Principles and Applications of NMR spectroscopy
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Module 3
Lecture No 12

In the last class we looked at how to a setup an NMR experiment and we looked at the different pre-sample preparation steps and how the sample should be prepared and what should be the typical concentration. So, today we will continue with that and we will see the different steps which we are required to setup an NMR experiment. So, once you prepare a sample once the sample is prepared then we have to insert the sample in the magnet and this is shown here in this slide.

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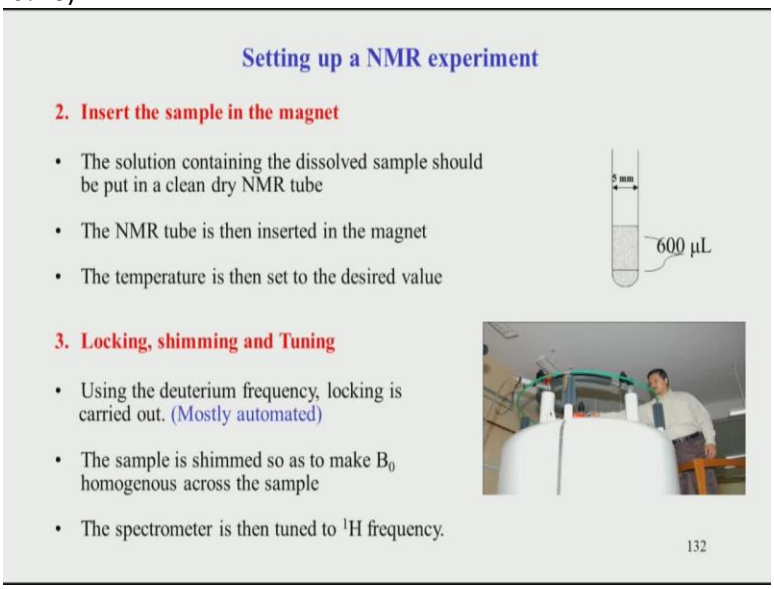

You can see that what typically is done is the first you take the sample in a clean dry NMR tube and this is shown on the right side. This is typically the picture of NMR tube here, it consists of 5 mm diameter glass tube and this is 5 mm diameter it can also be smaller 3 mm and depends on the spectrometer, the standard overall you everywhere is basically using a 5 mm NMR tube and the sample volume, which is shown here the sample exactly about 600 micro litres.

So, this is height of the sample volume this is typically about two one one inch one and a half or two inches, not more than that about one and half . And so, what is done is, this NMR tube is now taken and inserted into the magnet. So, this shown in the picture here. So, this the magnet

and the sample goes right at the top. So, you have to put it in what is called a spinner, a spinner is something which holds the magnet. So, holds the tube, so, the tube is not directly put as such we put it in a spinner and the spinner along with that is inserted into the magnet at the top. And then the once the sample is inserted and it is setting inside so, typically the magnet sample will come all the way down here, somewhere at the centre of the magnet that is at the probe.

So, we are looking in the middle of the magnet is remember white is a board is a open board and that is called that is where we put the probe and the sample goes in sit inside the probe and then it goes somewhere in the middle of the magnet. So, then after the sample is put the system the temperature of the system is set to the desired value. So, this is the temperature is typically controlled in NMR by air.

So you what is done is let us say you want to set a temperature to 25 degree celsius you pass cool air. So, the air will be the cooler than 25 it will be about 15 to 20 degrees and this cooling is done by like a refrigerator which is kept down on the floor and the cool air is then heated to the desired value. So, if you want to go to 25 degree or 30 degrees you heat that air and the air is circulated. So, typically if you look at this picture here this is a sample tube and the air is is given from bottom and there is thermocouple, there is thermocouple resets and monitors the temperature of the sample but remember this is the temperature what you set is not the temperature actually inside the sample.

That that is difficult to monitor, because there is no thermometer or thermocouple possible to insert into the sample and therefor what you are actually measuring is the desired temperature around the sample. To measure the exact temperature inside the sample now there are different temperature calibration methods which are used. we will not go into the detail for those methods. Typically a standard sample like a methanol is used and in the methanol you get two chemical shifts to peaks, one corresponding to the methyl and other corresponding to the OH.

