Chapter 2
Chapter 2
Experimental Protocols and Data Analysis

This chapter elaborates the methods of purification and isolation of GM1, preparation and characterization of membranes containing GM1; followed by description of the spectroscopic, biophysical and molecular modeling techniques and analysis used throughout the study regarding structural characterizations and interactions of selective proteins/peptides in the environment of GM1 containing model membranes, micelles and bicelles.

2.1. Purification of GM1

2.1.1. Isolation of Total Gangliosides from Goat Brain

Mixture of Gangliosides was isolated from goat brain following the extraction protocol previously published from our laboratory [1]. Briefly, about 150 gm of clean brain tissue made completely free from blood vessels and homogenized in a glass-warring blender with 3.0 liters chloroform-methanol (2:1) solvent (1 mg/20 ml solvent). The homogenate was filtered through Whatman large filter paper and clear filtrate was collected separately. The filtrate was mixed with 0.1 M KCl (aqueous) solution having about 1/5th volume of the filtrate. The final solution was vigorously shaken and kept overnight for phase separation. Clear upper phase was collected and the KCl concentration was adjusted to below 0.04 M by appropriately diluting the solution with methanol. The final solution was passed through sephadex G-25 column (2 x 15 cm, acetate form) that
separates elute according to molecular weight. After the sample completely passed through the column, the column was washed with three column volumes of methanol. The column bound mixture of gangliosides was eluted first with 0.075 M ammonium acetate and then with 0.4 M ammonium acetate in methanol. After the column eluted with three volumes of 0.075 M-ammonium acetate, two volumes of methanol was passed through the column to reactivate it. Elute after four bed volume of 0.4 M ammonium acetate passed through the column was discarded after test for the presence of sialic acid by resorcinol-HCl reagent.

Ganglioside in the eluted solution was primarily checked using the sialic acid estimation assay with resorcinol-HCl reagent developed by Svennerholm [2], where solution of N-acetyl neuraminic acid (NANA), from Sigma Aldrich was used as the standard (10^-8 mole solution of sialic acid). The standard sialic acid sample was heated in boiling water with freshly prepared resorcinol reagent (method of preparing resorcinol reagent is described in subsection 2.1.2) and after 20 minutes of heating each of the solution was cooled. The purple color appeared in solution was extracted in n-butyl-acetate/butanol (85:15 v/v). The absorbance was checked at 580 nm wavelength. Then 10 ml each of the ganglioside elutes (0.075 M and 0.4 M respectively) were pooled, concentrated, and dried under vacuum. 20 μl water was added to hydrate both the dried samples and resorcinol-HCl test was performed to check the purple coloration due to presence of NANA in both.

The total ganglioside elutes and methanol washing were assembled and dialyzed against double distilled water. Dialyzed sample was then lyophilized to fluffy white powder containing a mixture of mono-, di- and tri sialo gangliosides (GM1, GD1 and GT1). From
150 gm tissue we obtained nearly 120 mg of gangliosides mixture. Presence of mixtures of gangliosides was evidenced by thin layer chromatography (TLC) on thin glass plates coated with silica gel G (activated by heating at 110°C for 45 min) using a solvent system chloroform-methanol-0.2% aqueous calcium chloride, composition 60:40:9. Gangliosides on the developed plates were revealed with iodine vapor.

### 2.1.2. Preparation of Resorcinol reagent and Determination of Sialic Acid

A standard $10^{-8}$ mole solution of sialic acid (MW 309.2) was prepared. Stock solution of 2 gm of resorcinol (crystallized from hot benzene) in 100 ml of water was prepared and kept refrigerated in a dark bottle. Just before use, 1 ml of resorcinol stock solution was mixed with 8 ml of concentrated HCl and 0.025 ml of 0.1 M CuSO$_4$ and the final volume made up to 10 ml.

20 µl of a stock solution containing sialic acid along with 730 µl water and 750 µl of resorcinol reagent were mixed together and put in screw-capped tubes. Sample in screw-capped tube was put on boiling water bath for 20 minutes to get purple coluration. After cooling, the colour was extracted in 3.0 ml of n-butyl acetate/n-butanol (85:15 v/v). Absorbance was checked at 580 nm.

### 2.1.3. Standardization of NANA solutions

NANA solutions were varied over the range usually used i.e. 0.02 to 0.06 µmoles. To each of the samples containing NANA in a volume of 0.2 ml, 0.1 ml of 0.2 M sodium metaperiodate solution was added and allowed to stand at room temperature for 20 minutes. Then, 1.0 ml of 0.755 M sodium arsenite solution was added to each of the tubes.
and the solutions are shaken until a yellow brown color disappeared. Thioburbituric acid solution (twice crystallized form in 0.5 M sodium sulfate, supernatant only used in the assay) 3.0 ml 0.6 % was added; the tubes were shaken, capped with a glass bead, and then heated in a boiling water bath for 15 minutes. The tubes were then removed and cooled in ice-cold water for 5 minutes. The total 4.3 ml solution was transferred now to another tube, which already contained 4.3 ml of cyclohexanone. The samples were shaken in a vortex, and then centrifuged for 3 minutes in a clinical centrifuge. The clear upper cyclohexanone phase was red and the red was more intense in cyclohexanone than in water. Absorbance of the organic phase at 549nm ($A_{549}$) was read versus a water blank. The molecular extinction coefficient of NANA is 57000. The amount of NANA present in a sample can be determined using the following equation

$$\mu \text{moles NANA released} = \frac{V \times [A_{549}(\text{sample}) - A_{549}(\text{blank})]}{57} \quad (2.1)$$

$$4.3 \times \frac{[A_{549}(\text{sample}) - A_{549}(\text{blank})]}{57} = A_{549}(\text{sample}) - A_{549}(\text{blank}) \times 0.075 \quad (2.2)$$

![Figure 2.1. Standardization of NANA and Estimation of Gangliosides (red box)](image-url)
2.1.4 Neuraminidase Treatment and Preparation of GM1 from Ganglioside Mixture

GM1 was prepared from the ganglioside mixture by the treatment of the enzyme neuraminidase of Clostridium Perfringens (from Sigma Aldrich). The coupled enzyme assay for determining neuraminidase was previously described by Ziegler and Hutchinson [3].

2.1.5. Neuraminidase Assay of Gangliosides

One unit of neuraminidase causes release of one micromole of sialic acid per minute at 37°C and pH 5.4. 40 mg of the ganglioside mixture was dissolved in 2 ml of 0.1M sodium acetate (pH 5.4) buffer and 20 μl of BSA stock (1mg/1ml buffer) was added to this ganglioside solution. 1.6 unit of the enzyme (in 100 μl buffer) (approx. 0.2 mg) was dissolved in the mixture and the final mixture was incubated at 37°C for 30 minutes. A volume of 0.2 ml sample from a 1 mg/ml of the ganglioside mixture was taken as the test sample wherefrom released NANA was primarily estimated by thiobarbituric acid assay. Absorbance of pink colored organic phase was checked at 549 nm (Figure 2.1). The enzyme treated entire solution was then diluted with 10 ml methanol, passed through again acetate treated sephadex G-25 column and eluted by washing with 0.4 M ammonium acetate. The solution was concentrated to evaporate excess methanol and then dialyzed in a 1kDa cut off Sigma dialysis bag and lyophilized.
Total sialic acid content of the ganglioside after neuraminidase treatment (Figure 2.2) was determined by the resorcinol-HCl reagent ($A_{580}$ nm) as mentioned earlier for the estimation of NANA. The pooled solutions that give positive resorcinol test were again dialyzed and lyophilized. TLC with the finally purified GM1 gives only a single band with relative mobility 0.85 for GM1 when kept in contact with iodine vapour. From 40 mg of ganglioside mixture, we recovered 22 mg of GM1.

