Probing aggregation of human insulin in solution using pulsed-field gradient NMR spectroscopy

Aditya Mishra

A dissertation submitted for the partial fulfilment of BS-MS dual degree in Science

Indian Institute of Science Education and Research Mohali
April 2018

Certificate of Examination

This is to certify that the dissertation titled **Probing aggregation of human insulin in solution using pulsed-field gradient NMR spectroscopy** submitted by **Aditya Mishra** (Reg. No. MS13109) for the partial fulfillment of BS-MS dual degree programme of the institute, has been examined by the thesis committee duly appointed by the institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Sandeep Kumar Goyal Dr. Samir Kumar Biswas Dr. Kavita Dorai
(Supervisor)

Dated: April 12, 2018

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr.Kavita Dorai at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Aditya Mishra
(Candidate)

Dated: April 12, 2018

In my capacity as the supervisor of the candidates project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Kavita Dorai (Supervisor)

Acknowledgment

I am greatful to my supervisor, Dr.Kavita Dorai for giving me a chance to explore the field of Diffusion-NMR spectroscopy and being a wonderful guide throughout my project.
I would like to acknowledge Dr. Sandeep Kumar Goyal & Dr. Samir Kumar Biswas for being my committee members.
Special thanks to Ms. Jyotsana Ojha for helping in sample preparation & doing experiments on spectrometer. I am thankful to Dr. Harpreet Singh for clearing my doubts in analytical calculations in NMR. I am thankful to Satnam Singh, Navdeep Gogna, Amandeep Singh, Rakesh Sharma, Sumit Mishra, Dileep Singh, Akshay Gaikwad, Amit Devra, Akanksha Gautam, Ankit, Agrim gupta & Guru Gaurav Bhatt for being supportive lab members.
My sincere thanks to my friends Ankit Somani, Om Prakash Chaudhary(OP), Ankit Kumar Pandey, Rahul Rana & Rakesh Saini. I am very thankful to Mr. Achyut Pandey, Mr. Praveen Shukla, Dr. Rajkumar Vadla for the wonderful memories in intial days of IISER, Mohali. I am very much thankful to Kunal Sharma, Saayak Halder, Nikhil Jaisinghani for the support despite of the distance.
Last but not the least my parents and siblings for bearing me during the ups and downs.
I want to dedicate this thesis to my beloved friend Ms.Rati Upadhyay(Deceased 31st Janauary, 2017)

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Abstract

Insulin plays an imporatant role in biological systems and it has been extensively studied as a model of protein structure and function. It's ability to exist in different forms make its an intereting model therefore it has been extensively studied through circular dichroism(CD) spectroscopy, 1D-1H NMR spectroscopy, dynamic light scattering(DLS), mass-spectroscopy etc. The insulin produced and stored in the pancreas is in the active Zn hexamer in which three dimers are surrounded by Zn^{+} ion, but when it released into blood serum by the pH change, this hexamer dissociate into dimer and subsequently monomers which is its physiologically active form. However, monomers is less stable than hexamer exposing it to heat and motion it tend to aggregate. In this project, we are trying to study about insulin (with and without EDTA) aggregation at different pH, EDTA, temperature and amount of sucrose.

Contents

Chapter 1

NMR Spectroscopy

1.1 Motivation

Insulin plays a key role in biological systems and has been studied as a model of protein structure and its function[17],[13]. It is stored in as granules in pancreatic β cells as hexameric di-zinc complex. So, insulin is released from the β cells into the bloodstream, insulin dissociates into the monomeric insulin which is its physiologically active form[17],[13]. Therefore, production of active form from the storage form must involve number of steps whose mechanism, kinetics can be probed by the NMR spectroscopy. The motivation behind doing this work is to know about the dynamics of insulin behaviour with various pH and crowding agents like EDTA and Sucrose and it can benefit pharmaceutical industries[17],[13].

1.2 NMR Spectroscopy

Spectroscopy is the branch of science which deals and study with the interaction of radiation with matter. In particular, NMR spectroscopic technique deals with electromagnetic interaction between the sample(matter) and magnetic field component of electromagnetic waves, in particular, radio waves(λ=1mm-100km). In general, spectroscopy can be represented by flowchart in figure 1.1 Matter is composed of atmoms. Atoms are made up of electrons and nuclei. Each atomic nuclei has four important physical properties: *mass, electric charge, magnetism and spin*. In NMR spectroscopy, sample consists several nuclei, and we use spin property of those nuclei. Although, the concept of spin a bit difficult to understand at first glance but it was forced upon

