NMR Spectroscopy for Chemists and Biologists

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Lecture No. 23

Distance and NOE

Welcome to today's class, so we were discussing polarization transfer and today will focus on how we can measure distance using NOE. So will connect the concept of distance and NOE in today's class. So what we have done previously?

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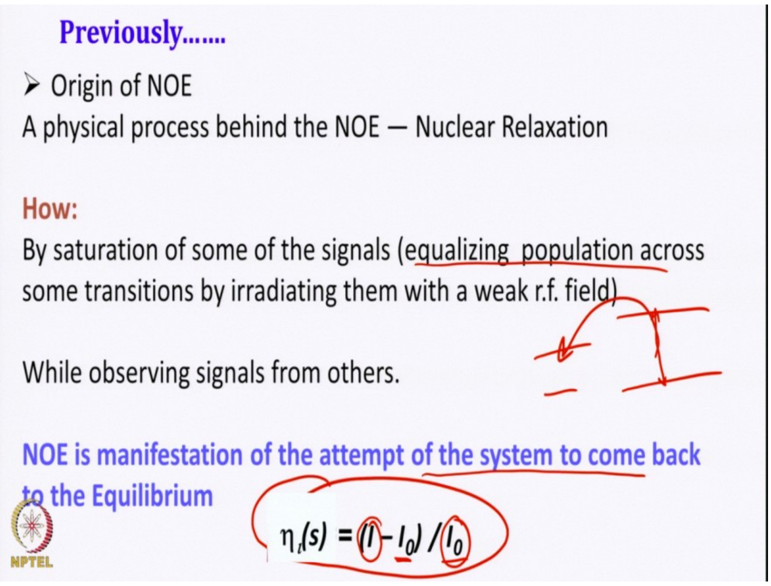

Previously…….

- Origin of NOE

A physical process behind the NOE — Nuclear Relaxation

How:

By saturation of some of the signals (equalizing population across some transitions by irradiating them with a weak r.f. field)

While observing signals from others.

NOE is manifestation of the attempt of the system to come back to the Equilibrium

$\eta_i(s) = (I - I_0)/I_0$

We explained the original of NOE, what is a physical process behind NOE, and as we said that it is a nuclear relaxation which cause nuclear overhauser effect. So, how it is done? So we were saying that there are two coupled spins, they are coupled by dipolar coupling or through a space. What we are doing? We are applying a saturation pulse on one of the signal, so this saturation pulse actually equalizes the population across this transition and then by irradiation we equalize this population. And then effect of that of that equalisation of population by irradiation of a particular signal. We look at what is happening other spin.

So if 2 spins, here we are equalizing population and we are looking effect of this on another coupled spin. So NOE essentially is manifestation of attempt of the system to come back to the equilibrium and that is we have defined at the $\eta_i(s) = (I - I_0)/I_0$. So this is after perturbation, this is equilibrium magnetization and that ratio with equilibrium magnetization. That is called NOE enhancement factor.

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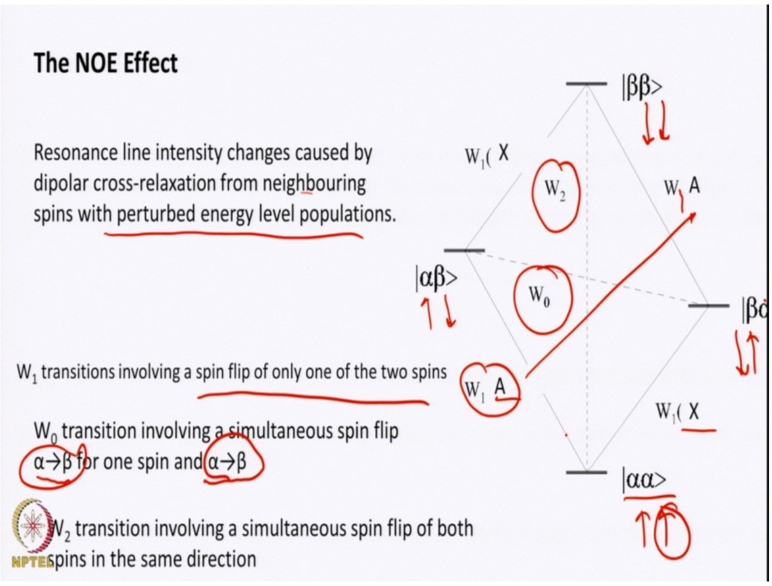

The NOE Effect

Resonance line intensity changes caused by dipolar cross-relaxation from neighbouring spins with perturbed energy level populations.

W_1 transitions involving a spin flip of only one of the two spins

W_0 transition involving a simultaneous spin flip α→β for one spin and α→β

W_2 transition involving a simultaneous spin flip of both spins in the same direction

So we discussed the NOE effect and just to remind you what we have discussed earlier? We have two spin, say spin A and spin X. These are say weakly coupled spins A and X. So there are four energy level, here both spins are say up, up alpha, alpha. Here, one spin is up, another spin is down. Here one spin is down, another spin is up and here both spin are down.

So now, there are four transitions possible. These are called ω_1, single quantum transition. That means only one spin is flipping, so this spin is flipping. That is why α is becoming β, this spin is flipping α again become a data. So these two belongs to ω_1 of A spin and these two belongs to X spin. So these are single quantum transition. Then we had looked here both spins are flipping X and A both spin are flipping. This is called double quantum transition. And here both spins are flipping simultaneously. If you look at alpha goes to beta, beta goes to alpha. So net magnetization change is 0, so that is why this is zero quantum transition.

So resonance line intensity changes caused by these dipolar cross relaxation. So this is happening of various transition and then there is a dipolar cross relaxation. Because A and X spins are connected by dipolar coupling. So from the neighbouring spin with a perturbation energy level populations. That is what we now it changes and we have seen previously because of this relaxation, the population redistribution happens.

So ω_1 to only these two spins are flipping and ω_2 α goes to β, β for a one spin and another spin is this. So that is ω_2 and here both spins are going. So actually, that is what is the transition probability for ω_1, ω_0 and ω_2, that is what we are discuss in previous class.

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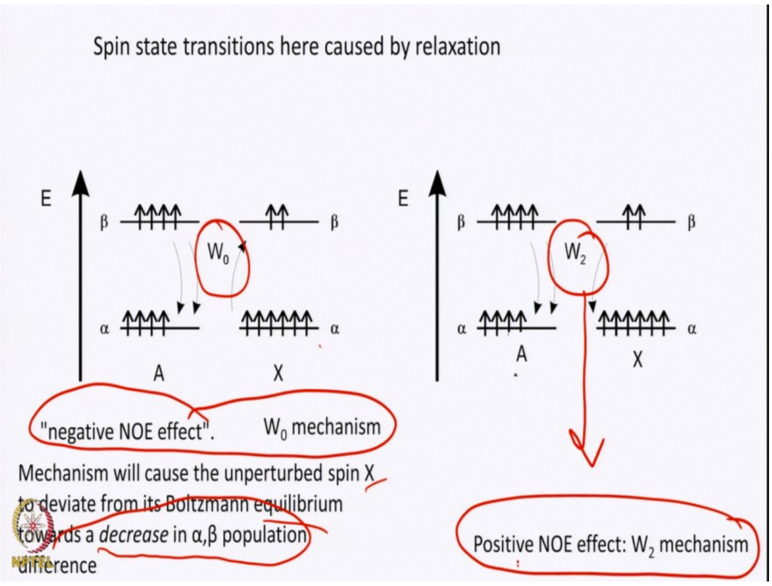

Spin state transitions here caused by relaxation

"negative NOE effect". W_0 mechanism

Mechanism will cause the unperturbed spin X to deviate from its Boltzmann equilibrium towards a *decrease* in α,β population difference

Positive NOE effect: W_2 mechanism

So now, to give you more clearer picture that what is happening here. So I draw a population diagram. So let say we have two spins again A and X spins. Here are α state and β state and here again in X spin, α state and β state. So if you saturate one of the population say A, there are two mechanisms that will relax and equalize the population and both of these contributes to the NOE. One we have discussed is ω_0 and another we have discussed that is ω_2.