### 2.2. Preparation of GM1 Containing Bicelles

Small isotropic DMPC/CHAPS (1:4) and DMPC/CHAPS/GM1 (1:4:0.3) bicelles were prepared. The protocol to prepare GM1 containing bicelles was standardized [4]. To prepare the bicelles, first a 75 mM stock solution of DMPC was prepared by suspending 5.0165 mg of DMPC in 100 μl of H$_2$O. The solution was vortexed and centrifuged at
room temperature, and the pellets of DMPC were re-suspended with vortexing. Repeating
the cycle for at least ten times, a uniform homogenous dispersion of DMPC was obtained.
Now CHAPS from a 500 mM stock solution (in water) was added to the DMPC
dispersion to achieve q = 0.25 with total lipid content in solution 92.5 mM. The sample
was vortexed until the solution was clear and transparent. The solution was again
centrifuged and no pelleted lipid was separated from the sample after the centrifugation
step. GM1 was incorporated in the bicelles at 30 mol% of the phospholipid (DMPC) to
prepare bicelles with GM1. GM1 and DMPC were weighed first to prepare a
homogenous of 3.46 mg GM1 and 5.0165 mg DMPC and then CHAPS was added in
appropriate amount to make the solution clear following the protocol as stated above. The
final dilution was done by deionized water of pH 6.6. The pH was adjusted by adding
small volumes of 1M HCL or 1M NaOH. No buffer was used in order to keep the ionic
strength minimum (Characterization of bicelles is discussed in Chapter 4).

2.3 Experimental Principles

2.3.1. Fluorescence Spectroscopy

2.3.1.1. Principle and Jablonsky Diagram

Fluorescence spectroscopy is a spectroscopy which analyzes the fluorescence from a
sample. Absorption of light causes the excitation of electrons when the electrons are
moved from lower to higher energy levels. The phenomenon of Fluorescence Emission is
recognized as to be the deactivation of the excited molecule in an emissive pathway
having spin multiplicity conserved (ΔS = 0); typically fluorescence emitted frequency is
red-shifted with respect to the excitation frequency. In a typical experiment, the different
wavelengths of emission by the sample are measured holding the excitation source at a
constant wavelength. Fluorescence spectra are presented by plotting the intensity of
emission on the ordinate and emission wavelength at the abscissa. The device that
measures the fluorescence is called fluorimeter and the four components that are essential
for fluorescence measurement are an excitation source, a fluorophore, wavelength filters
to isolate emission photons from excitation photons and a detector that accepts emitted
photons and produces recordable output, usually in form of a Gaussian line shape signal.
Fluorescence is illustrated by simple electronic-state diagram (Jablonski Diagram Figure
2.3) which illustrates stages 1) Excitation: A photon of energy hv is absorbed by the
fluorophore, creating an excited electronic singlet state (S₁) 2) Excited-state lifetime:
During the excited state lifetime, the fluorophore undergoes possible conformational
changes and performs possible interactions with its molecular environment. Collision
with other molecules cause the excited state to loss vibrational energy (internal
conversion or vibrational relaxation) until it reaches the lowest vibrational energy state of
the excited singlet (S₁) (Other processes such as collisional quenching, fluorescence
resonance energy transfer (FRET) and intersystem crossing (ISC) may also populate the
relaxed excited singlet (S₁)) 3) Fluorescence Emission: While returning to the ground
state (S₀), a photon of energy hv is emitted. Due to the relaxation of energy in the excited
state, the energy of photons emitted is lower, therefore emitting at a longer wavelength.
The difference in wavelength is called Stokes shift. Intensity of fluorescence is
quantitatively dependent on parameters like excitation source intensity, molar excitation
coefficient of the fluorophore, optical pathlength, solute concentration, and detector
sensitivity of the instrument [5].
2.3.1.2. Fluorescence Quenching

Fluorescence quenching may be referred to any process that reduces the fluorescence intensity of a fluorophore. Fluorescence quenching is customarily classified into two categories, namely, static and dynamic quenching. Static quenching is the case where quenching is the result of formation of a weak, non-fluorescent ground-state complex between the fluorophore and the quencher so that fluorescence intensity is reduced as a result of depletion in total population of the fluorophore. Collisional quenching requires the diffusion of the quencher to the fluorophore within the excited-state lifetime of the latter, resulting in quenching. Another case of decrease of fluorescence intensity of a fluorophore has been observed in connection with, fluorescence resonance energy transfer (FRET), in which an excited state interaction exists like in collision quenching,
but the two participating molecules do not have to collide; only excitation is transferred by electronic coupling to an acceptor molecule.

In general, a quenching process can be represented by the following equation:

\[ A^* + Q^* \rightarrow A + Q \]  

(2.3)

Or,

\[ A^* + Q \rightarrow A + Q^* \]  

(2.4)

Where, \( A \) is one chemical species, \( Q \) is the quencher and * designates an excited state.

Fluorescence quenching experiments measure the fluorescence intensity as the function of concentration of quencher. If, \( I_f^0 \) and \( I_f \) are, respectively, the intensities in the absence and presence of the quencher, and \( \tau_0 \) and \( \tau \) are the fluorescence lifetimes of the fluorophore in the aforementioned conditions and \([Q]\), the concentration of the quencher; then, the bimolecular quenching rate constant \( k_q \) is evaluated from the gradient of the Stern-Volmer plot as [5],

\[ \frac{I_f^0}{I_f} = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q] \]  

(2.5)

Or, \( \frac{\tau_0}{\tau} = 1 + k_q\tau_0[Q] \)  

(2.6)

2.3.1.3. Fluorescence Lifetime

The fluorescence lifetime refers to the average time the molecule stays in its excited state before deactivation to the ground-state. The lifetime of a fluorophore can be femtoseconds to hundreds of nanoseconds, which is measured either by time domain method or by frequency domain method. In the time domain method or the pulse method, the sample is illuminated with a short pulse of light and the intensity of the emission
versus time is recorded. If the decay is a single exponential and the lifetime is long compared to the exciting light, then the lifetime can be determined directly from the slope of the curve. In the frequency domain method or the phase modulated method, the sample is illuminated with sinusoidally modulated light and the phase shift and demodulation of emission, relative to the incident light, is used to calculate the lifetime.

According to time domain method or pulse method, lifetime measurement is the time required by a population of $N$ excited fluorophores to decrease exponentially to $N/e$ by losing excitation energy through fluorescence and other deactivation pathways. Since the intensity of fluorescence is proportional to $N$, the definition can be written as:

$$ F(t) = F_0 e^{-t/\tau} $$  \hspace{1cm} (2.7)

Where $F$ and $F_0$ are the intensities at any time $t$ and $t=0$ assuming $t$ is the excited state fluorescence lifetime. The definition assumes that the fluorescence arises from a single fluorophore and decay occurs as a single exponential.

Frequently, the decays are not adequately described by a single exponential. Then the observed decay is generally fitted to a sum of exponentials

$$ F(t) = \sum_{i} \alpha_i e^{-t/\tau_i} $$  \hspace{1cm} (2.8)

Where, $\alpha_i$ is the pre-exponential factor representing the fractional contribution to the time-resolved decay of the component with lifetime $\tau_i$ and $\tau_i$ represents the lifetime of individual species $i$. For heterogeneous samples, where there are several different fluorophores or one fluorophore in different environments with each species decaying exponentially, the fractional intensity ($f_i$) of each species is given by

$$ f_i = \frac{\alpha_i \tau_i}{\sum_i \alpha_i \tau_i} $$  \hspace{1cm} (2.9)
Here, the average lifetime is calculated as,

\[ \langle \tau \rangle = \sum_i f_i \tau_i \]  

(2.10)

The lifetime plot has time in its abscissa and intensity at its ordinate [5].

2.3.1.4. Fluorescence Anisotropy

Upon excitation by a polarized light, the light emitted by a fluorescent sample should be polarized. However, movement of particles in solution scrambles the polarized light, and forces the sample to emit light at a different angle than the incident one. The "scrambling polarization" effect is greatest with fluorophores freely tumbling in solution and decreases with decreased rates of tumbling. Upon binding to a partner molecule which is larger, or in a viscous solvent, the fluorophore should tumble more slowly (thus, increasing the polarization of the emitted light and reducing the "scrambling" effect). Anisotropy is directly related to the polarization, and is the ratio of the polarized-light component to the total light intensity.

For a vertical polarized emission, \( I_x = I_{\parallel} \) and \( I_y = I_{\perp} \). The anisotropy \( \langle r \rangle \) is defined as the ratio

\[ r = \frac{I_x - I_y}{I_x + I_y} = \frac{I_x - I_y}{I_x + 2I_y} \]  

(2.11)

when \( I_x = I_y \) since both are perpendicular to the polarization axis.

