Principles and Applications of NMR spectroscopy
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Module 8
Lecture No 39

In the last class we started with looking at what is DOSY, this is an acronym for diffusion ordered spectroscopy. One of the very useful experiments in an NMR not only in drug discovery but also for studying small molecules aggregation proteins and etc. So let us continue with that today.

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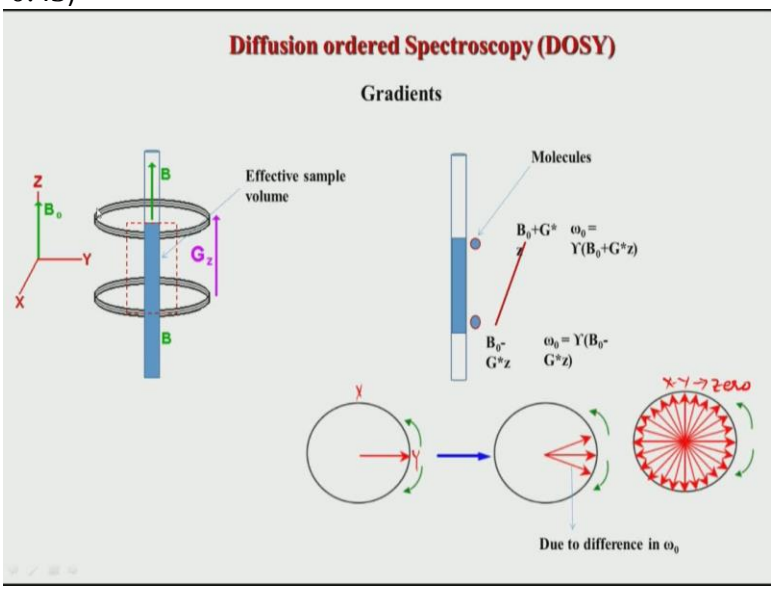

So this is that picture which shows how this doozy experiment what kind of things are is used. So the most important things in DOSY is the use of gradients, so we saw in the last class that gradients are basically coils which are present in the probeh near the RF coils and they work by circulating current in this coil and that current generates a gradient in the vertical in the centre of the coil in the vertical direction. So depending on the current the movement of current the direction of the current, the magnetic field can be either upwards or it can be downwards.

So typically what happens in all spectrometers we have what is called z gradients, so we call it as Gz. And that generates current in these coils and if you put it in one direction it will be a upward towards the Bo this is the main magnetic field or if you do it in anti-clock wise or in a different

direction it will be in the negative direction. So now imagine that you have a sample like this and this is the what is called as effective coil RFh coil volume effective volume of the sample because this is the volume which is basically seen by the RF coil.

So there will be sample above and below which is not available to the RF coil and the gradients. So our focus will be only on this portion. So this is typically two centimetres to twenty millimetres or eighteen millimetres long. So suppose we have a molecule which is at this end of the sample and the same molecule anotherh copy of the molecule is sitting at the bottom of the sample.

Now if I apply this gradient it is it experiences a different magnetic field, so the molecule sitting here experiences Bo + a certain field which depends on its Z axis coordinate meaning how far it is from the centre. Now the one which is at the bottom experiences a Bo - this gradient into this coordinate which is at the -, that is coming from here, that this coil carries the bottom coil carries current which is opposite in direction compared to what is in the top.

So therefore top and bottom oppose each other, so typically the top portion will be along the magnetic field be zero and the bottom magnetic field generated will be oppose in the main magnetic field. So therefore we can write the bottom part of the sample as Bo - Gz and top part of the sample as Bo + Gz. So now remember that according to the standard NMR concept that the precessional frequency or we use a word Larmor precision, is defined by gamma into Bo.

But now because of this additional magnetic field for the top part of the sample the molecule has Bo + Gz and for the bottom half part of the sample it is Bo - Gz. So the same molecule suppose let us say we are looking at water molecule or any molecule the same molecule now has different precessional frequency along the sample. So this is what the gradient does. It creates a linearly varying magnetic field across the sample. So when I apply a pulse the signal will first come to the XY plane.

