NMR Spectroscopy for Chemists and Biologists
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Lecture 60
Diffusion Ordered Spectroscopy

Hello! So welcome to today's lecture, so today we are going to discuss shape and size of a molecule macromolecule and how NMR can be used for getting some information about shape and size of a molecule. So the experiment, that is called DOSY Diffusion Ordered Spectroscopy. So what we are going to measure is diffusion, diffusion by NMR Spectroscopy and that is why it is called diffusion ordered spectroscopy.

So diffusion is a natural process that happens for all the molecules that are in the solution and that diffusion can be measured by NMR Spectroscopy and there are some dedicated pulse sequence for measuring the diffusion and then we can like there are some problem associated DOSY if the sequence overlap happens that but that can be edited and taken care of that.

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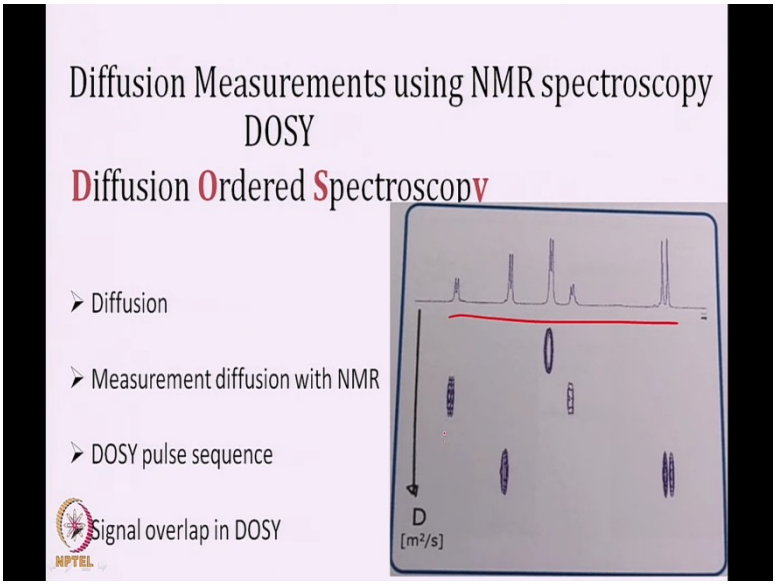

So what DOSY does essentially is correlate the diffusion in one dimension, so this is diffusivity *D* and chemical shift information in another dimension so directly you are now detecting the chemical shift information and indirectly you are detecting the diffusion of a particular molecule therefore from each peak you can find it out how this molecule is diffusing if it is a mixture of the molecules. That is what actually DOSY does, that is why it

is called diffusion ordered spectroscopy or NMR can be used to measure the diffusion of a molecule.

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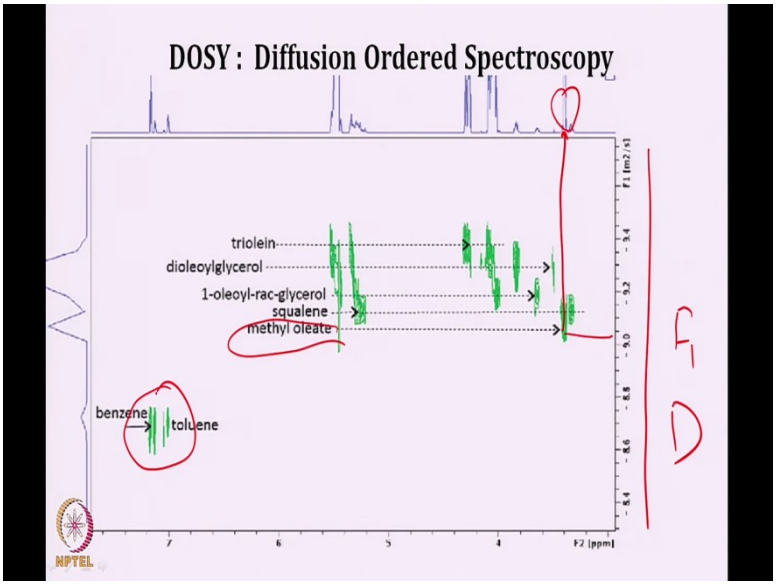

So essentially suppose we have a mixture of molecules with a different molecular weight therefore we can get a different chemical shift like here you can see I mean the F_1 dimension like indirect dimension, F_1 dimension is indirect dimension you have the parameter D. Now here if you look at the molecule like dioleoylglycerol or another glycerol molecule or say methyl oleate molecule have different diffusion depending upon their shape and size and that can be very well correlated with the chemical shift of each of this moiety like here can be correlated with this so now we know that these peaks belongs to say methyl oleate and their diffusion coefficient is somewhere around minus nine.

So we can differentiate on the basis of their diffusion rate and chemical shift to find that what these molecules are and what all in a mixture of molecules in solution it has and those molecules have a different diffusion than a solvent diffusion like benzene or toluene has. So let us see what actually diffusion is.

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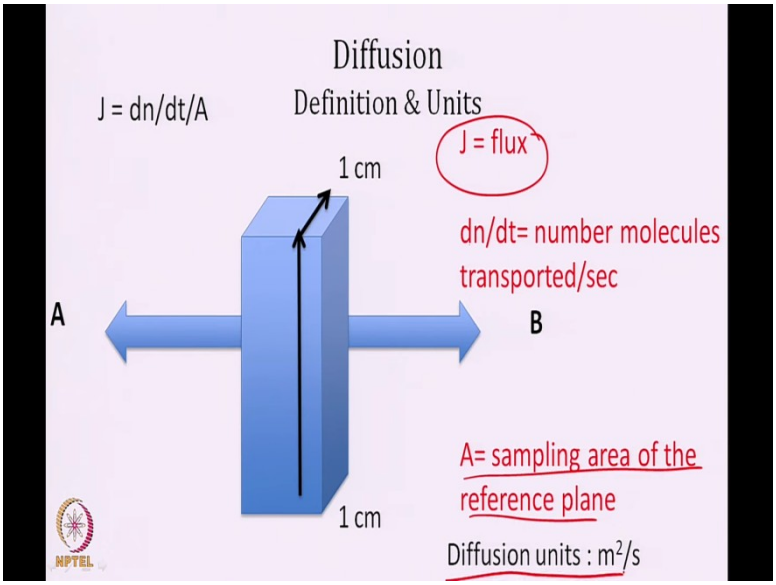

As we discussion diffusion is a natural process, so if say something is flowing through a box. So say A is going through b of a box of say 1 centimeter by 1 centimeter, so and the flowing rate you define as flux J, so the number of molecules that is transported per second is $\frac{dn}{dt}$ okay. So

$$J = \frac{dn}{dt} / A$$

So 1 centimeter by 1 centimeter how many molecules per second passing that is called diffusion. So A is sampling area of the reference plane like this and the rate with which they are passing that will be called flux. So diffusion is basically the this event of diffusing through particular area with some flux and the unit of diffusion is meter square per second or centimeter square per second, how many molecule are diffusing for like a meter square per second. So in this area how many molecules are diffusing that is diffusion.