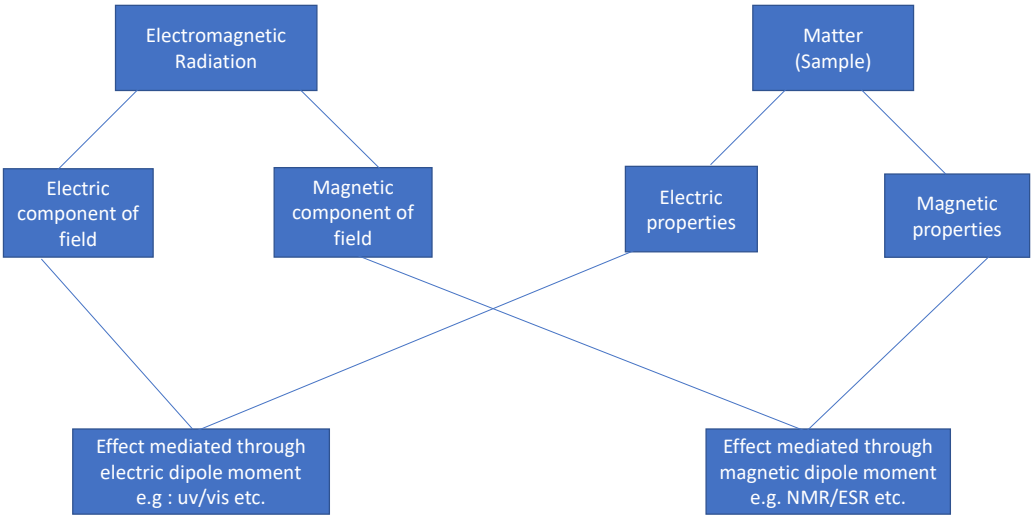


Figure 1.1: Spectroscopy

scientists with experimental evidence. It's worth trying since it involves rigorous manipulations of nuclear spins[1, 3, 2].

1.2.1 Spin Angular Momentum

A spin is a form of angular momentum but it's not produced by rotation of particle, but it's an intrinsic property of particle itself. Therefore, each elementary particle is characterized by nuclear spin angular momentum quantum number(I). For some particles, I is given by an integer and for the other I is given by half integer. Particles with integer spins are called *bosons* whereas with half-integer spins are called *fermions*. In NMR, nuclei, with given nuclear spin quantum number I has (2I+1) nuclear spin states with projections m_I = -I to +I. These nuclear spin states are also the eigen states of 2-component of nuclear spin angular momentum[2, 1]. As from elementary quantum mechanics formalism, $[I^2, I_z]$=0 & $[I_i, I_j] \neq 0$ if i≠j

$$I^2 \left|I, m_I\right\rangle = I(I+1)\hbar \left|I, m_I\right\rangle \tag{1.1}$$

$$I_z \left|I, m_I\right\rangle = m_I \hbar \left|I, m_I\right\rangle \tag{1.2}$$

for instance, the nucleus of the main isotope of hydrogen has 1H single proton therefore $I = 1/2$ subsequently has two nuclear spin states $|1/2, -1/2\rangle$ and $|1/2, 1/2\rangle$[1].

1.2.2 Larmor Precession : Classical Description

A nuclear state with thespin I is $(2I+1)$-fold generate. If magnetic field is applied, then the degeneracy has been broken, consequently spin states get split into two states $|1/2,-1/2\rangle$ and $|1/2,1/2\rangle$. Therefore, splitting of nuclear spin state when the spin is subjected into an external magnetic field then this phenomenon is called as Zeeman-Splitting[2, 1]. In physics the relation between magnetic dipole moment(μ) and its angular momentum is described by

$$\vec{\mu} = \gamma\hbar\vec{I} \tag{1.3}$$

By adopting analogy from classical electrodynamics, interaction energy of a dipole after placing in an external magnetic field is

$$E_{\text{interaction}} = -\vec{\mu}.\vec{B} \tag{1.4}$$

Therefore, by using equation (1.3) and (1.4) we can calculation the zeeman hamiltonian when the field is along z-direction

$$\hat{H}_{\text{zeeman}} = -\gamma\hbar\vec{B}\hat{I} \tag{1.5}$$

$$= \hbar\omega_0\hat{I}_z \tag{1.6}$$

where $\omega_0 = \gamma B_z(B_0)$ is called larmor freqency. Therefore, corresponding energies of non-degenerate states are $E_{\text{lower}} = E_1 =$ -$\hbar\omega_0/2$ and $E_{\text{upper}} = E_2 = \omega_0\hbar/2$. As a result $\Delta E = \hbar\omega_0$.