Now because of the gradient the different molecules will now start processing not according to one frequency but different frequencies and this different frequencies have been created by this gradient that because the same molecule which has all phased together now start de-phasing at

different speeds because each sampleh each molecule along this sample has different precessional frequency.

So you can also say that we have create an additional chemical shift along the sample. So the same molecule now is spread over a large chemical shift range because each part of the sample has different omega zero. So therefore the molecules do not go in phase they start to de-phase similar to what you see in T2 relaxation. So this is also similar to that but this is now a induced relaxation kind of a de-phasing and not a random de-phasing which happens in the T2 case.

So therefore this de-phasing now continues and after sometime all the magnetization has fanned out in all the XY direction because each volume you can see from here, this end of the sample to this end of the sample each of the spins or molecules have different omega zero. So they go out of phase. They are not moving together and they become completely de-phased. So therefore if we record a spectrum at this point we will not get any signal from the sample. Because you see the total vector addition of the magnetization is now equal to zero.

So therefore you apply a pulse, then you apply a gradient, then your signal is completely gone. And typically this gradient is applied for a very short duration. It is similar to the pulse but pulse is about microsecondh gradients are about hundreds of microseconds, hundred or five hundred or sometimes up to milliseconds. So they are not very long but at the same time they are short in time. So we use the word gradient pulse.

So we say pulse field gradients, this is a typical terminolo0gy is used in when we describe gradients.

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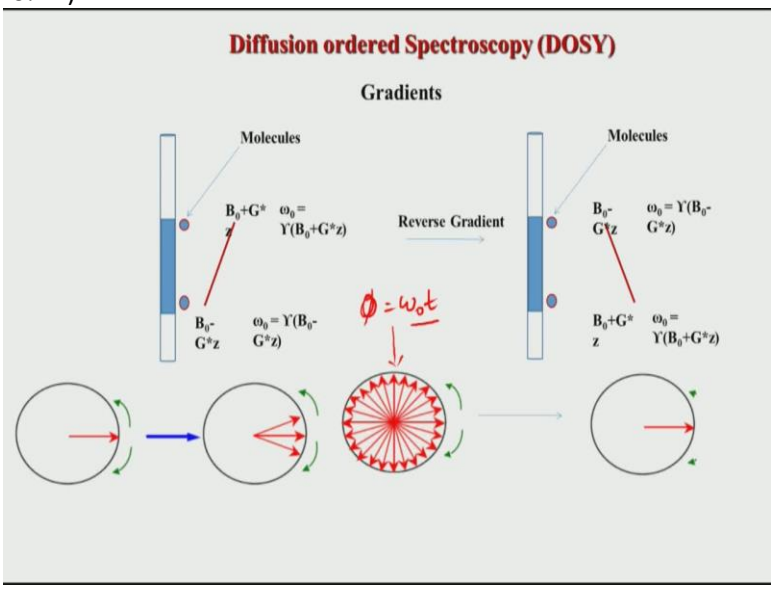

So now let us say how how is all this connected to DOSY let us continue more. So now suppose I have done this exercise I have excited the magnetization I have applied a gradient and because of the gradient after some time the complete de-phasing has taken place.

Now I can undo this, I can undo this whatever I have done the de-phasing by applying now a reverse gradient. So now you can see here if I apply a reverse gradient now it is in the other way around. Here it was Bo + Gz, now the top part of the sample becomes Bo - Gz and the bottom half bottom part as + Gz. So you see now the precessional frequency has completely reversed. So whatever was positive Bo + Gz now it is Bo - Gz.

So that means if this vector suppose this vector which is where I am pointing now suppose it has moved by 40 degrees because remember how is this degree coming from, the degree is called phase and the phase is nothing but omega into t. So let me show that here. So phase is equal to omega into t. So after sometime t this phases have accumulated by each spin and that phase depends on the magnetic field because it depends on omega zero.

So now suppose this omega zero for this sample has accumulated a phase of let us say hundred degrees but when I apply now a reverse gradient, the same phase will go in the opposite direction because now its frequency is - this earlier frequency. Therefore how much ever degree it has gone from this side it will now go in the opposite direction.

