Structural Biology

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Week – 06

Lecture - 05

Hi everyone, welcome again to the course of structural biology. Today we are discussing structural biology techniques; this is the last class of this module of NMR spectroscopy. And today we will discuss about 2D and 3D NMR in detail and how those techniques are helping in solving protein structure. So I was talking about routine 2D NMR experiments, the homonuclear through bond correlation method, heteronuclear through bond correlation methods, and through space correlation methods. Example of 2D homonuclear experiment through bond: (a) COSY (the correlation spectroscopy), which also called homonuclear correlated spectroscopy. Its scalar coupling, it is through bond identifies all coupled spin systems correlate between protons that are coupled to each other.

TOCSY (total correlation spectroscopy), it uses spin lock for coherence transfer. During the spin lock all protons of the coupled system become strongly coupled leading to cross peaks between all resonances of a coupled system. INADEQUATE (the incredible natural abundance doubly quantum transfer experiments), here it is 2D ^{13}C-^{13}C INADEQUATE is useful for determining which signal arise from neighbouring carbon. However, it is very intensive as 0.01% of the carbon are excited at natural abundance, use this experiment as a last resort when all the other correlations fails.

Correlation spectroscopy (COSY), here both the axis corresponds to the proton NMR spectra. The COSY spectra indicates which hydrogen atom are coupling to another. The information on the hydrogen that are coupling with each other is obtained by looking at the peaks inside the grid. These peaks are usually shown in a contour type format like height intervals on a map.

In order to see where this information comes from we consider the example of ethyl 2-butenoid. So if you see, there is a cross plot which is A there is a peak. This peak indicates a coupling interaction between hydrogen at 6.9 ppm which you will see here from the values that it is come here from 6.9 and another from 1.8. This corresponds to the coupling of the CH_3 group which is at 1.8 and the adjacent hydrogen which is at 6.9 in the alkene. Similarly the peak mark B indicates the coupling interaction between the hydrogen at 4.15 and the hydrogen at 1.25 which is here. This corresponds to the

coupling of the methyl group and the CH_2 group. Very interestingly if you see the diagonal in the symmetry, you will get another B peak as well as another A peak. Notice that there are a second set of equivalent peaks also marked A and B on the other side of the diagonal.
Example of other 2D homonuclear experiments which are through space. NOESY (nuclear overhausser effect spectroscopy). This is dipolar coupling, identifies neighboring spin system (within 5 angstrom distance). It identifies chemical exchanges because it goes through space. This is single most powerful NMR technique for determining the 3D structure of molecules from conformation of small molecule to the 3 dimensional structure of small proteins.
ROESY (rotating frame overhausser effect spectroscopy) it is very similar to NOESY, except that the initial state is different instead of observing cost relaxation from an initial state of z magnetization the equilibrium magnetization is rotated into the x axis and thus spin locked by an external magnetic field so that it cannot process.

The nuclear overhausser effect: NOE is caused by dipolar coupling between nuclei. They are not connected through bond but they are coming closer to space. This is very interesting because it will give you 3D idea. Suppose you have a protein, this is a protein sequence but when you want to understand the protein structure you want to understand the fold. So by getting this hydrogen, and this hydrogen coming closer you will get idea about which residues are closer. So that is what NOE does. The local field at one nucleus is affected by the presence of the other one. The result is a mutual modulation of resonance frequencies.

In NOE the intensity of the interaction is the function of distance between the nuclei according to the following equation.

$I = A(1/r^6)$

where I is the intensity, A is the scaling constant and r is the inter nuclear distance. So if you see there are three hydrogens, the arrows denote cross relaxation pathways,

$r_{1,2}$ distance between proton 1 and 2 and their interaction

$r_{2,3}$ distance between proton 2 and 3.
The NOE provide a link between an experimentally measurable quantity I and the internuclear distance. NOE is only observed up to 5 angstrom.