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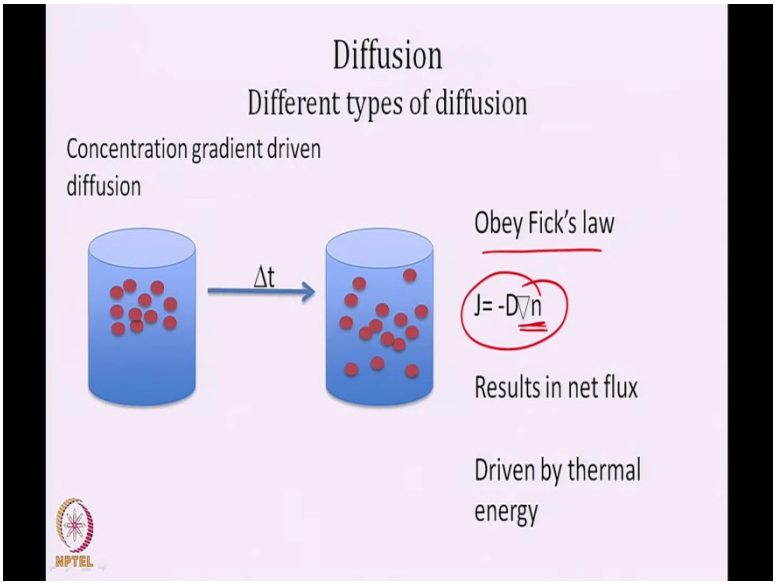

And diffusion can be of different type so and the concentration gradient is driven by diffusion. So suppose here in the same box here you have a concentrated molecule and with certain time t if you leave it then it will diffuse through whole volume so diffusion of these molecules generally follow the Fick's law which is like here the flux diffusivity D and change in the concentration of the $\frac{dn}{dt}$ is this terminology.

So that result into net flux flow of the molecule from the concentrated to the diluted and this is generally defined by say thermal energy. If you say it is just a simple example, if you take a pinch of salt put in the water it diffuses through the water that is what is diffusion.

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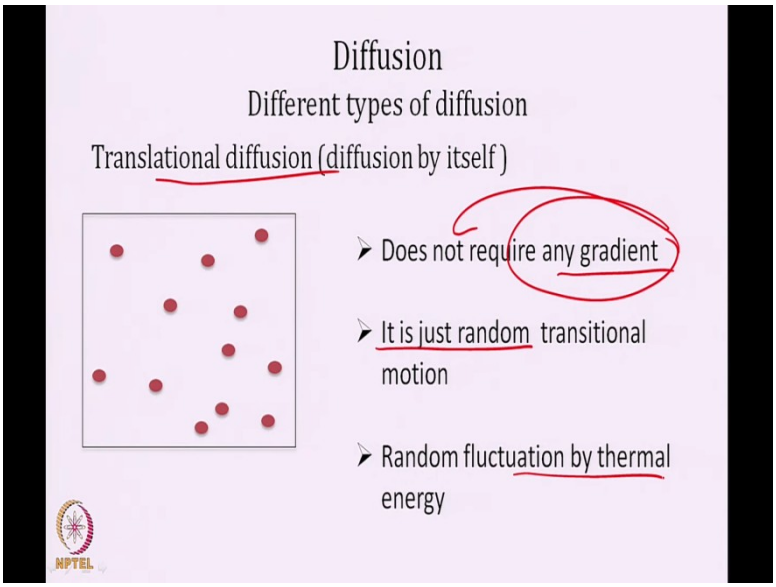

So there are different kind of diffusion: Translational Diffusion like just diffused by itself in linear dimension, then there are Rotational diffusion how the molecules rotate and then diffuse so the most common one is Translational diffusion and that is typically measured using NMR okay. So this diffusion basically can be measured translation diffusion we can measure, the molecules are randomly translating in the solution and that can be measured by something called like a gradient.

So this diffusion to happen you do not need any gradient but to measure it in NMR you need gradient of the magnetic field that I am going to come how we are going to use it and basically this random fluctuation happens by the thermal energy of the molecule how they are getting dispersed in the solution.

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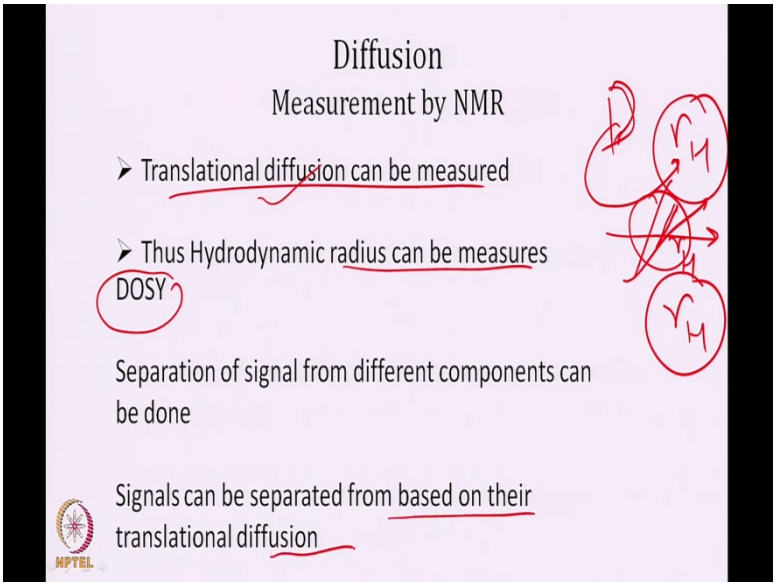

So how we measure diffusion by NMR spectroscopy that is I am going to discuss now, so if you look at other than NMR spectroscopy typically for shape and size of a molecule there are various technique that people use like say fluorescence correlation spectroscopy. This is also a technique where diffusion of the molecule is measured so fluorescence correlation spectroscopy in a small focal volume how the molecule diffuses at that small focal volume and it auto correlates so you plot autocorrelation signal or function of a molecule that diffuses trough the vocal volume and that gives an idea about the shape and size of the molecule.

