuss NOESY and ROESY.

But here we will be looking at another type of diffusion that is called translational diffusion. So, how the molecules move in space, as a function of distance? Ok. This is an experiment which is useful for that. And typically DOSY is used for studying interaction of small molecules with proteins. So, this is a very popular technique used in drug discovery for screening, a large number of molecules to see whether they bind to the target protein or not.

And it is a useful for identification of individual compounds in a mixture. So, I suppose let us say your mixture has two or more molecules. You do not know whether their number of molecules are formed two or three or so on. So, if they are different in size, then one can actually use a DOSY to differentiate or separate the molecules.

But the main thing is they should have a difference in the size. Because translational diffusion as you will see later is very critically dependent on the size of the molecule. So, the higher the size, the bigger the size of a molecule it will diffuse or it will move slowly in a solution. Similarly, if the molecule is small in size it will diffuse faster in solvent. So, that is what basically the idea is the diffusion is order spectroscopy tries to exploit the differences in the molecular diffusion based on their size.

Now, therefore, if size becomes an important parameter then one can also use this approach for studying aggregation of molecules. So, let us say your molecule is aggregating from a monomeric state to a dimeric state to a tetrameric state and so on. Then one can use DOSY to study or track how the aggregation is going on. Because as aggregation proceeds your molecular size will increase and if the molecular size increases your diffusion will slow down and if the diffusion slows down it can be captured by this experiment. So, these are basically the a few reasons or a main reasons why DOSY is very popularly used in NMR.

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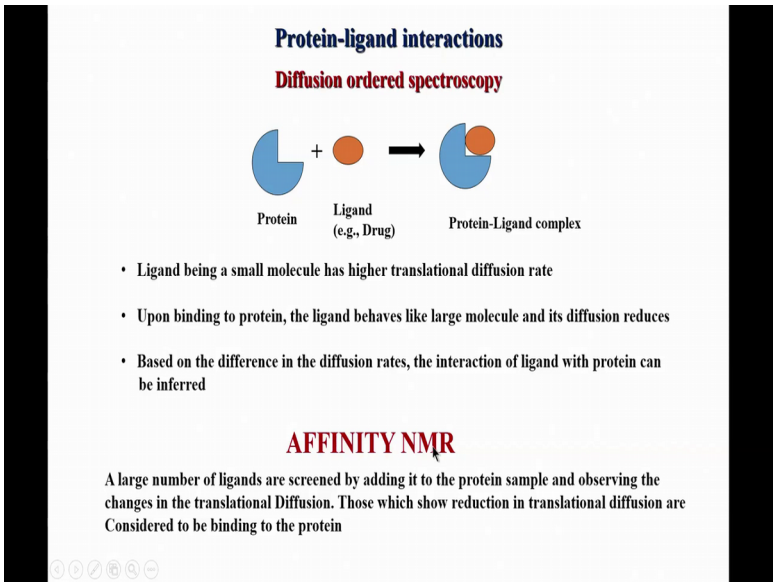

So, we will go through this and look at how we can study interaction of ligand with protein using this technique. So, we will again look at this picture which we have seen earlier. So, suppose let us say you have a small molecule ligand, it is some kind of a drug which interacts with protein. So, you have this complex which is formed. Now let us assume that the complex is strong, which means the ligand has a high affinity for the

protein. Which are in other words may implies, that the protein is the ligand as tightly bound to the protein. So, in such a scenario the ligand being a small molecule, the free ligand will have a high diffusional or higher diffusional rate in the unbound form, means in the free form.

So, in the free form the ligand will have a higher diffusional rate. Because it is now not bound to the protein, but upon binding to the protein the ligand behaves like a large molecule and therefore, its translational diffusion reduces. So, this is the main point here is that we are trying to differentiate or difference take the difference between the ligand in the free form, versus the ligand in the bound form. So, this is a point here which is written mentioned here also. That based on the difference, in the diffusion rates the interaction of ligand with protein can be inferred ok. So, now, if the ligand binding is not strong then, there will be a set another complications. So, there are two possibilities or let us say three possibilities.

