Multidimensional NMR Spectroscopy for Structural Studies of Biomolecules
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Lecture – 42
Understanding Protein ligand interaction by NMR Diffusion ordered Spectroscopy (DOSY) – Part II

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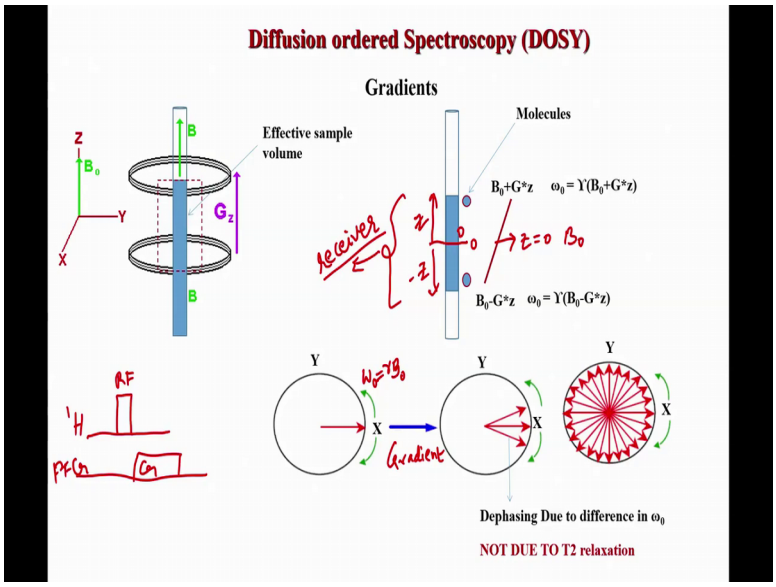

So we will see why is gradient. So, useful we will see that now you will see how for diffusion we can use this approach.

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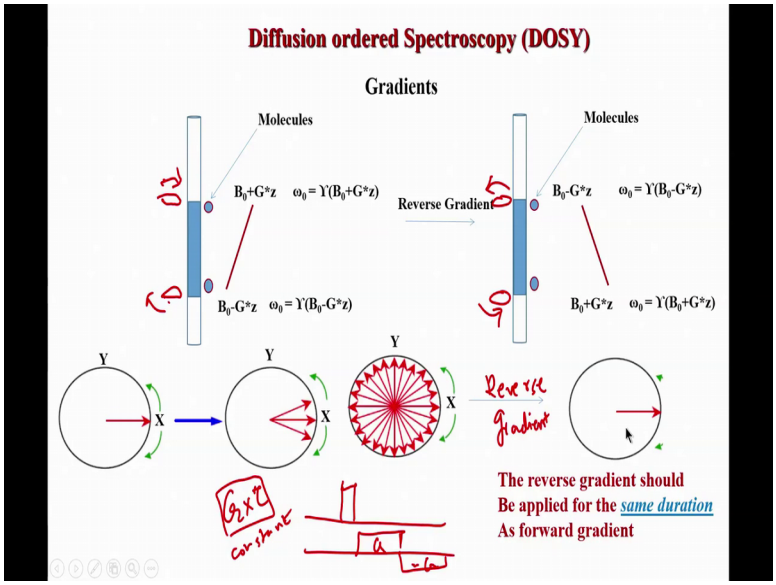

So, now, let us look at it back again the same picture that I have a sample and I have a molecule at one part of the sample on the top and molecule at the bottom of the sample. And you see this same which I wrote in the earlier slide these two molecules are experiencing difference in the magnetic field because, the magnetic field at top is plus G z and magnetic field at the bottom is minus G z.

So, now the same picture that it starts de phasing ok. Now suppose I apply a reverse gradient a reverse gradient means I change the current; I change the current in the top part of the coil. So, if you recall there is a coil here and there is a coil here and in this coil I am supplying current suppose in this direction and in this coil I am supplying in this direction. Now what I do is I reverse it, I reverse the gradient and I oppose the current opposite make the current opposite.

If I do this then what will happen is now this molecule will experience minus G z and this should experience plus G z. So, this is exactly the opposite of what is written here which means the molecular precession what was positive plus G z is opposed or reversed and it becomes minus G z. So that means, if I the complete defacing has happened after this gradient; now when I apply a reverse gradient.