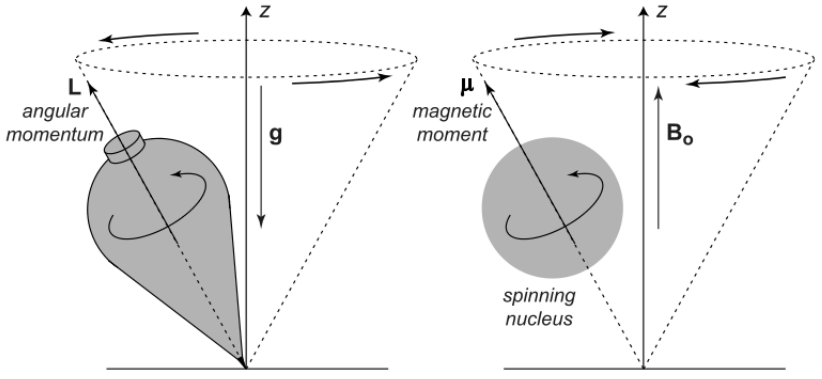


Figure 1.2: Analogy between a spinning top in a gravitational field and a magnetic moment in a magnetic field.[7]

1.2.3 Larmor Precession : Quantum Mechanical Description

In genreal, for single spin nuclear spin state can be described as

$$|\Psi(t)\rangle = C_{1/2}\,|\phi_{1/2}(t)\rangle + C_{-1/2}\,|\phi_{-1/2}(t)\rangle \tag{1.7}$$

where time evolution of basis states is given by

$$|\phi_m(t)\rangle = e^{\frac{-\iota E_m t}{\hbar}}\,|\phi_m(0)\rangle \qquad \text{with} \qquad E_m = -m\hbar\omega_0 \tag{1.8}$$

by using equation (1.1), (1.2) & (1.3) we can calculate expectation values of μ_x, μ_y & μ_z as

$$\langle\mu_z\rangle = \frac{\gamma\hbar}{2}[C_{1/2}C_{1/2} - C_{-1/2}C_{-1/2}] \tag{1.9}$$

$$\langle\mu_x\rangle = \frac{\gamma\hbar}{2}[C^*_{1/2}C_{-1/2}e^{-\iota\omega_0 t} + C^*_{-1/2}C_{1/2}e^{\iota\omega_0 t}] \tag{1.10}$$

$$\langle\mu_y\rangle = \frac{\gamma\hbar}{2\iota}[C^*_{1/2}C_{-1/2}e^{-\iota\omega_0 t} - C^*_{-1/2}C_{1/2}e^{\iota\omega_0 t}] \tag{1.11}$$

By assuming $C_{1/2} = ae^{\iota\alpha}$ & $C_{-1/2} = be^{\iota\beta}$ We get,

$$\langle\mu_z\rangle = \frac{\gamma\hbar}{2}[a^2 - b^2] \tag{1.12}$$

$$\langle\mu_x\rangle = \gamma\hbar ab[cos(\beta - \alpha - \omega_0 t)] \tag{1.13}$$

$$\langle\mu_y\rangle = \gamma\hbar ab[sin(\beta - \alpha - \omega_0 t)] \tag{1.14}$$

Therefore by choosing our nuclear spin state to be normalized $a^2 + b^2 = 1$ we can conclude that as we keep the nuclear spin, having magnetic moment μ, then it will precess around the z-direction since the expectation value of μ_z is time independent whereas x-component and y-component oscillate with sine and cosine function with frequency ω_0 i.e. larmor frequency.

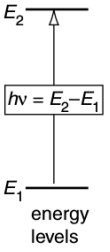


Figure 1.3: Splitting of energy levels of a spin in B_{ext}[2]

1.3 NMR Interactions

1.3.1 External Interactions

Zeeman Interaction

If a magnetic field is applied, the degeneracy of nuclear spin state is broken. The splitting between the nuclear spin levels is called nuclear-zeeman splitting. The zeeman splitting of a proton nucleus is about 10 times larger than zeeman splitting of ^{15}N since ^{1}H is 10 times more magnetic than ^{15}N in the same magnetic field[2, 1].

$$\hat{\mathcal{H}}_z = -\hbar\omega_0 I_z \tag{1.15}$$

Radio Frequency Field Interaction

By splitting the nuclear spin state is not enough to get a signal therefore to make a transition between the these splitted energy levels one has to perturb a system using electromagnetic waves in such a way that frequency matches wih that of splitting between the energy levels[1].
In order to disturb the system application of oscillating rf-field in the x-direction which in mathematical form is written as

$$\vec{B_{\mathrm{rf}}} = 2B_1 cos(\omega_{\mathrm{rf}} t + \phi)\hat{I}_x \tag{1.16}$$

where ϕ is the phase associated with the rf-field. Now this oscillating rf-field can be broken into two parts one which rotates as that of spins which we can state as resonant component and other which is opposite to that of spin is called non-resonant component of rf-field. Mathematically, this can be written as

$$\vec{B}_{\text{resonant}} = B_1 \cos(\omega_{\mathrm{rf}} t + \phi)\hat{x} + B_1 \sin(\omega_{\mathrm{rf}} t + \phi)\hat{y} \tag{1.17}$$

$$\vec{B}_{\text{non-resonant}} = B_1 \cos(\omega_{\mathrm{rf}} t + \phi)\hat{x} - B_1 \sin(\omega_{\mathrm{rf}} t + \phi)\hat{y} \tag{1.18}$$

