NMR spectroscopy for Structural Biology
Prof. Ashutosh Kumar and Prof. Ramkrishna Hosur
Department of Chemistry
Indian Institute of Technology - Bombay

Lecture: 59
Basics of solid state NMR spectroscopy - IV

Okay, good morning. So welcome to today's lecture. So we were discussing about basics of solid state NMR and how we can apply this in structural biology. So if you remember in the previous class, we have discussed the differences between solid and liquid state NMR. One of the major difference we have is that in solution state most of the polarization transfer happens through J-coupling, because of the fast tumbling of the molecules, these interactions that are present are averaged out and that is how the dominant mode of magnetization transfer is via J-coupling. However, in solid because of lack of motion, we have a dipolar coupling and quadrupolar coupling and that leads to anisotropic interactions.

However in liquid state or solution state, these anisotropic interactions are averaged out, we have only isotropic interaction. Then we looked at that if we want to achieve high resolution we have to adopt one technique called magic angle spinning, which mimics the tumbling like situation what we have in solution and due to this magic angle spinning we can achieve sharper line. Other important differences that we looked at that inherently we detect on proton in liquid state, however because of the broad lines of proton we detect on carbon. Nowadays with fast spinning we can also detect on proton or when we spin dilute it with deuterium then we can also detect on proton.

At moderate speed the mode of detection is 13C, however mode of detection in liquid state is proton. Because of low γ, the sensitivity of 13C is low and that is why we have a low sensitivity. So 13C detection and broad line are kind of signature of solid state NMR. However, in liquid state we have a proton detection and that is how we have a high sensitivity. So just to summarize what we discussed, the cross polarization and magic angle spinning, these are the two basic building blocks of solid state NMR.

Then we discussed about decoupling that enhances the resolution and then we use recoupling to reintroduce the lost interaction that we had because of anisotropy. So then we use discussed how we can employ this 2D correlation spectroscopy for resonance assignment. We briefly looked at the carbon-carbon correlation 2D spectrum and carbon-

nitrogen 2D experiment, and today we are going to now discuss in more detail how we can use carbon-carbon correlation, carbon-nitrogen correlation for resonance assignment.

Just to remember, at the moment, we are talking about spinning at moderate speed, not very high speed more than 60 kilohertz. So we are talking about 10 kilohertz, 15 kilohertz, 20 kilohertz where dominantly we are detecting on carbons and therefore we are trying to look at the carbon-carbon, carbon-nitrogen correlation spectrum. So, we introduced to you the 2D carbon-carbon correlation which is known as proton driven spin diffusion PDSD or DARR which is Dipolar Assisted Resonance Recoupling Sequences. So what we are doing, we are transferring the proton magnetization to respective carbon and we are trying to establish the carbon-carbon correlation. That is what these two experiments does.

So just to walk through you to the PDSD experiment, we start with a 90° magnetization, which we see here. Now using 90° magnetization we first polarized spins into xy-plane. Then using this cross polarization condition, we transfer the magnetization on carbon. Now my magnetization at this stage is on carbon.

For doing this cross polarization just to remind you again we are just getting this Hartman-Hahn matching condition in the rotating frame so that we can transfer the magnetization from proton to carbon. So at this stage my magnetization is on carbon. So then we are introducing this 2D formalism, where we are incrementing it, to achieve another indirect dimension and that is how you see t_1 here. During that time, we are decoupling the protons.

So TPPM is two-pulse phase modulation pulse sequence is one of the decoupling sequence that we had discussed earlier, one of the decoupling sequence that decouples the proton-carbon coupling. So after that we now encoded indirectly into carbon dimension. Here during this period we are mixing the carbon-carbon magnetization. It is similar like NOESY. We are mixing it in the z-direction.

So here is a 90° pulse and here again a 90° pulse. So we have seen three pulse experiment, in liquid state you have one pulse, two pulse and three pulse. This is similar three-pulse experiment but here we are using dipolar coupling for mixing. Actually, in NOESY also we use dipolar coupling. So here, we are mixing the magnetization and because of that the

magnetization can transfer say from C αto CO, Cα to Cβ, Cβ to Cγ, Cα to Cγ depending upon how long we mix it.