So, when I apply a reverse gradient if I apply a reverse gradient now all this pins which are de phase which are gone suppose let us say this one particular spin consider this molecule each arrow remember is like a one molecule or one proton. So, imagine this as

one proton located at some part of the sample this proton located at some other part and so on. So, now, this proton which has gone by some angle suppose let us say 30 degrees; now will go back in the opposite direction by 30 degrees because of the reverse gradient ok. So, this opposition or coming opposing it makes it come back to the original position.

So, when I apply a 1 D pulse. So, let us look at the how this pulse will be there I apply a 1 D RF pulse and apply a gradient this is the forward gradient then I reverse the gradient ok. So, you can see this is the reverse means opposite, but this gradient and this gradient are equal and opposite; they are not the same I mean they are applied one after the other, they are not applied at the same time, but one is positive G and another is negative G.

So, when I apply this positive G I have got this defacing complete, but when I apply the negative G it will be opposing means whatever has gone to this direction by 10 degrees or 20 degrees or 50 degrees will exactly come back by that much because, of the reverse gradient ok. So, my time should be same see if I apply this for 10 milliseconds for example, I should also apply the reverse for the same time the strength the z value also should be same ok.

So, the strength and the time of a gradient are very important parameters; see if I apply two gradients opposite to each other for the same time and same strength they can I can make everything come back to the original position. Why; because whatever has happened in the forward direction is reversed by applying in the reverse direction.

So, this is a very beautiful approach where in you apply a forward and a reverse and you can actually undo what has been done by the forward gradient, using the reverse gradient. So, this is basically trying to erase the memory; that means, whatever happened here you are erasing that memory by reversing it. So, we will use this approach now in a DOSY experiment to capture what happens during (Refer Time: 04:38) diffusion.

So, let us go further. So, as I mentioned reverse gradient should be applied for the same duration as the forward gradient. So, the duration is also important now; I cannot apply this 1 millisecond long and here half a millisecond or make it 5 here and 2 here it has to be the same duration not only that it has to be the same strength. That means, the area the total area under the gradient should be the same in both. I can change the strength if I want here, but the area should then reduce I mean the length should reduce.

So, what is important basically is let me write down here G into the time; this should be constant means for forward and reverse ok. So, if I increase the G for the forward increase means I have a higher G value then the reverse then my tau for the reverse should be longer than the tau for the forward. So, keep in mind that this product has to be kept constant for forward and reverse; then only you will get a complete reversal means complete coming back in face. So, you start from in face; you de phase because of a gradient apply reverse gradient and reface back.

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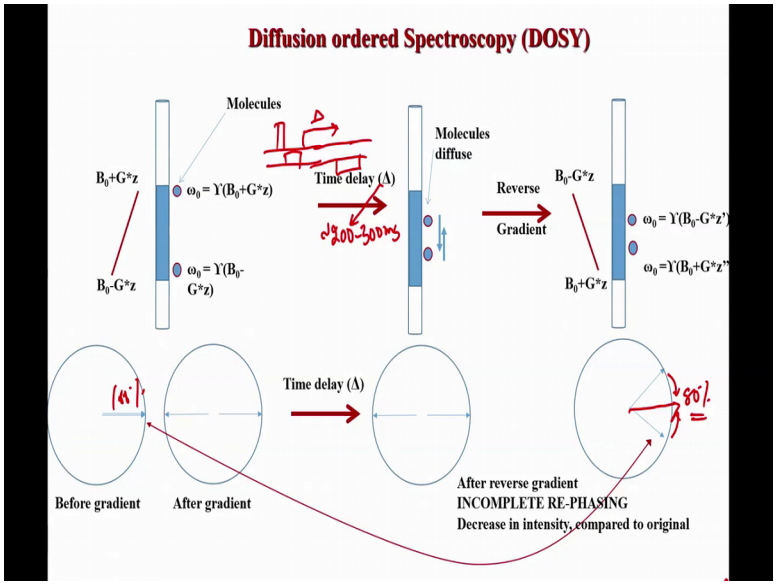

So, let us see how this can be exploited for DOSY. So, this is the same picture again we have a molecule we apply a gradient and the molecules on the top and bottom are experiencing different magnetic field because of the gradient. Now let us say I give a time gap some delay some just I do not do anything I apply a delay. So, these delay for example, let us say I have a pulse; I have a pulse and then I apply a gradient then I give some delay ok. So, this delay is typically of the order of 200 to 300 milliseconds this is delay delta.

