**Principles and Applications of NMR spectroscopy**
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**Module 8**
**Lecture No 38**

In the last class we looked at the different chemical exchange processes. we saw that there is what is called slow exchange and fast exchange and the slow and fastness of this exchange is based on the chemical shift difference between the peaks. So therefore we say that the exchange is fast or slow on the chemical shift time scales. But that is not only the chemical shift time scale that is important, we can also characterize the chemical exchange based on another time scales that is of relaxation time scales or we can also call it from the diffusion time scales.

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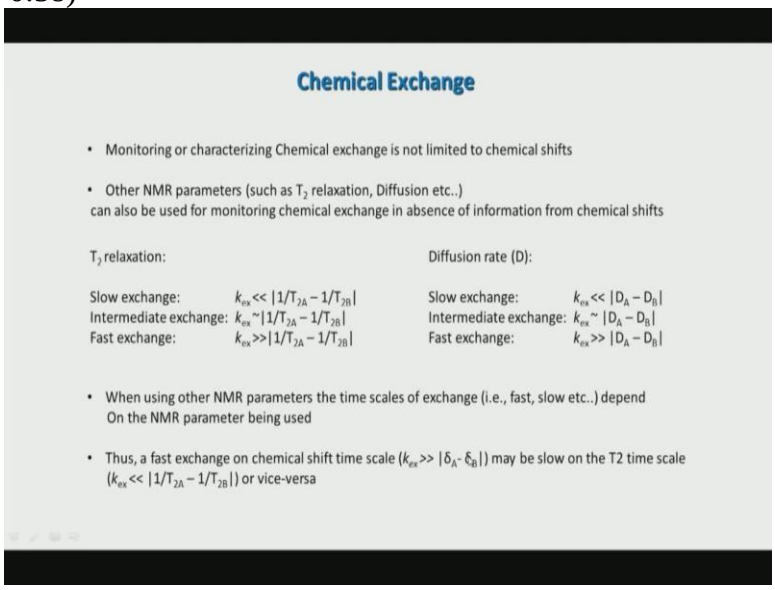


So this is what is shown here that we can use other NMR parameters, such as T2 relaxation, diffusion, etc which can also be used for monitoring chemical exchange because what may happen is in certain systems your peaks the chemical shift which we need for the two different (pro) states A and B that chemical shift information may not be available. So it may not be possible to measure the chemical shift of state A and state B.

And therefore we may not be able to characterize the dynamics from the chemical exchange chemical shifts. So in such type of systems we will then have to go for the T2 relaxation or

diffusion. So this is how what is shown here that we can also look at these different exchange processes based on these criteria. So we can say that an exchange process rate of exchange chemical exchange is slow if it is much smaller than the difference in the relaxation rate between the two states.

So here we are measuring one over T1 not T T1 directly T2 directly one over T2 because one over T2 is the rate and that is what is this is also a rate. So the exchange rate is very slow if one it is much smaller than this difference in the 1 by T2 of the state A and the state B which we are trying to characterize. And as we saw in chemical shift case we say that this is intermediate exchange if the exchange rate falls almost similar range as the difference in the relaxation rates. And we can say that it is a very fast exchange if the exchange rate is much higher than the difference in the relaxation rate. So we can see here that based on these relaxation properties we can characterize whether an exchange is fast or slow.

Similarly, we can do it in the if you have the diffusion rate for the molecule. So this is the translational diffusion rate which we are looking at here and you will see later in the next part in the next class how it will happen but let us continue, suppose we know that there is a diffusion rate available to us translational diffusion of the two states A and B and we use characterize the diffusional rate by using a constant known as diffusional rate constant for that state A and state B, then if the difference between the two is much higher than the exchange rate, than we say that the exchange rate is a slow process.

That particular chemical exchange is slow. For example, this can happen let us say you are trying to study and exchange between a monomer and a dimer of let us say a molecule, and if there is an exchange between these two states that is monomer and dimer, than we can call we can characterize that by an exchange rate and that is slow or fast will depend on the difference in the diffusion between the two. Now we can say that the intermediate exchange if the exchange rate between the monomer and dimer again for example, comes very close to the diffusion difference in the translational diffusion coefficients of the two states.