With excitation and emission polarizer mountable, the \( \langle r \rangle \), anisotropy is measured as
\[ r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2 \cdot G \cdot I_{VH}} \]  \hspace{1cm} (2.12)

Where, 'G factor' is \( \frac{I_{HV}}{I_{HH}} \) \hspace{1cm} (2.13)

\( I_{VH}, I_{VV}, I_{HV}, I_{HH} \) denote different excitation and emission polarizer condition, e.g. \( I_{VV} \) means the condition where excitation and emission polarizers both are mounted vertically [5].

2.3.1.5 Fluorescence Red Edge Excitation Shift

The phenomenon of as Red Edge Excitation Shift (REES) is the shifting of fluorescence maxima to the red upon shifting of the excitation wavelength to the red end of the absorption band. A shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of absorption band, is termed as red edge excitation shift (REES). In a bulk non-viscous solvent, the dielectric relaxation of the solvent dipoles around the excited-state fluorophore is much faster than the fluorescence lifetime. Wavelength of maximum emission, in such a case, is independent of the excitation wavelength, since fluorescence occurs from the lowest vibrational state of the excited singlet state, irrespective of the excitation wavelength (according to Kasha's rule). However, the dependence of the emission maximum on excitation wavelength arises if the relaxation of the solvent molecules occurs at a slow rate around the fluorophore in the excited state, such that the relaxation time is comparable to or longer than the fluorescence life time. Under this condition, if a fluorophore is excited by a light quantum whose energy is lower than the
average energy of the electronic transition, there should be selective excitations of certain chromophores within the ensemble, whose interaction energy are maximum in the excited state and minimum in the ground state. These are the fluorophores around which the solvent molecules are oriented in such a way as to be more similar to that found in the solvent-relaxed state. Thus, in the REES situation, fluorescence occurs from various partially relaxed states. Utilizing this approach, it becomes possible to probe the mobility parameters of the environment itself (which is represented by the relaxing solvent molecules) using the fluorophore merely as a reporter group. The essential criteria for the observation of the red edge effect can be summarized as: (i) The fluorophore should normally be polar so as to be able to suitably orient the neighboring solvent molecules in the ground state; (ii) The solvent molecules surrounding the fluorophore should be polar; (iii) The solvent reorientation time around the excited state dipole moment of the fluorophore should be comparable to or longer than the fluorescence lifetime; and (iv) There should be a relatively large change in the dipole moment of the fluorophore upon excitation. The observed spectral shifts thus depend both on the properties of the fluorophore itself (i.e., the vectorial difference between the dipole moments in the ground and excited states), and also on properties of the environment interacting with it (which is a function of the solvent reorientation time). The unique feature about REES is that while all other fluorescence techniques such as fluorescence quenching, resonance energy transfer, and polarization measurements yield information about the fluorophore itself, REES provides information about the relative rates of solvent (water and organized molecular assemblies such as micelles, membranes in biological systems) relaxation dynamics, which is not possible to obtain by other techniques [6].
2.3.2. CD Spectroscopy

Circular dichroism is the difference in the absorption of left-handed circularly polarized light and right-handed circularly polarized light if the chromophore is optically active. Circular dichroism spectroscopy is a spectroscopic technique where circular dichroism of molecules is measured over a range of wavelengths. The measure of circular dichroism (CD) is depicted as (Figure 2.4)

\[ CD = \Delta A(\lambda) = A(\lambda)_{LCP} - A(\lambda)_{RCP} \]  

(2.14)

Where \( \lambda \) is the wavelength at which the absorption occurs. However, when one component of light is absorbed to a greater extent than the other, the resultant will trace out an ellipse, which has the relation
\[ \theta = 32.98 \, \Delta \Lambda \]  

(2.15)

\( \theta \) is the ellipticity (in degrees) of the resultant ellipse and \( \Delta \Lambda \) is the absorption difference.

Molar ellipticity is defined as

\[ [\theta] = \frac{M_0}{100c_l} \]  

(2.16)

Here, \( M \) designates the molecular weight of the sample, \( c \) is the concentration in g/cm\(^3\), and \( l \) is the sample length in decimeter.

CD spectroscopy is suitable to study many aspects of the molecular solution structure of biocompounds. This technique is used extensively to study chiral molecules of all types and sizes, but in the study of large biological molecules it finds its most important applications. A primary application relies in analyzing the secondary structure or conformation of macromolecules, particularly proteins and nucleic acids, and because of secondary structure is sensitive to its environment, e.g. temperature, denaturant concentration or pH, CD can be used to observe how secondary structure changes with environmental conditions or on interaction with other molecules.

For a protein, the concentration or protein (\( C \)) in molar unit is multiplied by the number of amino acids (\( n \)) in the protein to provide the mean residue concentration (\( C_{MR} \))

\[ C_{MR} = C \times n \]  

(2.17)

\[ [\theta]_{MR} = \frac{100 \times \theta}{C_{MR} \times l} \]  

(2.18)
CD spectrometers measure alternately the absorption of L- and R-CPL (Circularly Polarized Light) at a modulator operating frequency, and then calculate the CD signal. A number of algorithms exist which use the data from far UV CD spectra to provide an estimation of the secondary structure composition of proteins. Most procedures employ basis datasets comprising the CD spectra of proteins of various fold types whose structures have been solved by X-ray crystallography. The widely used algorithms include SELCON (self-consistent) [7], VARSLC (variable selection) [8], CDSSTR [9], K2d [10], and CONTIN [11]. An online server DICROWEB [12] has been developed, which allows data to be entered in a number of formats including those from the major CD instrument manufacturers, and to be analyzed by the various algorithms with a choice of databases [13,14].

2.3.3. Dynamic Light Scattering

Dynamic Light Scattering (DLS) is the methodology where scattered light is used to measure the size of particles to study the dynamics of particles in solution based essentially on two assumptions: i) particles are in Brownian motion (also called ‘random walk’) and ii) particles are spherical with diameter smaller than compared to the molecular dimensions [15].
If particles are illuminated with a laser, the disperse particles or macromolecules suspended in a liquid medium undergo Brownian motion which causes fluctuation in the scattered light intensity at a rate that is dependent upon the size of the particles (see Figure 2.5). From the time-dependent correlation function of the scattered light i.e. \( \langle I(0)/I(t) \rangle \) vs. \( t \), the decay is calculated; and the diffusion coefficient of the particles is determined to be inversely proportional to the decay time of light scattering fluctuations. The linewidth of the light scattered spectrum \( \Gamma \) (defined as the half-width at half-maximum) is proportional to the diffusion coefficient of the particles \( D \)

\[
\Gamma = Dk^2 \tag{2.19}
\]

\[
k = \frac{4n}{\lambda} \sin \frac{\theta}{2} \tag{2.20}
\]

Where, \( n \) is the refractive index of the medium, \( \lambda \) is the laser wavelength, and \( \theta \) is the scattering angle [16].
With the assumption that the particles are spherical and non-interacting, the particle size is calculated in accordance with the Stokes-Einstein formula relating the particle size to the diffusion coefficient and viscosity.

\[ R = \frac{k_B T}{6\pi \eta D} \]  

(2.21)

Where, \( k_B \) is the Boltzmann constant, \( T \) is the temperature, and \( \eta \) is the viscosity of the solvent. Typical applications of Dynamic Light Scattering are the measurement of the size and size distribution of particle emulsions and molecules dispersed or dissolved in a liquid, e.g. proteins, polymers, micelles, carbohydrates, nanoparticles, colloidal dispersions, emulsions, microemulsions [17].

2.3.4. Electron Microscopy

In the early 1930's there came up a scientific desire to see the fine details of the interior structures of organic cells (nucleus, mitochondria etc.) which required 10,000 x plus magnification which was just not possible using Light Microscopes. This led to the discovery of electron microscopes (EMs) which function exactly as their optical counterparts except that they use a focused beam of electrons instead of light to "image" the specimen and gain information about its structure and composition. A stream of electrons is formed (by the electron source) and accelerated toward the specimen using a positive electrical potential [18]. This stream is confined and focused using metal apertures and magnetic lenses into a thin, focused, monochromatic beam. The beam is focused onto the sample using a magnetic lens. Interactions occur inside the irradiated sample, affecting the electron beam - and the interactions and effects are detected and
transformed into an image. These are the basic steps involved in all types of electron microscopes; however, there are two main types, namely Transmission Electron Microscope (TEM) and Scanning Electron microscope (SEM).