Possibility number one is that the ligand does not bind to the protein at all. If that happens then they should not be any change in the diffusion of the ligand, because it remains in the unbound form whether if the protein is present or not the diffusion of the ligand should not change; if it is not binding to the protein. But imagine another scenario where it binds with the protein, but does not bind very tightly, a loose binding means a weak interaction. In a weak interaction case scenario the protein now and the ligand remains in the free form as well as in the bound form. So, both these forms will exist in a dynamic exchange with respect to each other ok.

So, this dynamic exchange therefore, will cause an average diffusion of the ligand between the free form and that bound form. So, the diffusion what you will measure or observe in the spectroscopic technique will not be the free ligand diffusion rate neither will it be the diffusion in the bound form. What you will observe in a loose binding situation that the diffusion will be an average of these two states ok. So, that is case with a weak binding scenario.

And the third scenario is a tight binding, means it binds very tightly and therefore, the population or the amount of free ligand in the solution will be very less. And so, if that is the scenario then the ligand will diffuse like a protein because it is now belonging to this

bigger complex and therefore, is diffusion will be considerably reduced and it will diffuse like a protein size.

So, in such scenario very easily we can now distinguish the bound form and the free form. So, let us see how this diffusion order spectroscopy helps to differentiate the two states by using the diffusion rates. So, before we go into the DOSY details, we have to first understand the basic concept in NMR which is required for DOSY. Now this idea of using diffusion to decide whether the ligand is binding or not is known as affinity NMR and a large number of ligands in the drug industry or pharmaceutical companies are screened in the initial stages by adding the ligands to the protein sample and then observing whether the translational diffusion has changed or not.

Those molecules when we add to the protein, those which show reduction in translational diffusion are then considered to be the binders or considered to be binding to the protein. So, this is the standard definition or approach in a drug discovery process and the word affinity NMR you will see being used in literature to determine whether diffusion can be used or not for binding of ligand to proteins.

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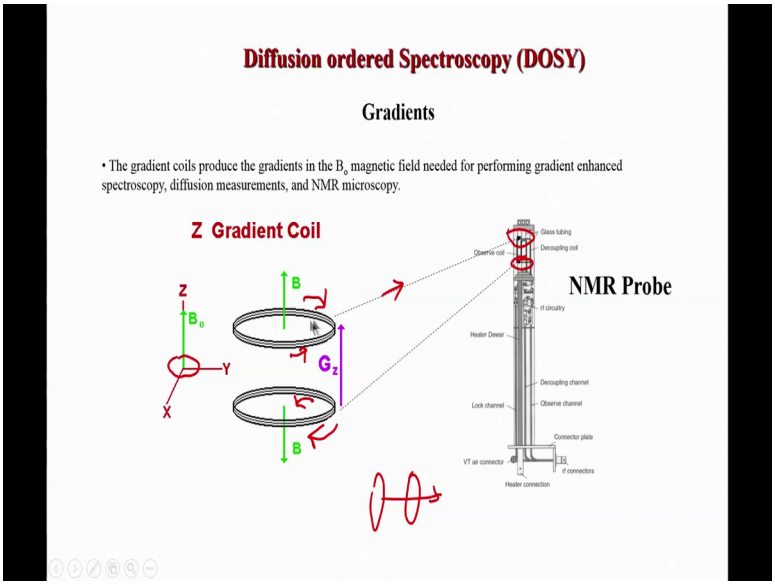

So, let us now move in to how diffusion DOSY experiment works? For that you have to first understand a basic concept in NMR that is known as gradients. And this is something we have not covered in extensively in this course. So, I will give a very brief introduction to the gradients and then we will go on to how gradients are used in DOSY.

So, what are gradients? Gradients are coils, you can see these are like coils circular coils and these coils are present here. You see this is called as a NMR probe this whole cylindrical thing here is an NMR probe and the probe goes into the magnet. So, if you recall the how the NMR system means spectrometer is build?

N M R spectrometer consists of a magnet, it consists of a console; console means electronic components it consists of probe. Probe is basically where you put your sample. So, you see where this arrow is pointing right now you have a sample typically sits there. So, there is this R F coil you can see this observe coil and decoupling coil these are this coils which are kept there I mean are there. And above and below this coils R F coil you will see this circular coils called gradients. So, what happens in a gradient is a current; current is supplied in this coil. So, a current flows in a direction like this, in the coil this coil is sitting there in the top position here and in the down coil will go in the opposite direction and that will be in the bottom of this R F coil ok.