The other one is by analytical ultracentrifugation, it is commonly used in many labs or man companies to find it out the shape and size of a molecule. Suppose a molecule is aggregating one percent so you can find it out by analytical centrifugation that what is the fraction of those one what is the fraction of those aggregated sample, then there is another technique called dynamic light scattering that is also commonly used for finding it out the size of the molecule but those are different techniques, many of them are the light based technique or the mass based like ultra centrifugation is the centrifugal force based technique

Here we are just going to exploit the concepts of NMR and then we can measure how the translational diffusion happens. So what we can measure is essentially translational diffusion and if we measure this translational diffusion we can measure like hydrodynamic radii of a molecule. So suppose this molecule is diffusing in solution and then we can measure what is the hydrogen r_H, hydrodynamic radii of this molecule. Smaller molecule can diffuse fast like you can simply say a small ball can float or can move in crowded environment fast and the bigger ball can move slow, that is what actually it is. If the diffusion coefficient is very well correlated with the hydrodynamic radii and in a moment we are going to look at how this is D and r_H are correlated hydrodynamic radii and diffusivity.

So using this diffusion based NMR technique one can measure the translation diffusion or diffusivity D and by measuring this D one can measure the hydrodynamic radii r_H. So this technique in NMR is called DOSY and actually it is useful for separation of signal of different component, different component as we given the example of second slide. If say I have five small molecules that are mixed together I can do the diffusion experiment and we can find it out the signal coming from all these five molecules and how they are differently diffusing in the solution.

So signal can be separated best on their translational diffusion and that can be correlated with the chemical shift of each of the individual compound and that is how one can use it.

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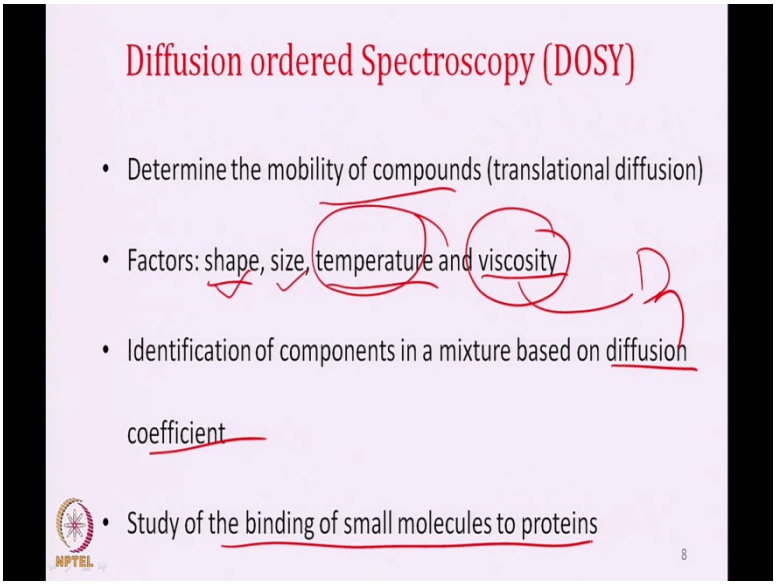

So DOSY basically determines the mobility of the compounds, how fast or how slow they diffuse or they actually they have a translational diffusion in solution but this diffusion rate of these molecules varies on basis of shape. So suppose a molecule is elongated right so its hydrodynamic radii is different and that is how the diffusion is different. So shape determines size determines smaller molecule can diffuse faster bigger molecule can diffuse slower.

The another important temperature like as you said the translation diffusion depends on the thermal energy. So if you increase temperature diffusion rate can change, also viscosity in a like if the it is very viscous solvent then you have a viscosity is high then molecule can diffuse slowly if the viscosity is low it is a thinner solvent molecule can diffuse fast.

So viscosity also plays an important role, temperature plays an important role, shape size of course plays an important role for diffusion so D varies on all of these component. But if suppose I keep temperature and viscosity same then one can get an idea about the shape and size of the molecule. So using this diffusivity or using DOSY experiment one can identify the component in a mixture based on this diffusion coefficient D.

And of course if we can measure how they are diffusion and this can be diffusivity can be also used for binding of the smaller molecule to proteins. In the last class we have looked at the line shape changes right and we have also looked the STD concept. We also looked at the T_2 relaxation time changes and that is what line shape changes, similarly diffusion can also change.

So suppose the molecule is diffusing very fast diffusion is very fast translational diffusion. When it binds to macromolecule now its effective side is increasing and there its diffusion rate can change and then its like it can diffuse very slowly compared to whatever it was doing.

So actually it can also be used for binding of small molecules to a protein and suppose like you have a mixture of 4-5 compounds and one of them which are more likely to bind to a macromolecule you can find it out by recording simply DOSY experiment that which is binder and which is the non-binder, similar like concept that we discussed in last class.

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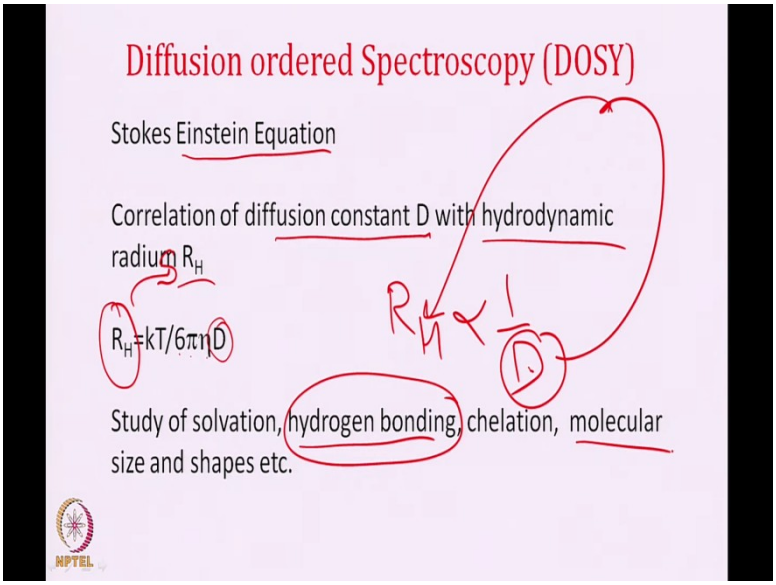