So we also discuss that positive NOE that comes because of this ω_2 mechanism this we have shown quantitatively in the previous class and negative NOE which happens for bigger molecule that is coming because of ω_0 mechanism, right. So mechanism which cause unperturbed spins say X to deviate from Boltzmann equilibrium distribution a decrease in alpha, beta population difference happens and that actually cause what is positive NOE or negative NOE.

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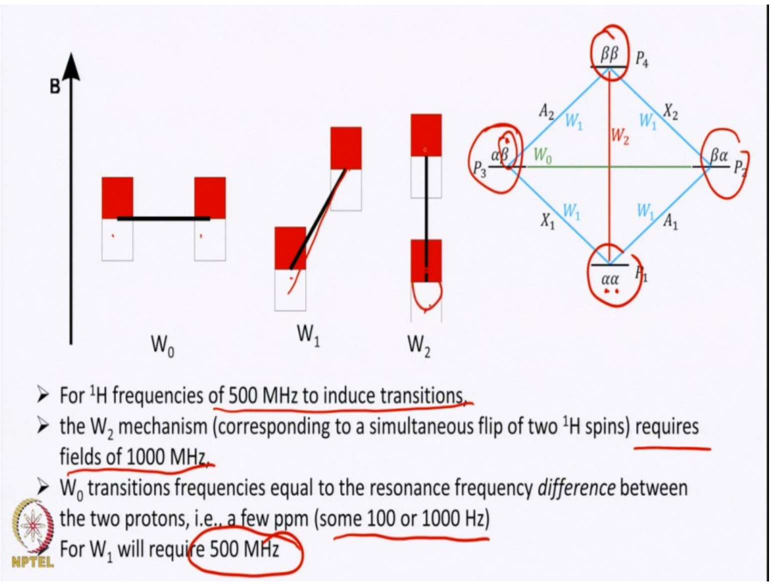

- For ^{1}H frequencies of 500 MHz to induce transitions,
- the W_2 mechanism (corresponding to a simultaneous flip of two ^{1}H spins) requires fields of 1000 MHz,
- W_0 transitions frequencies equal to the resonance frequency *difference* between the two protons, i.e., a few ppm (some 100 or 1000 Hz)

For W_1 will require 500 MHz

To make it even more simpler, let me try to consider these spins as a magnet. So $\omega_1$spin and ω_2 spin is what like if you say, we have H_1 frequency. So, say we are doing proton resonance at 500 Megahertz and transition is happening at 500 Megahertz as per a proton. So that means one transition here say α is this α is flipping to β here, this one. So one spin is flipping, so that means that 500 megahertz induces a transition.

Now for both spins flip you see this 500 Megahertz and this 500 Megahertz, so total transition say will be equal to field 1000 Megahertz to flip and for 0 quantum you just need couple of hertz difference because both spins are at 500 Megahertz, but there chemical safety is little bit different and that difference in terms of hertz. So that will be around 100 to 1000 hertz. So $\omega_1$requires 500 Megahertz, say ω_2 will required 1000 Megahertz. However, ω_0 will require approximately 100 to 1000 hertz.

So this is zero quantum transition, here what I show bar magnet kind of simplicity cartoon representation we have shown. So flipping is happening just from spin here to spin here, both are up, down happening. In ω_1 transition flipping is happening from one state to another state and here double quantum flipping is happening from this state to this state. So that is what we require for this flipping is to happen.

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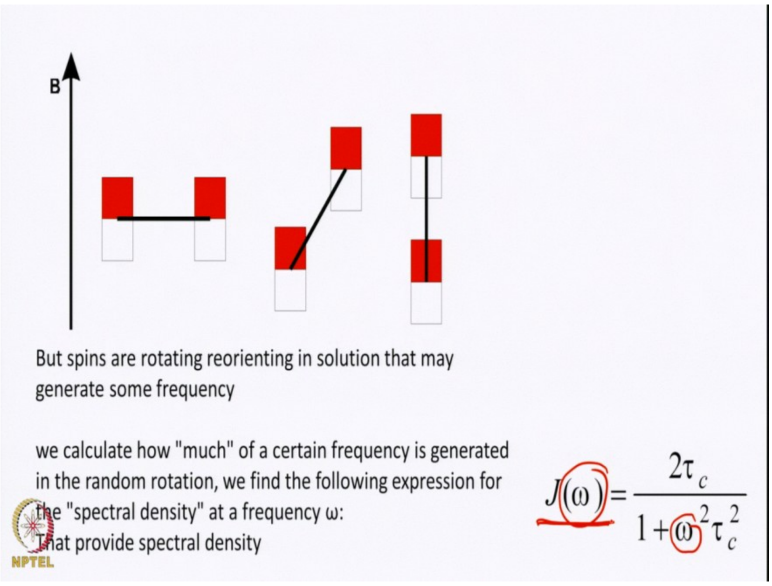

But spins are rotating reorienting in solution that may generate some frequency

we calculate how "much" of a certain frequency is generated in the random rotation, we find the following expression for the "spectral density" at a frequency ω:
That provide spectral density

$$J(\omega)=\frac{2\tau_c}{1+\omega^2\tau_c^2}$$

But this is static case, whatever I have showed from here to here, zero quantum this transition is single quantum, this transition is double quantum. But this is happening for like here it is cartoon is showing for static case, but suppose spins are reorienting in solution. So they may generate some frequency and we can calculate how much frequency is generated by this random rotation of a spins.

So if we calculate, then we can find an expression for something called spectral density at any frequency omega and spectral density is given by

$$J(\omega)=\frac{2\tau_c}{1+\omega^2\tau_c^2}$$

So τ_c is the tumbling time of that particular system or spin. So this J omega is called spectral density and we can calculate how much certain frequency is generated by this random rotation of spins, because of all this relaxation that is coming.