So if I apply a reverse gradient for the same time t so suppose this gradient is time t and I apply the same time t it will completely come back to the original position. So all the spins here what you see here which have all got de-phased they have all de-phased because they have gone by different angles and that different angle is because they are along the different length part of the sample. So because they are at the different part of the sample they have experienced different omega zeros .

But t is same for all because this is the gradient time that is same for all but their omega zeros are different so their phases are all different and because phases are all different they underwent a complete de-phasing but now by applying reverse gradient I can undo that by making now a opposite side so all these phases will be negative because you can see here now they are subtracted and they here it is added.

So they will come back to the go back by the same rotational angle. So if something has moved by 50 degrees will come back to - by - 50 to 0. Similarly something which has gone by 72 degrees will come back with - 72 back to zero. So all of them finally come back to the same position and now they have come back in phase. So this is the advantage of gradients that you can de-phase and re-phase simply by applying an equal and opposite reverse we use the word reverse or you can say opposite gradient.

And therefore it has come back to the original phase. So therefore you can play a play around with magnetization by simply de-phasing, re-phasing and whatever angle you want we can choose that by applying this kind of gradients. So what has basically the take home from this the take home from this is we create a specially varying magnetic field and because of that each spins in the different parts of the sample now acquire different phases because they now have different frequencies.

So this phasing it results in each part of the sample is different phase so results in total de-phasing which can be now reversed by applying a opposite gradient which also creates a specially varying field but it is now undoing what was done before. So how do you create a reverse gradient? simply reverse the direction of the current which we saw in the previous slide, you apply at the reverse direction and the gradient magnetic field is changed.

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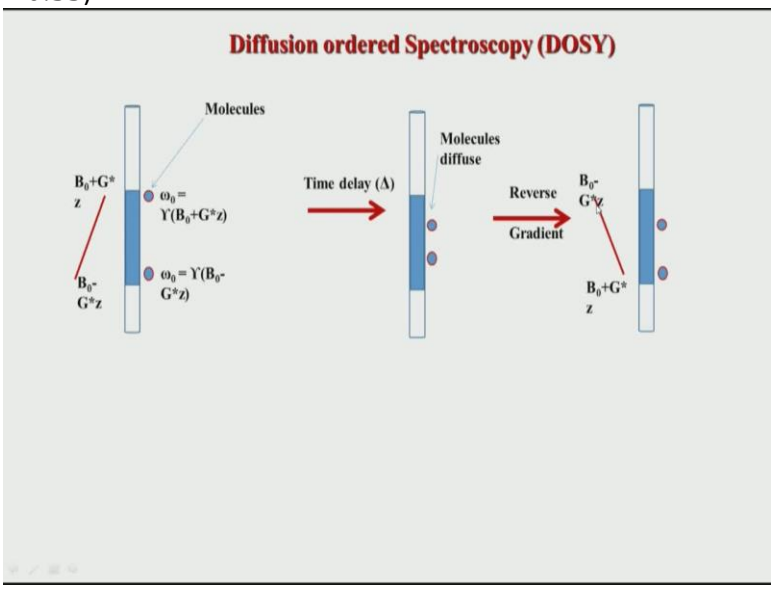

Now let us see how this helps in forgetting the DOSY experiment going. So this is shown here so you can see in a DOSY what we do is the following. You first apply a gradient like we saw earlier. So when you apply a gradient now the molecules are all de-phased because as we just discussed they are all in different locations. So then what you do is you give some time. So after applying a gradient you allow some time delay means you do not do anything just let the system idle.

That delay is called delta some delay delta. Now during that delay delta the molecules are physically translating along the sample. They are diffusing because this is a random motion of the sample random diffusional motion. So the molecules are diffusing, that is the standard diffusion process, now because of this diffusion the molecules now are no longer in the same position so this molecule for example has moved from here at the top part of the sample it has moved to the centre

Similarly one with the bottom part has moved towards the centre. This is just an example which is shown but you can imagine that for all molecules they are randomly moving around so they now start going to different locations. So therefore that depends on this value delta, if you give too much delay too long delta the molecules would have completely gone from one end of the sample to the second part of the sample end of the sample but if you give very short delay they would not have moved at all.