2.3.4.1. Transmission Electron Microscopy

Transmission Electron Microscope (TEM) was the first type of Electron Microscope to be developed and is patterned exactly on the Light Transmission Microscope except that a focused beam of electrons is used instead of light to "see through" the specimen. It was developed by Max Knoll and Ernst Ruska in Germany in 1931 [19].

The transmission electron microscope uses a high energy electron beam transmitted through a very thin sample to image and analyze the microstructure of materials with atomic scale resolution (Figure 2.6). The electrons are focused with electromagnetic lenses and the image is observed on a fluorescent screen, or recorded on film or digital camera. The electrons are accelerated at several hundred kV, giving wavelengths much smaller than that of light. Since, TEMs use electrons as “light source”, their much lower wavelength make it possible to get a resolution a thousand times better than with a light microscope. A TEM can magnify up to about 500,000 times. The brighter areas of the image represent areas where more electrons have passed
through the sample. The darker areas represent areas where fewer electrons have passed through. Using TEM, one can study small details in the cell or different materials down to near atomic levels and illuminate the morphology (size, shape and arrangement of particles) and crystallographic information (arrangement of atoms in a specimen). This possibility for high magnifications has made the TEM a valuable tool in medical, biological and material research [20].

2.3.4.2. Scanning Electron Microscopy

The first Scanning Electron Microscope (SEM) debuted in 1942 [21] with the first commercial instruments around 1965 [22]. Its late development was due to the electronics involved in "scanning" the beam of electrons across the sample (Figure 2.7). The scanning electron microscope (SEM) uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens, and predicts about the topology, morphology, composition and crystallographic information of the sample. In most applications, data are collected over a selected area of the surface of the sample, and a 2D image is generated that displays spatial variations in these properties. Areas ranging from approximately 1 cm to 5 microns in width can be imaged in a scanning mode using conventional SEM techniques (magnification ranging from 20X to approximately 30,000X, spatial resolution of 50 to 100 nm). Field emission scanning electron
microscope (FE-SEM) images a sample surface by faster scanning over it with a high-energy beam of electrons. The electrons interact with the atoms comprising the sample to produce signals that contain information about only surface topography, composition and other properties, such as electrical conductivity. Features can be characterized at length scales from millimeters to around 10 nanometers. FE-SEM can even produce clearer, less electrostatically distorted images with spatial resolution down to 1 to 0.5 nm, which is 3 to 6 times better than conventional SEM. FE-SEM results in both improved spatial resolution and minimized sample charging and damage [23].

2.3.5. Nuclear Magnetic Resonance (NMR)

NMR Spectroscopy is one of the most powerful techniques for structural investigations, which is indispensable for the synthetic chemist, materials chemist or structural biologist alike. By basic definition, NMR is an analytical technique which deals with the active nuclei which have nuclear spin (I) equal to non zero. If an external magnetic field is applied to a nucleus, it absorbs the energy and radiates the energy back out that allows observation of specific quantum mechanical magnetic properties of an atomic nucleus, which in order yields an NMR spectrum for the nucleus concerned. In comparison to other spectroscopy like UV, Raman, Fluorescence, NMR can detect very fine structural components, works for organic and inorganic, qualitative and quantitative in a versatile manner. Much of the recent innovation within NMR spectroscopy has been within the field of protein NMR, which has become a very important technique in structural biology. The common goal of investigations in NMR is to obtain high resolution three dimensional structures of the protein, similar to what can be achieved by X-ray
Crystallography. However, in X-ray experiments, we can get only one parameter-set so we are able to observe only one conformation and there is no possibility to examine small parts in the molecule. There is also no chance for direct determination of secondary structures and especially domain movements which is a big disadvantage of crystallography compared to NMR. The maximum usable NMR experiments regarding proteins involve $^1\text{H}$, $^{13}\text{C}$, and $^{15}\text{N}$ nuclei. Experiments are homonuclear (TOCSY, NOESY, ROESY, DQF-COSY) as well as heteronuclear (HSQC and HMBC).

2.3.5.1. 1D Experiments

$^1\text{H NMR}$

The simplest and most often applied NMR experiment is the one pulse $^1\text{H}$ experiment. The pulse sequence consists of the recycle delay $d_1$ (preparation period) followed by a radio frequency (rf) pulse $p_1$. The pulse excites all the proton spins of a molecule and generates transverse magnetization (coherences) evolving in time and carrying the spin information i.e., its chemical shift, its scalar couplings to other spins and its relaxation properties. Data is collected following the rf pulse (detection period). The length of this detection period is denoted as the acquisition time. In practice a value for $p_1$ close to $90^\circ$ is normally used and the recycle delay $d_1$ is set long enough to avoid problems with partial saturation. Commonly used pulse program is zgpr using presaturation in f1 channel, where a low power irradiation on water is applied before the first $90^\circ$ pulse usually during the relaxation delay. Because of spectral bleaching of signals near the large dispersive tail of water, sometimes WATERGATE 1D water suppression (p3919gp) is also used where suppression of water signal is performed using 3-9-19
pulse sequence with gradients (Bruker Avance-Version "/opt/ts13/exp/stan/nmr/lists/pp/zgpr or p3919gp"). WATERGATE uses gradient pulses to create nulls in the spectrum. Though presaturation is the easiest option for suppression of one residual solvent peak, presaturation adversely affects signals from protons exchanging with the water.

\[ \text{zgpr: } \text{d1: relaxation delay} = 5 \text{ sec, p1: f1 channel - 90}_x \text{ degree high power pulse} = 12.50 \mu\text{sec. } \text{p3919gp: } \text{d1: relaxation delay} = 1.00 \text{ sec, p1: f1 channel - 90}_x \text{ degree high power pulse} = 12.50 \mu\text{sec, p16: homospoil/gradient pulse} = 1500.00 \mu\text{sec, d16: delay for homospoil/gradient recovery} = 0.00010000 \text{ sec, d19: delay for binomial water suppression} = (1/(2^*d)) = 0.00025000 \text{ sec, used gradient ratio: gp 1 (G): 20} \]

\(^{13}\text{C NMR} \\
\textit{Distortionless Enhancement by Polarization Transfer (DEPT)}

The DEPT experiment (Bruker Avance-Version "/opt/ts13/exp/stan/nmr/lists/pp/DEPT") is a polarization transfer technique and is useful for the observation of low-\(\gamma\) nuclei (most
commonly $^{13}$C) which are J-coupled to $^1$H. DEPT is a spectral editing sequence, that is, it can be used to generate separate $^{13}$C subspectra for methyl (CH3), methylene (CH2), and methine (CH) signals. DEPT makes use of the generation and manipulation of multiple quantum coherences to differentiate between the different types of $^{13}$C signals. The pulse angle ($\theta$) of the final $^1$H pulse is the basis of spectral editing with DEPT. CH3 and CH2 groups have maximum intensity when $\theta = \pi / 4$ and 0 intensity when $\theta = \pi / 2$; CH groups have maximum intensity when $\theta = \pi / 2$; and CH2 groups have maximum negative intensity when $\theta = 3 \pi / 4$. Quaternary carbons are missing from DEPT spectra because the large one-bond heteronuclear J-coupling ($J_{\text{CH}}$) is used for polarization transfer. Quaternary carbons, by definition, are not directly bonded to any $^1$H's, experience only small n-bond heteronuclear J-coupling ($^nJ_{\text{CH}}$), and so undergo no polarization transfer.

This $\theta$ angle is set to 45° in the sequence DEPT-45, which yields spectra with positive CH, CH2, and CH3 signals; to 90° in DEPT-90, which yields spectra with only CH signals; and to 135° in DEPT-135, which yields spectra with positive CH and CH3 signals and negative CH2 signals.
2.3.5.2. 2D Experiments

**Total Correlated Spectroscopy (TOCSY)**

TOCSY also known as HOHAHA – Homonuclear Hartmann Hahn spectroscopy is useful for correlating many spins in a set of mutually coupled spins, especially when the multiplets overlap (have very similar chemical shifts) or there is extensive second order coupling. In TOCSY, the transfer of coherence proceeds through the entire coupling network, so that there can be net magnetization transfer from one spin to another spin even without direct coupling. TOCSY is usually used in large molecules with many separated coupling networks such as peptides, proteins, oligosaccharides and polysaccharides.