So, the difference in the ppm value between the two peaks can be is related to the temperature. So by recording a methanol spectrum one can actually calibrate the temperature. So, the temperature is very important parameter in NMR experiments, because this is a sample dependent and this is a experiment dependent parameter. So, one has to make sure that the temperature what you need is actually what is inside the sample.

The temperature calibration forms a very important step in setting up the NMR experiment. And once you put the sample and set the temperature the next three things are to be done one is what is called locking and locking is something which we saw in the last class and where we had said that you need to monitor the deuterium frequency. The reason being that the magnet continuously drifts we used the word drift, which means the magnetic is lost and because of this drift we have to correct it.

So that it is not affecting it is not affect the spectrum and that correction it is carried out by small current which is applied in a coil which corrects the, current is adds to the frequency and the amount of frequency to be added is based of deuterium signal and this is silently carried out in the background and you will never come to know that there is deuterium spectrum we required there but it is definitely required for locking and therefor your solvent sample should have a small amount of deuterium. Typically the amount of deuterium is used is about 0.5 % if the solvent is protonated. But you can also use deuterated solvents like CDCL3, CD3OD and deuterated water DLD2O, where you do not have to add the deuterium is already present in the solvent.

But in case you are working with protonated solvent such as H2O, there you have to add a small percentage of deuterium to for locking. So once the locking is done as it is said here is mostly automated and then you go the next part, where you actually shim the magnet. Shim basically the word is a has a historical origin, it basically means that you want to homogenise the magnet homogenise the magnetic field across the sample.

So if you look at this picture again here of the NMR tube typically what happens is this is a sample but all the molecules of this sample do not experience the same magnetic field. That is because the magnetic field may not be homogeneous or same across the whole sample. So, to homogenise to equalise that magnetic field across whole sample, we need to do this process called Shimming. So we have seen in this previous class where we saw that what homogeneity, how it is equalised? How it how that results in a very sharp line?

So the sharper the line, the more is a resolution in NMR. So one of the main requirement in a NMR is a line should not be broad. So, and why it is broad many attempts it is broad because the magnetic field B 0 not homogeneous and therefore one has to do Shimming. So, Shimming again

in the todays spectrometers is done primary in very automated manner. So, this is simple command based operation in within few minutes. So, this operation takes about few minutes, few seconds this operation taken another minutes. So, within very quickly you can Shim and Tune lock the system.

The next important thing is to tune the spectrometer to this frequency. So, this is something analogous to what I was telling the other day that suppose you want to tune a radio. So if you are listening to a radio and you have different FM channels and you want to tune to a particular channel, which of your interest. So, you turn the knob and the knob basically Tunes the frequency for the best reception to the frequency which you desire. So, if you slightly turn the knob more or less you can see you will see the blurring of the sound.

So only at the exact frequency at the exact frequency of the radio channel, we get the best reception and audio quality. The same thing is applicable to NMR that at the best frequency what NMR the frequency of a spectrometer you have to tune exactly to the frequency so that the maximum signal to noise you can obtain the maximum signal can be obtained otherwise the signal to noise deteriorates very rapidly.

So tuning is a again a very important similar to what is done in locking and Shimming. Nowadays this is also automated. So this whole operation of locking , Shimming, Tuning typically takes about 5 minutes to 10 minutes and after you put that sample within that period of time and the sample is now stabilized to the temperature and we are ready to go to the next step that is recording the spectrum.

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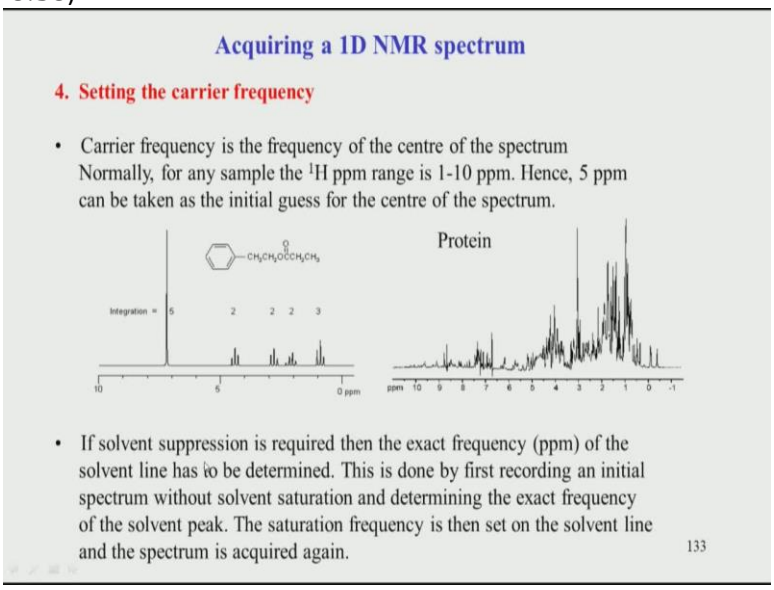