And we can say that the exchange rate is fast if the translational diffusion between them is much smaller than the exchange that is the exchange rate is faster than the difference in the translational diffusion rates. So these are also the other parameters which we can use for

characterizing the slow exchange regimes, so we used a word "regime" because whether it is a slow exchange regime, intermediate, or fast not only based on chemical shifts but also other parameters. So this when you can use other parameters, then we have to measure them by some methods, so for example T2 exchange can be measured by different experiments which we call we will not go into detail in this course.

Similarly, your diffusion rates can be measured by what is called "DOSY" which we will see in the next part and so on. So therefore we can also use not only chemical shift but also the exchange rates. But one thing you have to keep in mind is that a particular exchange, let us say a fast exchange on the chemical shift time scale may be fast, means it may fall in this particular regime and you can say that in the chemical shift time scale they are fast, but it may be slow on the T2 time scale because it may come into this condition. So it is not that if something is fast on chemical shift time scales is also fast on relaxation time scales or vice versa it all depends on this differences. So our exchange rates become accessible based on what we are trying to measure.

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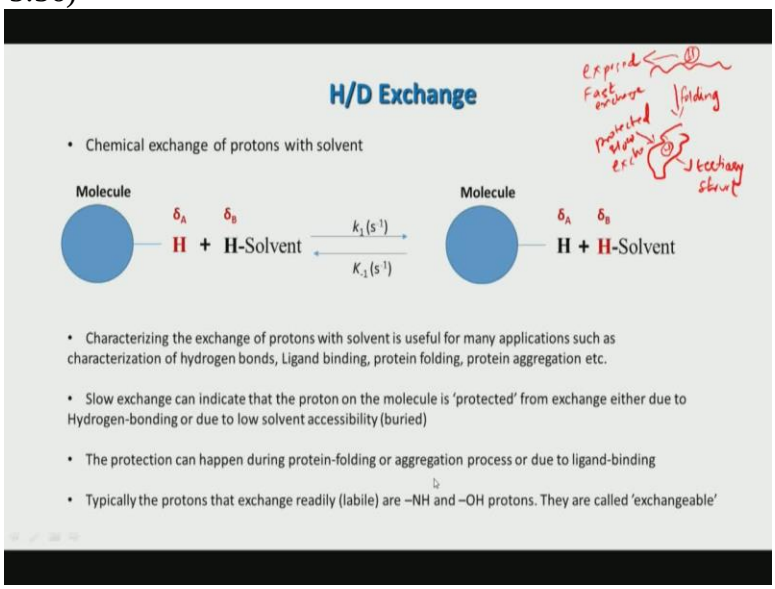


So now let us move to the next topic which is again based on chemical exchange is what is called hydrogen deuterium exchange H/D exchange for in short, and this is a very routine experiment measurement which is done on many peptides and proteins because it helps us to tell whether that particular hydrogen is involved in a hydrogen bond or is it exposed to solvent and so on. So what is done in a hydrogen exchange? We do the following:

You take suppose this is a molecule and this has a hydrogen molecule attached to it, let us call it as chemical shift delta a of that hydrogen. Now with this molecule solute will be dissolved in a solvent. So suppose solvent is a protic solvent, means it has a hydrogen which can be exchanged and that hydrogen, let us chemical shift let us call it as delta b, so this will be for example, water or methanol all the protic solvents.

Now what will happen is if suppose there is an exchange between the two, that is a solvent hydrogens and the molecule hydrogen, this is typically happens in peptides and proteins when we dissolve in water, and what are those hydrogens typically it's the backbone amide hydrogen which we are going to look at. So the backbone amide hydrogen nhco nh, that nh is called a labile hydrogen because it is exchangeable with this hydrogen of the solvent.

And this exchange now is a reversible process it goes and exchange it with a solvent so you can see by the color here that this hydrogen which is black we comes to this now and it has become come to the molecules, so it has got now this chemical shift but this hydrogen which was on this molecule has migrated to the solvent and it has become a different chemical shift. So therefore there is a constant interchange between these two hydrogens and that is a reversible process and we can call it by we can denote it like K1 and K - 1.

So this is again the similar to or same as a chemical exchange process which we saw earlier. So now why is it usefull, why is this experiment useful? This is few reasons are given here, so one of them is that when you characterize the exchange is very useful such as, as we discussed for hydrogen bonds, ligand binding and so on. So for example let us say that there is a very slow exchange between them, that means this two hydrogens do not exchange rapidly very slow, let us say it could be even hours or days that means in one hour it may exchange only once. So it may be very slow exchange and why is that very slow?