Examples of 2D heteronuclear experiments through bond and through space: HSQC (heteronuclear single quantum correlation or coherent spectroscopy), HSQC experiment is one of the most used experiments in biomolecular NMR. The HSQC experiment is one of the fundamental building blocks of course of multidimensional heteronuclear and triple resonance NMR experiments. The HSQC experiment correlates

chemical shifts of one nucleus to another. The 1H-15N pairs in amide groups of amino acids in proteins are convenient reporters of each amino acids, the 1H-15N HSQC spectrum of a protein is a fingerprint can be used to monitor structural changes, you could study ligand binding, you could study variance of pH, temperature, a solvent, a salt anything and these are very critical experiments. For highest sensitivity, uniform 15N labelling is used but for more concentrated samples even natural abundant sample can be analyzed with modern high sensitivity instrumentation and cryogenic probes. They are not at all limited to 1H-15N, 1H-13C important for organic chemistry as well as biomolecular NMR.

One study was performed using HSQC experiment of a protein (calmodulin) in unbound state and bound state of a drug called W7. The HSQC experiment is fundamental building blocks to record scores of multidimensional heteronuclear and triple resonance NMR experiments. Chemical shift of amide 1H correlated to directly bonded 15N for each amino acid. So this is the drug W7 which is also called N6-aminohexyl-5-chloro-1-naphthalene-sulfonamide. If you know sulfonamides are typically used for inhibiting folate biosynthesis pathway. So this enzyme is bound to calmodulin. The work is done by Ikura and co-workers. And if you see the black spots in the spectra, is actually showing the unbound calmodulin whereas when this drug W7 is bound, there is shift which is shown by the red spots you will see clear spots and this will help you to characterize the shifting of the amino acids. So it is a very convenient way, you have a drug library, you could screen it, and you could take the help of HSQC spectrum.

HMQC (heteronuclear multiple quantum correlation), information content compared to HSQC is more or less same. They are same type of process only the single and multiple nuclei. The HSQC versus HMQC analysis of protein had make by Backs and co-workers to find out that the information coming up are more or less same. There are differences based on the relaxation of multiple quantum magnetization as opposed to the single quantum. So the information coming is same but there are differences based on the relaxation. There are differences based on the dipolar broadening of multiple quantum coherence as opposed to the single quantum. There are differences based on unresolved coupling that broadened signals in the directly detected dimension. Multiple quantum magnetization does not evolve with scalar coupling which could be used as an advantage in some cases. HMQC can be different from 1H-15N versus 1H-13C and the size of the molecule. HMQC can be subtle and difference depends on the application.

The HMBC experiment gives correlation between carbon and proton that are separated by 2, 3 and sometime in conjugated systems 4 bonds. Direct 1 bond correlations are suppressed. This gives connectivity information much like a proton-proton COSY. The intensity of cross peaks depends on the coupling constant, which for 3 bond coupling follows the Karplus relationship. For dihedral angle near 90 degrees the coupling is near 0. Thus the absence of cross peak does not confirm that carbon-proton pairs are many

bonds apart.
Also there is HECTOR which is heteronuclear correlation usually correlate between ^{1}H and ^{13}C resonances mediated by the J_{C-H} coupling constant. Proton NMR spectra on one axis and the 13C NMR spectra on the other. The Hector spectra matches the hydrogen to the appropriate carbon. The information on how the hydrogen and carbon are matched is obtained by looking at the peak inside the grid. Again these peaks are usually shown in a contour like format like height intervals on a map.
This is a Hector representation and we are again looking at ethyl 2 butanoate. Here we are looking at again 2 peaks A and B. A is near the middle of the grid, this peak indicates that the hydrogen at 4.1, and attached to the carbon at around 60 ppm, this correspond to the OCH_2 group. Similarly the peak B is indicate hydrogen at near 1.85, is attached to the carbon at 17 ppm. Since the hydrogen is a singlet we know that this correspond to the CH_3 group attached to the carbonyl in the acid part of the ester and not the CH_3 group attached to the CH_2 in the alcohol part of the ester, you could also notice that the carbonyl group from the ester has no match since when the carbonyl part is interacting there is no hydrogen present.