In NMR, we can neglect the non-resonating component by using a approximation called rotating wave approximation. Therefore, by using rotating wave approximation, interaction energy of spin and rotating magnetic field can be written as

$$\hat{\mathcal{H}}_{\mathrm{rf}} = -\hbar\gamma B_1 \hat{I}_z (B_1 \cos(\omega_{\mathrm{rf}} t + \phi)\hat{x} - B_1 \sin(\omega_{\mathrm{rf}} t + \phi)\hat{y}) \tag{1.19}$$

After simplifying $\hat{\mathcal{H}}_{\text{rf}}$ by using $\omega_1 = \gamma B_1$

$$\hat{\mathcal{H}}_{\text{rf}} = -\hbar\omega_1 \hat{I}_z (B_1 \cos(\omega_{\text{rf}} t + \phi)\hat{x} - B_1 \sin(\omega_{\text{rf}} t + \phi)\hat{y}) \tag{1.20}$$

1.3.2 Internal Interactions

In NMR spectroscopy, various interactions i.e. among the spins or between spin and magnetic field can be described as follows

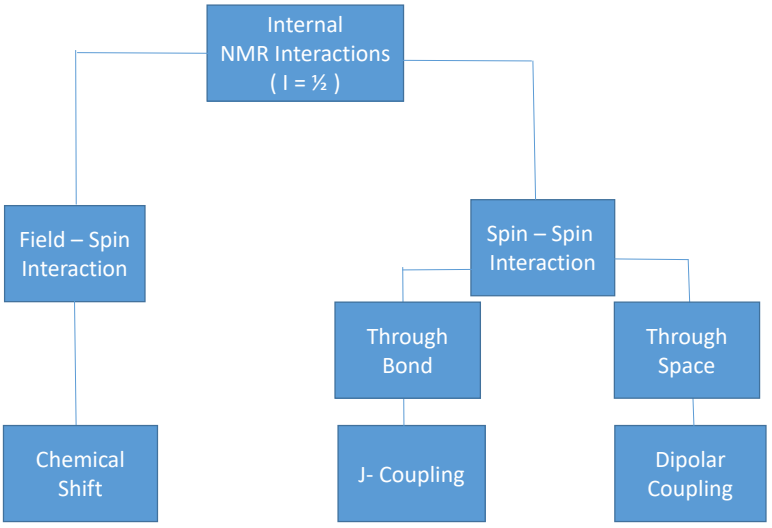


Figure 1.4: Internal Interactions in NMR

Chemical Shift Interaction

The interaction between nuclear spin and external magnetic field mediated by electron cloud around that nucleus is called chemical shift interaction. In other words, we can say that circulating electrons aroud the nuclear spin generate local magnetic field (B_{local}). In mathematical terms, this local magnetic field can be written as

$$\vec{B}_{\text{local}} = \bar{\bar{\sigma}} \vec{B} \tag{1.21}$$

where $\bar{\bar{\sigma}}$ is a chemical shift second-rank tensor. So, generally in matrix form

$$\begin{bmatrix} B_{\text{local,x}} \\ B_{\text{local,y}} \\ B_{\text{local,z}} \end{bmatrix} = \begin{bmatrix} \sigma_{xx} & \sigma_{xy} & \sigma_{xz} \\ \sigma_{yx} & \sigma_{yy} & \sigma_{yz} \\ \sigma_{zx} & \sigma_{zy} & \sigma_{zz} \end{bmatrix} . \begin{bmatrix} B_{\text{ext,x}} \\ B_{\text{ext,y}} \\ B_{\text{ext,z}} \end{bmatrix}$$

Therefore, if $\vec{B} = B_0\hat{k}$ and $\sigma_{xx}\&\sigma_{yy}$ is negligible then

$$\hat{\mathcal{H}}_{CS} = -\hbar\omega_0\sigma_{zz}B_0 \tag{1.22}$$

Scalar Coupling

The interaction between two spins which are covalently bonded is called Scalar coupling or J-Coupling. This interaction is mainly through bond[8, 9]. Mathematically, Scalar coupling hamiltonian can be written as

$$\hat{\mathcal{H}}_J = 2\pi\bar{\bar{J}}(\text{Hz})\hat{I}.\hat{S} \tag{1.23}$$

Where $\hat{I}$ & $\hat{S}$ corresponds to nuclear spin angular momentum operators belongs to spin-I and spin-S respectively.