So you see the short and long all depends on the diffusion rate. If the diffusion is slow molecules even if you give long time they would not have moved. So if the diffusion is fast even if you give a short time they would have moved a lot. So this relative term, moving a lot less short time and so on now all depends on the diffusion co-efficient of the molecule. So we do allow some delay, after this delay we now apply this reverse gradient ok.

So what do you expect? you expect that if I apply a forward gradient that is normal gradient and if I do a reverse I should get back to the original complete signal ok but during that will not happen because of this delay here. Because of the delay, the magnetization have lost their original position. So remember this de-phasing, re-phasing works only because if they have not moved at all.

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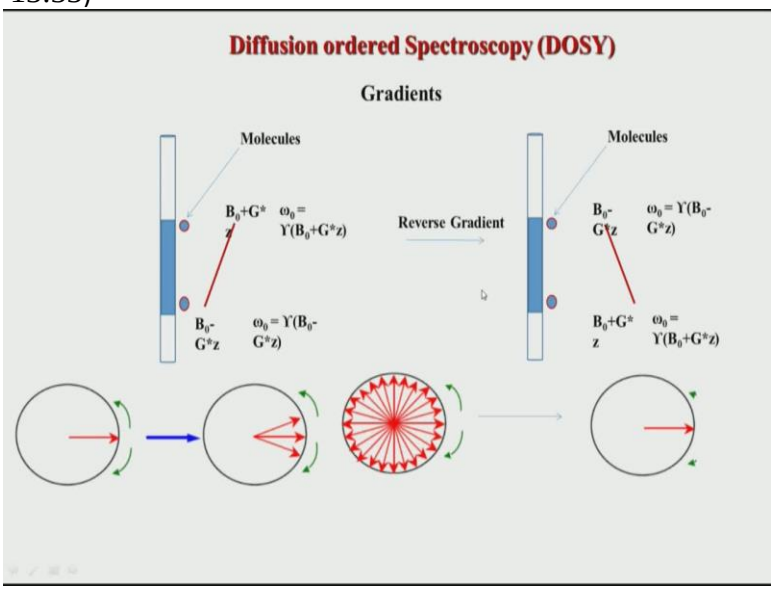

So if you recollect in the previous slide, so let us go back to the previous slide. Here we saw that this re-phasing which is going from complete de-phasing to original phasing I mean original in phase happens only if the molecules has retained its original position because whatever you see the term z comes here. So the omega not depends very critically on the z value means how far it is from the origin.

Suppose I put the origin as centre I can say this much is the distance or this down is the distance, so the phase it accumulates the phase phi we saw it is equal to omega into t but that phase will

depend therefore on the z value. So if it has to re-phase means it has to exactly come back to the original position it should not move at all because if it moves then this z will not be will be different from this z here.

The molecule will be at a different location. So therefore its phase will not be completely comeback to original, it will continue to be slightly de-phased. So this perfect re-phasing what is shown here therefore will work only if the molecules have not moved from their position, means the molecules have not diffused. So therefore this gradient should be immediately applied as soon as you apply the first gradient.

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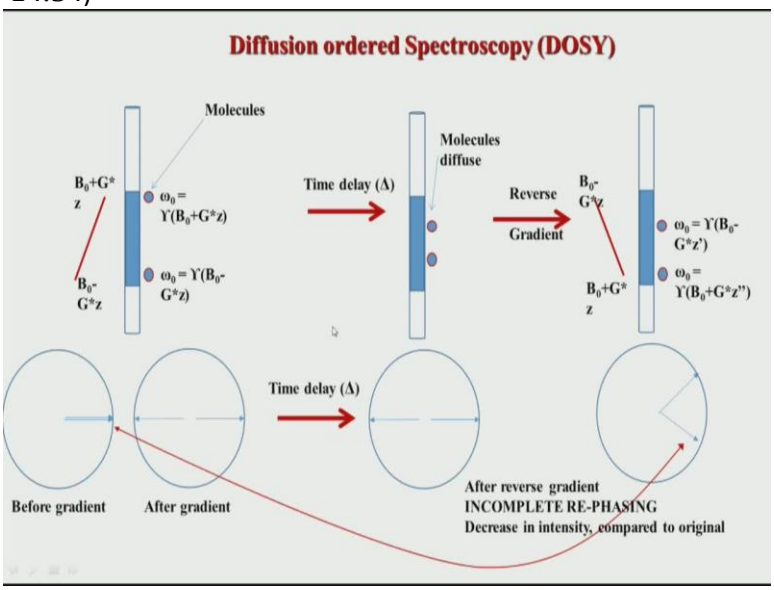