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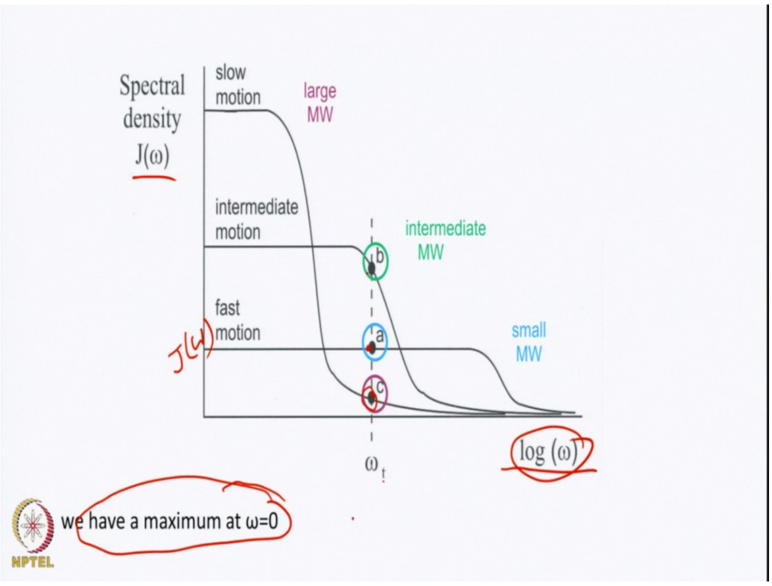

we have a maximum at ω=0

Now, so that has been calculated and plotted spectral density that is $J(\omega)$ versus $\log(\omega)$here. So this is the frequency, so one can see if a molecule is large, large molecule has a slow motion and that is how their spectral density will be plotted like this. So it decreases fast and intermediate motion, which is for intermediate molecule, you have a decrease which is relatively slow and for a fast motion for small molecule you have a quite broad range of $J(\omega)$.

So $J(\omega)$value is less, but it varies for a long ω_t. So one can see for any particular $\omega_t$for all this molecules, for say intermediate molecular weight you have value here and for a small molecule you have $J(\omega)$ value here. But for a large molecule you have a very short $J(\omega)$. So we have a maximum at $\omega=0$right. So that is a spectral density and it is obvious intuitive that big molecule will relax fast and small molecule will relax slow. So τ_c, here whatever we have defined is a molecular rotation correlation time. So correlation time in short, it is a time that molecule takes to rotate in the solution.

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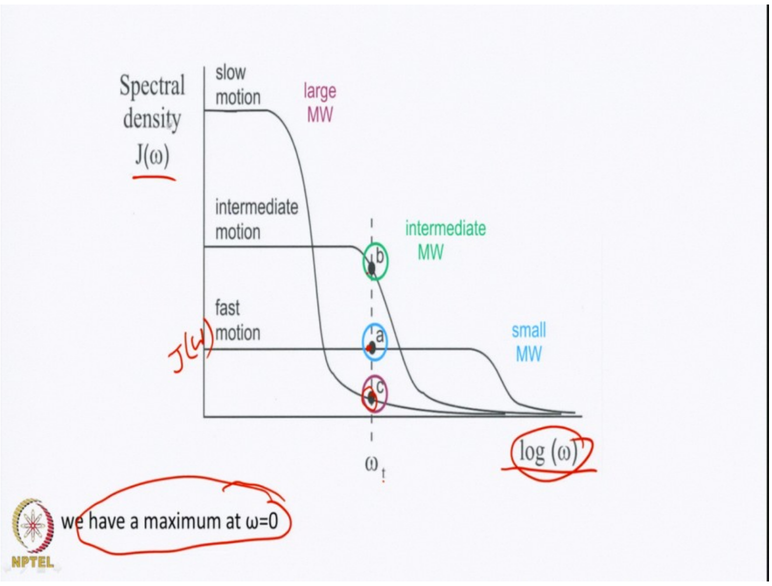

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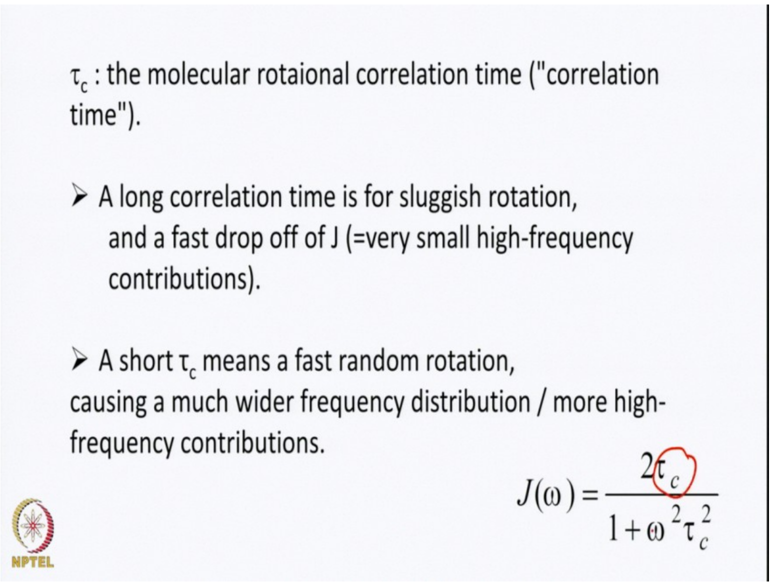

τ_c : the molecular rotaional correlation time ("correlation time").

- A long correlation time is for sluggish rotation, and a fast drop off of J (=very small high-frequency contributions).

- A short τ_c means a fast random rotation, causing a much wider frequency distribution / more high-frequency contributions.

$$J(\omega)=\frac{2\tau_c}{1+\omega^2\tau_c^2}$$

Along correlation time is for sluggish rotation and it fast drop. So if you go here, if you look at here what is happening? For a large molecule and $J(\omega)$spectral density is rapidly actually decreasing. That is what we meant that it is a sluggish rotation and it drop fast. So J value very small at high-frequency contribution. For short τ_c that means fast rotation causing much wider frequency distribution or more frequency contribution if you look at here. So for a small molecule you have a broad range of frequency at a particular $J(\omega)$. So that is the spectral density with correlating with your τ_c or ω.

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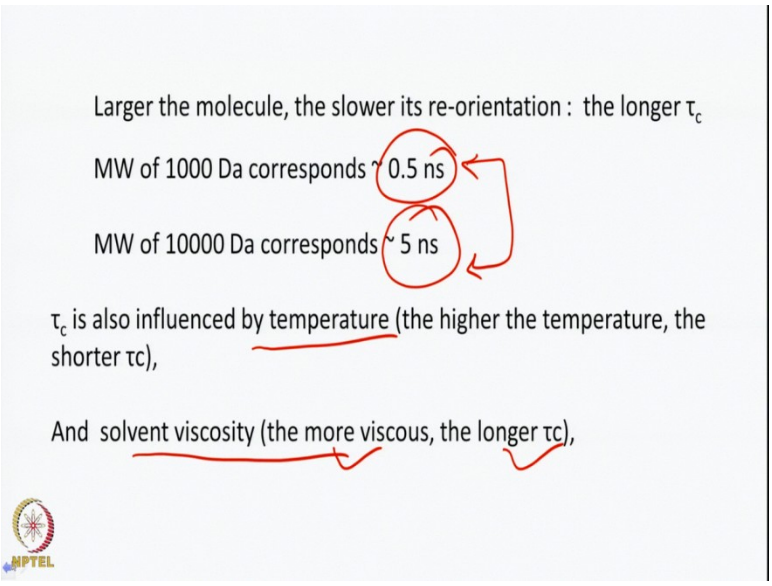

Larger the molecule, the slower its re-orientation : the longer τ_c

MW of 1000 Da corresponds ~ 0.5 ns

MW of 10000 Da corresponds ~ 5 ns

τ_c is also influenced by temperature (the higher the temperature, the shorter τc),

And solvent viscosity (the more viscous, the longer τc),

So give some value for this rotation. So larger molecule generally as we say slow reorientation time. Therefore longer τ_c, say molecular weight of 100 Dalton that is 1 kilo Dalton will corresponds to typically like 0.5 nanosecond. If molecular size increases like it becomes say 10000 Dalton that is 10 kDa. Tau C will correspond to 5 nanoseconds. So you see as molecular size increases the correlation time increases. So for typical bigger size protein the correlation time is 5 nanoseconds.