Another one the last one is HOESY, which is heteronuclear overhausser effect spectroscopy it makes correlation between proton and heteronuclei that are close in space like noisy and rosy.

Now we are shifting to 3D or multidimensional NMR. A 3 dimensional NMR experiment can be developed from a 2 dimensional one by incorporating an additional indirect evolution time. In addition to that we also need a second mixing period between the first mixing period and the direct data acquisition. Each of the different indirect time periods (T1 and T2) is incremented separately. There are two principal classes of 3D experiments. One, experiments consisting of two 2D experiments merging their results, second, the triple resonance experiment.

2D spectra like NOESY or TOCSY of larger proteins are often crowded with signals because higher the size of the protein there would be more signal as well as less tumbling. Now if you consider small molecule it is always tumbling in a very high speed so it is not a problem. For macromolecule like protein when it increases its size, the tumbling speed would be decreased. This is very critical for NMR experiments of protein. So when you have up to 100 to 120 amino acids they are okay. But with higher ones, that tumbling becomes slow and that push towards lower resolution. One of the solutions is you go for higher megahertz power. Also, you cannot study the folding of the protein at high temperature because at high temperature, the protein denature, so you need cryoprobe. The spectra spreading out in a third dimension (usually ^{15}N or ^{13}C), so that the signals are distributed in a cube instead of a plane. This spread out is achieved by combining HSQC and NOESY in a single 3D experiment. The noisy experiment is extended by an HSQC step. Acquisitions start after this HSQC step instead of at the end

of the NOESY mixing time. The resulting experiment is called 3D NOESY-HSQC which is very critical for protein. In a similar way a TOCSY HSQC can be constructed by combining the TOCSY and the HSQC experiments.

The triple resonance experiments are the method of choice for the sequential assignment of larger protein greater than 150 amino acids. These experiments are called triple resonance because three different nuclei ^{1}H, ^{13}C and ^{15}N are correlated. The most important advantage of triple resonance spectra is their simplicity; they contain only a few signal of each frequency of an only one. The problem of spectral overlap is therefore, markly reduced this is the main reason why proteins of more than 20 kilo Dalton can be assigned with triple resonance experiment. However, the coordinates of clearly separate signals from different amino acids can accidentally be identical which is called degeneration of signals. The correct choice of connectivity between amino acids is the main problem in the assignment of triple resonance spectra. Another advantage of triple resonance spectra is their high sensitivity which is caused by an efficient transfer of magnetization. The magnetization is transferred via ^{1}J or ^{2}J coupling directly via the covalent chemical bonds. Therefore, the transfer times are shorter and the losses due to relaxation are smaller than in homonuclear experiments. The disadvantage of all triple resonance experiments is the necessity of doubly labeled protein, the preparation of which is often expensive and challenging. There is a whole bunch of triple resonance experiments which cannot be covered in one of this in this one model of my study. I will explain only the general nomenclature of triple resonance experiments and also I will talk about HNCA which is the prototype for all these experiments.

Coming to the nomenclature of triple resonance experiment, the names of triple resonance experiments sound little odd at first glance but they are very descriptive. The names of all nuclei which are used for magnetization transfer during the experiment are listed in the order of their use. Bracketing the names of nuclei which are used only for transfer and whose frequencies are not detected. I will use two experiments as example to illustrate this HNCO and HN (CA) CO.

HNCO experiment, in the HNCO experiment the magnetization is transferred. You will see here this is the (I-1)th residue of the protein and this is the ith residue of the protein they vary with the Rs. Transferring from $H^{N}(i)$ via the N(i) means atom to the directly attached CO(i-1) carbon atom and return the same way to the $H^{N}(i)$ nucleus which is directly detected. The frequencies of all the three nuclei are detected.