In TOCSY, the coherence transfer occurs during a multiple-pulse spin-lock period and one of the most popular multiple-pulse-spin-lock schemes in TOCSY is the MLEV-17. The length of the spin-lock period determines how “far” the spin-coupling network should be probed. Duration of the spin lock depends both on coupling constants and the number of bonds separating the spins to be correlated. Spin-lock should be applied for between 20 and 200 ms with a pulse power sufficient to cover the spectral width. The general rule is that $1/(10J_{HH})$ should be allowed for each transfer step, and five transfer...
steps are typically desired for the TOCSY spectrum. Here, the typical pulse program that has been used is **mlevgpph19** (Bruker Avance-Version 
"/opt/ts13/exp/stan/nmr/lists/pp/mlevgpph19"), which is designed for mixing spins using two power levels for excitation and spinlock. mlevgpph19 is phase sensitive and water suppression is done using 3-9-19 pulse sequence with gradients. TOCSY spectrum contains a diagonal as well as cross-peaks. The diagonal consists of the 1D spectrum with single peaks suppressed. The cross-peaks correspond to coupling correlations through bonds between various spins in the system. The TOCSY spectrum should be phased so that all cross peaks and diagonal peaks have positive intensity.

![Diagram of mlevgpph19 pulse program](image)

[d1: relaxation delay = 2.0 sec, d0: incremented delay (2D) = 0.00009912 sec, d9: TOCSY mixing time = 0.06 sec, p6: f1 channel - 90 degree low power pulse = 35.0 μsec, pl10: f1 channel - power level for TOCSY-spinlock = 8.94 dB, pl18: f1 channel - power level for 3-9-19-pulse (watergate) = 0.0 dB, d19: delay for binomial water suppression = (1/(2*d)) = 0.00025000 sec, used gradient ratio: gp1 (G1) = 20, FnMODE: States-TPPI]
Nuclear Overhauser Effect Spectroscopy (NOESY)

NOESY is one of the most useful homonuclear NMR techniques aiming to identify spins undergoing cross-relaxation. Direct dipolar couplings provide the primary means of cross-relaxation, and so, spins undergoing cross-relaxation are those which are close to one another in space. The strength of the NOE signal ($I$) is proportional to the inverse sixth power of the distance ($r$) between the atoms, i.e. $I \propto 1/r^6$. Comparison of the cross-peak integrals in a quantitative NOESY is used as a measure of the distance between the protons that are close to each other in space even if they are not bonded.

For a pair of protons, $I$ and $S$, with two rotating frame precession frequencies $\Omega_I$ and $\Omega_S$, the basic NOESY sequence consists of three $\pi/2$ pulses. The first pulse creates transverse spin magnetization, which processes during evolution time $t_1$, which is incremented during the course of 2D experiment. The second pulse produces longitudinal magnetization equal to the transverse magnetization component orthogonal to the pulse direction. Thus, the basic idea is to produce an initial situation for the mixing period $\tau_m$ (the time during which cross relaxation occurs) where the longitudinal polarization of each spin is labeled by its resonance frequency. It may be pointed out that the optimum mixing time is different for different compounds depending upon the molecular weight of the compound. More is the molecular weight less is the mixing time. During the mixing time $\tau_m$, spins $I$ and $S$ undergo cross-relaxation and some of the $I$ spin $Z$-magnetization is transferred to the $S$ spin via NOE. Finally, the $S$-magnetization is rotated onto the $y$-axis by a third pulse whence it precesses at frequency $\Omega_S$ during $t_2$. For a basic NOESY experiment, $\tau_m$ is kept constant throughout the 2D experiment. The NOESY spectrum is generated by a 2D Fourier transform with respect to $t_1$ and $t_2$. 
The experiment we have used here is **noesyppph19** (Bruker Avance-Version 
"/opt/ts13/exp/stan/nmr/lists/pp/noesyppph19"), which is an advance-version 
pulseprogram with 2D homonuclear correlation via dipolar coupling (dipolar coupling 
may be due to the noe or chemical exchange). The pulse sequence is phase-sensitive and 
water suppression is performed using 3-9-19 pulse sequence with gradients.

![noesyppph19](image)

[d1: relaxation delay = 2.0 sec, d0: incremented delay (2D) = 0.00009517 sec, d8: mixing 
time = 0.30000010, pl18: f1 channel - power level for 3-9-19-pulse (watergate) = 0.0 dB, 
d19: delay for binomial water suppression = (1/(2*d)) = 0.00025000 sec, used gradient 
ratio:cnst21 (G1): cnst22 (G2) = 20 : 20, FnMODE: States-TPPI]

**Rotating-frame Overhauser Effect Spectroscopy (ROESY)**

ROESY is an experiment in which homonuclear NOE effects are measured under spin- 
locked conditions. ROESY is especially suited for molecules with motional correlation 
times ($\tau_c$) such that $\omega \tau_c \sim 1$, where $\omega$ is the angular frequency $\omega = \gamma B$. In such cases the 
laboratory-frame NOE is nearly zero, but the rotating-frame NOE (or ROE) is always 
positive and increases monotonically for increasing values of $\tau_c$. In ROESY, the mixing
time is the spin-lock period during which spin exchange occurs among spin-locked magnetization components of different nuclei (recall that spin exchange in NOESY occurs while magnetization is aligned along the z axis). Different spectral density functions are relevant for ROESY than for NOESY and the ROE is positive for all values of $\tau_c$. ROESY spectra can be obtained in 2D absorption mode. This is also useful for the identification of certain artifacts. Spurious cross peaks, both COSY-type and TOCSY-type, can be observed due to coherence transfer between scalar coupled spins. COSY-type artifacts (anti-phase) arise when the mixing pulse transfers anti-phase magnetization from one spin to another (the long spin-lock pulse acts like the mixing pulse in COSY). TOCSY-type artifacts (which have the same phase as the diagonal peaks, while ROESY cross peaks have the opposite phase) arise when the Hartmann-Hahn condition is met (e.g., when spins A and B have opposite but equal offsets from the transmitter frequency or when they have nearly identical chemical shifts). In general, to minimize these artifacts, it is suggested to limit the strength of the spin-locking field.

The ROESY pulseprogram used in our experiments is roesygppph19 avance-version (Bruker Avance-Version "/opt/ts13/exp/stan/nmr/lists/pp/roesygppph19"), with constant width spinlock for mixing. The pulse sequence is phase sensitive, and water suppression is performed using 3-9-19 pulse sequence with gradients.
Double Quantum Filtered Correlation Spectroscopy (DQF-COSY)

The DQF-COSY pulse sequence consists of three pulses, where the third pulse converts part of the multiple-quantum coherence into observable single-quantum coherence, which is detected during the acquisition period.

Advantages of DQF-COSY on COSY

1) The phase-sensitivity, i.e., the cross peaks can be displayed with pure absorption lineshapes in both the F1 and the F2 dimension. In general, a phase-sensitive spectrum has a higher resolution than an otherwise equivalent magnitude spectrum because the magnitude lineshape is broader than the pure absorption lineshape.
2) Partial cancellation of the diagonal peaks in a QF-COSY spectrum. Thus, the diagonal ridge is much less pronounced in a DQF-COSY spectrum than in a normal COSY spectrum.

3) The elimination of strong signals, e.g., the solvent $^1$H which do not experience homonuclear J-coupling.

The pulse-sequence used here in our experiments is **cosydfphpr** (Bruker Avance-Version "/opt/ts13/exp/stan/nmr/lists/pp/cosydfphpr") with double quantum filter. It is an avance-version pulse sequence with 2D homonuclear shift correlation, having presaturation during relaxation delay. The pulseprogram is phase sensitive.

Heteronuclear coupling (such as from $^1$H to $^{13}$C) is used to assign the spectrum of another nucleus once the spectrum of one nucleus is known. It may also be of help in assigning the spectra of both nuclei even when a partial assignment of one or both is available.
Such an experiment may also provide chemical information which the spectra of neither nucleus can provide alone.