Dipolar Coupling

This kind of coupling is in between two spins which are spatially apart. To understand this, we can adopt the analogy from classical spin-spin interaction[8, 9]. Therefore, classical spin-spin interaction between the two magnetic dipoles which are spatial apart can be given by

$$E^{ij}_{\text{interaction}} = \frac{\vec{\mu_i}.\vec{\mu_j}}{r^3} - 3\frac{(\vec{\mu_i}.\vec{r})(\vec{\mu_j}.\vec{r})}{r^5} \tag{1.24}$$

where $\vec{r}$ is pointing from dipole i to dipole j and by defining $\vec{r_{ij}} = \vec{r_i} - \vec{r_j}$

$$E^{ij}_{\text{interaction}} = \sum_i\sum_{j>i}\frac{\vec{\mu_i}.\vec{\mu_j}}{r^3_{ij}} - 3\frac{(\vec{\mu_i}.\vec{r}_{ij})(\vec{\mu_j}.\vec{r}_{ij})}{r^5_{ij}} \tag{1.25}$$

Therefore, Hamiltonian can be written by using $\vec{\mu} = \gamma\hbar\vec{I}$ as ,

$$\hat{\mathcal{H}}_{\text{DIP}} = \sum_i\sum_{j>i}\frac{\gamma_i\gamma_j\hbar^2}{r^3_{ij}}[(\hat{I}_i.\hat{I}_j) - \frac{3}{r^2_{ij}}(\hat{I}_i.\vec{r}_{ij})(\hat{I}_j.\vec{r}_{ij})] \tag{1.26}$$

simplifying it by using a unit vector $\vec{e}_{ij}$ along the direction of r_{ij}

$$\hat{\mathcal{H}}_{\text{DIP}} = \sum_i\sum_{j>i}\frac{\gamma_i\gamma_j\hbar^2}{r^3_{ij}}[(\hat{I}_i.\hat{I}_j) - 3(\hat{I}_i.\vec{e}_{ij})(\hat{I}_j.\vec{e}_{ij})] \tag{1.27}$$

$$\hat{\mathcal{H}}_{\text{DIP}} = \sum_i \sum_{j>i} \frac{\gamma_i \gamma_j \hbar^2}{r_{ij}^3} [(\hat{I}_i.\hat{I}_j) - 3(\hat{I}_i.\hat{e}_{ij}.\hat{I}_j)] \tag{1.28}$$

where $\hat{e}_{ij}$ is a dyadic product of $\hat{e}_{ij}$ with itself. Therefore, for a single pair,

$$\hat{\mathcal{H}}_{\text{DIP}} = \frac{\gamma_i \gamma_j \hbar^2}{r_{ij}^3} [\hat{I}_i.\bar{\bar{D}}.\hat{I}_j] \tag{1.29}$$

where $\bar{\bar{D}}$ is called dipolar coupling tensor.

Quadrupolar Coupling

All nuclei with spin I ¿ 1/2 have non-spherical charge spherical distribution and so they are subjected to electrostatic interaction with neighbour electrons and ions. Mathematically, quadrupolar coupling is described by the following, written in principal axis frame associated with the electric field gradient[7].

$$\hat{\mathcal{H}}_Q = \frac{e^2 Q q}{4I(2I-1)} [3I_z^2 - I^2 + \eta(I_x^2 - I_y^2)] \tag{1.30}$$

1.4 Quantum Mechanical Description of spin placed in static and oscillating field

In elementary quantum mechanics, the schrodinger equation is given by

$$\iota\hbar \frac{d\,|\psi\rangle}{dt} = \hat{\mathcal{H}}(t)\,|\psi\rangle \tag{1.31}$$

if $\mathcal{H}$ is time-independent then solution is given by

$$|\psi(t)\rangle = e^{-\frac{\iota\hat{\mathcal{H}}t}{\hbar}}\,|\psi(0)\rangle \tag{1.32}$$

otherwise, it's given by

$$|\psi(t)\rangle = e^{\int -\frac{\iota\mathcal{H}(\hat{\sqcup})dt}{\hbar}}\,|\psi(0)\rangle \tag{1.33}$$

In NMR, generally we deal with the time-dependent hamiltonians, therefore, it's a bit convenient to write the hamiltonians in different reference frame to cancel out the time dependency. There are two frames of references which we can see to make time-independent hamitonian.