So the first thing so, when you start when you record a spectrum. So, remember in a NMR proton typically the scale for many molecules almost all the molecules, you will see that the chemical shifts always will always come somewhere between 0 ppm to around 10 ppm. So this is typically the range for all the many molecules in chemistry and biology. So, what is done is we have to set what is called a centre frequency so the offset.

So, this is a technical jargon which is used in NMR pulse recording NMR spectrum. So, we say that what is the offset value, the offset value basically represents the centre of the spectrum. So, is very simple idea, centre is around 5 ppm. So, what a typically is done practically is that we take the water. Suppose let us your recording sample in D2O or H2O then the H2O signals comes somewhere around 4.7 at room temperature. So you take that as a centre. So centre is taken as the water frequency but if you are doing it in some other solvent like in CDCL3, which is shown here that is case you take the centre to be around 5 ppm.

So once the this centre is set to 5 ppm you roughly set the whole spectral width. So this is what you have to do next, the spectral width, it is range of chemical shift. So, here is about 1 to 10 ppm. So, what you can do is take the centre as 5 ppm and then take the range of chemical shifts as 10 ppm and then you can record the spectrum.

So, typically for a small molecule like this, you will see the different peaks and will see later how do you interpret this peak pattern. But this just to get the given idea that that the peaks comes

somewhere like this and if you go to a little large, larger molecules like biomolecules-proteins, it is more complicated looking. But again you see here, the peaks are typically coming somewhere between 0 to 10, slight deviations will be there. But more or less, the range is 10.

So, in the beginning when you do not know the chemical shift range, the spectral width of your sample. You can choose about 15 ppm. So, 15 ppm will cover surly the full rang normally absorbed absorbed in molecules and then once you have record a data with 15 ppm, you can shorten the spectral width to a desired value and avoid the extra regions where there is noise. So, this how typically a spectrum is recoded and one of the major things in NMR is what is called solvent suppression.

When we go to the advanced applications of NMR in this course we will see how, what are the different techniques for solvent suppression and this is very important area of research and many papers are published in literature for achieving a good solvent suppression. Now the question is why we do need solvents suppression? You can consider this in following way, suppose let us say, you have a water sample ignore for the moment that we have a deuterated chloroform.

Let us say, you have a sample dissolved in water H2O. Now what is concentration of H2O, concentration of H2O remember is 55 molar. That means if you take a glass of water you are having 55 moles of the protein in one litter, that is 55, sorry 55 moles of water in one litter. So, water has concentration of 55 molar. but typically the concentration of our sample the com. we take is about the order of milli molar. You may take let us take 10 milligram 20 milligram in dissolved in the half an ml or 500 micro litres and that if you calculate you may get around typically about a milli molar or let us say 5 milli molar.

So, now if you compare 5 milli molar with 55 molar, there is factor of 10 to the power of 5 difference. That means the water is 10 to the power of 5 1 lac times stronger compare to the signal of their com.. So, this is called a dynamic range problem. So, dynamic range problem means that you have a huge range of concentration in your sample. One molecule which is your com. has having a concentration about let 5 mill molar. But in the same NMR tube you have water which is sitting at the at the concentration of 55 molar, which is 10 to the power of 5 times.

So when you have this kind of a huge difference, NMR spectrometer cannot work properly. It fails to record a good signal spectrum for your sample, if the solvent is at a huge concentration.

So therefore it is very important to suppress the solvent peak. So, that when if you suppress solvent peak all the way from 10 to the power of 5, let us say to 10 to the power 1 means instead 10 to the power of 5 times larger than the com., suppose it is 10 times larger. Then it is coming within the range at which the two molecules that is water and their com. have a similar order of concentration ok.