Now mixing without any additional irradiation if it is done that is called PDSD, proton driven spin diffusion. Proton driven because it is driven by protons. Protons are contributing to the magnetization and spin diffusion because spin is diffusing, transferring its magnetization to other spins like a Cα is transferring to CO, Cα is transferring to Cβ and Cγ. So that is a PDSD. However, many times this transfer is not that effective.

So what we do? We apply additional pulse on proton which is of order of spinning speed, half of the spinning speed that assist the recoupling. That is how it is called dipolar assisted resonance recoupling. So it recouples, this facilitates recoupling and the transfer of magnetization from one spins it becomes better Cα to CO, Cα to Cβ, Cβ to Cγ. So using this simple carbon-carbon correlation spectrum, we are now establishing the correlations.

This is kind of the parameters that are used. So here, we have two axis, say proton axis we are starting from here because proton has a high γ, so it has high sensitivity. So we excite protons using 90° pulse. What is the typical power? Like if you are using 2.5 μs of pulse that corresponds to 100 kilohertz.

So what we are doing in experiment little more experimental details. We are exciting protons with a 90° pulse. The duration of this pulse is 2.5 μs. That means the power that we are applying on proton is 1/(2.5)*10*4 μs that goes here.

So that will be $10^6/10 = 10^5$ hertz = 100 kilohertz. So this is the power that we are applying on proton for exciting proton and why we are exciting proton because it has high γ it is more sensitive. Next, our job is to establish a cross polarization condition. Now cross polarization simultaneously we are applying a pulse on proton and on carbon with varying power.

If you look at here we are typically having 67.5 kilohertz, here we are having 50 kilohertz and duration of that cross polarization is 700 μs. Now if you remember here ω_C = 50 kilohertz and ω_H = 67.5 kilohertz.

So if ω_R we are keeping 17.5, so that becomes $\omega_H - \omega_C = \omega_R$, so that is the Hartman-hahn matching condition that we are achieving here. Duration of this cross polarization is 700 µs. Now our magnetization is on carbon during this cross polarization. This is called ramped CP because of RF inhomogenity, we ramp it say 70 to 100 or 80 to 100, this is called ramped CP, right.

So we are ramping, we are changing the power, typically power applied on proton and power applied on carbon that should have matching this condition $\omega_H - \omega_C = \omega_R$, then we have a Hartman-Hahn matching condition in rotating condition. We are back on carbon, we are doing the t_1 encoding the frequency here on carbon while applying a decoupling. So decoupling power is 90 kilohertz, this is called high power decoupling. The TPPM is one of the pulse sequence that we discussed in the last slide. So applying this 90 kilohertz we are decoupling protons while encoding on carbon and then we are applying here again a pulse of 90° on carbon of 50 kilohertz which duration is about 5 µs. During this time, we are mixing the carbon-carbon polarization magnetization of about 20 ms.

So, this can be done without this pulse or it can be done with this pulse. If we are doing without this pulse that is PDSD, if we are doing this pulse that is called DARR. So the power we are applying is ω_R = 17.5 kilohertz and the extra power that we are applying on proton facilitates the mixing, it enhances the mixing capacity and then finally we apply a 90° pulse on carbon of 50 kilohertz. We decouple the protons here using high power decoupling and we acquire on carbon. So this is the typically we are establishing carbon-carbon correlation using one of these two pulse sequence either PDSD or DARR.

Wonderful, so what we get? Here we are getting now carbon-carbon correlation spectrum. It is similar like NOESY spectrum. Here you see 13C carbon frequency, here 13C carbon frequency, here we have a diagonal peak and these are off diagonal peak that shows correlation. So here, if you remember your chemical shifts, here are threonine Cα-Cβ. So this is threonine Cβ, here we have a threonine Cα.