**Heteronuclear Single Quantum Coherence (HSQC)**

The HSQC experiment is in fact a double INEPT experiment. This experiment correlates protons with their directly attached heteronuclei (e.g. Carbon, Nitrogen). Proton magnetization is detected during t2 - detection time while the low-gamma nuclei evolve during the evolution time - t1. Because of the detection of the high frequency nuclei, this sequence is very sensitive. The enhancement in sensitivity in this experiment is much greater than the enhancement obtainable by simple NOE (Nuclear Overhauser Effect). This is why this experiment has been referred to as the "Overbodenhausen" experiment.

A typical HSQC experiment starts with proton magnetization. Therefore the recycle time is based on proton relaxation time ($1.26T_1$). The first INEPT step is used to create proton $^1H$ antiphase magnetization ($2\pi$ delay) which is then transferred to the directly attached heteronucleus X. This X magnetization is left to evolve with its chemical shift during t1 - evolution time. The effect of proton coupling and chemical shift is removed by the use of a $180^\circ$ proton pulse applied at mid evolution time. The double $90^\circ$ pulse applied to both nuclei H-1 and X in the beginning of the last INEPT step transfers the magnetization back to proton as an anti-phased magnetization, which is then refocused during the last ($2\pi$ delay). The proton in-phase magnetization can then be detected in the presence of the X-nucleus decoupler.
hsqcetgp (Bruker Avance-Version "/opt/ts13/exp/stan/nmr/lists/pp/hsqcetgp") is a ge-2D phase-sensitive HSQC using echo-antiecho. ge-2D HSQC is the gradient-enhanced version of the conventional 2D HSQC experiment in which 2D $^1H/X$ coherence selection is achieved by means of pulse field gradient. Thus, clean 2D HSQC spectra can be recorded in a single scan per $t_1$ increment without need for phase cycle when sample concentration is high. Other advantages are the optimal dynamic range, improved water and artifact suppression, and reduced $t_1$ noise in the minimally required experiment time. Important aspects when PFG is incorporated in the HSQC pulse sequence are the sensitivity and resolution requirements. The echo-antiecho version of ge-2D HSQC, i.e. hsqcetgp, uses the same pulse-sequence as HSQC, but intensity of the refocusing gradient G2 becomes separately inverted on alternated scans. After proper processing, this approach allows obtaining phase-sensitive spectra though sensitivity losses to a factor of square with respect to the classical phase-cycled experiment.
ge -2D J-Resolved HSQC

Experiments were performed using pulse-sequence hsqctgpsisp2 (Bruker Avance-Version "/opt/ts13/exp/stan/nmr/lists/pp/hsqcergpsisp2"), which is a phase-sensitive ge-2D HSQC using PEP (preservation of equivalent pathways) - coupled with inversion and refocusing adiabatic pulses with gradients used in back-inept where 2D H-1/X correlation occurs using sensitivity improvement. Here instead of rectangular pulses we have used Chirped shaped pulse with 0.5 truncation (Crp60,0.5,20.1) for all 180 degree pulses i.e. both for inversion and refocusing. Use of the PEP methodology is the best option to record phase-sensitive 2D HSQC spectra with maximum sensitivity. The second back-INEPT block is used here in order to select both the orthogonal components of the magnetization ($I_2S_x$ and $I_2S_y$) present during $t_1$. In addition, impressive improvements are achieved in broadband heteronuclear decoupling using X nuclei-inversion and X nuclei-refocusing adiabatic pulses. Provided that the adiabatic condition is satisfied, the inversion efficiency is independent of the intensity of the radiofrequency field.
Hence, careful calibration is no longer required, and that the inversion efficiency is insensitive to any spatial inhomogeneity of the radiofrequency field.

Heteronuclear Multiple Bond Correlation (HMBC)

HMBC experiment is the modified version of HSQC, which is suitable to determine long range heteronuclei coupling sensitivity. This technique is very valuable to detect indirectly quaternary carbons coupled to protons - especially useful if direct Carbon-13 is impossible to obtain due to low amount of material available. This is an useful sequence which provides information about the skeleton of a molecule. It is also very useful in
carbohydrate area as a sequence analysis tool that provides unique information concerning connectivities across glycosidic linkages. Another area of interest for using HMBC is in the peptide-protein area - especially when applied to an isotope labeled protein/peptide to get connectivities between the amide nitrogen (HN) and Alpha carbon (HCα) of the next residue.

In a basic HMBC sequence, the first 90° pulse on Carbon-13 serves as a low-pass filter that suppresses one-bond correlation and passes only smaller coupling. This pulse creates multiple quantum coherence for the one-bond coupling which is removed from the spectra by alternating the phase of the Carbon-13 pulse. The second 90° pulse on 13C creates multiple quantum coherence for the long-range couplings. After the evolution time t1, the magnetization is converted back into detectable single quantum proton magnetization. The carbon decoupler is never used in this sequence: therefore, the protons displays homonuclear as well as heteronuclear couplings.

The heteronuclear HMBC experiment, hmbcgplpndqf (Bruker Avance-Version "/opt/ts13/exp/stan/nmr/lists/pp/hmbcgplpndqf"), is a ge-2D HMBC experiment that is gradient-enhanced version of the conventional 2D HMBC. Here, 2D 1H/X correlation via heteronuclear zero and double quantum coherence selection is achieved by means of PFG. Therefore, clean 2D HMBC spectra can be recorded in a single scan per t1 increment without need for phase cycle when sample concentration is high. Other advantages are the optimal dynamic range, improved water and artefact suppression, and reduced t1 noise in the minimally required experiment time. A low-pass J-filter (d2-90°(X) where d2=1/2(JXH)) is optionally inserted in the pulse-sequence after the first
pulse to minimize direct responses. The last refocusing delay is omitted and the X-band broadband decoupling during acquisition is not applied. PFGs are usually applied for coherence selection and only one of two desired coherence-transfer pathways are selected, thereby producing magnitude-mode spectra. Defocusing gradients are usually applied during the variable evolution period and the refocusing gradient is applied just prior to acquisition.

\[ \text{[di: relaxation delay} = 1.5 \text{ sec, d2} : 1/(2J)XH = 0.00344828 \text{ sec, d6: delay for evolution of long range couplings} = 0.07142857 \text{ sec, d0: incremented delay (2D)} = 0.00000300 \text{ sec, d16: delay for homospoil/gradient recovery} = 0.00010000 \text{ sec, p16: homospoil/gradient pulse = 1500.00 } \mu \text{sec used gradient ratio: gp1 (G1) : gp2 (G2) : gp3 (G3) = 50 : 30 : 40.1 for C-13, FnMODE: QF]} \]

2.3.5.3. Diffusion NMR Experiment

Self-diffusion is the random translational motion of molecules or ions that arises from the thermal energy under conditions of thermodynamic equilibrium. The random jostling of
molecules leads to the net displacement of molecules over time, and it is the Stokes-Einstein relationship that relates the diffusion coefficient to the molecular size:

\[ D = \frac{kT}{f} \]  

(2.22)

Where, \( D \) is the self-diffusion coefficient, \( k \) is Boltzmann’s constant, \( T \) is absolute temperature, and \( f \) is the friction factor. Self-diffusion \( D \) is related to the hydrodynamic volume diffusing through. For a spherical particle with hydrodynamic radius \( r_s \) in a solution of isotropic and continuous viscosity \( \eta \), the friction factor \( f \) is given by,

\[ f = 6\pi \eta r_s \]  

(2.23)

However, molecular shapes are more complicated and may include contributions from other factors such as hydration, aggregation etc. Therefore, friction factor \( f \) is also influenced accordingly and as a consequence, diffusion should also provide information about the shape of the diffusing molecules. Due to its noninvasive nature, NMR [24] has unique capability to provide valuable information regarding a mixture of molecules which diffuse in solution individually; but unfortunately, NMR is sensitivity-limited with respect to other analytical approaches - which results in the fact that for a long time, it has been preferred to isolate each mixture component prior to its analysis by NMR. The preferred methods for mixture analysis are chromatographic methods coupled with NMR, e.g. LC-NMR, HPLC-NMR. However, there is indeed ‘a pure NMR’ method that allows precise analysis of a complex mixture without any prior separation of the different components; the Diffusion Ordered SpectroscopY (DOSY) method [25,26].
2.3.5.3.1. Principle of DOSY