But in this in DOSY we apply we give a delay and because of this delay the molecules when they move they do noth located in this they are not located in the same locations so they do not completely re-phase back and this is exactly what we want. We do not want it to be re-phased completely because if it is re-phased everything is back to the original position that is not useful for us. What we want is some amount of de-phasing because of the diffusion and that will give information on the diffusion process.

So let us see why vector picture what is happening. This is what you saw. If you have let us say two spins so I have put two arrows and this corresponds to these two spins. Then after the

gradient is applied suppose they are out of phase means their one is exactly opposite to the other. So there is no signal. If you add the two signal it is opposite so it is zero.

So now when I apply the gradient delay its phase does not change because molecules are moving but the phase has nothing to do with the molecule movement. It is just remains like this but now when I apply a reverse gradient because the positions have changed so we can see here it is z dash and z double dash. Means just saying that these two molecules are no longer at the same location are positioned as before. Because of this when you do the undoing by reverse gradient it does not bring them back in phase because they are no longer as the same position so this phase which was here perfect together is not together now.

So what is the consequences of this? The consequence of this is there is incomplete rephrasing and that consequences there is a decrease in the intensity of the signal compared to the original signal. So you can see this is the original signal, it is hundred percent phase. So suppose it is hundred + hundred two hundred or x + x 2x signal, if there is x is one signal one spin but here it is less than 2x because if you have to take the component of this along the X axis.

Remember always we take along one of the axis because the detector is sitting either along X or Y. So if you take along X axis this is a component of this signal is less than the original full signal. So therefore along X axis the signal has reduced and along Y axis anyways they cancel each other. So signal intensity has now reduced and this is exactly what we want because why do you want a decrease in signal we want because we will understand how much diffusion has happened and that should be proportional to the diffusion.

So the diffusion is very fast then the signal should be remained fully opposite to each other. They will completely be de-phased but if the diffusion is very slow then the signal molecules would not have moved from here. So they would be still close to their original locations and the signal would still be very close to this full value. So based on this we can figure out whether the diffusion has taken place or it has not taken plac. In other words what is a diffusion co-efficient of the molecule.

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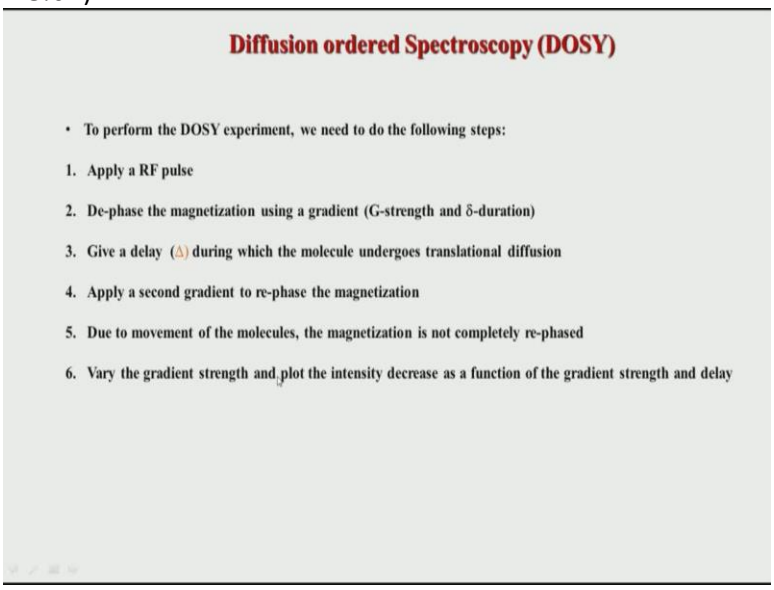

So this is the flow chart this is typically again what is done in DOSY, so we first apply an RF pulse then we because of and then we de-phase the magnetization by using a gradient and this gradient when were to apply we we have to give specify the how the gradient is measured. So gradient is measured by two parameters. One parameter is the strength of the gradient, how strong it is and second is the duration how long the gradient was applied.