So diffusion actually takes account of Stokes Einstein relation and as we discussed it has a correlation with a diffusion constant D with hydrodynamic radii R.

$$R_H = \frac{kT}{6\pi\eta D}$$

So R_H hydrodynamic radius that will be k is Boltzmann constant, T is temperature and $6\pi\eta$ is the viscosity and D is the diffusivity. So one can see this D and R_H is inversely correlated, so if the hydrodynamic radii is more that means diffusion value so R_H is inversely proportional to D. If D is high that means R_H is low and if D is small R_H is more.

So if we notice R_H and D so we can calculate the D from DOSY and therefore we can calculate the R_H. Now by doing this one can study the salvation of a particular compound so a

compound is getting solvated or not if it is solvated its hydrodynamic radii can change. It can become it can effectively become bigger molecule, you can study the hydrogen boding suppose this molecule in one solvent or in normal case it is not hydrogen bonded but in some case it forms hydrogen bond so its effective size changes.

And then one can study that whether it is hydrogen bonding or not, you can also study the chelation and of course molecular shape and size of a molecule how they are diffusing in a particular solution.

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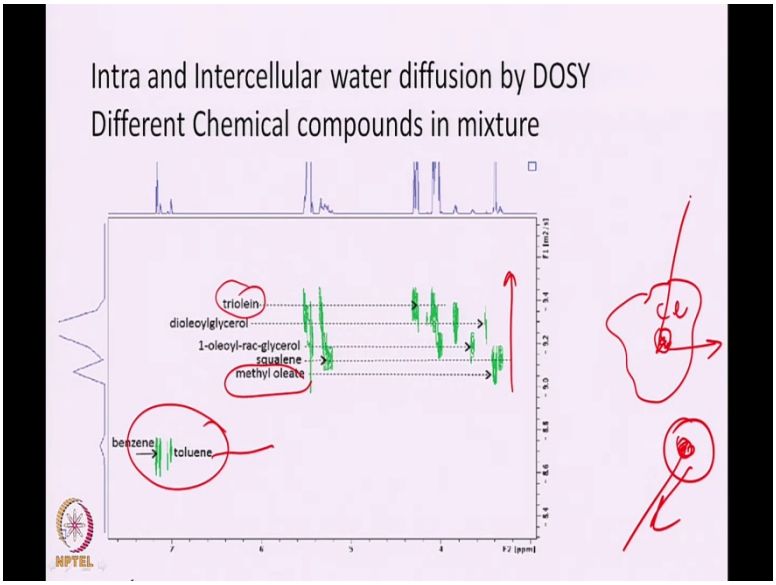

So same example now one can see it and that this different molecules are diffusing differently, so here like a solvent diffusion is around say 8.7 and if you increase the size here the diffusivity is going to increase. So this is negative value so one can see like this molecule has a higher diffusion rate than this molecule and therefore the different size and shape can vary. Now this also can be used to simply understand the water in the cell and outside cell.

You know cell can have two kind of water; one water is here one water is here. This is say cell and this is your water molecule. Now the water here can diffuse fast and because this water is compartmentalized so its diffusion property will be restricted compared to this water. So now you can get by just taking cell and doing diffusion experiment you can get two diffusion rates, one coming from the free water one coming from the cellular water. And you can find it out which is the free water and which is the cellular water looking at the diffusion rate okay.

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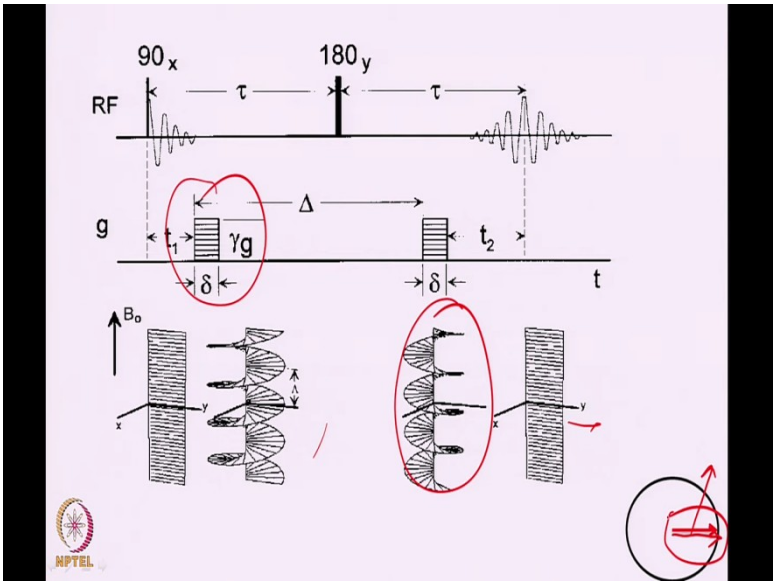

So now how these experiments are done in NMR? So we have studied this sequence called spin echo. You know we apply a 90˚ pulse then we wait for some time, then we apply 180˚ pulse and then you wait for some time and you form an echo right. So suppose we do same experiment and here we introduce a gradient pulse. So this is kind of dephasing gradient and this is called dephasing gradient.

So here we start with something like this and then with this time it will dephase and then if you do not do anything of all the molecular diffusion perfectly fine it can be rephase, so after some time all can come in a phase like here. This is a vector result in vector. So if there is no diffusion all molecule can come here at the same time just like in spin echo okay so no diffusion, everything is diffusing together you get it.