Now, during this time period that is during this delay delta the molecule start diffusing the same molecule what is shown here they will start diffusing because their translational diffusion causes them to move ok. So, this is let us say coming down and let us say this is going up. Now after some time after this delay is over I apply the reverse gradient I apply the reverse gradient ok; so, that is opposite gradient.

So, you see there is a gap here I have not immediately applied the gradient reverse. Remember in the last slide we applied it immediately, but here we are not applied immediately; we have given some time gap and during the time gap the molecules have moved means this molecule is no longer in this position, it has come to some other position.

Now, when I apply the reverse gradient I should get a rephasing of the signal right. So, this is what is shown here. So, this is a before the gradient I apply after a gradient I apply let us say these two molecules which are at the extreme end of the sample; they will now be opposite in the phase because one has x gone by some angle and another fellow has gone by an exactly opposite angle because they are equal and opposite ok.

Now, I apply some time delay; so during the time delay this frequency will not change. Now when I apply the reverse gradient I expect them to come back to in phase, but that is not going to happen now; why is that? Because the molecules have moved from their positions; so therefore, when you apply the reverse gradient it will not be exactly the same Larmor precession opposite as this. Because you see here now the z value has changed; z is now z prime you see this z value of this molecule here is different from the z value of this molecule here.

So, between the first and the second gradient their molecule has moved to a new position and that a new position is because it diffuses during this delta period. So, during the delta period when it diffuses it has reached a new position. So, that position now the Larmor precession of the molecule will be corresponding to that position. So, when I apply a reverse gradient it will not be able to cancel the value which it started from beginning.

So, therefore, the Larmor precession is not anymore the same as before and therefore, they do not come back in face means it does not exactly cancel the one which applied earlier. See if you recollect the previous slide again what we did was we basically this was exactly equal and opposite, but there was no delay given here if you recall the previous slide. So, when we do not give any delay the molecule are have not moved at all they are still in the same position. See if the molecules are in the same position then I can reverse the gradient and completely cancel the phase because whatever has gone in the positive direction will be in the opposite now because of negative.

But if the molecules move then my positional information has changed and now I cannot reverse completely because the molecules no longer on the same place. So, this is what we exploit in DOSY that when you give a time gap between two gradients opposite gradients, the molecules have moved and therefore, when they do not come back your signal has come down in intensity. Why? Because now the component of this here and component of this here suppose this is 100 percent here if I write it as 100 percent this will be something like 80 percent.

So, my signal has come down by 20 percent because it could not come back to the face completely. So, this reduction from it 100 to 80 is basically proportional or dependent on how fast the molecules move. Suppose the molecules are diffusing very slowly; so imagine a situation where the molecules do not diffuse at all they are very small they are in a gel formation or they are in a solid state. So, there is diffusion is very slow; in such a scenario my signal intensity will be almost 100 percent because everybody will come back to phase because they have not moved from that positions.

But if the molecule diffuse is very fast; then this molecule would have completely come down this side this would have come up and maybe the signal intensity may remain like in not come back at all and then my diffusion intensity will gone down to may be 30 to 20 percent. So, there will be a more dramatic reduction from 100 percent if the molecule moves very fast.

So, you see the reduction in the signal intensity by applying a gradient and the reverse gradient with a delay use me an information about how fast or how slow the molecules are defusing. So, that is what is basically used in a diffusion ordered spectroscopy that the incomplete re phasing is related or dependent on the diffusion value of the molecule.