It is never possible to suppress a solvent 100%. This for practical reasons which will see in the advance part. But it is not possible to suppress water 100 completely. So, therefore at least if you can reduce it by a factor of 10,000 then you come down to a level where it is similar signal to noise compare to your solve com. and that that process of supressing this peak is called as solvent suppression and there are as I said there are varieties of methods available in literature to supress the solvent and we will see some of the as we move along.

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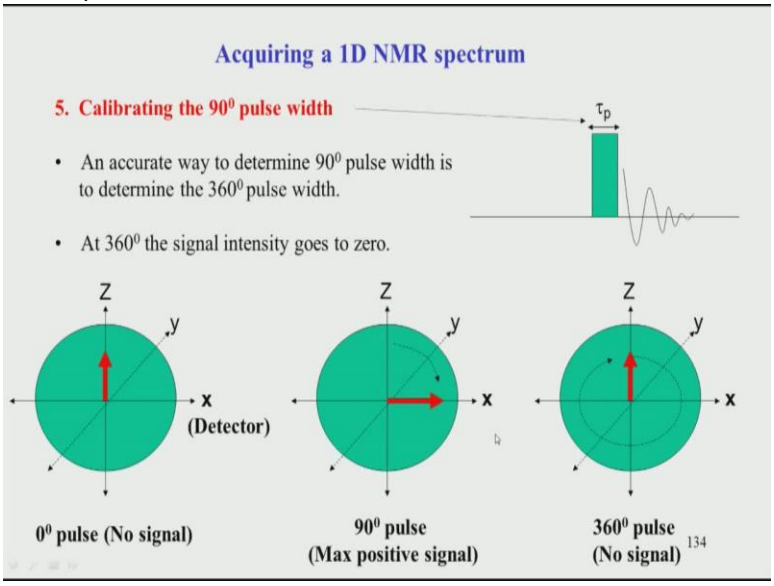

So, once the solvent suppression is taken place the next step is to calibrate the pulse width. Typically what happens is we actually do the solvent supersession after doing the pulse width calibration. So what is the pulse width calibration which is shown here. So, what we are trying to do is remember I showed you a pulse sequence of our 1 D experiment. 1 D experiment consists of a single pulse, which is typically a micro seconds and then after immediately after the pulse you start recording or acquiring physically detecting the signal and that is called F I D.

So, this pulse which is generates the FIDhas to be well calibrated and when we say calibration what it means? It should be a 90 degree pulse.

So, if you remember back the picture remember a 90 degree pulse brings a magnetisation from this z axis if you apply let us say along x axis, suppose this pulse this pulse is applied let us say along x axis then it will come to y axis this red colour vector will rotate by 90 degrees and it will come to the y. If you apply along y axis the pulse, it will go the x axis. So, it will always go to the perpendicular to where the pulse is applied. So, let us assume that let us say this pulse is applied along x axis, x direction. So, we apply this pulse remember pulse is nothing but a magnetic field, a second magnetic field which is applied perpendicular to the main magnetic field.

So main magnetic field is in this direction and we are applying a perpendicular magnetic field, which is much much smaller. It is a very small magnetic field of the order of kilo hertz compared to what you get here, this is megahertz. So, this kilo hertz magnetic field we apply perpendicular the magnetic magnetization, we rotate and will come to this direction the 90 degrees. When it comes to the 90 degrees and suppose you kept a detector here, where the arrow is pointing now. At that point the signal will be maximum because the detector will get the maximum vector signal pointing towards in that direction ok.

So therefor it important to calibrate the spectral the pulse width. That is how long does the pulse take to bring the magnetization from z axis to this y axis the 90 degree. So, the pulse width means how long does the pulse take to bring the magnetisation from z axis by 90 degrees to y axis. So that is very important because if I do not calibrate this properly. Suppose let us say it is not 90 degree I make it 70 degrees. In that case this red colour magnetisation would not have come 100 %.

It would have been sitting somewhere in this direction and then the component of that along this direction is what will be detected. Because detector remember only detects what is coming in this line, it does not look at any other direction. So if the component of this vector along this line is reduced then your signal will be reduced ok. So, therefore it is important to determine 90 degree and that is practically what is done that instead of recording a 90 degree we measure a 360 degree.

So, if you look at this here picture if I record a 90 degree the magnetisation comes here. Which is maximum positive signal which I mentioned. Now but if you do a 360 degree rotation the whole magnetisation has come back full circle and is back to z axis. So, obviously you do not expect any signal at after the 360 degree pulse. Why? because the signal is exactly along z back to z and therefore its component along the y where the detector is kept is 0.