So, you see there are two opposite gradients they supply current in the opposite direction, and they will therefore, cancel each other. But not always i will see that how it gets cancel how it gets added up and so on. But this gradients when you supply a current in a coil what happens it have what happens is a magnetic field is generated perpendicular to this plane of this coil. So, you suppose you imagine this plane as a X Y plane, so, this is in the X Y axis X Y direction. So, you can think of the ring of the coil like this. So, whenever you have a coil like this current is flown in this coil and this for generates a magnetic field in the Z axis ok.

This is something very similar to what we do with a main magnetic field. Main magnetic field also consists of superconducting coils which carry the current and that generates the main magnetic field. And the gradients are also magnetic field, but the magnetic field they generate is very very small. This magnetic field typically is of the order of gauss ok; is a not a very big remember one gauss is 10 to the power minus 4 Tesla. So, we are generating a very weak magnetic field, from the gradients. So, gradient generate an additional magnetic field on the sample ok. So, this is in addition to the external magnetic field which is already present.

This is something we do not have to think about because this is the main magnetic field, but by turning a gradient applying a current in this coil it generates a magnetic field.

Now, suppose I apply in the opposite direction in this coil down then the magnetic field will be in the opposite direction ok. So, this is called the right hand rule ok. So, one has to keep that in mind that when you supply a current it basically generates a magnetic field like this and if, you oppose the I mean apply an opposite current it will basically go to this opposite direction ok. So, basically you have to look at clockwise versus anticlockwise. So, we can think of it this as this direction also and this as this direction ok.

So, any direction you apply it will generate a magnetic field corresponding to the right hand rule and the next the other coil which here will be opposite in this direction. So, now let us see what is the effect of this on the sample? Why do we, what happens when you apply a gradient current in a coil like this and what happens when into the sample. Now one thing is the this gradient coils are nowadays standard in all NMR spectrometers.

But olden days 20-30 years ago this gradient coils were not there in the probe. So, now, second point is that this gradient is along Z axis. Why it is along Z axis we will see that shortly. But you can also apply gradients in the X Y direction. So, if I turn this coil around if I turn this coil if I keep the coil like this, then the gradient will be like this in this direction.

So, it depends on what direction you put the gradient will be depending on that. So, in our case in a standard NMR spectrometer, routinely typically you will see that this gradient coils are situated like this in the X Y plane. So, that magnetic field which is generated is in the Z axis that is G z ok. So, this G z is known as a gradient we will see this gradient how it looks shortly, but keep in mind again we have a small coils in the probe, where we apply current in opposite directions and that generates magnetic field additional magnetic field.

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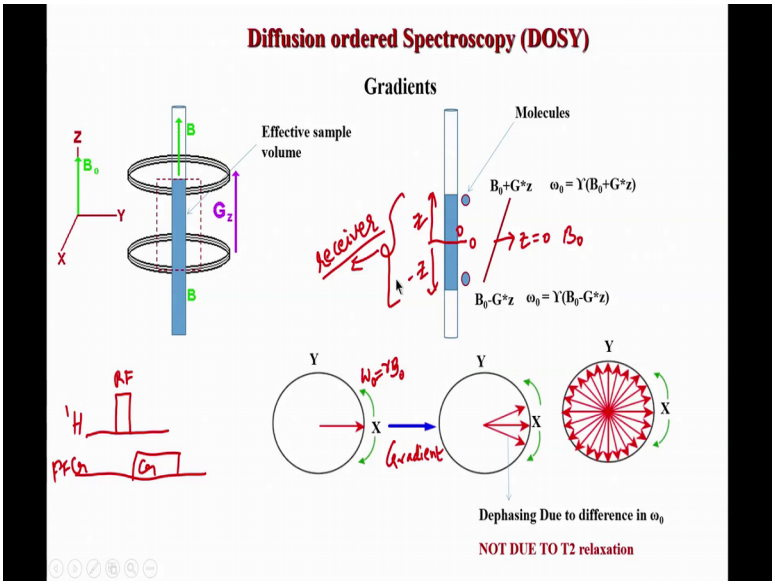

Now, let us see what is the effect of additional magnetic field, on the sample? So, now, look at this picture here. So, let us say this blue colour, what you see in the blue is our sample kept in the sample tube. So, sample tube is this rectangular portion shown here, this full rectangle out of which let us say we have filled the sample like this.