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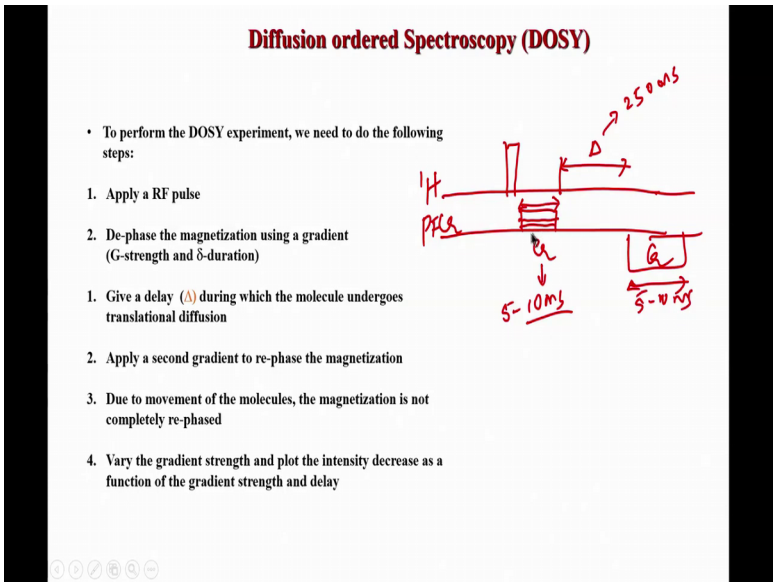

So, this is basically what we do here we first apply an RF pulse in a DOSY experiment and this is proton channel suppose and this is a gradient channel; we call it as I said PFG; I apply a gradient some value G then I wait for some delay some delta delay, then I apply a reverse gradient. So, when I do this way I de phase this first the first gradient the first gradient de phase is the magnetization and because of as I showed earlier because of the difference in the Larmor precession.

Now, once we give this delay the molecule undergoes translational diffusion; that means, it moves from one position to another position during that period. Now you apply a second gradient to re phase means to undo the phase which has accumulated because of the diffusion; not diffusion because of the Larmor precession. But because a diffusion takes place they will not be complete magnetization is not completely re phased. And that tells me how bad the diffusion is there in the molecule means how strong is a diffusion or how weak is the diffusion based on how much the signal has decreased ok.

So, what typically is done in a DOSY experiment is the apply this value. So, as I said this typically is of the order of 250 milliseconds and this gradient strength is vary. So, this is your time di graph. So, this let us say is 10 milliseconds. So, we keep that also fixed 5 to 10 milliseconds and then we used to vary the strength ok. So, what we do is we record one experiment where the strength is some value; then we change the strength of this

gradient and at each time each point in we record the spectrum. So, how does the spectrum look; let us look at that.

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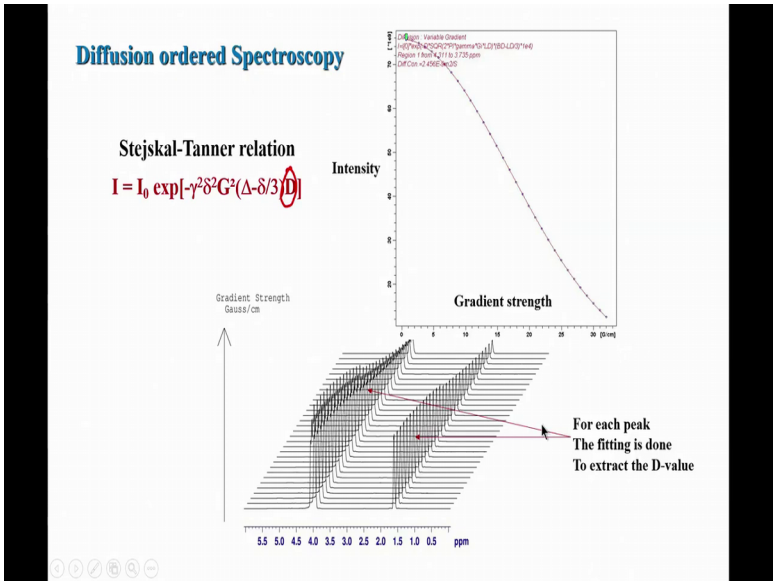

So, this is how it looks. So, as you change the intensity of the peak changes as you increase the gradient strength. So, which gradient strength are we talking about? We are talking about this value. So, I will change from here the next gradient will be here next gradient will be here and so on so, forth.