That is because, this hydrogen may not be available for exchange and that is what is written here that it is protected from exchange because for either because of two reasons, one is it could be because of hydrogen bonding that is this hydrogen is bonded to some other hydrogen some other oxygen in the molecule and therefore it is tightly protected, it cannot be released for exchange. Or it could be that this hydrogen is buried inside the molecule, deep inside the molecule for the

solvent molecule cannot penetrate or this cannot come out of the molecule to exchange. So therefore that could be another issue or reason why the exchange can be slow.

So when does this kind of a protection from exchange happen? Suppose let us say you are studying protein folding process in which the molecule is a linear molecule a linear peptide starts to form a tertiary structure. So let us let me schematically show you by this picture drawing here, so suppose I have a linear peptide like this, and when I start folding it, or when it starts folding in an environment it may form now a secondary 3D structure tertiary structure.

So during this process suppose there is a hydrogen atom here, that hydrogen atom comes here and it may be it gets protected and it comes into the system inside the structure. So therefore, it becomes protected so here we can say it is exposed. So it will be fast exchange means it will exchange rapidly. But here it is protected, so we can say it is slow exchange because it is not available to exchange. So this can happen during protein folding, so hydrogen exchange therefore is a very popular experiment to study this folding process and we people look at how this hydrogen exchange rates chemical exchange happens between solvent and the proton during this process, so this is important.

Similarly, you can also have a situation where a protein or a peptide or a molecule is aggregating means it is forming oligomer, dimer, trimer, tetramer and so on. And during that process the hydrogen which is exposed in a monomer may start now getting buried because it is now in the interface, interface means between the two dimers it is forming an interface between monomer and dimer between one molecule and other in the dimer. So it now gets protected from exchange so that also can result in a slow exchange from fast. So initially it is fast because it is monomer but it becomes a dimer or trimer it may become slow.

So therefore that also helps us to hydrogen exchange helps us to characterize this aggregation process. And typically the protons as we discussed are the one which we are studying are the nh protons and the oh protons, oh is a side change protons in the case of sarin enthreonine and so on tyrosine, but normally they exchange very fast the most standard protons which we use for this characterizing this hydrogen deuterium exchange are this nh backbone amide they are called exchangeable hydrogens.

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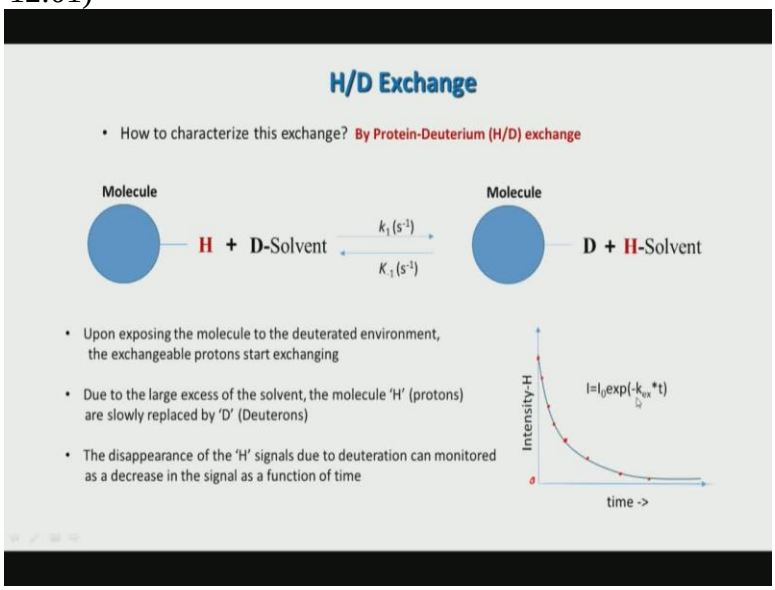


So let us see how we characterize this exchange, we use this word called "protium deuterium exchange". So here what we do is we take the molecule again which is shown here but this time we will use a deuterated solvent, we use a deuterated solvent, the reason is that when the hydrogen exchanges with deuterium which is as shown here then this molecule the deuterium comes to the molecule and hydrogen has gone to the solvent this because of this deuteration we use the word deuteration because of the deuteration the signal from this hydrogen is gone.