However, for small organic molecule correlation time is 0.5 nanoseconds and that is what we see that spectral density accordingly changes and for any particular frequency. Like τ_c correlation time is not fixed, for a molecule, it can also vary depending upon temperature. So like if temperature is higher or like if you increase the temperature molecule tumbles fast. So even a bigger molecule can give you sharp line or actually spectral density, if you record spectrum at higher temperature.

And the other thing is viscosity, if you keep increasing the viscosity of solvent. Now small molecules can also experience slower motion. So although the spectral density and their frequency depends upon temperature and solvent viscosity because more discuss longer τ_c, if you have a longer τ_c like we have for larger molecule spectral density decreases fast. Now we correlated your spectral density with the frequency as well as with the correlation time.

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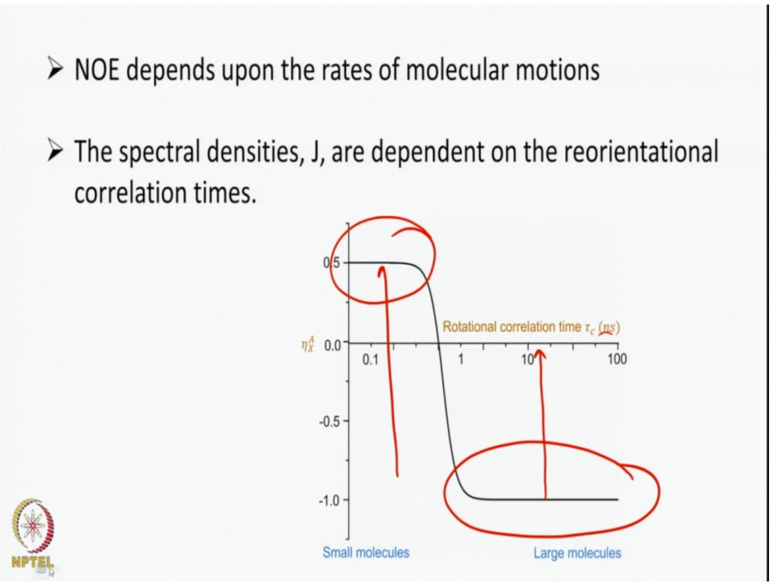

- NOE depends upon the rates of molecular motions
- The spectral densities, J, are dependent on the reorientational correlation times.

Next we move and let see, we have seen this earlier as well. That NOE, now NOE enhancement, coming back to NOE, NOE we have seen that depends upon molecular motion, which is τ_c and spectral density J are dependent of the re-orientational correlation time it depends upon omega. So we have seen earlier that correlation times and NOE enhancement is like this.

So for large molecule, we have a negative enhancement, for small molecule, we have a positive enhancement and correlation time τ_c in nanosecond typical protein like large molecules comes here and typical organic molecule comes here, for a small molecule that we have seen earlier as well.

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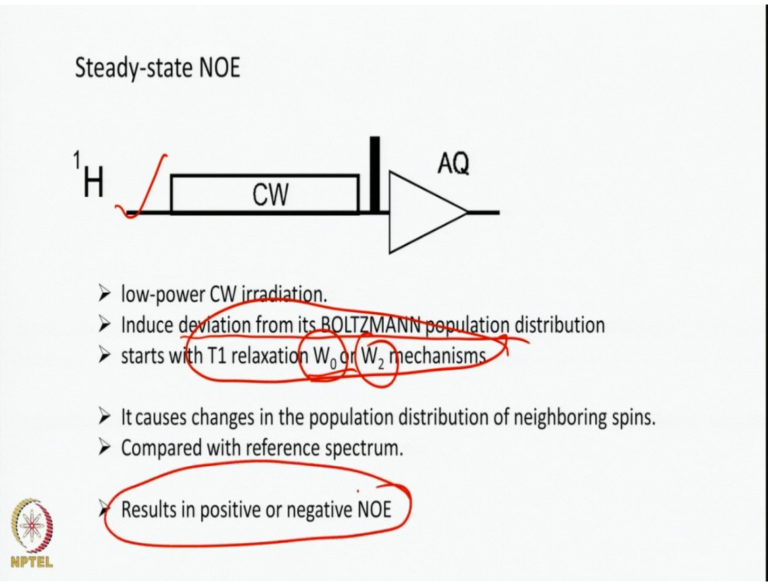

Steady-state NOE

- low-power CW irradiation.
- Induce deviation from its BOLTZMANN population distribution
- starts with T1 relaxation W_0 or W_2 mechanisms
- It causes changes in the population distribution of neighboring spins.
- Compared with reference spectrum.
- Results in positive or negative NOE

So just to refresh you again how we are doing these experiments? In case of a steady-state we were taking two coupled spins, one spin was perturbed with a CW like weak irradiating pulse. Then we were applying 90°pulse and we are acquiring and we are seeing what is the signal change and comparing that with non-irradiated or reference signal.

So we have seen that this long low-power CW irradiation which is shown here actually induces deviation from Boltzmann population distribution and to then relaxation have happens that relaxation because of ω_0 and ω_2. This also we have seen and this happens because of T_1 relaxation.

So we have seen T_1 relaxation is the main cause of these two relaxation ω_0 and ω_2 and it causes population distribution in the neighbouring spin. So that population distribution because of irradiation is compared with a reference spectrum and then we get positive and negative NOE. This, we have understood earlier as well.