Then if you go around this axis, we have a threonine Cγ. Here are the region for serine and we can have here isoleucine, you can see isoleucine δ, γ, γ2, β, α. So isoleucine you can establish all the way like here say if you go to diagonal peak, here is α of isoleucine, then β of isoleucine here, then γ2, γ1 and δ. Looking at these chemical shifts you can identify

spin systems and if you start mixing more and more the neighboring residue will start contributing. So you can even get near-neighbor effect, so like a Cα of magnetization of this i residue can be also transferred to i-1 residue CO or Cα depending upon how long we mix it.

So it not only gives spin system specific assignment but also near neighbor assignments or even little medium range assignment depending upon how we mix it. So we need to just vary this mixing time, 20 ms is short distance correlation, if we increase to 150 ms, it will be neighboring residue, if we increase to 500 ms even more some long range, 800 ms even long range. So we can establish all these carbon-carbon correlation using DARR or PDSD that helps us in resonance assignment of this spectrum. So I am taking another example of say here K59, K is lysine 59.

So we have a Cα here, if you just follow these lines, so Cα here in red you can get a Cβ, lysine has Cγ, Cδ, Cε. So we can assign all those and Cε comes around 41, 42 ppm, Cβ comes around 35 ppm. So if you know BMRB values of different amino acid, you can essentially analyze it. You see here epsilon of lysine is coming around 42, β is coming here, δs, γs and all those are coming there and which can be assigned. So I am showing you one of the publication from Adam Lange group where they have used this *BacA*, Bactrophyllin, they got a beautiful spectrum, carbon-carbon correlation spectrum and you can see it is as beautiful as NOESY spectrum.

You can use this PDSD based experiment or DARR based experiment for resonance assignment. So these are all short range correlation. You can see here is isoleucine Cα-Cβ, serine Cα-Cβ, then V and Q Cα-Cβ. If you go here this region phenylalanine, this region is for alanine Cα-Cβ, here are threonine Cα. So, all these correlation one can easily establish from Cα to Cβ to Cγ to Cδ, here Cβ to Cγ, Cδ. You can see all the way going from top to bottom, we can assign the spin system specific resonance assignment or near-neighbor assignments by doing couple of months of assignment.

This is beautiful spectrum and can be used for starting the assignment. But that is not enough, this was only carbon-carbon spectrum. So we have to establish heteronuclear assignments for a like a carbon-nitrogen experiment like we have a typically HNCO or HNCA kind of experiment in liquid state. Similarly, in solid we can do this by again polarization transfer. So to explain to you how we do it, I am just explaining you see.

This is i-th spin system, this is i-1 spin system. We start polarization from H^N, using dipolar coupling we transfer to through bond connected N15 of amide, then we transfer to Cα and we establish the correlation between nitrogen and Cα, this will be direct dimension, here will be indirect dimension. So we are establishing a correlation N-Cα and we are detecting on carbon while nitrogen is on indirect dimension, so we have a 2D of N-Ca. Similarly rather going in this direction we can go in backward direction, so this will give i-1 correlation, H^N we are transferring to N15 going to CO. Now i-1 correlation can be established here and that will be kind of HNCO experiment that we have in liquid.

So this is HNCA and this is HNCO, but in solid since we are not utilizing proton so we called it NCA and NCO. Here we are two times transferring the magnetization from H^N to N15 and N15 to CO or Cα. This is called double CP experiment. Ek CP yaha, dosra CP yaha.

First CP here, second CP here. That is what it is called double CP, DCP, double CP experiment. So schematic is something like this. We are starting with proton and transferring here to carbon here, we are starting here. So we can get i-th correlation, we can go back and we can get i-1 correlation in NCO and the pulse sequence is something like this.

So let me walk through you to the pulse sequence. We are again exciting the protons using 90° pulse. So then we are doing the first CP, we are transferring the magnetization from proton to nitrogen. So first CP, directly attached protons magnetization are transferred to nitrogen. Now magnetization is on nitrogen. Then in indirect dimension we are encoding nitrogen.