So a simple component analysis if you look at that component of this is zero. Therefore the signal is zero along this axis and the detector cannot detect any signal. So by recording the signal at different angles I can figure, out I get the maximum signal and where I get the minimum signal and that will give me two different values and I can figure out the exact 90 degree pulse value from this exercise. So this exercise is done for every sample whenever you put in a magnet again this is done now a days in a automated manner.

Therefore it takes about 5 minutes to(record) to calibrate to find out the exact 90 degree pulse.

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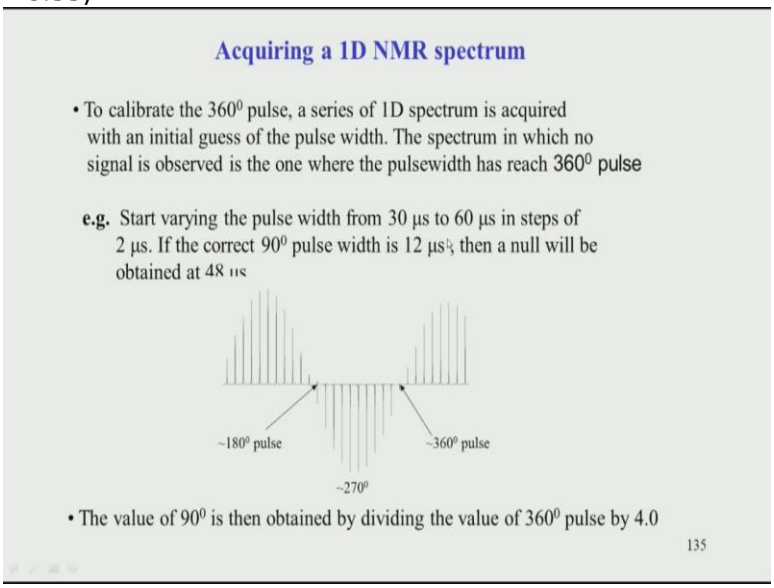

So we can go on to the this is what is shown here, how it is recoded? So typically what is done is that to calibrate 360 degree pulse we go like this, we start from 0 degrees, go to 90 degrees, go to again 180 degrees to 0 ok and then if you look back in the slide. See remember zero is there is no signal at zero. Because the component along y is zero, then if you do 90 degree it is maximum. If I go to the 180 degree is again zero, because 180 degree is perpendicular is exactly perpendicular to this y axis. So, again thus detector is cannot receive any signal because the component of this vector along this direction is zero.

Then it goes to 270 degrees which is here and then 270 degrees again it is perpendicular to y. You will not get but if it is detector is along sorry it goes from here to here. So first 90 degree comes here then 180 is here. Then it goes back to 270, which is in opposite direction. So, when the signal when the vector this red coloured vector is sitting in this direction along this - y. Then the signal is negative because the detector is remember along this line. So, anything which comes along this line, it can detect as a signal. So, whatever the component is along this side will be negative.

When you go back to 360, it is back to 0, because again there is no signal along y. So, this is what is shown here that when you start recording NMR spectrum you will first take nothing zero. Because of zero degrees, then next slowly it is the signal is increases this corresponds to 90 degrees maximum comes down become zero at 180 degree then again goes to negative comes back so on and so forth. So, this is how the pulse width are calibrated.

For example if I know the 0 degree value is this much I just take it by half I mean 0 degree means 180 degree is here. Then I take it by half and that half will give me the 90 degree pulse width. So, typically the value of pulse width in NMR range from about 10 micro seconds to 20 micro seconds. So that is the typically the range will see in NMR. In fact pulse width is a very important parameter to figure out if everything is right with the sample and the system.

So, it is like you know when you go to a doctor he looks at the pulse width of a patient and based on that he can figure out if there is a person has having a particular problem ailment or not. Similarly an NMR expert by looking at the pulse width we can by this exercise, we he can figure out if there is a problems. So, let us say if a sample if you gets about 20 micro seconds value instead of 10 then that means there is something wrong.

So, there could be either the sample has lot of salt in it and if you have a very salty samples. Remember in biomolecules you have to always add some salt to keep the molecule stable and that salt increases the value of pulse width. Because of certain effects we will see later if time permits. But mainly it is because of this loss in resistance and so on.