The product multiplication of these two gives you the total gradient strength. So for example I can reduce the gradient strength I can increase delta or I can increase the gradient G, reduce delta. The product G into delta is what is what matters. Then after we apply the gradient the molecules spins have completely de-phased, then I give a delta delay, a simple delay where nothing goes on a simple system is completely at idle ok. During that process, during that delay the molecule now undergoes translational diffusion movement. Then after this delay we apply another gradient which is opposite to the first gradient because we want to re-phase the magnetization.

Now this re-phase will work hundred perfectly if there was no diffusion but because of diffusion during this delay delta the magnetization is not completely re-phased. So therefore the amount of decrease in the intensity I can measure by simply two experiments, one I do not apply any delay and I apply a delay or I keep the delay same. I simply in one experiment I do not apply gradient and another experiment I apply the gradient.

So when I do not apply the gradient there is no question of de-phasing. So when there is no question of de-phasing there is no question of worrying about re-phasing. So the signal remains in phase throughout the experiment. That is my first point but then if I am put on switch on the gradient and if the gradient is small then the diffusion effect is less. So I keep changing the gradient the diffusion effects on the signal intensity will keep increasing.

So what the experiment therefore involves changing the gradients strength so you record a series of experiments not just one experiment, DOSY consists of a series means a number of 1D. In each 1D spectrum you keep changing the gradient strength and start and look at the intensity, plot the intensity which should decrease as a function of the gradient strength.

So this is very much in captured by the famous equation the Stejskal-Tanner equation relation which gives you is derived way back in sixtys and that tells you this, its says that their original intensity where there was no gradient which is the standard signal which we expect now is attenuated means decreased because of the application of this gradient and the delay and that is given by this relation.

So in this relation what you can see what are the parameters. D is the diffusion co-efficient which we want to extract which is what we want to find out. Delta is the delay the diffusion delay which we gave long delay here and that is typically of the order of hundred or two hundred or three hundred milliseconds, that all depends on two factors one is how strong is the diffusion and second what is the t1 relaxation of the sample or t2.

This is the gradient strength, this is what we described then this product G into delta come together. So normally what we do is we keep the delta fixed that means the duration of the gradient when when during de-phasing and re-phasing both the delta values are kept same and constant and what is changed between the experiments is this value of G. so this whole experiment is carried out and gamma is off course a constant that is for hydrogen.

So the whole experiment is carried out by simply recording the spectrum at various G values so you keep G zero that is your first signal then keep changing G to something else then you get another signal which is called I like this you keep changing the G values and you monitor the I values.

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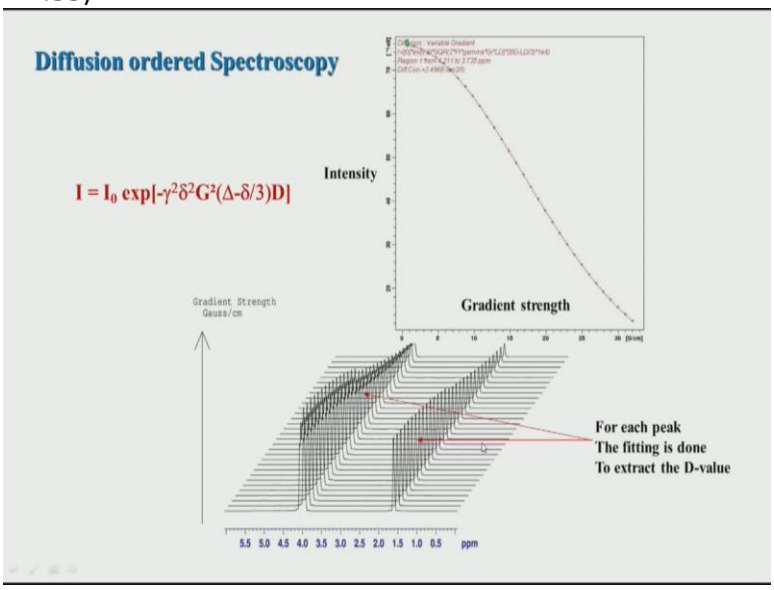