So this is the t_1. Then we are using another CP. here for transferring. But before that we are just decoupling protons as well as using 180° pulse we are decoupling carbon as well when the nitrogen is evolving. So then when we are at this stage we will use the second CP and we transfer the magnetization from nitrogen to carbon while decouple proton and then we detect on carbon, so this is our t_2 and we are again decoupling proton. So these are the two high power decoupling we are using. One when t_1 is being encoded, when t_2 is being encoded and during this transfer we are using the CW decoupling.

So that is why we have a one dimension N15, another dimension C13 and depending upon how we want to transfer the magnetism, the same pulse sequence where we shift our carrier frequency, the NCA correlation can be established or NCO correlation can be established. Same pulse sequence, just you have to shift the frequency. If you are shifting on CO, we can establish the CO correlation, i-1 NCO correlation. Whenever shifting to 55 ppm then we can establish the CA correlation, so that would be NCA correlation, and when we are shifting to say 175 ppm it would be NCO correlation. So that is how we do it and we get a beautiful spectrum.

This is from Christ Jeroniec group. They have done on some fibril protein. You can see now beautiful NCO correlation you are getting. This dimension is N15. This dimension is C13 and here for each amino acid i-1 correlation we are getting, here for each amino acid i-th correlation we are getting.

So here is like you know this is CO of say 137, this is CA of 137. Similarly, we can get for valine, so you can see CO of 122 valine. So similarly you can establish i-1 correlation coming from here and i-th correlation coming from here. Just by looking at this and using couple of these carbon-carbon correlation spectra you can assign, you can identify all of these correlations. But that is not enough right, we just did 2D, we need to establish further correlations to get a resonance specific assignment.

So what we can do? Can we fuse this? First we perform the carbon-carbon correlation, now we are performing the nitrogen-carbon correlation. Can we fuse little bit this and get a more magnetization transfer? That is what these experiments do, NCACX and NCOCX. So what we are doing here? Starting with a proton we are transferring to N15 encoding here and then we can transfer to CO, but we do not stop it here, let it mix whatever we are doing in PDSD or DARR. So we can fuse these two sequence, the 2D HNCA correlation with PDSD or DARR correlation. So now my magnetization does not stay at CA, it can go all the way to Cβ, Cγ, Cδ, so that will be called NCACX, CX means Cβ, Cγ, Cδ and all those.

NCOCX analogous to like our HN, this is like HNCACB, this is like HNCOCACB. So here, we are transferring starting from proton, transferring to nitrogen going to i-1 CO and then we are mixing that so we can get the i-1 Cα, Cβ, Cγ and Cδ. The third one can be also

depending upon how we are starting. So starting from say Hα-Cα-N then going to CO and then mixing with CX. So this is like HCA NCOCACB something like that.

So i-1 all the correlation we are getting when we are starting from Hα to Cα to N15 going to i-1 residue, Co, Cα, Cβ, Cγ, Cδ. So these can be 2D version as well as 3D version depending upon how many axis or frequency we are labeling it. Now using all these series of experiments, we can almost achieve a liquid like correlation spectrum, which can be used in a similar manner for resonance assignment. Just one of the example that I am showing here. So NCOCX and NCACX, so if you look at here it was NCA experiment but when we did NCACX-PDSD, now we are getting also correlation from Cβ, Cγ and Cδ.

Take any amino acid that we want, K60 here, you see it is Cβ is coming here, Cγ and all those. Similarly, one can take valine here, valine you get valine 53 or something like that, Cγ, Cδ and all those. So glycines, just one peak coming here, right. So you can establish putting NCOCX, NCACX, even using 2D you can achieve lots of lots of resonance assignment because 2D can be done in few hours, 3D will take days. So you can collect a good 2D and that should be enough for resonance assignments of few of these peaks.

So here, I show an example how you can use your DARR or PDSD with NCACX and NCOCX and establishing the correlation. So you can start from say threonine Cα, Cβ, then you can go to Cγ, here you come find its Cβ, then from here Cα. Here you can find the NCα, we can go here and find it out CO of i-1 residue. So this helps us, facilitates us in walking. You can take another example, like here in this region we can get an alanine, so alanine Cα is here, Cβ is here. Now we can come all the way, we get N15 of this, we can get i-1 of this and similarly using NCACX, NCOCX, we can assign each of these resonances that are present.