1.4.1 Interaction frame

In general, Suppose total hamiltonian of a quantum system comprised of two hamiltonians with one of the component is too large as compared to other.

$$\hat{\mathcal{H}}_{\text{total}} = \hat{\mathcal{H}}_0 + \hat{\mathcal{H}}_1 \qquad \text{with} \qquad H_0 >> H_1 \tag{1.34}$$

to get a better picture of the dynamics of system in presence of $\hat{\mathcal{H}}_1$ alone let us define a new state $|\tilde{\psi}\rangle$,

$$|\tilde{\psi}(t)\rangle = e^{\frac{\iota}{\hbar}\hat{\mathcal{H}}_0 t} |\psi(t)\rangle \tag{1.35}$$

by differentiating this equation we get,

$$\frac{d|\tilde{\psi}\rangle}{dt} = \frac{\iota}{\hbar}\hat{\mathcal{H}}_0 e^{\frac{\iota}{\hbar}\hat{\mathcal{H}}_0 t} |\psi(t)\rangle + e^{\frac{\iota}{\hbar}\hat{\mathcal{H}}_0 t}\frac{d|\psi(t)\rangle}{dt} \tag{1.36}$$

multiplying from both sides by $\iota\hbar$ and by solving further we get

$$\iota\hbar\frac{d|\tilde{\psi}(t)\rangle}{dt} = \hat{\tilde{\mathcal{H}}}_1 |\tilde{\psi}(t)\rangle \tag{1.37}$$

1.4.2 Rotating frame

For a single-spin external hamiltonian can be written as

$$\hat{\mathcal{H}}_{\text{lab}} = \hat{\mathcal{H}}_{\text{Zeeman}} + \hat{\mathcal{H}}_{\text{RF}} \tag{1.38}$$

$$\hat{\mathcal{H}}_{\text{lab}} = -\hbar\omega_0\hat{I}_z - \hbar\omega_1[\hat{I}_x\cos(\omega_{\text{RF}}t) + \hat{I}_y\sin(\omega_{\text{RF}}t)] \tag{1.39}$$

By combining above equation by using rotations-sandwitch relations as

$$\hat{\mathcal{H}}_{\text{lab}} = -\hbar[e^{-\iota\omega_{\text{rf}}t}(\omega_0\hat{I}_z + \omega_1\hat{I}_x)e^{\iota\omega_{\text{rf}}t}] \tag{1.40}$$

Transforming the new state by following rule as

$$|\psi'(t)\rangle = e^{\iota\omega_{\text{rot}}t\hat{I}_z} |\psi(t)\rangle \tag{1.41}$$

whose dynamics is given by

$$\iota\hbar\frac{d|\psi'(t)\rangle}{dt} = \hat{\mathcal{H}}' |\psi'(t)\rangle \tag{1.42}$$

where effective hamiltonian is given as $\hat{\mathcal{H}}'$

$$\hat{\mathcal{H}}' = \hbar(\omega_o - \omega_{\text{rot}})\hat{I}_z - \hbar\omega_1\hat{I}_x \tag{1.43}$$

Therefore, at $\omega_o = \omega_{\text{rot}}$ this is called on resonance condition and therefore, magnetization in rotating frame will be in x- direction. Pictorially, the concept of rotating frame can be depicted as

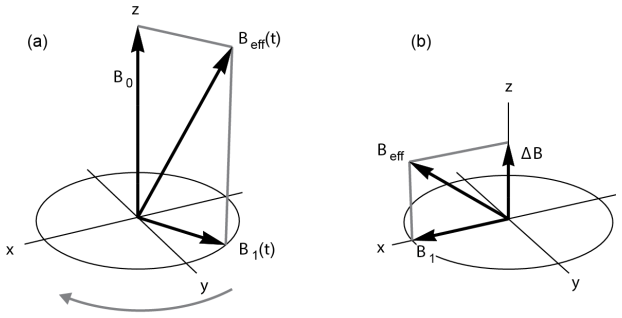


Figure 1.5: Magnetic fields in an NMR experiment(a)in the laboratory frame (b) in the rotating frame[4, 5]

1.5 Relaxation

1.5.1 Longitudinal Relaxation

At equilibrium, net alignment of the spins is along z. As we have previously discussed, to perturb the system net magnetization must be rotated away from +z axis. In order to do that, application of 90^0_x or 90^0_y pulse,which rotate the magnetization to the transverse plane. As soon as, we remove the pulse, net magnetization goes back to z-direction again. The time taken by the net magnetization to return back to equilibrium is known as *Longitudinal relaxation time or spin-lattice relaxation time(T_1)[4, 5].*

Measurement T_1 Relaxation : Inversion Recovery Experiment

In order to measure T_1 equilibrium magnetization is first inverted by the 180^0 pulse that creates -z-magnetization, which will eventually back to +z. If the equilibrium magnetization along the +z-direction is M^0_z then immediately after the 180^0 pulse the magnetization is -M^0_z. Ralaxation with time τ occurs acoording to the following equation[4, 5]

$$M_z(\tau) = M^0_z(1 - 2e^{-\frac{\tau}{T_1}}) \tag{1.44}$$

But, net magnetization must be rotated into the xy-plane in order to be detected. Thus, in order to measure T_1 a series of experiments with different τ values must be performed and at the end by fitting the above equation we can get T_1.