DOSY NMR is a two-dimensional NMR experiment where chemical shifts ($\delta$ in ppm) on the horizontal axis correspond with diffusion coefficients ($D$ in $\mu$m$^2$s$^{-1}$) on the orthogonal axis. The idea of diffusion in DOSY originates from the variation of gradients. Principle of DOSY is based on typically a pulsed field gradient (PFG) stimulated spin-echo experiment, where a series of 1D PFG-STE experiments is acquired with systematic variations of the gradient pulse amplitude and the rate of decay of amplitude of each signal correlates directly to the diffusion coefficient $D$ of the molecule, which depends on the molecular weight and other hydrodynamic properties (size, shape, charge) as well as on the surrounding environment (temperature, aggregation state). The signal contribution of each component from the DOSY experiment is described by the equation

$$I_{(g^2)} = I_{(0)} \exp \left[ -D_i K^2 (\Delta - \delta/3) \right]$$

(considering $K = \gamma \delta g$) \hspace{1cm} (2.24)

Where, $I_{(g)}$ and $I_{(0)}$ = Intensities with and without pulsed field gradient (PFG)

$\gamma$ = Magnetogyric ratio of the nucleus (radS$^{-1}$T$^{-1}$)

$\delta$ = Gradient duration (s)

$g$ = Strength (T)

$\Delta$ = Duration (s) between gradient pulses (the diffusion time)

And, $D_i$ = Diffusion coefficient ($m^2/s$) of the ith species or the ith signal in the sample

In case where $\Delta$ and $\delta$ are experimental constants, the signal of a DOSY attenuates depending on the square of gradient strengths ($g^2$) and the diffusion coefficients ($D$) of individual components. Here we suppose to deal with constant amplitude gradient pulses.
also called as rectangular pulses. For a mixture of three compounds, at each gradient
level, the measured spectrum is the sum of three combinations as in following equation

\[ I_{(x,y)} = \sum_{i=1}^{3} I_{(lg2)} \]  

(2.25)

The above equation can also be represented in a bilinear way,

\[ I = C S^T \]  

(2.26)

Where \( I (r \times c) \) represent a matrix with \( r \) rows and \( c \) columns, \( C(r \times n) \) represents the pure
decay profiles of the \( n \) components, and \( S^T (n \times c) \) contains the corresponding pure NMR
spectra. Fourier transformation of the NMR signal and the inverse Laplace transformation
of the decaying signals lead from here the DOSY spectrum. Thus, DOSY is, in otherwise,
a mixed Fourier-Laplace spectrum. Aim of DOSY is to find out pure decay profile \( C \) and
spectra \( S^T \), given the measure data set \( I \) and appropriate constants [27].

2.3.5.3.2. DOSY Pulse Scheme

We have used the 2D Pulse scheme ledbgpppr2s (Bruker Avance-Version
"/opt/ts13/exp/stan/nmr/lists/pp/ledbgpppr2s") for DOSY diffusion measurement that
possess Longitudinal Eddy Current Delay (LED) bipolar gradient pulse pairs (to
minimize effect of eddy currents) for diffusion with two spoil gradients using
presaturation during elaxation delay. Notation and numbering for pulses and delays are
specified considerably as:
[d1: relaxation delay; 1-5 * T1 = 1.5 sec, d21: eddy current delay = 0.00500000 sec, p19: gradient pulse 2 (spoil gradient) = 600 μsec, p30: gradient pulse (little DELTA * 0.5) = 2500 μsec, d16: delay for gradient recovery = 0.0002 sec, d20: diffusion time (big DELTA) = 0.15 sec, used gradient ratio: gp 6 : gp 7 : gp 8 = 100 : -17.13 : -13.17, The gradient ramp from 2% up to 95% in several steps in DOSY].

### 2.3.5.3.3. Analysis of DOSY

Though the NMR machines are capable to analyze DOSY spectra by themselves, the software **DOSY Toolbox** [27] stands for reliable open source software independent of brand of spectrometer specialized in DOSY processing. It handles raw data from the major manufacturers (Bruker, Jeol, and Varian); processes data, and recall them in the DOSY Toolbox (.nmr) format. The processing is performed here in a user-friendly graphical user interface which includes Fourier transformation, zero filling, phasing, apodisation, baseline correction and reference deconvolution. The most effective and basic way to process DOSY data is by high resolution DOSY (HR-DOSY) approach. Here, the decay of each signal is filtered to a single exponential decay and the decay and the data are presented in a pseudo 2D-DOSY plot. Each peak, in the diffusion dimension,

![Diagram of DOSY analysis](image)
is Gaussian centered on the fitted diffusion coefficient with a width determined by the statistics of the fit. HR-DOSY is capable of detection of significantly small change (0.5%) in diffusion coefficient.

There are two main classes of DOSY data processing: univariate and multivariate. In univariate processing, each signal is treated as independent; whereas multivariate methods exploit the covariance by fitting the entire spectra or the chosen parts of the spectra simultaneously. The main processing multivariate methods are respectively - Direct Exponential Curve Resolution (DECRA), Speedy Component Resolution Software (SCORE) and Multivariate Curve Resolution (MCR). DECRA is fast, but relies at best on experimental data conforming to pure exponential decays. A known or assumed decay shape is a prequisite for SCORE. MCR makes no assumptions on the decay shape but is truly influenced from good starting guesses and constraints. MCR in principle is able to accommodate polydispersity, whereas, SCORE is able only when correct decay function is chosen and DECRA in general cannot accommodate polydispersity. In MCR, DOSY data are first analyzed by Principle Component Analysis (PCA); the loading obtained from PCA are used as the abstract spectra, and are rotated by VARIMAX rotation - which maximizes the simplicity of the abstract factor. The rotated factors hereby produced are used as the initial guess to start the alternating least square (ALS) which finally produces the decay profiles. MCR only depends on the change of intensity with the increase of the square of gradient strength \( g^2 \), and therefore, neither an assumption of exponential decay profile nor the specific requirement of equidistant \( g^2 \) values are necessary in MCR.
The DOSY Toolbox used here is written in MATLAB(R) language and the version is 8.0. The Toolbox can be run directly on any platform that has an appropriate MATLAB(R) version installed, or the compiled versions of the Toolbox run independently of any MATLAB(R) version for Windows, Mac, or Linux. We have used Windows XP version of DOSY Toolbox 8 [27].

2.3.5.3.4. Determination of the Peptide Diffusion

The analysis of the peptide diffusion data is based on a two-site model, which is applicable only when exchange between the peptides in free form and bound form is fast compared to the pertinent NMR time scale. The measured diffusion coefficient $D_{obs}$ [28] obtained from the decay of the peptide signals is given by

$$D_{obs} = f_b D_b + (1 - f_b) D_f$$  \hspace{1cm} (2.27)

Where $D_f$ and $D_b$ denote the diffusion coefficients of the peptide in the free and bound forms, respectively, and $f_b$ is the fraction of the bound peptides. Since the dimension of the peptide in the bound form is much smaller than the dimension of the membrane mimics i.e. micelles, bicelles or vesicles; the $D_b$ can be taken as equal to the diffusion coefficient of the membranes only. $D_b$ can be measured with the LED experiment from the signals of the selective regions of the membrane molecules directly. However, expression of $D_f$ is corrected as small molecules are obstructed by other spherical particles in solution as:

$$D_f = \frac{D_f^*}{(1 + \phi/2)}$$  \hspace{1cm} (2.28)
Where $D_f^*$ is the diffusion coefficient of free peptide and is measured for peptide in water. 

$\phi$ is the volume fraction for obstructing particle which is usually approximated by using 
the weight fraction of the other spherical component in solution [29].

2.3.6. NMR Structure Calculation

An NMR method for protein structure determination [30] could be based on (the following four principal elements:

I) Sequence-Specific Resonance Assignments

There are closely spaced pairs of hydrogen atoms in neighbor residues of a polypeptide 
chain, which can be connected by the observation of "sequential NOEs". Each amino 
acid residue represents a "spin system" and it consists of an array of hydrogen atoms 
including an amide proton (HN), an alpha proton ($H_\alpha$), and the side chain protons, which 
can be connected by steps over three or less covalent bonds through the observation of 
scalar spin–spin ("through-bond") couplings. Sequential NOEs enable progressive 
resonance assignments while walking along the polypeptide backbone.