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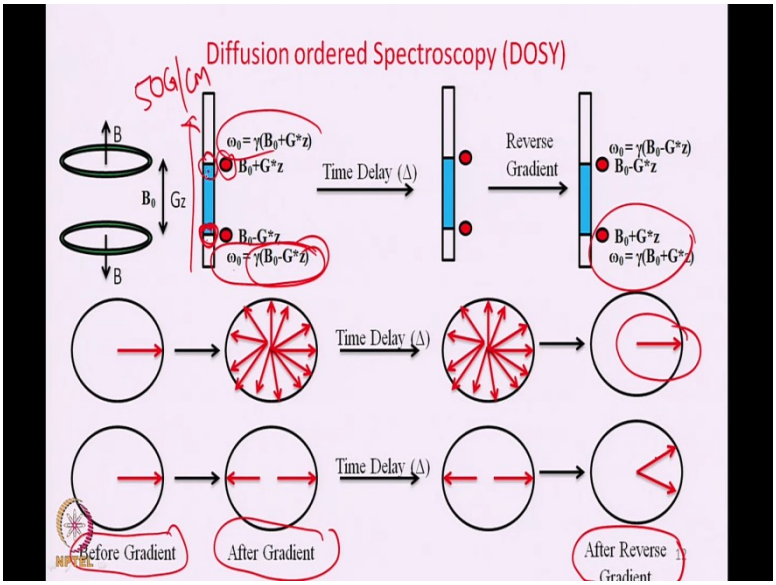

But if you have suppose mixture of compounds, now everything is not diffusing together there is some difference and that you can do create a different filed experience by the molecule by creating this gradient. So what essentially we do, so say this is our homogenous magnetic field B_0 and then we are applying a gradient across the magnetic field. So here if you look at so gradient is in Z direction, so gradient say in our typical case is 50 gauss per cm, like we are changing 50 gauss per cm in Z direction.

So that means the Larmor frequency experienced by the molecule here and Larmor frequency experienced by the molecule here are going to be different.

$B_0 - G * z$

Larmor frequency will be

$\omega_0 = \gamma(B_0 - G * z)$

this will be the gradient strength and it will be plus here. This is our NMR tube we are putting in the magnet, we are applying a gradient at some time. So the molecule that experiences gradient here and gradient here and gradient here are going to be different.

So if you leave it for time delay they are diffusing differently and then we leave it for time delay, then we apply a reverse gradient, so the first gradient dephases it then we wait for some time and then we apply a reverse gradient. So if there is no diffusion everything will

come together here just with a reverse phase it will come here. But suppose molecules are diffusing so by diffusion you can say before gradient we have here after gradient it is here and then with time delay after reverse gradient so because of diffusion molecules are not coming in the phase so they are diffused little bit out of the phase.

So the diffusion for each of this vector is different and that is what is actually captured in DOSY experiment. So that means if the molecule have a different diffusion rate they will not come at the same position they will come at the different position and this in homogeneity in the magnetic field along the height of the tube we are creating by applying the gradients. So magnetic field gradients which actually changes the magnetic field here and here therefore local magnetic experience at these two points are different and now this molecular diffusion therefore they come at the different position and that is what actually you measure by change in the intensity.

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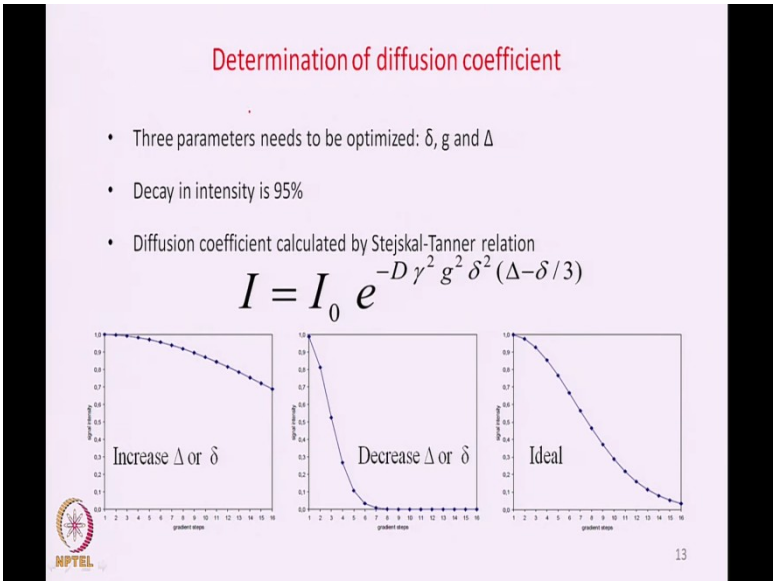

So what how the experiments are done essentially? So essentially now we take a mixture of molecules and then we apply some sequence like this.

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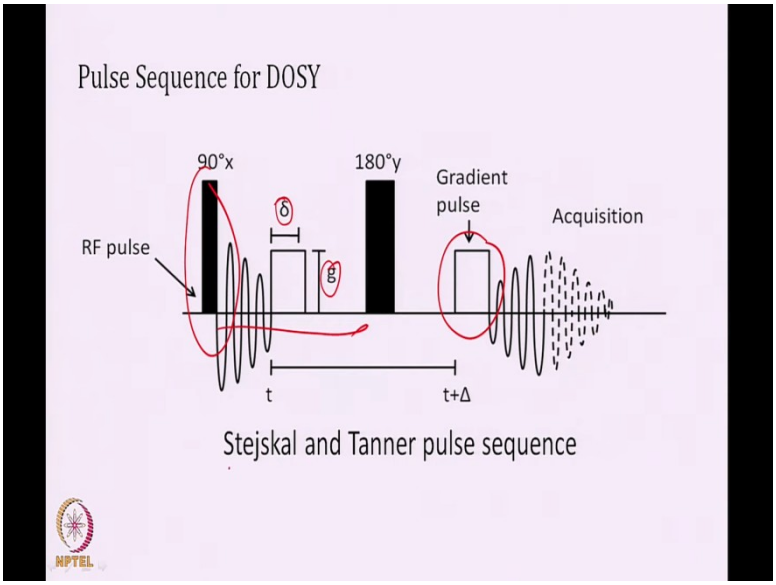

Here where we apply a 90˚ pulse and then we apply a gradient of duration δ and strength g and then there is a time tau here and then we have a 180˚ pulse to change the direction and then we have a refocusing gradient in a position and then we acquire. So this is called Stejskal-Tanner pulse sequence for the DOSY experiment.