So therefore if there is a higher salt in the sample, it causes a longer pulse width and that causes loss in a signal to noise or it could that the spectrometer is having a problem the transmitter which provides the R F signal the transmitter has gone bad and therefore it is not able to provide enough power. So, if the power is not enough it what it means that it the pulse width goes up so and so on so forth.

So, as a for an expert NMR a pulse width is very important number which gives a lot of id insight or idea about the system of the spectrometer.

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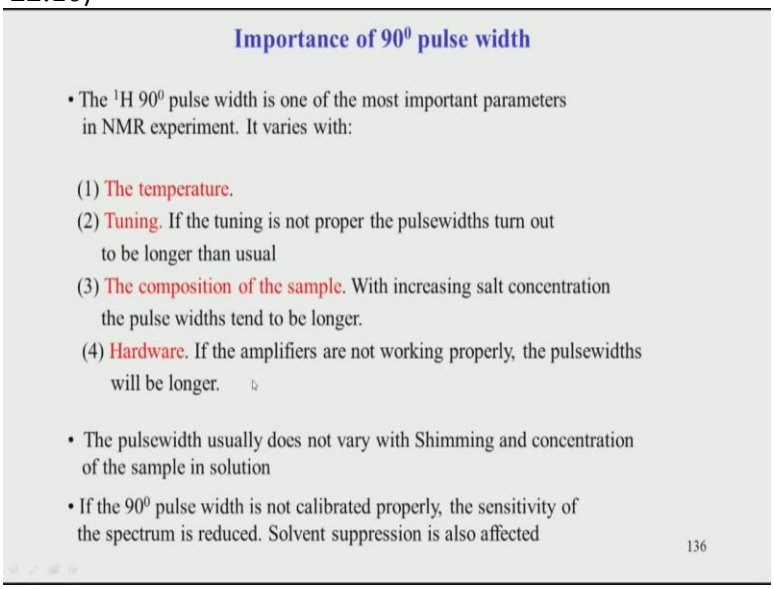

So, this is what I shown here that there are different reasons why the pulse width can go wrong and one of the thing is, let us say if you change the temperature, if you are at a very long, very high temperature alone the spectrometer may not give exact pulse width what you expect. It may be that your tuning was wrong, you did not tune the spectrometer. So, remember in the beginning I said this is an automated step but sometimes one may forget to tune the spectrometer and that will be reflected in the pulse width of the sample.

It could be that the a salt, it has lot of salt is there in the sample and that can cause increase in pulse width or finally as I saying there it could be hardware problem that the transmitter or amplifier not able to provide the enough power to the system to get a very accurate pulse width value. So these are the different reasons why with that pulse width can go wrong.

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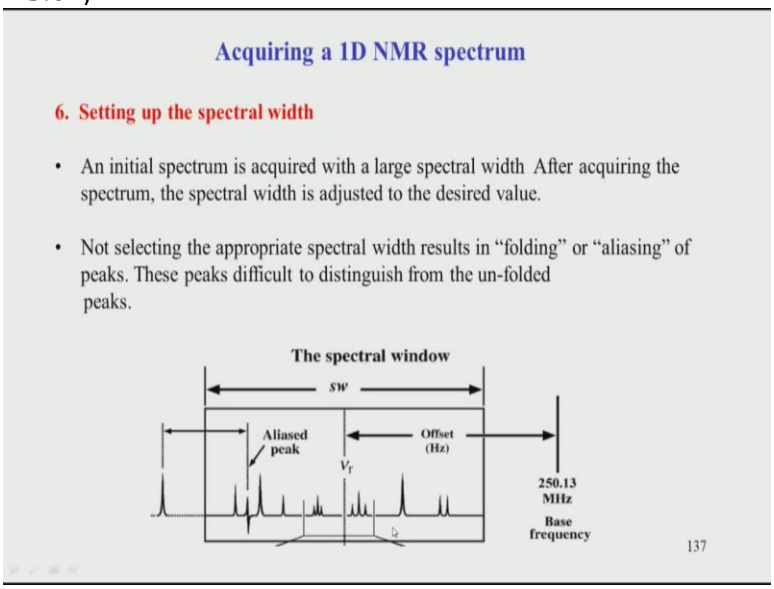

So, the once the pulse width is set properly the next step is is setup what is called as a spectral width. So, these are the parameter. So, we are going basically through this exercise because idea is that when you a record a NMR spectrum one should be aware of what are the different parameters which one should use because a many time what happens is that it is all automatically set and the users of the students do not get idea about what is happening behind the screen. So, this in the whole thing we are going through now is basically to give an idea that what is involved in recording data.