That means the chemical shift delta A which was coming from here the peak now this disappears, where does it disappear? Because now it is replaced by a deuterium deuteron. So therefore, because of that the signal is lost so the more the exchange takes place more is the signal lost. So we can actually monitor the loss in the signal intensity of the molecule of this hydrogen and with time, so as it is reducing with time it means it is exchanging with slowly with time. If it does not exchange at all if it is very slow exchange then this hydrogen intensity will not reduce and it will be almost constant or very slow decay.

But if it is exchanging rapidly it will decay very fast. So based on the decrease or decay of the intensity of this hydrogen signal we can characterize its exchange whether it is a slow exchange or a fast exchange. So this is what is written here that one we expose the molecule to a deuterated environment the exchangeable protons will start exchanging, and because of the large excess of

the solvents so this remember this is a millimolar, a very less whereas solvent is in molar, very high.

So the molecules protons now are slowly replaced with deuterion deuterium, and therefore the proton signals starts disappearing or reducing in intensity due to the in deuteration. So therefore as the signals disappear in terms of the intensity the decrease as a function of time that can be monitored and if it is slow, we have can we can measure up to a large time long time, but if it is fast it has to be done rapidly. So this is typically the profile which we will get that the intensity of that hydrogen atom which you are looking will slowly decrease with time and this is typically an exponential decay and that can be fit to a exponential decay equation like this intensity.

So we intensity is measured at various points so you may have intensity measures at let us say initially zero before you put it as soon as you put it you may have intensity here, you may have intensity here, you may have measure here, you may have measure here, you may have measure here, and so on and after this it is almost zero. So by measuring this kind of large number of intensity points in between, we can actually characterize the you can measure these points and fit it to this equation and from that equation we can extract the exchange rate, K exchange, and that is very useful because if K exchange is slow we can say that it is protected and if this is fast we can say it is exposed. So there are lot of methods which are in known in literature to measure the exchange rates.

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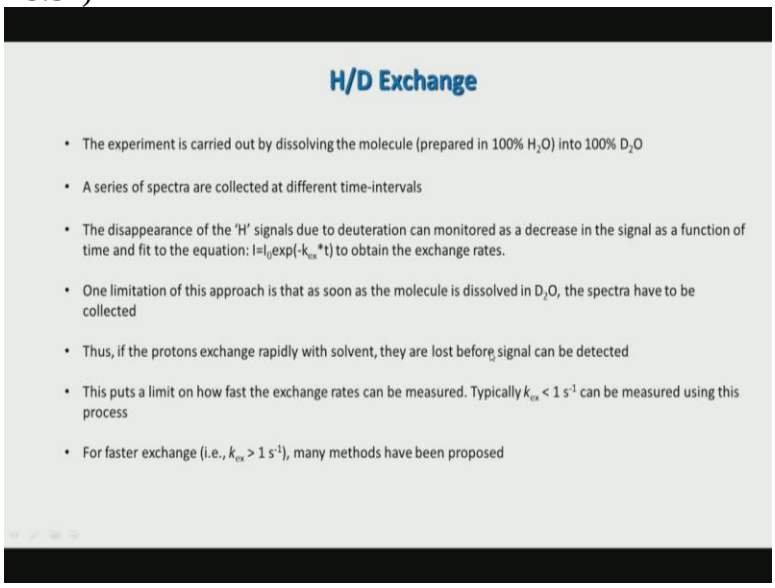

So typically this is how the experiment is done the experiment is carried out by dissolving the molecule in D2O 100% D2O. And then a series of spectra are collected at different time-intervals, so every five minutes or ten minutes you measure and then as we saw you fit that decrease in the intensity to this particular equation to measure or obtain the exchange rate. But what is the limitation of this approach? The limitation of this approach is that as soon as you dissolve in D2O, the protein will, the spectra has to be recorded.

Why? Because as soon as you add the D2O to the sample, all the nhs will now start exchanging with the D2O. So if there is a very fast exchange by the time you record the first spectrum after dissolving, your signal would have been already gone to zero because that exchange would have been very fast for that proton and that proton will be completely absent disappear from the spectrum. So when it goes completely absent you cannot measure this profile because you do not have any intensity measurement.