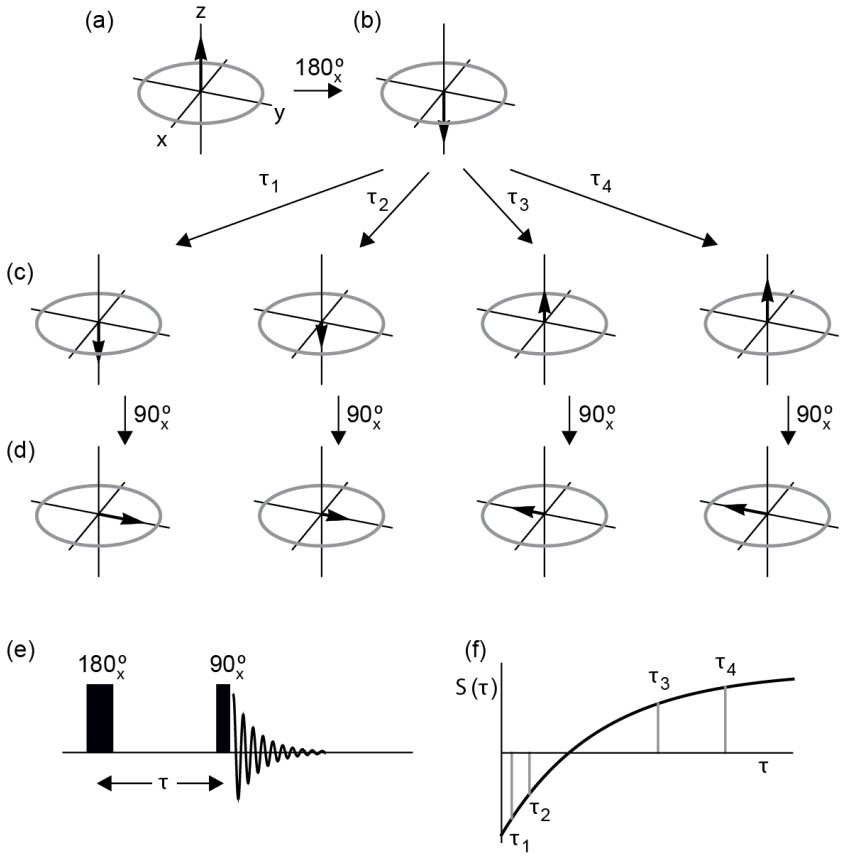
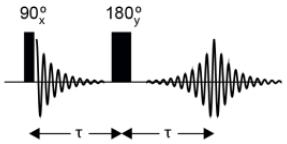


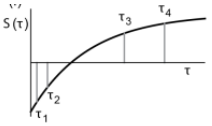
Figure 1.6: Inversion Recovery Experiment(a) Equilibrium Magnetization (b) Magnetizaton after 180^0 pulse (c) Magnetization after different τ(d) Magnetization after different τ after 90^0_x pulse.(e) Build-up Profile of Magnetization[4, 5]

1.5.2 Transverse Relaxation

After perturbing the system from equlibrium, net magnetization in the transverse plane spins dephases and as a result the net magnetization in the x-y plane turns zero. The total time taken by the system in such a way that net magnetization is zero is knon as *Transverse relaxation time or spin-spin relaxation time(T_1)[4, 5].*



(a) CPMG-Sequence[4, 5]



(b) Decay Profile of Magnetization M_{xy}[4, 5]

Figure 1.7: Carr-Purcell-Meiboom-Gill Sequence[4, 5]

Measurement T_2 Relaxation : Carr-Purcell-Meiboom-Gill(CPMG) Sequence

T_2 can be measured by rotating the equilibrium magnetization to the x-y plane via a 90^0_x pulse. After waiting for various period of time t we can measure the spectrum. Such an experiment yields a sinusoidal decay of magnetization that decays with τ and follows the equation

$$M_{xy}(\tau) = M_z^0 e^{-\frac{\tau}{T_2}} \tag{1.45}$$

In order to remove experimental source of decay caused by rf-inhomogeneity we can use the spin-echo sequence$(\tau - 180^0 - \tau)$(discussed later). so that dephasing of spin in the forst τ period can be refocussed back in the second delay. Pictorially, the working of sequence can be depicted as