But as I said, as a user you may not even worry about all this, in the end you may just record whatever is the standard default values. But it is important to understand and know what is goes behind goes on behind recording a spectrum. So the next parameter we come to this is spectral width. Spectral width is basically the range of chemical shifts, where you expect to receive your peaks. For example as I said in the molecule typically we expect somewhere between 0 to 10 ppm. So, some 0 to 15 ppm.

So, that is the typical value we set. But now the question arises is suppose I make a mistake, I do not set the spectral width properly or you also use the word spectral window, I do not correctly set it. What happens to this spectrum? So, this what happens is the following. What happens is let us say that you have a peak sitting outside the window and let us say you gave this window to the spectrometer to record a data. But by mistake or by chance which you did not realise there may be a peaks sitting outside this window, what happens to that peak?

Typically what happens is if it is too much away from this window it is simply cut out by electronic devices called filters. The filters will setup a range there will a cut chop any signal out of this or this side. So, what happens is most of the time you may just not see this peak at all. But in the NMR there is a fundamental thing what happens sometimes you will see in this more is 2D NMR. That if you do not set the spectral width correctly this peak which was expected to be seen outside this window comes and appears in the spectrum at this position.

So what is this position? This is exactly this distance, so you take this distance from here, as shown by this arrow. So take this distance and at the same side this distance it will come as a mirror image. This is like a reflection and this for this is called aliasing. This is very a standard terminology used in signal processing. It says the signal is got aliased or in other words we use the word folded. It is like folding.

So imagine that are say you fold around this axis, this is a mirror image this peak will come here. So you may see x a very spurious peak here and you will not know whether that is coming from the actual salt sample or is it a folded peak. So, in that you will only come to know if you open the spectral width. So, let us say you expand the spectral width to now include this peak. Then this peak will disappear and the actual peak at the real position will appear.

So one of the ways to figure out or find out if the peak has got folded or not or aliased or not is to expand the spectral width. So, this is what typically we do, we start from a very large spectral window in the beginning and just scan the region where the peak should come and then second time we record again the data but with a shorter spectral width which will exactly cover all the peaks.

So the first step is done to get the idea about where the peaks are there and after that the second experiment is repeated with everything the same sample all the same setup, except that the

spectral width is then narrowed down to the range which you need for actual data. So therefore if you forget to do this expanded spectral width if you end up folding a peak, the peak will appear in this side. So, this is called aliasing. So, commonly in fact in multidimensional NMR, when we see who will in 2D NMR we deliberately we purposely have to do the sometimes.

Because reducing a spectral width helps you to save time and thus those details we will see that later. But in the spectral width and when we deliberately reduced the spectral width and you miss a peak the peak does not go away it comes back or comes back in a alias manner. And there are different ways to figure out whether this particular peak is aliased or not. So, it does not matter so much if it is folded, provided you know that it is folded.

So as long you know this is a folded peak, you do not care because you can calculate the real value by simply looking the distance from here and taking it on this side. So, that will be the chemical shift actual value. So, this is the problem when you do not set the spectral width correctly.

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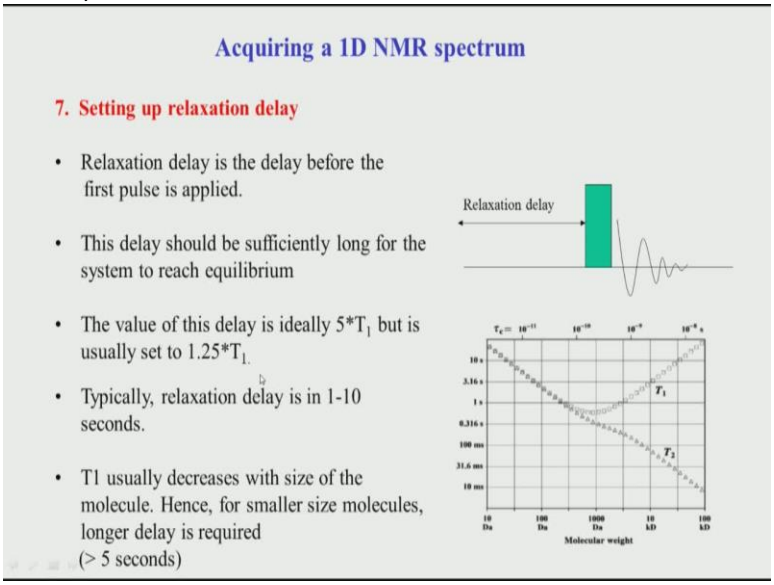