II) Two-dimensional NMR Spectra

A greatly improved separation of the individual cross peaks is best seen in contour plots 
of 2D NMR spectra which might be used for detailed structural analysis. 2D correlation 
experiments are TOCSY, COSY, NOESY and ROESY, DQF-COSY and the hetero- 
nuclear HSQC, HMBC, etc.
III) Integration and Calculation of Spectral NOE cross peak volumes

Each spectral peak is centered, shaped as Gaussian and integrated. The larger peak volume is selected for restraints.

IV) Calibration of Distance Restraints

Under the assumption of isolated spin pairs in a rigid molecule, the target distances $d_{\text{NOE}}$ can be obtained from the cross peak volume $V$ by a simple calibration function,

$$d_{\text{NOE}} = (CV)^{1/6}$$

The calibration constant $C$ can be set by the user or determined automatically as:

$$C = \frac{\sum_{\text{NOEs}} d^{-6}}{V}$$

Where, the sum runs over all NOEs with a corresponding average distance $d$ smaller than a cutoff of typically 6 Å. In the XPLOR-NIH algorithm, the lower bound of the distance is always 1.8 Å, whereas the upper bound is varied.

V) Determination of NOE upper Distance Limits as Conformational Constraints

NOE assignments can involve with two or more atoms, such that $r_{\text{NOE}}$ is averaged. Sum ‘averaging’ is the most common method for distance computation:

$$r_{\text{NOE}} = (\sum_{ij} (q_i - q_j)^{-\alpha})^{1/\alpha}$$

Where, $q_i$ and $q_j$ are the position of atoms i and j, i, j sum is over all atom pairs associated with the given NOE cross-peak, and $\alpha = 6$ is usually used. It is a center averaging method used in XPLOR-NIH which uses the distance between the centers of the two selections (NOEPot in XPLOR-NIH).
VI) Combination with Coupling Constants and Dihedrals

Three-bond J coupling experiments provide quantitative information about torsion angle values through the empirical Karplus equation, calculated from a structure as:

\[ J = A \cos^2(\phi + \theta) + B \cos(\phi + \theta) + C \]  

(2.32)

Where \( \phi \) is the value of the torsion angle in question, \( \theta \) is a phase lag, and A, B, and C are Karplus coefficients. For protein and polypeptides' backbone \( \phi \) dihedral, A = 6.98, B = -1.38, C = 1.72 and \( \theta = 60^\circ \) (JCoupPot in XPLOR-NIH). Torsion angle restraints derived from three-bond J couplings is available in the CDIH XPLOR potential.

VII) Other Restraints

1. J_{Ca-H_a} couplings are related to \( \phi \) and \( \psi \) angles by an empirical relationship. J_{Ca-H_a} coupling restraints are available via the ONEBond XPLOR term.

2. Cα/Cβ secondary shifts, which are empirically related to \( \psi/\phi \) values are available in the CARBon XPLOR term.

3. \(^1\)H chemical shifts restraints, which include the capability of dealing with non-stereospecifically assigned methylene and methyl groups, are available in the PROTon XPLOR term.

4. Backbone amide hydrogen bonding is encoded via two terms. The HBDA XPLOR term employs an empirical relationship between hydrogen–oxygen distance and the NHO angle. An alternative representation of backbone hydrogen bonding is encoded in the HBDB XPLOR term.

5. Also, paramagnetic relaxation enhancement (PRE), Residual Dipolar Couplings (RDCs), and heteronuclear relaxation ratios (T1/T2) are also included in XPLOR-
VIII) Removal of Erroneous Restraints by Violation Analysis

When building a three-dimensional structure from NOE data, most erroneous distance restraints are inconsistent with each other and with the correct ones. The erroneous restraints can therefore, in principle, be detected by analyzing the violations of restraints for a bundle of three-dimensional structures in a cycle of calculation. A violation analysis can be performed by counting the conformers in which a given restraint is violated by more than a cutoff. The solution is, either the upper distance bound may be increased, or the restraint may be removed from the input for the structure calculation in the current cycle. A detailed listing of the specific violations for each term is given in a separate file with a .viols suffix in XPLOR-NIH [30].

IX) Ensemble Refinement

In solution, molecules are in constant motion, so that NMR observables generally do not reflect the measurement of a single conformer, but rather a collection of structures which interconvert. Strongly simplified “soft” force fields are generally used for the de novo calculation of NMR structures to allow for a reasonably smooth folding pathway of the polypeptide chain from a random initial structure to the native conformation. The stiffness incurred by potentials during the initial stages of the simulated annealing procedure would result in most conformers being trapped far from the native structure in local minima with unfavorable energies.
**X) Quality Control and Validation**

Final validation of a structure may include cross-validation of NMR observables found in .viols files or .stats files for the ensembles form the external tools such as PROCHECK or Whatif, or PSVS 3.1 online server can be used (http://psvs-1_3.nesg.org/).

(www.ebi.ac.uk/thornton-srv/software/PROCHECK/) and (swift.cmbi.ru.nl/whatif/)

### 2.3.7. Molecular Docking (The Best Way Two Molecules Interact)

Molecular Docking may be defined as an optimization problem, which could describe the “best-fit” orientation of a ligand that binds to a particular molecule of interest such that the free energy of the overall system is minimized. During the docking process, receptor and ligand both perform some conformational adjustments resulting in overall binding which is referred to as “induced-fit”. Success of a docking program depends on search algorithm and scoring function. ‘Search’ explores the position and orientation of the ligand with respect to the macromolecule which includes systematic or stochastic torsional searches about rotatable bonds, molecular dynamics simulations or genetic algorithms to evolve new low energy conformations; whereas “scoring” evaluates each generated molecular configuration, where a low or negative energy indicates a stable system or likely binding conformation.

Autodock [31] is a suite of automatic docking tools. It actually consists of two main programs: AutoGrid pre-calculates the grids, and AutoDock performs the docking on grids. To note, AutoGrid considers the receptor as the potential grid, where the docking place of ligand in AutoDock is searched. Autodock can use several optimization methods to search for the best placement of the ligand. Methods are as following:
1. **Simulated Annealing (SA):** At each step of simulated annealing, position and internal rotational state of the ligand is adjusted and energy calculated. Because of its randomized method, different runs will produce different solutions.

2. **Genetic Algorithm (GA):** It represents the states of the degrees of freedom of a ligand as a string of digits, and this string is referred as gene. Population of different genes is generated at random, and each is scored using the AutoDock energy function. The better scoring genes are more likely to be selected.

3. **Lamarckian Genetic Algorithm (LGA):** This is different from the standard genetic algorithm in way that before the genes are scored, each gene is subjected to energy minimization. The next population is then founded by members of this energy-minimized population. The name “Lamarckian” refers to the genetic theory of Jean-Baptiste Lamarck. LGA is faster than both SA and standard GA, and it allows the docking of ligands with more degrees of freedom.

The distribution between docked conformations is carried out by the following empirical scoring function:

\[
\Delta G_{binding} = \Delta G_{vdw} + \Delta G_{elec} + \Delta G_{hbond} + \Delta G_{desolv} + \Delta G_{tors} \tag{2.33}
\]

Where, \( \Delta G_{vdw} \) is 12-6 Lennard-Jones potential, \( \Delta G_{elec} \) is Coulombic with Solmajer-dielectric, \( \Delta G_{hbond} \) is 12-10 potential with Goodford Directionality, \( \Delta G_{desolv} \) is Stouten Pairwise Atomic Solvation Parameters and \( \Delta G_{tors} \) is the number of rotatable bonds. Because, the SCORE is an approximation of free energy, lower scores represent greater stability and the lowest score corresponds to the docked conformation.
A graphical user interface has also been developed by the developers of AutoDock, the AutoDockTools or shortly ADT (autodock.scripps.edu/resources/adt), which amongst other things helps to set up the ligand as well as macromolecule flexibility and to analyze dockings. AutoDock can also be used to identify the binding site of a ligand without prior knowledge of active site; it is called 'blind docking' (http://autodock.scripps.edu).

2.4. References