In the HN(CA)CO experiment, the magnetization is transferred from the $H^{N}(i)$ proton via the N(i) atom and the CA nucleus ($C_{alpha}(i)$) to the CO(i) carbon and back the same way. The C^{alpha} atoms which are highlighted here in yellow acts only as relay nucleus, its frequency is not at all detected. It is only frequencies of H^{N}, N and CO(red) are recorded. This nomenclature has the advantage that the spectra can be easily imagined

by their names. In the HNCO, an amide proton is correlated with the carbonyl atom of the preceding amino acid, whereas the in HN(CA)CO the correlation to the intra residual carbonyl atom is also visible.

The HNCA experiment is the prototype of all triple resonance experiments. Starting at an amide proton if you see here you have the amide proton CONH the hydrogen, the magnetization is transferred to the directly attached nitrogen so from these it would be transferred to these which is measured as the first spectral dimension. Then the magnetization is transferred to the C alpha nucleus which is measured as second dimension. Afterwards the magnetization is transferred back the same way to the amide proton which is measured as the third dimension. In each step magnetization is transferred via strong ^{1}J couplings between the nuclei.

The coupling which connects the nitrogen atom with the C alpha carbon of the preceding amino acid $^{2}J = 7$ hz is only marginally smaller than the coupling to the directly attached C alpha atom which is 11 hz, thus the nitrogen atom of a given amino acid is correlate with both the C alpha its own and the one of the preceding amino acid. Therefore it is possible to assign the protein backbone exclusively with an HNCA spectrum. But usually more triple resonance experiments are needed because the cross signal of the preceding amino acid has to be identified and degenerate resonance frequencies have also be resolved.

The aim of the analysis of NMR spectra is to extract all available information about inter atomic distances and torsion angles. In the initial stage of investigation by NMR spectroscopy each resonance must be associated with a specific nucleus in the investigated molecule. The strategies employed for the assignment procedure depend on whether only a homonuclear 2D spectra are available which are unlabeled protein, whether ^{15}N heteronuclear spectra are available ^{15}N level proteins, whether triple resonance spectra both ^{15}N and ^{13}C proton is already there doubly level protein are available. But in general the assignment can be divided in two parts. The sequential assignment of amino acids in the protein sequence and the assignment of the amino acid side chain.

Secondary structure determination by NMR techniques does not require a full three dimensional structural analysis. Knowledge of the amide and alpha proton chemical shifts are in principle all that is necessary. But if this information is available it is also considered like when you see that the chemical shifts are available it is we consider that nearly complete assignment of side chain protons are also available. While obtaining the sequential resonance assignments in a laborious task the NMR method is perhaps the most powerful and certainly the most accurate method of secondary structure determination without a three dimensional structure.

The three bond coupling constant between the inter residual alpha and amide protons is the most useful for secondary structure determination. It is directly related to the backbone dihedral angle phi but unfortunately there is no three bond proton coupling that can be related to the angle psi.
So right hand alpha helix phi = -57 and 3J H_AH_N = 3.9 Hz.

Right handed 3_{10} helix phi = -60 and 3J H_AH_N = 4.2 Hz.

Anti-parallel beta sheet phi = -139 and 3J H_AH_N = 8.9 Hz.
Parallel beta sheet phi = -119 and 3J H_AH_N = 9.7 Hz and

left handed alpha helix phi = 57 and 3J H_AH_N = 6.9Hz.