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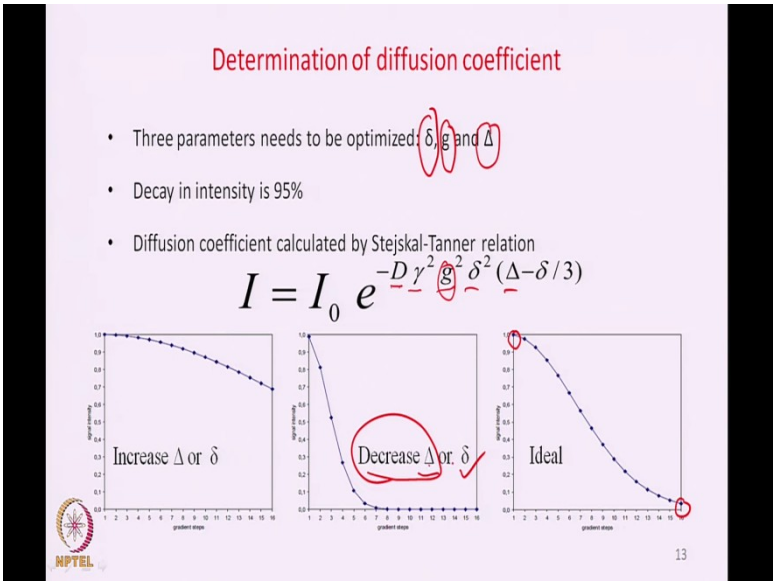

So now two things to be understood here, are we are applying a gradient now gradient has a duration and it has strength and then there is a time. So time of and the δ here so if I go here this is the δ time. So three things need to be, strength of the gradient then

$$I = I_0 e^{-D\gamma^2 g^2 \delta^2 (\frac{\Delta - \delta}{3})}$$

Three parameters have to be taken care and we apply the gradient.

So now experimentally what we need to do we have to vary the gradient in different experiments of how this experiment run, it runs as a pseudo 2-D. We are going to change the gradient strength in each experiment, experiment number one has a like one gradient strength, experiment number two will be different, three will be different, fourth will be different. So we are going to change it and B_0 changing it here we are measuring the intensity decay that is happening and that actually intensity decay follows this equation which is called Stejskal-Tanner relation.

So here I is the intensity at any particular gradient strength, I_0 without any gradient strength and these diffusivity this is the gyromagnetic ratio. These are g and δ and Δ are those time duration that we and this is the gradient strength that we discussed.

So if you fit this equation it will give us diffusivity, now for experimental point we need a nice point so that it is like at a very low gradient. We have intensity almost 100 percent and as we increase the gradient strength its intensity should go down. So we have to make sure that the curve comes like this. So this is the ideal case for this if the curve does not come out, then we have to play around with this duration of the gradient pulse and the time delay.

So this is in this case you have to increase these two parameter Δ and a δ, in this case you have to decrease it and this is the ideal case okay.

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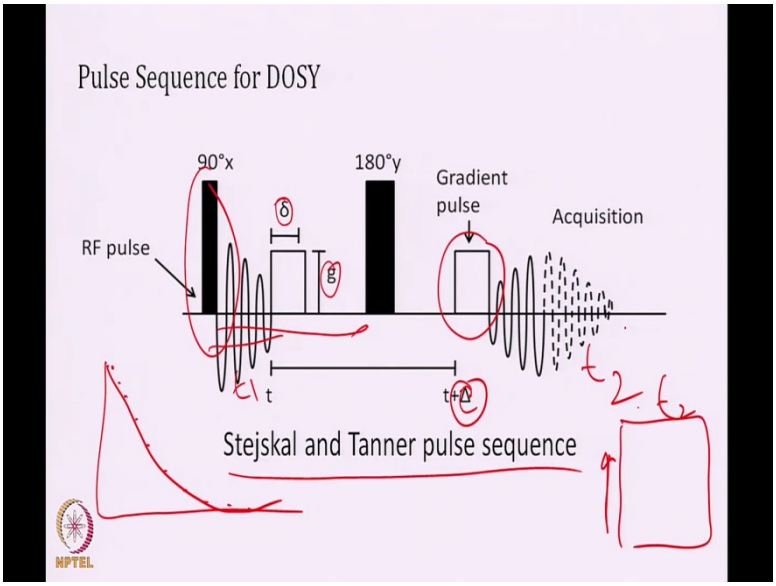

So now go back to again the pulse sequence here are these δ g and Δ, now as we said we are doing a pseudo 2-D so this is your t1 dimenison and this is equation is two dimension. So you have pseudo 2-D in one case it is just the gradient strength we are varying and here t_2 is our chemical shift. So as I discussed the gradient strength is going to change g, we are going to change it from say 5 percent to 95 percent. So we have a very less gradient you have almost highest intensity and as we keep increasing this gradient distance your intensity decreases okay and then you essentially you fit this intensity to the equation that is given here.

So if you fit it and you can simplify this declaration that I am going to come in a moment, if you fit this equation I by as you know get the value of D. Now this is your diffusivity now once you get the diffusivity you can calculate the hydrodynamic radii by a Stokes-Einstein relation. Great!

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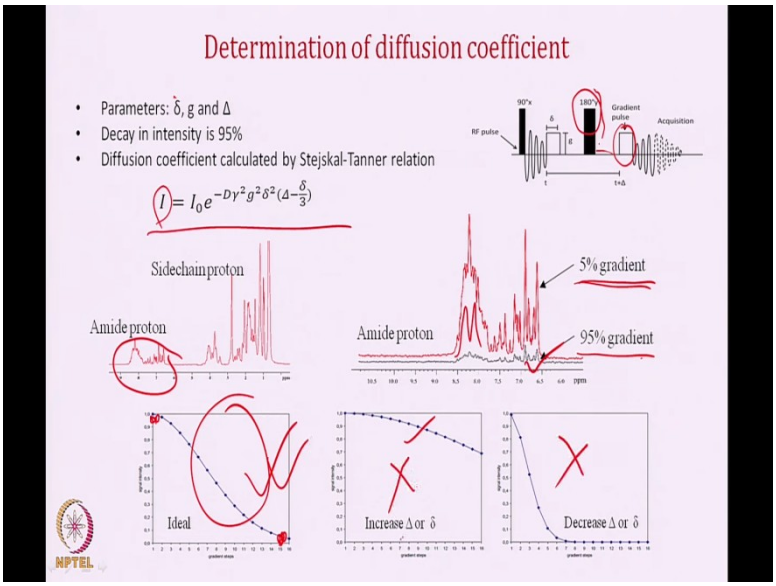

So the parameter to be understood is the δ the gradient strength g and the Δ that big time delay and we have to monitor the decay in the intensity that comes upto 95 percent. And the pulse sequence that we are going to do is this, we are 90˚x pulse we are applying force then we are encoding here instead of 2-D dimension then we are applying a gradient strength of duration say δ and strength g. Then this we are going to vary then we are have a refocusing 180˚ pulse and here we are applying a refocusing gradient and acquiring it.