So that is the big drawback so we have to increase or speed up how we do this and many protons exchange rapidly with solvent, so that is why those this typical standard method does not work and how was the length the limit of this method? The method typically works if your exchange rate is one per second. That means if the hydrogen and deuterium in the solvent exchange one per second, one time per second, once per second less than that, means if they less than once per second, than you can say that the exchange is slow and that can be measured by this kind of approach.

But your exchange rate is faster than that, then you cannot use this approach and there are many alternate methods have been proposed to measure exchange rates faster than one per second. So we will not go into that because that is a more difficult I mean more research oriented work, but if one should keep in mind that exchange rates slow exchange rates can be only measured if you use this approach where you just dissolve the (sol) molecule in 100% D2O and you monitor with time.

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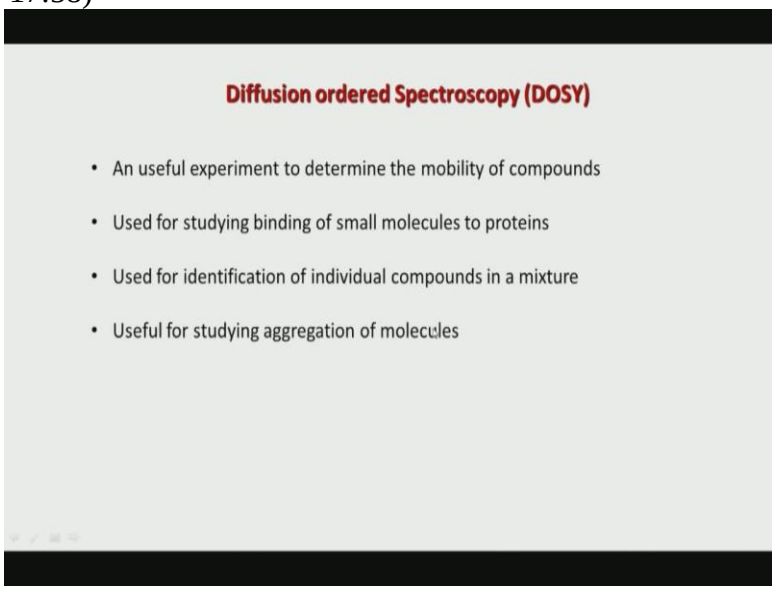


So this brings to the end of this hydrogen exchange part, we will now move to the next part of this advance lectures in NMR course, so this is next topic is what is called diffusion ordered spectroscopy or in short DOSY. So DOSY is a very standard technique very useful technique used in chemical research in anatomical chemical research for characterizing different com.s and few of the importance few of the applications are listed here. So typically we measure diffusion if you want to characterize the mobility of com.s.

So what is mobility? We are now talking about translational mobility not rotational, the rotational diffusion is another type of diffusion, translational diffusion is another type diffusion. So our experiments all this experiments under the word DOSY is based on the translational diffusion of com.s. And this DOSY is also useful for studying binding of small molecules to proteins because small molecules have a very fast diffusion, they diffuse very fast in your in the sample, but when they bind to the protein their diffusion is now reduced because proteins are big molecules so they are heavier and they do not translation diffusion is very slow.

So by change noticing the change in the diffusion we can characterize the binding and this is one of the standard approaches used in drug discovery where they also use a word affinity NMR where this screening a large number of molecules to proteins for a receptor or a target suppose this is a target protein against which you are trying to develop drugs in your screening com.s the

one which bind to the drug, one the one which binds to the protein target strongly will diffuse slowly, and the one which does not bind they will diffuse fast.

So by like this method you can screen or find out which of the huge number of molecules we have which of them actually bind to the target protein. You can also identify individual com.s in a mixture. Suppose you are given a mixture of com.s and not a pure com. and in that case each com. may have a different molecular weight. So but looking at the 1D spectrum, you cannot tell which molecule is which peak, because they may be similar and in the same range and there is no connectivity information in 1D.

So you can either do 2D NMR or you can also do DOSY in which it helps you to separate in the terms of spectrum which com.s are diffusing faster, which com.s are diffusing slower. So that way you can identify com.s from your mixture. Another very useful application of DOSY is for studying aggregation of molecules, so let us say I have a molecule which is undergoing aggregation, for example forming amyloid or so on. It will go from monomer, dimer, to tetramer and so on.