So this can be plotted now as you shown here so you can see this is the intensity that I as a function of gradient delay strength sorry gradient strength G value this G value. So if you plot this you will see you will get a curve like this why because this is like a Gaussian curve. So this is half of a Gaussian. So it looks like a Gaussian curve. So in reality if you see the spectrum will be like this. So let us say this is a 1D spectrum of some molecule and as you record the change the gradient as you change the gradient you will see that this signal is decaying.

Why is the signal is decreasing? Because as you increase the strength of the gradient two things one is you can notice from this equation that if I increase G value my I should decrease. This is simply coming from the mathematical equation here but if you want to understand intuitively what is going on. So what is going on is that when you increase the gradient you de-phase the signal a lot. so if you want to re-phase you have to undo it.

Then undoing will work only when the diffusion is not taken place at all but the moment diffusion is there, a small diffusion also will not allow you to undo or reverse the phasing of the signals which have got de-phased. The stronger the gradient harder it is to rephrase the signal which I have got de-phased. So if the but ofcourse if the D is zero suppose there is no diffusion at all suppose imagine that you have some sample which has no diffusion. if D is zero then the intensity should not change at all.

The whole thing will be zero and e to the exponential of zero is one, so I will always remain I0. So first if your D is not zero then the amount of the signal which comes back because of the re-phasing depends now on this G value. So if I increase the G value it becomes harder and harder to re-phrase them back and the signal becomes lesser and lesser. Why does it become difficult to re-phrase them back? Because a small diffusion D because of D will change the phase value so much that re-phasing will not help because now the locations have changed.

So you need a very accurateh re-positioning for the re-phasing so it would not re-phase because the molecules have moved away. So this is typically how the philosophy of DOSY is works as. So let us see what how does it manifests. So what you do is how do you calculate the D value now? So simply ploth equation plot the function like this and then you can fit this whole points to this equation and from there the D value can be extracted.

So what is done is for each peak in the spectrum a D value is calculate. It is not that D is calculated for the whole molecule, D is actually calculated for each and every peak in the spectrum. So if all the peaks belong to the same molecule, then obviously you will get the same D value for all the peaks but you may have a mixture of molecules which have different D values. So then the peaks corresponding to one molecule will show a different decay profile compared to the peaks belonging to the another molecule in the mixture.

And this is the strength of the DOSY. DOSY helps you now to differentiate between two peaks in the same spectrum that they do not belong to the same molecule. So if you recollect this was what we did using toxy. We identified spin system but here the spin systems are different because the molecules are different.

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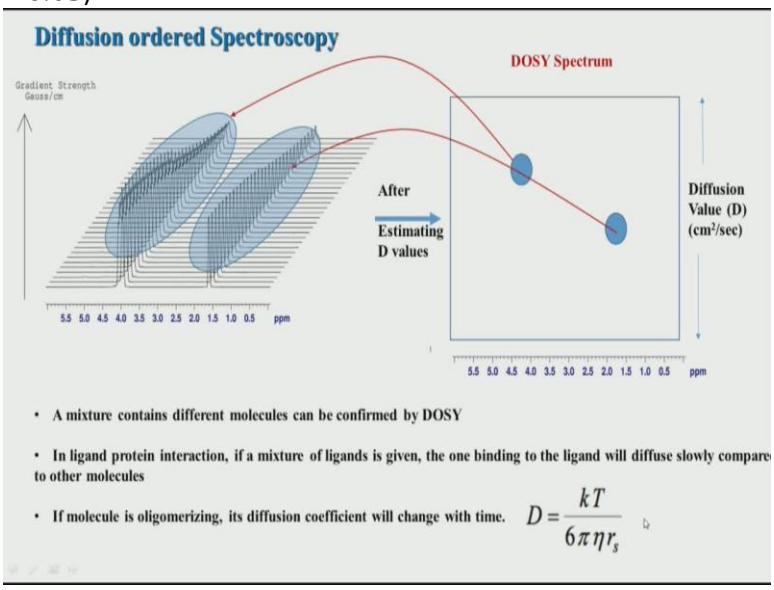