This is the relation that we are fitting the intensity decay, so now let us see how experimental data comes. So here suppose I take a proton so a proton spectrum for a protein we know that this is amide proton and this is the side chain proton. We record two experiments just to see

how much signal has decayed. So if we start with a 5 percent gradient we have high intensity and if we apply again 95 percent gradient just to experiment I did one where intensity decay whatever is at 5 percent gradient one at 95 percent.

You can see intensity has decreased drastically for the amide proton so that means my parameter seems to be okay. If the intensity has not decreased it was something like this, then I need to play around with each of them to make at the lower one. If I do that then essentially I should get an ideal curve for fitting this equation. So that means 95 percent should be more or less here and 5 percent should be more or less here. If we have this kind of curve that is a ideal decay for measuring the diffusivity, then one can fit it.

So in these two cases it is a non-ideal case so even if you fit it you get a wrong value. So essentially we need to get this kind of curve, this is correct this is wrong. So we need to play around with δ and Δ value.

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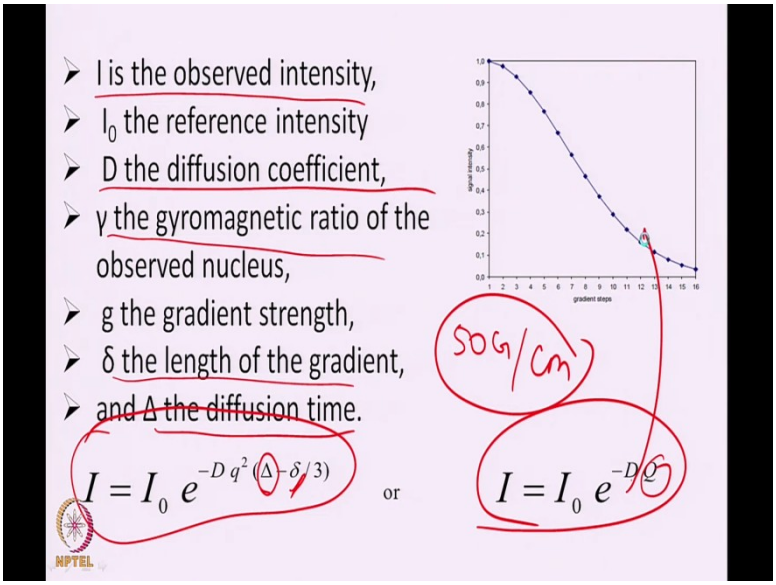

So now we are playing around with Δ, δ to get this nice fitting curve. Then we are measuring the intensity I observed intensity and I_0 is the reference intensity where we have not done anything. So now if we fit it you get diffusivity, as we discuss Υ is gyromagnetic ratio of the observed nuclei, g is the gradient strength, delta is the length of the gradient and D is the diffusion time that we are giving.

So here the time that we are giving here is D that is the diffusion time, now how much it is diffusing. So if we do that now you can fit this equation and this equation as I said this Stejskal-Tanner equation can be simplified. So how you can simplify? Υ is constant and g we

are keeping it like 5 percent 95 percent so typical say gradient strength for a spectrometer is 50 Gauss per cm. You can include that value and then your delta you are keeping diffusion time some constant like 120 millisecond or so.

$$I = I_0 e^{-D/Q}$$

Then here also you can vary it out, if we fit it you can essentially you need to fit this equation which can be easily fitted Q is a constant term and by fitting it one can determine the D value which comes from this curve fitting.

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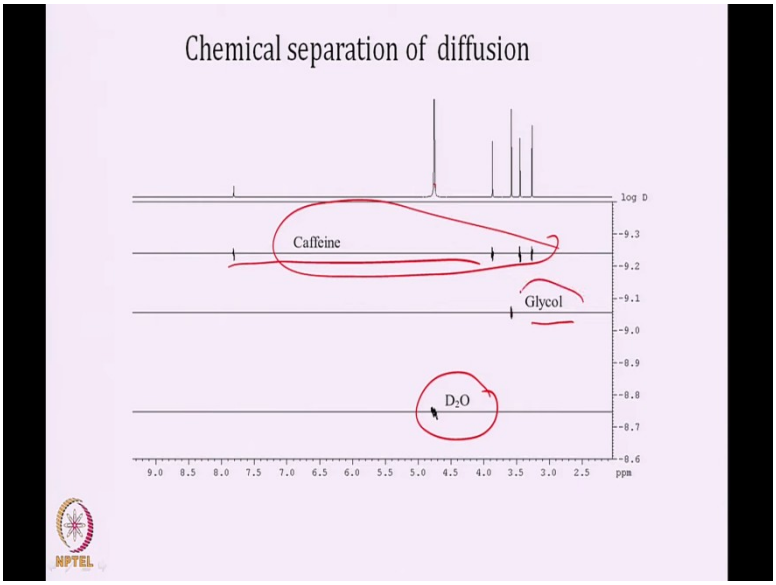

So nowadays actually many software does it but you can write an MATLAB code for fitting this equation. So if you do that here is a mixture of say three compound that we have now say one we have caffeine, one we have a glycol and one have a D2O. We recorded 1-D spectrum so here is your water signal water H2O- D2O. This is your glycol signal and these three are coming from caffeine but at priorly we do not know which signal is coming from which because this is a mixture of compound.

Now we recorded a diffusion experiment and we found that caffeine is diffusing different than glycol and which is diffusing different than D2O. So you can find it out the caffeine is here because we have a three signal and also its diffusion is higher than the glycol than the water. So here one can separate this compound not physically separate but spectroscopically we can separate the caffeine, glycol and D2O by recording DOSY experiment.