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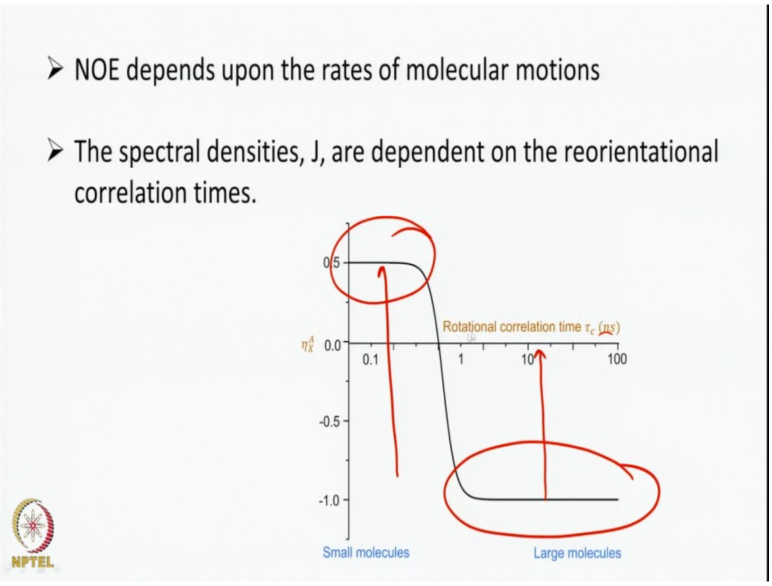
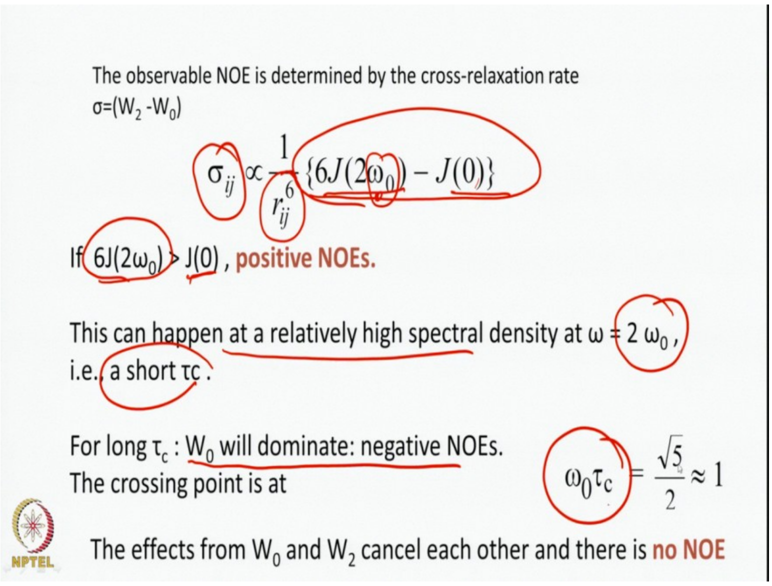


- NOE depends upon the rates of molecular motions
- The spectral densities, J, are dependent on the reorientational correlation times.



The observable NOE is determined by the cross-relaxation rate

σ=(W_2 -W_0)

$$\sigma_{ij} \propto \frac{1}{r_{ij}^6}\{6J(2\omega_0) - J(0)\}$$

If $6J(2\omega_0) > J(0)$, **positive NOEs.**

This can happen at a relatively high spectral density at $\omega = 2\omega_0$, i.e. a short τc.

For long τ_c : W_0 will dominate: negative NOEs.

The crossing point is at $\omega_0\tau_c = \frac{\sqrt{5}}{2} \approx 1$

The effects from W_0 and W_2 cancel each other and there is **no NOE**

So now, refreshing you so observable signal that is determined is given by the ω_0 frequency and spectral density at particular ω_0 because this is double quantum frequency and this is zero quantum frequency. So this is cross correlation rate depends upon our double quantum frequency and zero quantum frequency. So if this term is higher than J_0, we get a positive NOE and that is typical in small molecular case.

So their double quantum frequency is a higher than zero quantum frequency and cross relaxation rate actually depends upon that and it is also inversely proportional to distance between them. For small molecule, this is higher. Therefore, we have a positive NOE. We can have, that is for small molecule, where there is relatively have spectral density at $2\ \omega_0$that is double quantum and we have a short τ_c.

For long τ_c, which is typically seen in a macromolecule omega 0 dominates this whole thing can be negative and if that happens, this is negative, then cross correlation rate which depends upon this form this gives you negative enhancement, so that is what we have seen. So typically there is a crossing point between positive and negative NOE that is given by ω_0 τ_c, which is typically 1.

So if you have seen in previous slide here. So this is a crossing point which is typically around 1, $\omega_0\ \tau_c$is typically around 1, at some cases, like where ω_0 and τ_c is typically 1. So ω_0 relaxation rate and ω_2 relaxation rate will cancel each other and there will be no NOE.

So let me summarise again, ω_2, which is dominant mechanism of relaxation for small molecules gives positive NOE. ω_0, which is dominant relaxation mechanism for macromolecules gives negative NOE. The some cases comes where both of them cancels and that will cancel when ω_0 and τ_c becomes almost 1. So there will be no effect, so effect from ω_0 and ω_2 will cancel each other and there will be no net NOE enhancement for some cases.

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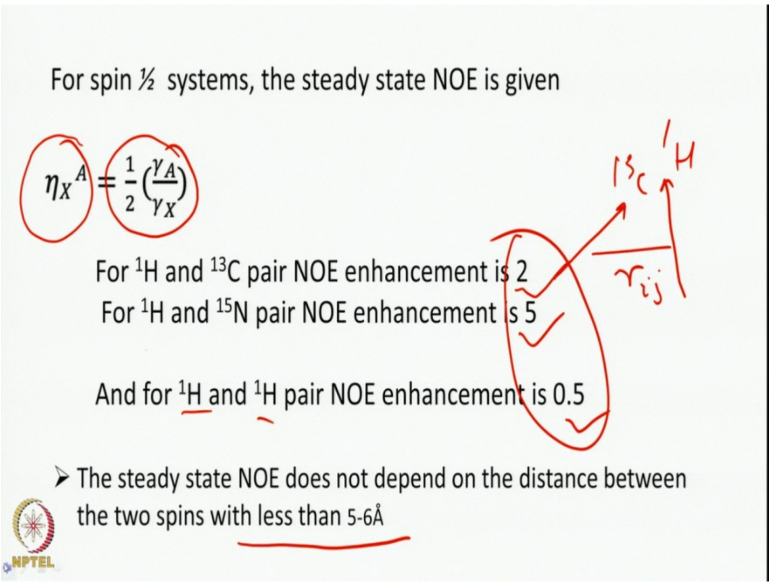

For spin ½ systems, the steady state NOE is given

$$\eta_X{}^A = \frac{1}{2}\left(\frac{\gamma_A}{\gamma_X}\right)$$

For ^{1}H and ^{13}C pair NOE enhancement is 2

For ^{1}H and ^{15}N pair NOE enhancement is 5

And for ^{1}H and ^{1}H pair NOE enhancement is 0.5

- ➤ The steady state NOE does not depend on the distance between the two spins with less than 5-6Å

So we had done detailed calculation to find it out that enhancement between *A* and *X* spin will be given by the gyromagnetic ratio of *A* and *X* spin. So like we have seen if two coupled spin proton and carbon, this is the distance between them r_{ij}. If there coupled proton and carbon, so if this ^{13}C and this is proton the gyromagnetic ratio of this is 4. So we have a enhancement of 2.

If they are proton and nitrogen the NOE enhancement will be 5 and if they are proton, proton. Then NOE enhancement that we get is 0.5. So we have seen steady-state NOE does not depend upon distance but if these two spins are less than 5 to 6 ˚A, you get an enhancement looking at this formula. However, as we said NOE is very, very important parameter to measure the distance quantitatively between two coupled spins. So for doing that experiment, we had seen earlier that there was another statement called transient NOE.