NOE is instrumental, a number of short less than 5 angstrom distances are fairly unique to secondary structural elements. For example, alpha helices are characterized by short distances between certain protons on sequentially neighboring residues between backbone amide protons, which is dNN, as well as between the beta protons of residue i and the amide protons of residue i+1 which is dBN. Helical conformation result in short distances between the alpha proton of residue I and the amide proton of residue i + 3 and to a lesser extent i + 4 and i + 2. Since i + 2 and i + 3, i + 4 NOEs are collectively referred to as medium range NOEs while NOEs connecting residues separated by more than 5 residues are referred to as long range NOE. Long range NOE is normally is weak but it need like separate experimental setup or special condition. Extended conformation for example, beta strand on the other hand are characterized by short sequential DAN means alpha and distances you could see all the parameters here. The formation of sheets also result in short distances between protons on adjacent strands dAA and dAN. So for alpha helix the sequential NOEs observed in 3D, 15N edited NOESY indicative of alpha helix. So if you look at the secondary structure assignment of this protein, there is amide proton exchange rate with solvent water which is filled with the diamonds here, sequential backbone dNN and dAN NOE connectivities are classified as strong, weak or absent and are represented by the thickness. So, you know if you look at you will see that how thick they are on the spot and you could understand them of a bar connecting the residues in question. Medium range NOE connectivities dAN (i to i + 3 and i to i + 4) are drawn as line segment connecting the residue contributing to the observed cross peak if it present. Coming to beta sheet, across strand NOE is observed in 3D ^{15}N edited NOESY and 3D ^{13}C edited NOESY, are indicative of beta sheet.
Alignment of the four stranded beta sheet observed in GRX3 deduced from inter strand NOE connectivities. The amino acid backbone is represented in stick format with residue numbers indicated above the C alpha position. Inter strand NOEs are drawn as double headed arrows connecting protons giving rise to the observed cross peaks. Slowly exchanging amide protons are identified in red. The turns sequential NOEs observed at

3D 15 nitrogen edited NOESY are indicative of turn. Similar to alpha helix, shorter amino acid stretches connect beta strand.

Amide proton exchange rates, the regular hydrogen bonded secondary structure protect amide proton involved in them as evidenced by their significantly reduced amide proton exchange rates with the solvent. Although nearly all polypeptide amide protons are involved in hydrogen bonds in a globular protein those in regular secondary structures appear to be longer lived. For example, after placing a lyophilized sample of BPTI, Bobhain protease trypsin inhibitor into water many amide protons are completely replaced with deuterium within 1 hour. Over the next several hours the amide protons in the N terminal and then the C terminal helix also completely exchange. Some amide protons participating in the central anti-parallel sheet are still present after some months.

Since the chemical shift of a nucleus is sensitive to the environment it should also contain structural information. Correlation between chemical shift tendencies and secondary structures have been identified. The alpha proton of all 20 naturally occurring amino acids has been shown to have a strong correlation with the secondary structure. Wissert et al have produced a simple method for secondary structure determination by analysing the difference between the alpha protons chemical shift for each residue and that reported for the same residue type in a random coil. So they are taking alpha proton chemical shift and then from the secondary structure and they are comparing them with a random coil. Helical segment have grouping of alpha protons whose chemical shift are consistently less than the random coil values whereas beta strand had values consistently greater. So you could get the standard and you could differentiate them. In this way the location of helix and strand segments are possible and quite reliable although the boundaries of the secondary structural elements are difficult to determine. So if you see chemical shift difference between C alpha C beta random coil values and experimentally observed values yields secondary structure chemical shift.
So if you see on the phi psi plot you will see that the helix $\Delta C\alpha$ is 3 ppm, $\Delta C\beta$ is -1 ppm whereas in beta strand $\Delta C\alpha$ is -2 ppm, $\Delta C\beta$ is 3 ppm and this is the chemical shift of the entire protein.

Selection of secondary structural segments, unfortunately low single criteria coupling constant should amide-proton exchange or short and medium range NOEs is sufficient for an unambiguous assignment of secondary structure. For example, short distance greater than 3.6 angstrom or greater than or less than 6.8 angstrom here between sequentially neighboring amide-proton DNN are a necessary consequence of a helical conformation but the presence of a short sequential DNN is not a sufficient criteria for helical prediction as 49% of such occurrences are not in helical conformation according to Urdreich's work in 1986. However sequential stretches of residues with consistent secondary structure characteristics NOEs coupling constant slowly exchanging amide-

proton and chemical shifts together provide a reliable indication of the location of this structural segment.