Now the effective sample volume, meaning the R F coil is somewhere is between these two coils it is not shown here you can think of this dotted line also as I have R F coil. But this remember if you go back to this picture here what you can see here is that this gradient coil are sitting at the ends of the R F coil. So, R F coil is like this the gradient coils on top and bottom of the R F coil.

So, if this is what is shown here we can think of this middle portion as R F coil and the top and the bottom is basically a gradient coils. Now this what is shown in this dotted box, are the one which basically is covered by the R F coil is our effective sample volume. What does this mean? It means that whatever NMR experiments you do, your experiment is basically a sample is what is generating the signal is coming only from this portion. The portion below and the portion anything above is no use why? Because it is not scene by the R F coil. So, R F coil and the gradient, I mean this portion essentially are the main part of the sample.

Sample is molecules in the sample in this portion, are the ones which are finally, giving you the NMR signal. So, this typically is about 250 to 300 micro liters in a 5 mm tube.

Suppose your tube is 5 mm in diameter and you have a 5 mm probe then your actual volume of the sample which is effectively seen by the probe is about 300 micro liters whereas, you take about 600 micro liters in a NMR tube. So, half the sample is essentially not visible to the NMR spectrometer or to the coil. But that is not a important point here the point here is that this effective volume is essentially the volume which is seen by the R F coil and the gradients. So now, see imagine; so, this is what is shown here in this picture on right hand side.

You have this effective volume this blue colour shaded portion. So now, imagine that there is a molecule at the top of the sample and imagine the same molecule another copy of it in the bottom. So, remember our sample contains a large number of molecules. So, if you take for example, 1 millimolar sample you have a huge number of molecules 10 to the power almost 20. So, when you have such large number of molecules obviously, you have them distributed throughout the sample. They are not sitting in one side of the sample they are spread throughout the sample. They are not sitting in one side of the sample they are spread throughout the sample, because we think we are assuming it is a homogeneous be soluble sample.

So, if we imagine a molecule which is at the top part of the sample and at the bottom part another is a molecule, you see these two molecules now when I apply the gradient means I apply this R F current in this R F coil here and here ok. What will happen is the following. The molecule at the top will experience an additional magnetic field in addition to B 0. Now, what is your additional magnetic field? Additional magnetic field is given like this it is space based on the strength of the gradient means how strong is the current in the gradient coil or how strong is a magnetic field generated. Multiplied by the z;z means its distance from the centre ok. So, imagine this is your centre this part is this Z.

So, centre is now considered as 0 and this is be minus Z. So, Z is basically just the distance, of the molecule from the centre of the sample ok. So, what happens is when I apply a gradient I am applying two opposite magnetic field ok. So, the magnetic field and this coil, remember I showed in the last slide is in the upward direction and the magnetic field and the lower coil is in the download direction. So, therefore, for a molecule which is at the bottom of the sample experiences an opposite magnetic field compared to the molecule at the top of the sample ok. So, this additional magnetic field

are in addition to B 0 which is our external magnetic field which is already present. So, remember B 0 is always present we are not disturbing or we are not changing that value.

What we are doing is, we are creating or we are adding an extra magnetic field at the across the sample where in the top most part gets this addition magnetic field and the bottom most part gets a subtracted magnetic field. What happens in between? In between there is a range of G value because the Z will change right. So, for example, if you take here Z equal to 0. So, the magnetic field is just B 0, there is no addition to the external magnetic field because Z is 0. So, when you put Z equal to 0 here, this becomes0. So, total magnetic field now for this molecule suppose i have a molecule sitting here, then that will experience a 0 additional magnetic field this is not 0 remember ok.

So, what have you create? What have you done here? What we have done here is that across the sample from the lower part to the top part, we have created a difference in the magnetic field. And the difference is a linear you can see this is a straight line. So, linear it goes from minus G z to plus G z linearly ok. So, this is a very important thing, we have deliberately means purposely we have created this difference in the magnetic field. If the G value was 0 then you can see that there wont to be any gradient. So, when is G equal to 0, g is going to B 0 when there is no current in the coil. So, when I do not apply any current in the gradient coil here and here it means my G value is 0.