So, we apply different gradient strengths and at each strength we measure the intensity how it falls with respect to time or with gradient strength. This is given by this equation this is very famous Stejskal Tanner equation and we will not be able to derive this, but the idea is the intensity of a proton signal as you vary the G value; it will go down like it this equation this is like a Gaussian equation. So, this is a not a straight line it is a Gaussian curve.

So, what are the constants here? This is constant delta value because that is not changing gamma is constant that is a gamma of proton delta of gradient it is the duration is kept constant and D is your diffusion value that is what we are trying to find out ok. So, this is what we are trying to estimate we do not know this value we are trying to estimate that, but we use this equation. So, how does a signal vary? So, you can see here this is a real spectrum that as I increase the gradient strength and I record a lot of experiments; I do

not do one experiment I do one experiment with one gradient strength then keeps change the gradient strength and repeat the experiment.

So, I will get like curve like this you can see the intensity is falling and that falling of intensity you can be fitted to this curve. And once you fit that intensity to this curve you can actually extract the D value because all the other values are known to us only unknown here is D value. So, that is how we extract the diffusion rate from the intensity of the proton signal as a function of time sorry function of gradient strength ok. So, the fitting is done for each peak. So, this peak is separately fit to this equation this peak is separately fit to the equation and so on.

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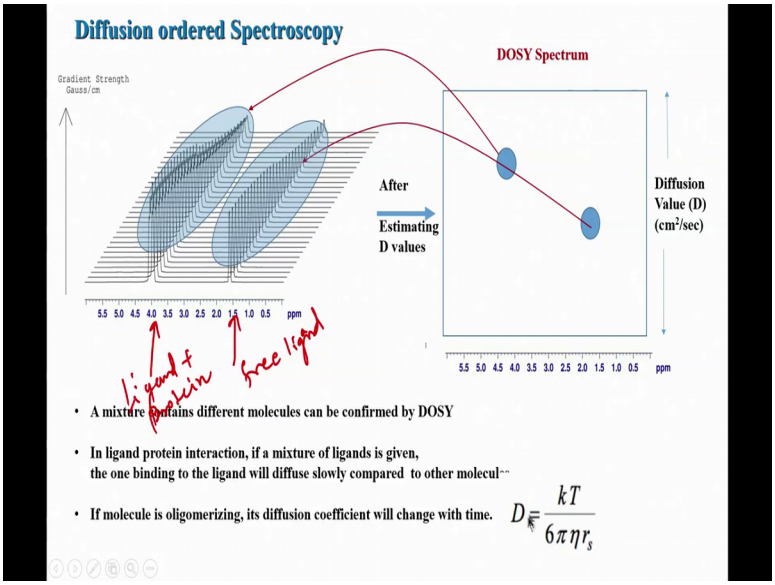

So, now suppose I have a mixture of two sample suppose this is one molecule and this belongs to another molecule. So, these two molecules do not have the same diffusion rate. So, when I plot the diffusion on the y axis and the x axis is a proton chemical shift. So, this chemical shift is x axis you can see here that the diffusion values are not same because the molecules are not the same.

So, imagine that you have a mixture of molecules then each molecule in that mixture will have experience a different diffusion rate. And therefore, your diffusion values which you extract from that equation; remember that equation which has showed in the previous slide that is a important equation which is used for calculating the D values ok. So, this is how basically you will see the diffusion changes.

So, this you can also think of as a protein and ligand suppose that this is a free ligand or let us say this is your free ligand and this could be ligand and protein complex. So, if that is the case then the ligand free ligand will have a different diffusion values; a lower diffusion value and ligand bound protein will have a higher or it can be opposite. You can say this is lower diffusion this is higher depends on how you plot this D value ok. So, diffusion difference will be there between a free ligand and a bound ligand and that will be useful for finding out if the ligand is binding to the protein or not ok.