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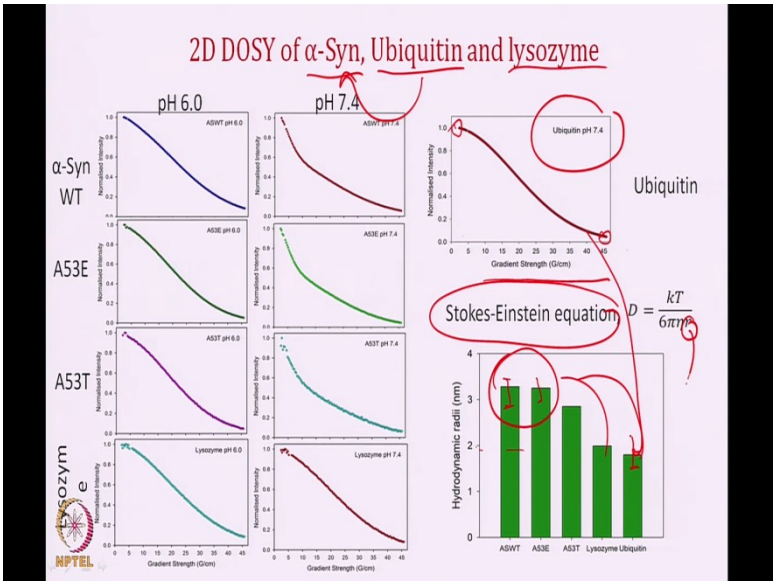

So now I will come to little bigger molecules, suppose I have a protein what we can do? So I have I am showing you here three experiments alpha synuclein is a natively unstructured protein of 140 amino acids. So natively on a structure means a long chain, then we have ubiquitin this is 76 amino acid long globular protein and we have a lysozyme which is also a little bigger than the ubiquitin.

So if you record similar kind of experiment, here I am showing you for ubiquitin. You can see here I am varying the gradient strength, so this parameter is varied from 5 percent to 95 percent and therefore say less than 5 gauss per cm to almost 46 gauss per cm and here is the normalized intensity. So here very less 5 percent gradient strength and here 95 percent gradient strength, you have a nice curve.

Now if I fitted that equation, Tanner equation and one can find it out that the hydrodynamic radii of say ubiquitin is approximately 2 nanometer, lysozyme is little big. But if you look at the α-Synuclein, α-synuclein as I discussed is an intrinsically disordered protein of not too big size like it is certainly twice of size of this ubiquitin this is 76 this is 140. But since it is disordered its hydrodynamic radii is quite a bit like 3.5 or so nanometer because this is disorder so hydrodynamic radii its like a little open chain. It is not completely disorder but it is an intrinsically disorder.

So its hydrodynamic radii is more than ubiquitin and lysozyme, just by recording dynamic hydrodynamic radii or diffusion experiment we now guess the shape and size of a molecule. This I was knowing to show just I have taken a known molecule to show you but you can

take unknown molecule and guess about the shape and size of a biomolecule. So essentially these are the data recorded at different periods of lysozyme and synuclein and one can find it out, all of them fit nicely and you can then translate that to hydrodynamic radii of the molecule.

So if you have a diffusion rate, so you can use this Stoke-Einstein relation and then one can find it out this R_H and that is what I represent here the hydrodynamic radii.

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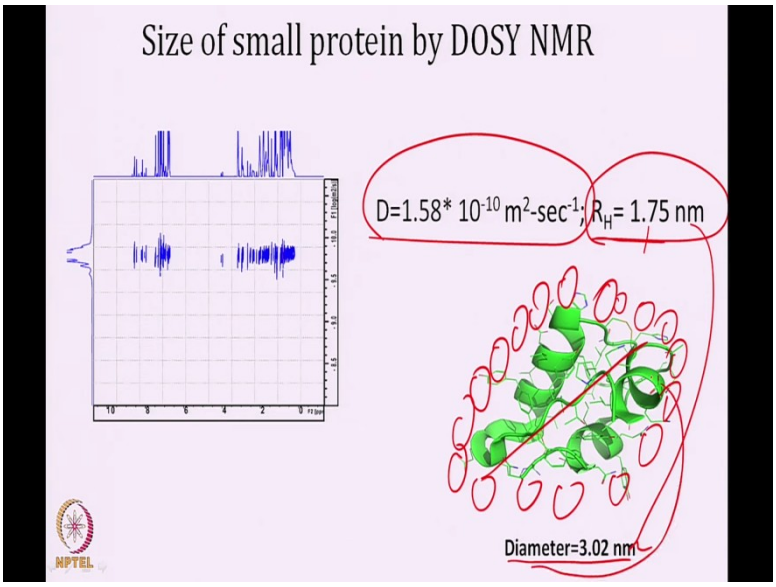

So another example I am just taking a small molecule small molecule its diffusion is calculated by DOSY and you get a hydrodynamic radii by putting like all those parameters. So your diffusion rate comes 1.58 into 10 to the power minus 10 meter square per second square, so hydrodynamic radii translates to 1.75. And if you convert to diameter by just measuring in PyMol you will get 3.02, this is very well correlated with the diameter that is measured from the experiment.

You see this molecule is solvated therefore some bigger hydrodynamic radii is coming because this molecule is not there, there are water molecules around it. So if you take a diameter that comes 3.5 and here we are getting very well correlation just by measuring from here to here it is 3.03 nanometer. Great so that is what I give you a brief background. How you can use diffusion based concept to measure the hydrodynamic radii from molecule using this NMR technique. So it is little bit different, we have to introduce the gradient here and one can measure in pseudo 2-D manner, one dimension is your diffusion rate another diffusivity another dimension is chemical shift.

So one can measure it and I showed you few examples of protein molecule, similar thing can be done even for the chemical macromolecule one can measure. So I hope you understood and got the idea of shape and size of molecule measurement by NMR.

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So at the end I would like to thank you and all those things that we discussed are related to this big magnet and big technology of this beautiful technology called Nuclear Magnetic Resonance, so as a curious student you have to stand in front of the magnet with the sample here and you can do a structure one can do dynamics you can get a shape and size. You can do lots of biopharma studies you can do chemical pharma study. This technique is going to append many avenues for you in future.

So I hope this course was useful for you, for depth we will try to float gain in new course where we discuss about protein and peptide NMR and let us see when we can do that. Thank you very much for being part of this course, looking forward to see you and perform well in you exam.

Thank you very much!