This is a few weeks or maybe a month job depending upon how much experience you have. This can be used for resonance assignment. Now people did not stop it there. They developed a series of sequence, so one of the sequence that I am showing from Tata-Gopinath and Veglia, they developed the C13-edited CC experiment and 15N-edited NCA experiment, NCO experiment, NCACX, NCOCX, NCACB and many of these where sometimes it is also labeling the protons. So this is a gallery of experiment that they have developed and these can be essentially used for doing resonance assignment.

Tata-Gopinath used multiple acquisitions, so in actually in the same sequence, he can detect various experiments like CXCX, NCACB, NCACX, NCO. So, in same experiment at the different time point you can detect it various experiments. So you save lots of time because these are low sensitive experiment. This is a beautiful exposition of Tata Gopinath and Veglia tried to attempt to cut short drastically the acquisition time and facilitating resonance assignments in a shorter possible time. Now if we record all these series of experiment, you can take a strips.

So you can take a strips and you can sequentially work Cα to Cβ, Cδ to Cγ using various experiment like NCACX, NCOCX. CANCO, NCACO all these are you can see in different colours. Similarly, like liquid state, you can use this strip for resonance assignment. So 128 we can walk to 127, we can walk to using these assignments to 126, 125, 124, 123. So if we assign all those now we have the value of Cα, Cβ, CO, then what is the obvious next step? Then we can get these assignments and we know that these chemical shifts are quite deterministic of the secondary structural information.

So we know from our chemical shift that Cα has only glycine, right. So however these spin system like alanine, threonine, serine, they have Cα, Cβ, and they have a characteristic Cα, Cβ. So it helps us in resonance assessment. Glycine has a Cα only at 45. Alanine has Cα around 52 and Cβ around 22, just two spin system.

Threonine Cβ is lower than Cα. Serine again Cβ is lower than Cα. So using all those signatures, we can start the assignment. These are start point or check point. Similarly, Cα, Cβ and Cγ gives most of other amino acids. Histidine, tryptophan, tyrosine, phenylalanine are difficult to identify because they have a broad line.

But then we can combine their aromatic chemical shift to find it out. Then looking at the distribution of different chemical shift for different amino acid that we have seen, it becomes quite easy for assigning using the spectrum of. You can start from threonine, it has a distinct chemical shift. Serine has a distinct chemical shift, alanine has a distinct, glycine again comes somewhere here. So you can use these distinct chemical shift for resonance assignments and then once you have the resonance assignment then subtract the sequence corrected random coil chemical shift of say Cα, Cβ plot along the sequence.

So here what I am showing you again from Adam Lange group, the ΔCα – ΔCβ, so these are Cα and Cβ subtracted the random coil chemical shift you plot along the sequence, and then if these values are negative, you know that these are β-sheet. So this protein that I am showing you is in rich of β-sheet, you can see using the secondary chemical shift, you can plot along the sequence and you can find it out what is the secondary structure topology. Like liquid state, we got a secondary structure topology, what next? Next is can we get inter-sheets or inter-strands distances? If we fix these distances then we are all set for structural determination of these long elongated fibrillary structures that we have.

So for that I said PDSD is also good enough because that gives you long range correlation. Some of these distances we have to measure using PDSD. But in the next class I am going to show you what else we can use for measuring the long range distances.

But PDSD is good enough for giving you long range distance. You can see 107 to V97. These kind of distances you can even get it from PDSD. Here 100 to 117 the long range distances can emerge from carbon-carbon correlation and that help us getting the topological arrangement of these β-strands how they will be arranged. That is how we can generate a structural model of any fiber. So with this I am going to stop it here today and in the next class we will be taking the idea of how we are going to use these structural information for getting the de novo structure of this difficult protein system whether they are amyloid fibers or membrane protein in their native state or the native setting. So, I hope to see you in the next class. Thank you very much.