So, this is what is shown here a mixture contains different molecules which can be confirmed and in the ligand protein interaction if a mixture of ligand is given then the one which binds with the ligand will diffuse slowly compared to the other molecules. So, the point here is that suppose I take a screening if I am doing a screening of a large number of molecules out of 20 only 1 or 2 may bind to the protein. So, for those 2 which bind to the protein their diffusion now, will look different from the other 18 which are not binding to the protein.

So, by using that approach by using DOSY I can find out which two are the one which are binding because their spectra or their peaks; I will be able to identify. Of course, you have to you should be separable it should be resolvable all the 20 molecules should have well separated signals; they should not overlap if there is an overlap here then there is a big problem. So, this DOSY will not work if you have an overlap of signals; then you have to do a 3 dimensional DOSY, this is 2 D DOSY a pseudo 2 D; it is not really a 2 D it is pseudo 2 D then you have to do a pseudo 3 D if there is an overlap.

But imagine suppose there is no overlap then each proton signal from each molecule is separated; then I can find out which molecule is binding because the proton signals of that molecule will be showing lower diffusion slower diffusion compared to other molecules which are not binding. This diffusion is related to this equation this is the Stokes Einstein equation.

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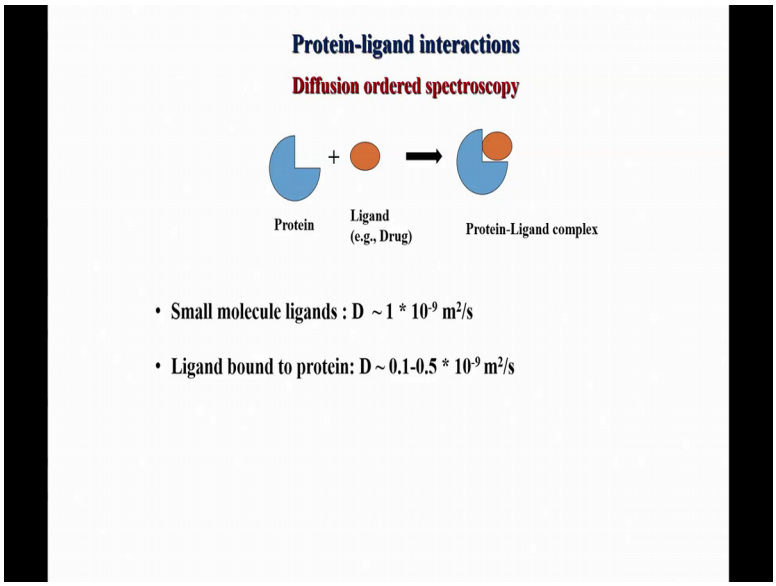

So, we can see that for a small molecule typically the D value is about 1 into 10 to the power minus 9 meter square per second. So, one has to keep in mind these units, one has to understand then values properly ok. Now, if you look at a ligand bound to a protein then the ligand we can see it is almost twice or 10 5 10 times smaller or slower diffusion. So, diffusion D decreases when D decreases; it means the molecule is diffusion slowly, if the D value is high the molecule is diffusing faster.

So, you can see that almost there is reduction typically when a ligand small molecule bind to protein the reduction is of the order of magnitude or it could be 2 to 3 times, but that is very important because that is sufficient for us to capture whether that ligand is bound to the protein or not. So, this is how basically DOSY works we come to the end of this interaction part it regarding the DOSY. So, this brings us to the end of this course.

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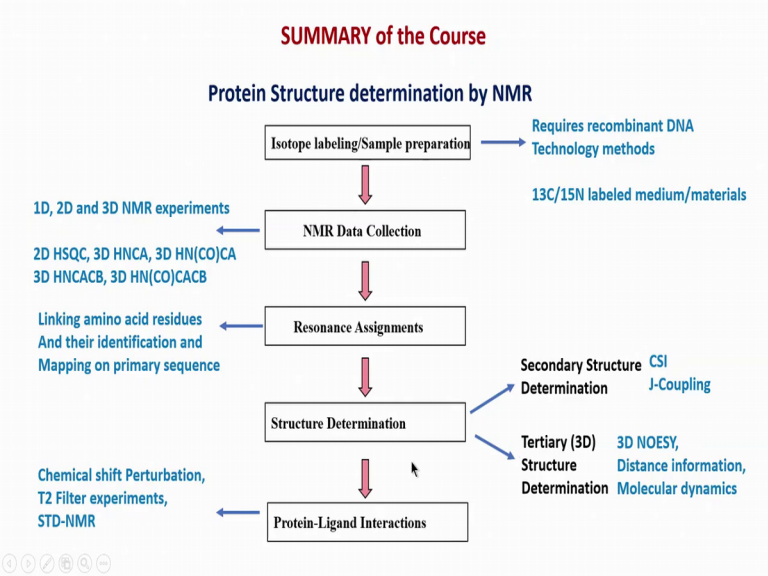