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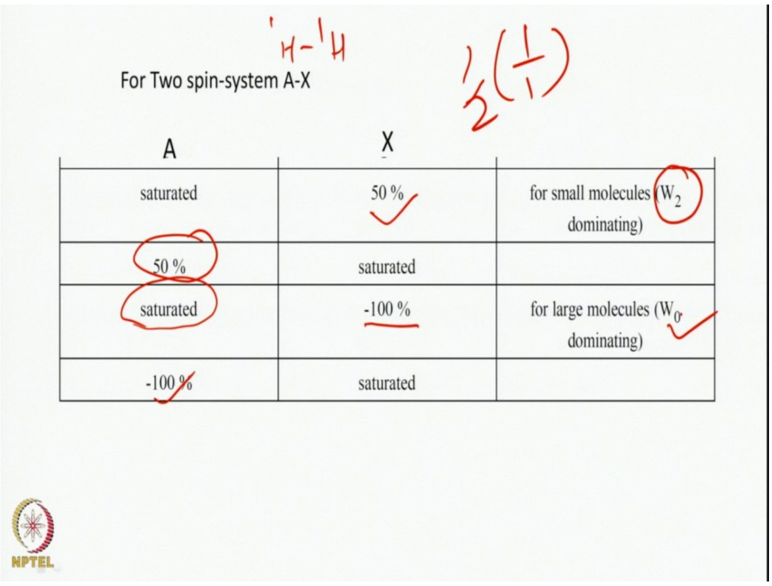

$^1H-^1H$

$\frac{1}{2}\left(\frac{1}{1}\right)$

For Two spin-system A-X

A	X	
saturated	50 %	for small molecules (W$_2$ dominating)
50 %	saturated	
saturated	-100 %	for large molecules (W$_0$ dominating)
-100 %	saturated	

But just look at two spins how much enhancement we get if you do a steady-state NOE. So if you have two spins system like *A* and *X*. So suppose we are saturating spin *A* we get a 50 percent enhancement in spin *X* and we have seen, what are these two spins? Say proton and proton. So their ϒ is equal. So we get $\frac{1}{2}\left(\frac{1}{1}\right)$ that is a 50 percent. So this is generally for small molecules, because ω_2 is dominating.

Now suppose, we are saturating *X* and we are looking at *A* again here enhancement will be 50 percent so both of this is for small molecules. Now suppose, we are saturating here say *A* and for a large molecule, where ω_0 is dominating, you can get the negative NOE enhancement of almost 100 percent because the mechanism is very different. And similarly, *X* spin is saturated then you can get enhancement of 100 percent because for these ω_0 dominating.

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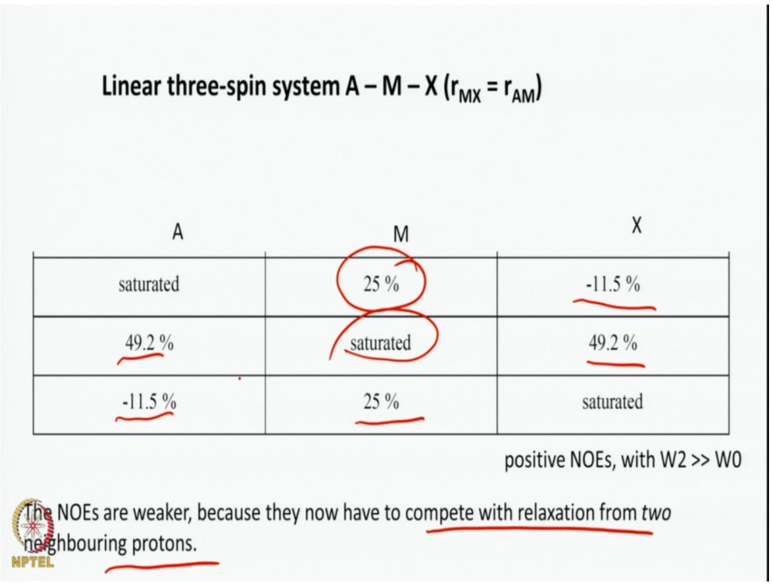

Linear three-spin system A – M – X ($r_{MX} = r_{AM}$)

A	M	X
saturated	25 %	-11.5 %
49.2 %	saturated	49.2 %
-11.5 %	25 %	saturated

positive NOEs, with W2 >> W0

The NOEs are weaker, because they now have to compete with relaxation from *two* neighbouring protons.

Now, let us take linear combination of three spins *A*-*M*-*X*. Now, here we are seeing the distance between *M* to *X* and *A* to *M* is same. So if we are saturating *A*. Now what is happening? Now we saturated *A* by applying a *RF* pulse and *M* and *X* are like their population is changed and they are going, undergoing relaxation now. So now they are competing, both of these are competing mechanism for the relaxation.

So they have to compete for two neighbouring protons and therefore, that NOE enhancement will be distributed. So for such cases, one can do calculation so that *M* will get 25 percent enhancement and *X* will get negative enhancement of 11.5 percent. Similarly, if we saturate *M*, now *M* is sitting in the centre *A* *X* and *A* are located so both of this will be get approximately 50 percent enhancement.

However, if we saturate *X*, now again *X* is located at one side and we are saturated here. So now enhancement will be similar like this 25 percent for *M* and -11.5 percent for *X*. So, in such case, this is three spin system, but now there is a competition of relaxation or redistribution of population between *M* and *X*. Therefore, this is the saturation 25 and 11 percent for both of this.

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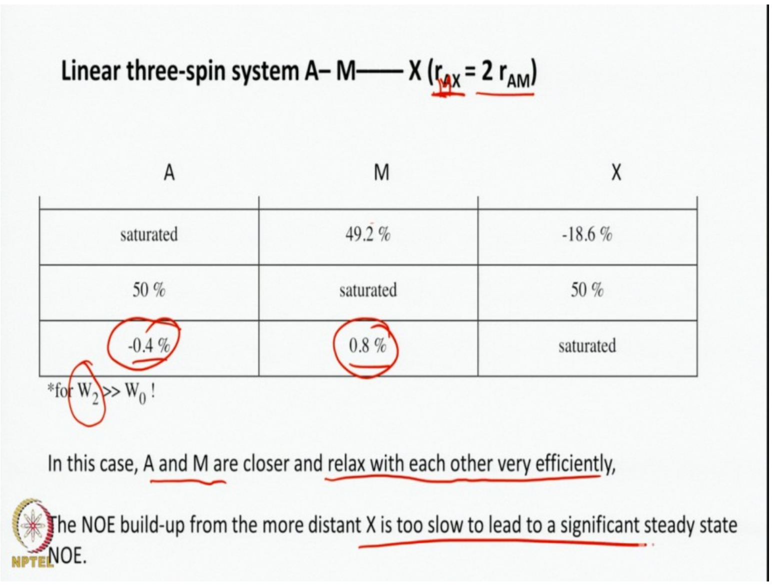

Linear three-spin system A– M—— X ($r_{AX} = 2\, r_{AM}$)

A	M	X
saturated	49.2 %	-18.6 %
50 %	saturated	50 %
-0.4 %	0.8 %	saturated

*for $W_2 >> W_0$!

In this case, A and M are closer and relax with each other very efficiently,

The NOE build-up from the more distant X is too slow to lead to a significant steady state NOE.

But let us see what happens if the distances are different between *M X*. So, say here, *MX* distance 2 time more than *A M* distance. So now, if we saturate *A*, so *M* will get around 50 percent and X will get around minus 18 percent, negative enhancement. And if we saturate *M*, then both will get 50-50 percent and if we saturate again X very less enhancement will happen for *M* and *A*. Here only 0.8 percent and here -0.4 percent and this is the case when ω_2 is much, much higher than ω_0 for small molecule case.

So in this case *A* and *M* are closer and relax with each other, very efficiently. Therefore, you see enhancement is quite a bit, if you saturate *A* almost 50 percent enhancement if you saturate *M* here 50 percent enhancement. So, now one thing is clear because here we change the distance, enhancements are going to be different. So NOE build-up can be measured depending upon distance and that is done in another way, which is called transient NOE.