So at each stage it is becoming higher and higher in molecular weight and at each stage therefore it is becoming slower and slower in diffusion translational diffusion. So therefore by monitoring the diffusion coefficient at each stage of the process I can figure out whether the molecule is become is a monomeric state or how it is going whether it is going to dimer or is going to trimer and so on. So aggregation processes very nicely can be monitored with DOSY.

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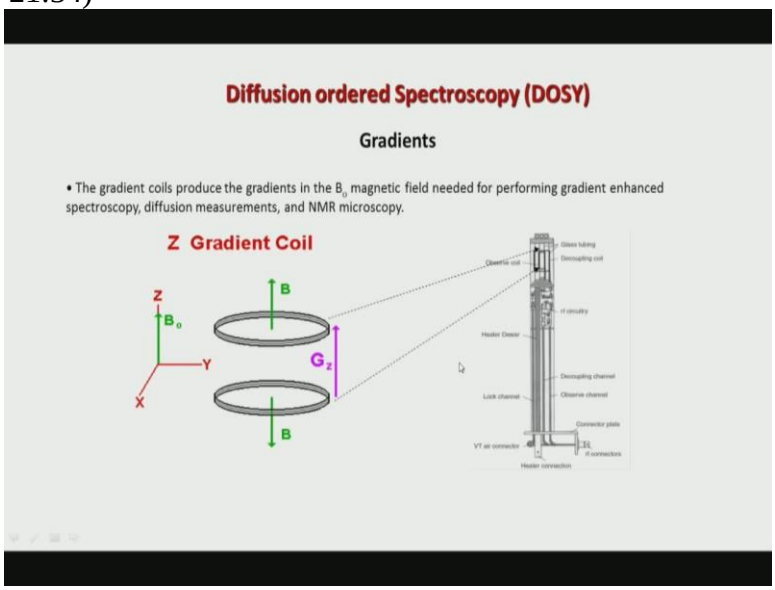


So now let us see how the DOSY experiment works, for this you have to understand what is called gradients. So this is what we have seen in the very first part of the course where we looked at how the hardware aspects when you looked at hardware aspects we had a quick look at what is called gradients. So basically gradients are like coils like this which is in located in this part of the probe. So this whole thing here here is a probe, the probe which goes into the magnet in the central portion of the magnet and this probe has a RF coil here you can see in black color which is the coil for pulsing and receiving signals from the samples.

But on the top of this coil top and bottom you have this circular another circular coils which we call it as gradient coils. She Now what are gradient coils? Gradient coils are nothing but then you apply a current in this coil so whenever there is a current in this coil remember a magnetic field is generated in the center of the coil. This is a standard laws in physics that when a current flows in a coil in perpendicular direction there is a magnetic field generated at the center.

So if I flow it in a clockwise direction I will get this direction, if I flow current in this coil in a opposite direction anti clockwise, then I will generate an opposite magnetic field, okay. So this is how we do that and here you see this is a main magnetic field, main meaning the big magnet which is outside which is where it is probe sitting in the magnet and that is the big magnet B zero. And this magnet field which is generated by putting current in this coil is a very small

magnetic field very small and it is in the milligauss millitesla level whereas this is in the tesla scale.

So you see this is thousand times less and that is a very small magnetic field, but what is happening now is what is happening is I am applying this current here, so my sample will be sitting in this coil, so then it will experience additional magnetic field on this direction towards the top this is a top part of the sample. Whereas the bottom portion of the sample will experience an opposite magnetic field because now it is now subtracted from B zero.

So this is like a chemical shift type of a thing where a molecule at the top have a different chemical shift because they are now B zero + B because they are now in the same direction and molecules sitting at the bottom of the sample have a different chemical shift because B zero - B because it is opposing the main magnetic field. So the whole idea of DOSY relies on this gradients, we can only do DOSY if we have gradients in our spectrometer and now a days all modern spectrometers have this gradient coils so it is not a big problem to do DOSY experiments on any standard spectrometer.