Going from calculation of tertiary structure, the idea of computer aided structure calculation is to convert distance and torsion angle data the constant into a visible structure. However the experimentally determined distances and torsion angles by themselves are not sufficient to fully characterize a protein structure as they are based on a limited number of proton-proton distances. Only the knowledge of empirical input data such as bond lengths of all covalently attached atoms and bond angles enables a reasonably exact structure determination. To do that a randomly folded starting structure is calculated from the empirical data and the known amino acid sequence. The computer program then tries to fold the starting structure in such a way that the experimentally determined inter-proton distances are satisfied by the calculated structure. In order to achieve this each known parameter is assigned an energy potential which will give minimal energy if the calculated distance or angle coincides with its input value. The computer program tries to calculate a structure with a possibly small overall energy. Without the experimentally determined distance and torsion angle constants from the NMR spectra the protein molecule can adopt a huge number of conformations around its chemical bonds in the N - C^{alpha} bond and the C^{alpha} - CO bond. All these possible conformations are summed up in the so-called conformational space to get a optimized conformation. Therefore it is important to identify as many constants as possible from the NMR spectra to restrict the conformational space as much as possible thus getting close to the true structure of the protein. In fact the number of constraints employed is more important than the accuracy of proton-proton distances so that the classification is sufficiently precise.

For calculation of tertiary structures there are various computer programs, employing two in principle different methods for calculating a protein structure. Distance geometry or DG: This method is based on a calculation of matrices of distance constants for each pair of atoms from all available distance constants, bond and torsion angles as well as Van der Waals radii. This set of distances is seen then projected from the N dimensional distance space into the 3 dimensional space of a Cartesian coordinate system. This would enable the user to determine the coordinates of all atoms of the protein. Another one is simulated annealing (SA), I have talked about simulated annealing before in X-ray. This is a molecular dynamics method which takes place directly in the Cartesian coordinate system. We have discussed this while talking about refining the crystal structures. In this method a stoting structure is heated to a high temperature in a simulation that is the atoms of the stoting structure get a high thermal mobility. During many discrete cooling steps the starting structure can evolve towards the energetically favorable final structure under the influence of a force field derived from the constraints.

So how we will do the protein sample preparation? We will do the structure determination by NMR typically requires a protein concentration of 0.5 to 3 millimolar or greater stable for several days at the desired temperature usually 20 - 40°C (the experimental temperature). Proteins and peptides should be dissolved in a suitable buffer 10 to 50 millimolars with 10 percent D2O. Ideally we would aim for an acidic buffer pH 4 to 7 but dissolve the sample in a buffer that provide maximum stability and solubility of the sample. As the sample needs to be stable for days the buffer content plays a critical role in protein peptide sample stability.

The NMR structure of a protein is presented as a bundle of conformer as you see this is the representation of a NMR structure of SS10352 protein from Synechocystis species and this is the crystal structure of the same. So you see that the dynamics of the loop is higher so you get a lot of conformation. Each conformer presents a good solution to the NMR restraint. First conformer usually is the best structure and finally typically a bundle of 20 conformers are deposited in the PDB.