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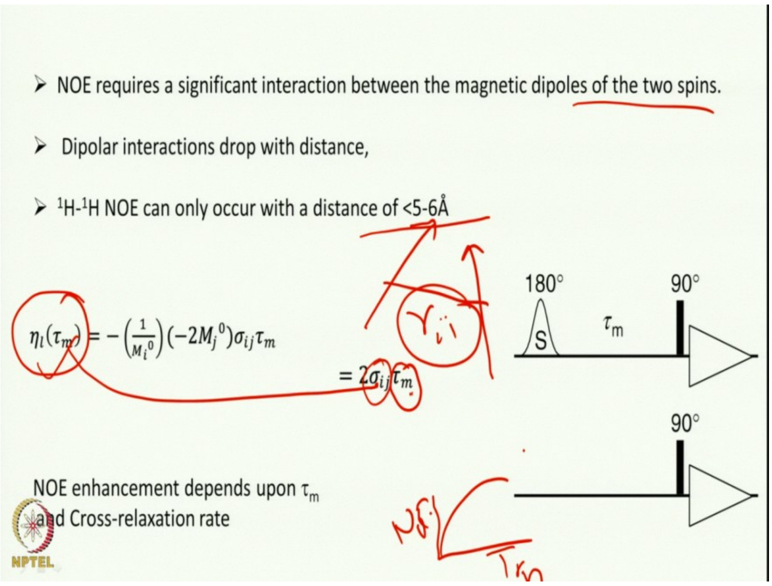

- NOE requires a significant interaction between the magnetic dipoles of the two spins.
- Dipolar interactions drop with distance,
- ^1H-^1H NOE can only occur with a distance of <5-6Å

$$\eta_i(\tau_m) = -\left(\frac{1}{M_i^0}\right)(-2M_j^0)\sigma_{ij}\tau_m$$

$$= 2\sigma_{ij}\tau_m$$

NOE enhancement depends upon τ_m and Cross-relaxation rate

So NOE requires a significant interaction as we have seen between the magnetic dipole and between two spins. So dipolar interactions that we have seen also varies upon distance. So if these two are dipoles it depends upon how much distance between them. So one can measure NOE between two protons up to 5 to 6 ˚A that we had seen earlier and this is that enhancement that depends upon this distance.

So one can vary in this case of transit NOE that can explained earlier. So it depends upon τ_m the mixing time, so to remind you how this experiments was done. There are two coupled spins say I and S or A and X, selectively we are inverting one spin by applying a 180˚pulse, then we are varying this τ_m, which is mixing time we apply a 90˚pulse and brings to XY plane and we detect it here.

In the other experiment we are not doing this, just applying a 90˚pulse, this is a reference experiment and we are detecting here. So because of the selective perturbation of coupled spin and varying this τ_m time, we can see the difference in the NOE enhancement. Because this NOE enhancement directly depends upon our mixing time and also directly depends upon cross relaxation rate. So if they are, both are coupled the two parameters that actually dictates, one is distance between these two spins and another in mixing time.

So if we vary say mixing time, we can get a build-up curve of NOE enhancement. So if we hear tm and we have a NOE so we can get a build-up and from this build-up one can measure how much distance between these two spins.

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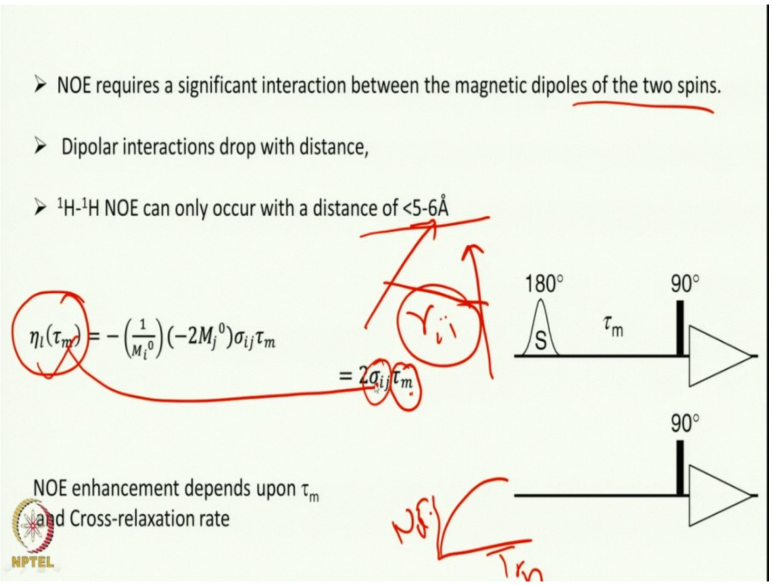

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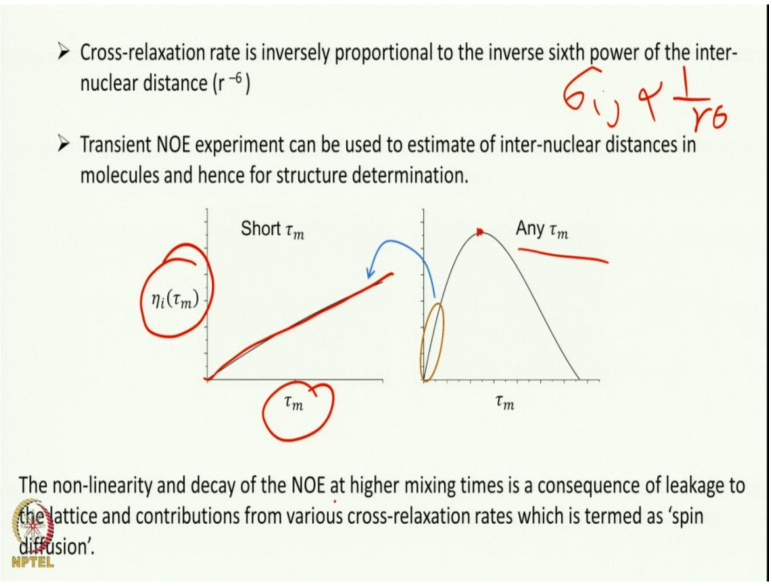
$$= 2\sigma_{ij}\tau_m$$

NOE enhancement depends upon τ_m and Cross-relaxation rate



- Cross-relaxation rate is inversely proportional to the inverse sixth power of the inter-nuclear distance (r^{-6})

$\sigma_{ij} \propto \frac{1}{r^6}$

- Transient NOE experiment can be used to estimate of inter-nuclear distances in molecules and hence for structure determination.

The non-linearity and decay of the NOE at higher mixing times is a consequence of leakage to the lattice and contributions from various cross-relaxation rates which is termed as 'spin diffusion'.

So that is what is done and that is what I will explain it here. So cross relaxation rate as we know how this, this term depends upon the distance between these two spins and it is inversely proportional to r^6. That means this term is inversely proportional to r to the power 6. Now in transient NOE experiment, we can use this to estimate inter-nuclear distance. So here is our enhancement factor and this is τ_m.