Another next important parameter to setup in a 1 D NMR experiment is what is called relaxation delay. So, if you can recollect in the last class we showed you the pulse sequence. A pulse sequence is basically a diagram a picture of how the different pulses are executed in NMR experiment and one of the first it consist of pulse sequence basically consist of pulses and delays. So, R F pulses and delays.

So, the first delay is called relaxation delay, it is just a simply a delay where noting is happens. Just a spectrometer is sitting silent during this portion of time and what is this period reason why it is required? This is required remember to sufficiently bring the magnetisation back to equilibrium. Because when we apply this pulse the FID is like this and the signal has come to the x-y plane. During this period the signal has come from z axis to the x or y axis.

So, after this period is over if I want to repeat this, if I want to repeat this whole process I cannot start from here because the signal is lying along x and y axis. I have to allow sufficient item for the whole signal to come back to z-axis before I apply a pulse So, to allow that duration for whole magnet magnetisation to come back to z axis, we take the sequence to this period. Means we give a delay between the next pulse.

So the delays applied between two pulses after the detection is over we use the word relaxation delay. So relaxation delay is now typically as us ideally the something like five times the T1 value. So, T1 value is something which you not know a priory of your sample but if you have a rough idea, if you know the molecule size and so on, which is shown here we will see this part again later when you go relaxation topic.

But T1 value can be roughly estimated for a given molecule based on the size and temperature. So based on that value you want to multiply it by five times and that is value ideally remember the word ideally it is required. But practically that becomes a not very good solution. Because let us say the T1 of your sample is 10 seconds so, 10 times 5 is 50 seconds, that is 1 minute. So, 1 minute of a delay is too long.

Because let us say, you want to repeat this cycle thousand times. Because your sample may be having a very low concentration. So, if you want to repeat this cycle thousand times you have multiply 1000 into 1 minute so, 1000 minutes, 1000 minutes is a huge time that to record 1 D spectrum. So therefore what typically is the done is that you do not take five times, roughly you take about 1.5 times or 1.25 times T1. So, this mathematically we can show it although we do not show it in this course but mathematically you can show that this is an optimal delay value which one can choose to get the best signal to noise.

Means the trade off between signal and time remember time is also signal to noise. We saw this in the last class that the longer the time you record the longer the time means longer the number

of scans, more the number of scans you use, better is your signal to noise. But if I have to give a long delay between scans then my times goes up very bad tremendously. But my signal to noise is not improved. Because this delay remember is doing nothing just ideal time ideal time.

So therefore to compromise we reduced this time by to 1.25 the time is also reduced. So, you can record record with more scans now. So, you can see that on one hand you lose signal because of reducing this delay but on the other hand you gain it because of more scans. So, overall optimally mathematically it has been shown that this is the ideal time. So, (()(31:53) let us say your T1 value is 1 second or let us say 10 seconds you just need to about 12 seconds. So, it brings down the time the factor of five from 60 seconds to 12 seconds.

So, this is the practically the value which is used in many experiments. So, typically the relaxation delay is in the range of 1 to 10 seconds for organic molecules. When you go to larger biomolecules there the relaxation delay can be further reduced because biomolecules have a very short T1, means the value will of the T1 is less compared to the value of T1 for organic molecule and why is that so, that comes from this whole theory of T1 relaxation, which will see at a later point.

So this is what is shown here the T1 decreases with the size of the protein increases with the size of protein as increases with the size of protein and T1 decreases with the size of molecule. So, therefore T1 is the parameter which depends on the molecule and once you have a rough idea you have to use a correct relaxation delay between the scans. So, these are the few parameters which we have seen in the setting of the experiment. We will continue little bit more on what are the other more parameter to be recoded or used in the next class.