But if I apply a current my G value will be some value. Now I can change the current strength. Strength means how strong the current is I can go from very low strength to very high strength or I can keep it for a longer duration I can apply a current of some value and keep it on for a long time. But what is the time typically we keep it on, the gradients are very short durations we typically keep it on for a few milliseconds ok, so, microseconds to milliseconds. So, therefore, it is like a pulse. So, gradient is also like an R F pulse, which is applied for a very short duration the current in this coil is applied for a short duration not for a very long duration. But in that time whatever has to happen we will see now what goes on.

But the g value is another thing g value is the strength of the current how strong is your current in the coil and that will decide how strong is your additional magnetic field. But another parameter is the time. That how long do you apply the gradient and that does not affect the magnetic field it only effects some other parameter. But it will tell you how

long the magnetic field is present. But as I mentioned, it is typically of the order of a few milliseconds. So, therefore, gradients are very short in duration and you can think of it as a pulse ok. So, now, let us see what happens when you apply this gradient to the sample. So, let us say I have a 1 d pulse I have applied a pulse and R F pulse, so, this is an R F pulse ok. Now this is for proton.

Now suppose after the R F pulse I apply a gradient gradience. So, gradience typically we show like this. We call it P F G is this called Pulse Field Gradient. P F G is pulse field gradient and this gradient let us say we apply after the R F pulse. So, when we apply the 90 degree R F pulse, then a magnetization is in the X Y plane ok. So, now I have apply this gradient. So, what will happen? The magnetization now is now precessing going to precess with the without the suppose gradient is not there. If the gradient is not there this will precess with omega naught equal to gamma into B 0. This is a standard NMR formula which all of us know.

But when you apply a gradient so, when I apply a gradient now, what will happen is, the molecules which is on this side of the sample and molecules which are on this side of the sample. They will not have the same omega value because, if you look at this form equation here the omega 0 or omega of this molecule has changed because now the total magnetic field experience by that molecule is not B 0. It is B 0 plus G into z, where z is the distance from the centre.

Now if you look at the in the same molecule, but now that is a copy means there is another molecule of the same type. Which is sitting at the bottom of the sample, it will have a different Larmor precession. Its precession frequency has changed, it is now b 0 minus G into z. So, now, what is happening is the same two molecule the same proton in a given molecule. For example, you take ethanol any molecule the particular hydrogen in that molecule if you are looking at that.

The same hydrogen because it has a copy on the top of the sample along the sample in bottom each part of the sample that is proton, experiences a different omega value. Because at every point in the sample you see here, the omega is not the same. Omega is change because of this additional factor. So, because of this, you see all this omega the different omega. So, they start a going in different speed, they start dephasing now because of the difference in omega 0. And this difference is around across the sample.

So, therefore, when you combine the signals, all of the signals are finally going to the receiver ok. But now the receiver is not going to get a single signal is going to get a signal which is completely dephase because each molecule in the sample is having a different omega value omega naught value precession normal precession value.

So, because of this complete change or spread of the omega value across the sample, there is a complete dephasing of the system. Because each velocity this can think of this arrow shown as a velocity or speed. The speed all changes the oscillation frequency changes for every sample here every molecule here, in the same sample and the same proton, but located at different region of the sample. So, therefore, when I combine all of this to get a single signal; remember receiver does not know where the signal is coming from the receiver takes the signal from the entire sample. So, the receiver now gets a 0 signal.

Because the complete signal this is all dephase and it has become going to 0. So, any signal here is cancelled by a signal here, any signal here is going to be cancelled by signal and so on. So, this is why a gradient completely kills the signal. A gradient is a very simple approach to simply destroy or remove or suppress any signal you want. And this suppression or dephasing you must keep in mind; it is not due to T 2 relaxation, because T 2 relaxation also causes dephasing.

But the T 2 relaxation is a slow process it takes 100 milliseconds, 200 milliseconds. Whereas, by applying gradient which is only 1 millisecond we can achieve this complete dephasing. So, gradient therefore, is a very fast way to remove any signal from your sample or destroy the or suppress the signal.