For sure τ_m it is very linear, so that is what in this linear regime we can vary and measure the distance and that is use for structural determinants. However, if you keep increasing this mixing time, then it becomes non-linear, here if you look at it keeps increasing and then it starts decreasing that is a non-linear behaviour. Now non-linearity and decay of NOE at higher mixing time is a consequence of leakage of the magnetization because of the lattice contribution and that is because of various cross relaxation rate comes into picture.

So to simplify you, if we keep increasing the mixing time, the NOE enhancement is not going linearly, starts decreasing. What is the reason for decrease? That is various relaxation rates takes over and this mechanism is called spin diffusion, means spin is diffusing and losing out its magnetization because we have kept it too long. So that is called spin diffusion. So in this case measurement is not very precise, so for all the distance measurement we must stick to this linear regime of the τ_m.

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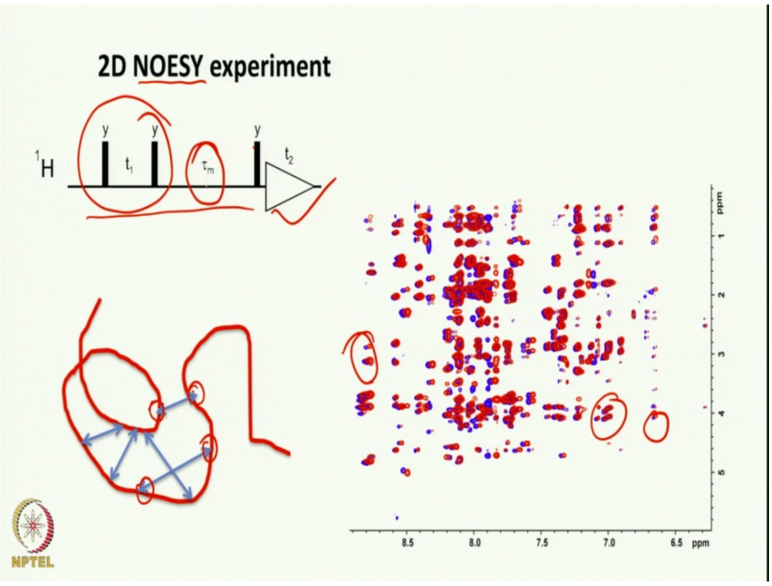

2D NOESY experiment

And precisely these experiments are used for measuring the 2-D experiment, which is called nuclear overhauser effect and this nuclear overhauser effect becomes the holy grail of structural biology because if you do same experiment, this is 2-D so little bit of I just introduced it, here for doing 2-D you added this module, which was not there and in the 1-D, but essentially it works on same formula, you perturb one of the spins and then mix it at various time and then you apply *y* pulse to bring it to *xy* plane and you detect it.

If you have this mixing time spin through dipolar coupling, it starts mixing it and in cross peak insensitivity that comes in this spectrum. So here is overlay upto NOESY spectrum, you can find that, there are various peaks that appears or this is the representation of distance between two coupled spins. So by measuring the intensity of each of these 2-D plots one can get an accurate estimate of distance between these two spins and this is used in twodimensional spectroscopy for getting the distance in protein structure and used for structural biology.

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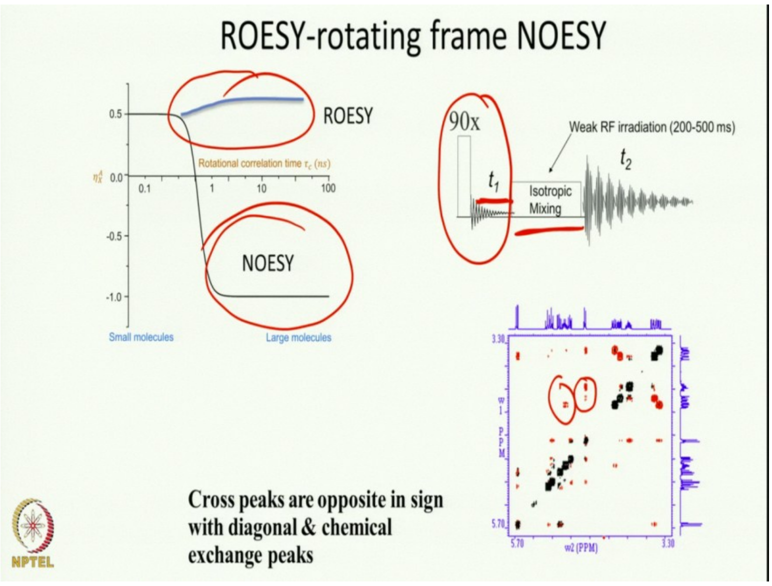
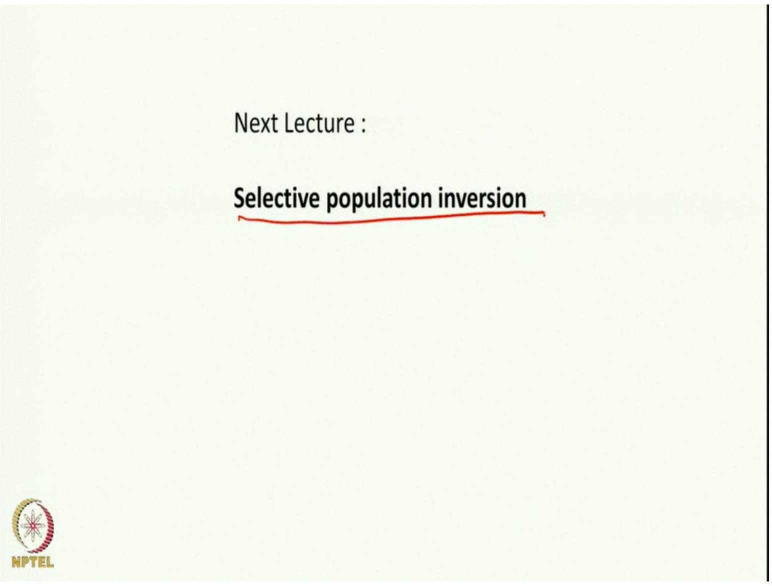


ROESY-rotating frame NOESY

Cross peaks are opposite in sign with diagonal & chemical exchange peaks



Next Lecture :

Selective population inversion

However, as we saw that for small molecules, this NOESY is not very efficient way and the ROESY is the more efficient way, so ROESY is little variation from the NOESY. What you do here? In case of ROESY you apply a mixing pulse, isotropic mixing pulse. So this is 2-D experiment, so when we go to 2-D we will explain little more detail. So here is just for introducing one-time domain for 2-D and then you apply a weak *RF* pulse for mixing the spins.

Similar like a NOESY concept, but here it is done in rotating frame and you mix the spin and you get these magnetization transfer through dipolar coupling and that is what the cross peaks gives the distance information. So if you look at the same curve NOESY is applied for these large molecules and ROESY is applied for small molecules in this correlation time.

So here interesting thing happens that cross peaks comes of opposite sign and then the diagonal peak and this is again used for distance information for small molecules and this distance information comes because these spins are coupled by dipolar coupling small molecules.

So thumb of rule ROESY is applied for small molecules distance determination, NOESY is applied for large molecules distance determination. So with this I would like to stop here and in polarization transfer, next class will discuss about the selective population inversion and how we do it and what we can achieve by doing this in case of heteronuclear system. So will continue with that, if you have any question, please write to us or ask us will be happy answer you. Thank you very much.