So suppose imagine that these two are not the same molecules these two peaks we have seen here may not be the same molecule then in that case if I extract the diffusion values this whole series profile we use the word profile may correspond to a different D value because it is a different molecule and similarly this whole profile may correspond to an different diffusion and totally different molecule.

So the DOSY spectrum is plotted like this, on the X axis you show the chemical shift value which is same as this 1D spectrum and on the Y axis you plot the diffusion value whose units is given like this. So you can see that if you have two different mixtures, two different molecules from the 1D you do not you cannot make out of course unless you do a 2D, 2D will help you but within 1 from 1D you cannot make out that there are two molecules.

But by simply doing this DOSY experiment you are able to (()) (27:03) them and you can say that this is one molecule and this peak is belonging to another molecule because if they were same molecule they should have been on the same line. Means they should have the same D value but the diffusion values are different and hence they can be called as different molecules. So this is how theh the application of DOSY is.

And DOSY helps you to figure out that the mixture the sample you have may contain a mixture of different molecules and this can be confirmed by DOSY and if you are let us say ,looking at

the ligand protein interactionsh what will happen is the ligand is will have a particular mixture of ligand is given to you ok and you want to find out in that mixture which ligand binds to the protein then you can use DOSY because in DOSY what will happen is all the small molecules will have the same diffusion values but the one which binds to the protein will be slow diffusion because it is now binding to the protein and it is behaving like a big molecule.

It is bound to the protein and protein is a large molecule so the diffusion will be slow. So in that ligand will now will have a smaller diffusion slower diffusion or less D value. So by that you can find out that that particular ligand is now binding to the protein whereas the other is not binding others ligands are not binding. So this is a typical strategy followed when when we try to screen a large number of com.s and drug discovery process where the com.s are huge from a library of com.s but the protein target is known.

So the goal is to find out which of these protein bind to the, which of the ligands bind to the molecules bind to the protein. This is also we use the word affinity NMR. Now if the molecule is oligomerizing, let us say that you are looking at some oligomerization process. For example a molecule is becoming a diamer a trimer or a tetramer and so on. So as it starts oligomerizing it becomes a bigger and bigger molecule and therefore its diffusion will start slowly reducing.

So by monitoring the diffusion co-efficient like this if you record this spectrum in real time as the process is happening in c2 then you will see the D value constantly will keep changing and the molecule will now show a shift along the time or you can also do it for equilibrium. We do not have to do it as kinetics experiment, we can also record slowly an at each stage of oligomerization the D values will change and that will be shift in the signal will be noticed.

Now according to the famous Stejskal equation it says here that the D value is proportional to directly temperature and inversely proportional to the radius, so this is the hydrodynamic radius of the molecule. So that means when that oligomerizes the hydro-dynamic radius will change and if that let us say, it changes and then that will affect it will reduce the diffusion value.

So and this ish viscosity of the sample. So that means the diffusion also depends on viscosity, larger the viscosity of a sample if you have a viscous solution the diffusion will be smaller, lesser. Similarly if you increase temperature diffusion will be faster, so these are different

parameters which determine what will be the D value. So this RS is the hydro-dynamic radius and that is now again remember is related to the molecular weight.

So higher the molecular weight the diffusion will be less, so typically if I double the molecular weight I expect the diffusion to go down by half and so on. So this D and if I double the viscosity again D will go down. So this completes the diffusion of spectroscopy DOSY. In the next class we will take up the last topic of this courseh which is very useful again used very routinely routinely in drug discovery and that is called saturation transfer difference and we will see how that works.