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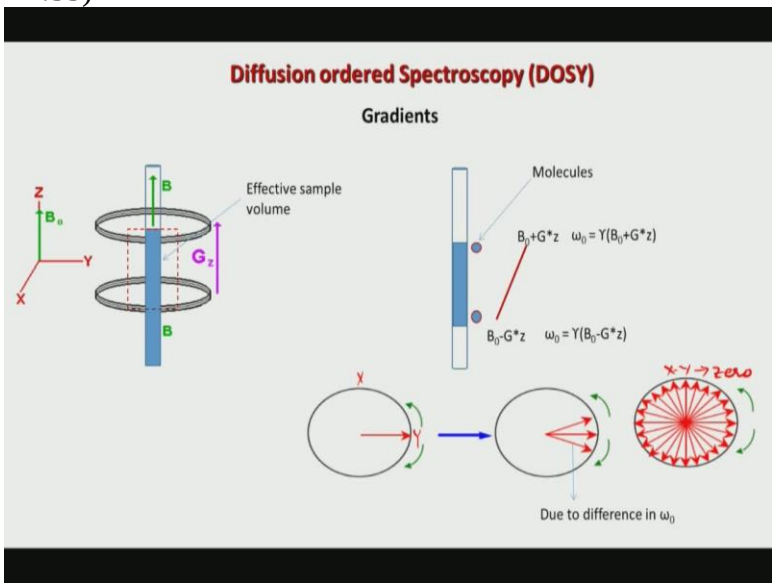


So let us see again this concept how this affects the sample, so this is what is shown here that I have now shown a sample here this is a sample tube and what happens is typically you have what is called an effective sample volume, means your sample which is sitting here this portion of the

sample is what is affective because all your coils RF coils is here your gradient coils are here and above and below has no effect on the sample. So our main focus will be on this rectangular portion of the sample which is exposed to this gradient coils.

So this is what is shown here, now as I said in the last slide because of application of this magnetic gradients now the magnetic field at this portion that is a top part this are the molecules shown here, two molecules let us take one at the bottom of the sample, one at the top of the sample. Now these two molecules do not have the same chemical shift or the precession flarmar prescession, Why? Because at the top portion of the sample I have added an extra magnetic field which is given by G into z, what is z? z is the distance from the center.

So at this point it is a maximum and it is now the chemical shift of that molecule which is sitting here is now slightly different value compared to the original omega not equal to gamma B zero. So we have additional addition of a magnetic field. Now if you look at the bottom of the sample it is opposite, remember we saw in the previous slide it is opposite to the main magnetic field so I can say it is subtracted so it is merged. And what happens in between? In between it is a range of values.

So for example if you look at the center, center this up magnetic field cancels the down magnetic field so at the center the magnetic field because of gradient is zero but this is still there B zero. So at the center the chemical shift will be omega equal to gamma B zero, there is no G and z, no G z is zero center means z is zero, but if I go down towards this side then the negative magnetic field dominates. If I go towards top of the sample the positive magnetic field dominates. So therefore we say that we have created a gradient, this is why we use the word gradient coils. We have created a gradient of a magnetic field so the magnetic field is no longer across the sample we have purposely deliberately created a difference and that is the gradient linear gradient and this is along the z axis, so this is around the z axis we have created this gradient.

Now this gradient, what it does? Now if I apply a pulse to this sample then the sample the magnetization as we saw will comes to this direction xy direction when I apply a pulse. Now when after the pulse when I apply this gradient, these two currents in this coil then the molecule which is here will have a different chemical shift compare to the molecule which is here. So all the molecules now do not have the same chemical shift, remember they are the same molecule

but, because of this change in the magnetic field across the sample the molecules here will have different chemical shift compared to here, compared to here. So therefore they will now start rotating or oscillating with different chemical shift.

So very similar to what you saw in the T2 situation, the T2 also because of the local magnetic field fluctuation that T2 we saw that there is what is called dephasing, but here also there is dephasing but this dephasing is not because of T2 it is because of the difference in omega not, and that difference in omega not is coming because we have purposely created a difference by applying a linear gradient and that is shown in this picture here there, that is completely dephased.

So after sometime suppose I keep the current on this coil for let us say for few milliseconds typically we record apply this current for a very short duration it is about 100 microseconds to 1 millisecond in that time the molecules completely get dephased and same molecule which is the same chemical shift before, but now because of the gradient has now acquired a range of chemical shift along the sample and has completely gone in all directions and it is dephased.

So that means if I now record a spectrum, I will not get any peak at all. Why? Because complete addition of this is zero. So let me point out that this is in the xy plane so this is let us say x axis and this is y axis, so in the xy plane the signal is zero. So this is called complete dephasing of the magnetization. So we will see in the next class how now this can be used for measuring the diffusion of molecules and how this will be very nicely we can calculate how the molecules are translationally moving in the sample.