Let me just summarize what we have done in this course; we started we basically the whole course was focused on how to determine structures of proteins using NMR and how to study the interaction of protein with ligands by different approaches. So, we looked at the, remember the in the flowchart the first thing which you need is a protein sample and for that you need to do isotope labeling that is what we saw. And we have looked at different techniques of isotope labeling we saw that it requires basically the concept of recombinant DNA technology of idea of cloning.

And for that you also for isotope labeling you need C 13 N 15 labeled medium and materials. Now this medium basically means it depends on what you are growing if you are growing e coli or yeast or if you have a cell free medium that medium will change and also what will change is: what is the source of nitrogen and carbon for the growth of the organism. So, for E. coli we saw that the standard molecule is glucose C 13 labeled glucose and ammonium chloride N 15 labeled is used as the source of carbon and nitrogen. Then the next part is NMR data collection although we dint follow this order, but here I am showing you through the flowchart.

And we looked at the first part of the course on NMR data collection where we looked at three experiments, three type 1 D 2 D and 3 D NMR experiments how they are performed and how what is the design principles and we looked at some pulse sequences of 2 D and 3 D. And in 3 D NMR especially we looked at very important set of

experiments known as triple resonance triple resonance means three different nuclei. So, the three nuclei are hydrogen nitrogen and carbon.

So, we this again therefore, needs isotope labeled materials sample or protein without which you cannot do that, but we looked at very interesting concepts here how they help us to assign a protein. So, essentially this data collection is all for finally, assigning the signals of the protein and that is what happens in the next step after a data is collected; you assign the protein and typically the assignment means linking amino acid residues and identifying them and then mapping them on the primary sequence.

So, we link the neighboring amino acid residues and then identify first we map them on the sequence. And also try to identify their type and for this we saw that you need c beta chemical shift carbon beta of the amino acid they are very strong identifiers of the amino acid type. So, this is a very important step in any protein NMR project that is resonance assignment this takes a lot of time, but is unavoidable has to be done.

Then once you have the resonance assignment you can solve the structure of the protein now. And this structure we saw that can be divided into two parts one is first you find out the secondary structure of the protein and this is done by typically the chemical shift index approach or using the J coupling approach which is measuring the backbone torsion angles both are combined now a days together. And there are software programs which combine these together and nicely give you the complete secondary structure information.

Then once you have the secondary structure you can then go on to determine the 3 D structure of the protein and for this we need few things first we need is a 3 D NOESY, for proteins this is what we saw a 3 D NOESY HSQC which can be either has to be carbon and or nitrogen 3 D experiments. From there we assigned all the NOE peaks first which are the long range which are the short range which are the medium range NOEs and from that those peaks we extract the distance by looking at the intensity of those peaks how strong and weak they are.

So, once the distance information between all the protons are known or not all we will never get every distance in the protein; we will get approximately 10 to 20 percent on that information of the distances is fed to a software which are typical very standard

established methods which use molecular dynamics calculations or simulations and they will give us the final 3 dimensional model.

Again remember this is not a structure what we finally, get we although use the one structure here what you are getting is a model of the protein because it is based on whatever distance information we have provided. And then having the structure the structure in our hand and resonance assignment, we saw in the last part we can study protein to ligand interaction using many different approaches such as chemical shift perturbation T 2 filter and STD NMR.

So, this was basically the entire course I am sure and I hope you enjoyed this course and you have benefited from learning more about protein structures.

Thank you.