So we started from a zero concept in the periodic table finding out NMR active nuclei and then come to depositing the PDB that is what the journey is. So why we study protein NMR, why it is important? Initial days people used to think that we are studying NMR for proteins that are hard to crystallize which is was definitely true which is true today also. But what is changed we have found many other application of NMR structure. For protein that can be dissolved at high concentration to study dynamics of protein conformational equilibrium, folding and intra intermolecular interactions. For proteins which are drug target to screen a protein drug interaction for DNA-DNA drug interaction but honestly with advent of new techniques, with advent of more sensitivity, with advent of more automation possibilities are increasing. If you look at so many different type of interactions are already you see so many different type of constructions of experiments, so many different setup and how differently they are giving information. This is definitely in a point from where we could go in such a state where we are getting more and more information regarding a protein. We will get information about more about the functional state of a protein, more about when a protein is binding to a substrate, more about transition state, more about its intermediates and what not. With that I will finish this module but before that I will go through a brief idea what we have learnt through NMR. I will talk about brief ideas of NMR. We have the first idea that some nuclei have non-zero spin quantum number making them NMR active. If current passed through a coil it induces a magnetic field and a changing magnetic field in the coil induces a current vice versa. Placing nuclei with a spin I of half into a magnetic field leads to a net magnetization aligned along the magnetic field axis. When the B1 field is turned on the external magnetic field the net magnetization rotates down into the XY plane. When the B1 field is turned off the net magnetization relaxes back to the z axis with the time constant T1. Individuals spin precess about the magnetic field axis.

After magnetization is rotated into the xy plane by the B1 field produced from a pulse through the cell it will precess in the XY plane. The individual magnetization vector whirling around in the xy plane represent a changing magnetic field and will induce a current in the sample coil which has its axis along the x axis. NMR signal is a Fourier transform of the oscillating current induced in the sample coil. Nuclear spins produce small magnetic fields. Electrons are spin I equal to half particles; they produce small magnetic fields which oppose the external magnetic field. So we talked about the nucleons, the nucleons are producing magnetic field because of the charge of the proton and the spin of the proton and neutron. Here electrons are also developing magnetic field which are opposed to the external magnetic field. The surrounding electrons still the nuclear spins from the larger external B0 field this results in a reduction in the energy spacing of the two energy levels and a lower Larmor frequency which is called chemical shift.

In a frame of reference that rotates at the Larmor precision frequency magnetization that is placed along the x axis does not move it simply relaxes back to the z axis via T1 process. The nuclei with different chemical shifts and Larmor frequencies will rotate around the z axis at different speeds. T2 is the time constant for the magnetization vector to de-phase in the xy plane. Idea 15 all the concept discussed above leads to different 1D NMR experiments combining them higher dimension like 2D, 3D experiments are possible which could be categorized into following groups. Homonuclear through bond correlation,

Heteronuclear through bond correlation,

Homonuclear through space correlation and

Heteronuclear through space correlation

A higher dimensional NMR experiment can be developed from a two dimensional one by incorporating additional indirect evolution times empowered with additional addition of NMR active nuclei if it is required. Like a 3D NMR experiment would be developed by performing experiment consists of two 2D NMR experiment or the triple resonance experiment.

The aim of the analysis of NMR spectra is to extract all available information about inter atomic distances and torsion angles for doing that each resonance must be associated with a specific nucleus in the investigated molecule which is called assignment. Analysis of 2D data would be performed by using chemical shift, nuclear overhausser effect, spectroscopy, coupling constants and amide proton exchange rates. Further in dimension computer aided structure calculation is to convert distance and torsion angle data constants into a visible structure mainly working in the principle of distance geometry and simulated annealing. After solving the structure, structure quality measures are

performed using knowledge based methods like comparing existing crystal structure, MVD, measurement, RPF score etc using bond length and bond angle distribution, dihedral angle distribution, atomic packing, hydrogen bond geometries and other geometric features. The last idea now you started from scratch you come to structure development you have already refined your structure you check the structure finally the structure would be deposited to protein data bank using 20 conformers first one with the highest resolution. So this is our journey about NMR as I told in the beginning of the course this is not a course to teach you an about the details of NMR this is a course which will give you basic knowledge how NMR works and I hope this will make you excited to know more about NMR because as I have tried to talk through the course through the module that this is one of the next generation technique with millions of potentials with that I will finish today.