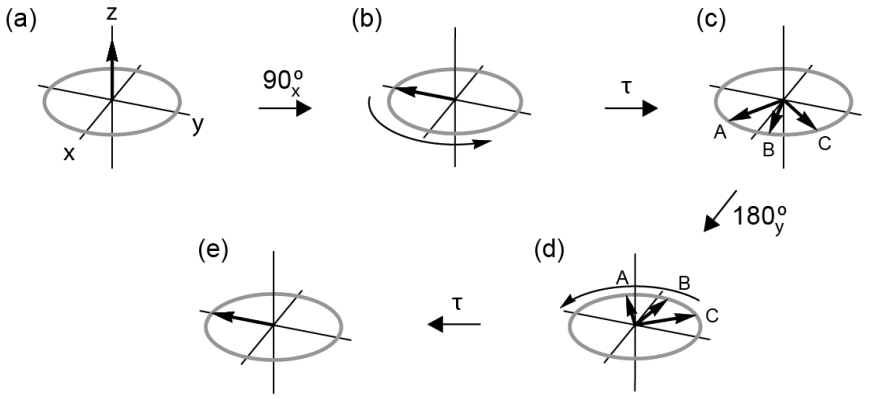


Figure 1.8: CPMG-Sequence Experiment(a) Equilibrium Magnetization (b) Magnetizaton after 90^0_x pulse (c) Magnetization after first τ delay(d) Magnetization 180^0_x pulse.(e) Magnetization after second τ delay[4, 5]

Chapter 2

Diffusion NMR

2.1 Diffusion : Introduction

Diffusion is the random translational motion of molecules or ions driven by the internal energy of the system. This is the basic mechanism by which molecules can be rearranged themselves after perturbing the system. Since, this translational diffusion is the most fundamental form of transport in chemical and biochemical systems. Therefore, to get the intuition about the translational diffusion coefficient can help in governing the process, constituents, system dynamics. But, generally molecular shapes are more complicated and it includes the contribution from hydration factor, friction factor etc. As a result, it also provides information on the interactions and shape of diffusing molecule[16, 18, 13, 28].

2.2 Types of Diffusion

2.2.1 Self Diffusion

It is a random thermal motion of molecule, therefore, this is the most fundamental form of transport. Intuitively, this can be loosely thought as a brownian motion without an applied force and therefore no net displacement is observed as a result molecules that are in proximity will be seperated. Therefore, the stochastic motion of molecules in a pure-liquid at thermal equilibrium is called as *Self-Diffusion*[13]. It is charecterized by self-diffusion coefficient $D(m^2/s)$.

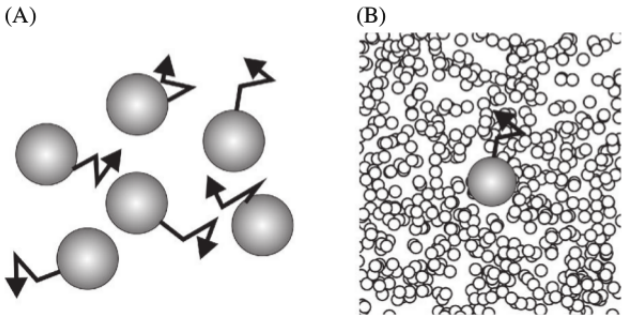


Figure 2.1: (A) Diffusion is the random thermal motion of molecules[13]. (B) Brownian motion of a particle in the liquid[13].

2.2.2 Mutual Diffusion

In a multicomponent system, inhomogeneity of any component in a multicomponent system will arise in mass fluxes to average out the inhomogeneities to achieve thermodynamic equilibrium. Therefore, the force behind mutual diffusion is a gradient of the chemical potential. This is also called as *interdiffusion, concentration diffusion or transport diffusion[13]*.

2.3 Brownian Model of Diffusion

The random motion of a small particle immersed in a fluid is called as *brownian process*. Diffusion can be thought of as a random walk of particles. for the simplest case , one-dimensional case we are going to get 2^n possible trajectories for n molecules. Therefore, no. of trajectories that gives R steps towards right side is

$$ {}^nC_R = \frac{n!}{(n-R)!R!} \tag{2.1} $$

herefore, probability of finding particle at k after n steps

$$ W(k,n) = \frac{n!}{2^n(\frac{n+k}{2})!(\frac{n+k}{2})!} \tag{2.2} $$

which can also be expressed as

$$ W(k,n) = \frac{1}{\sqrt{2\pi n}} e^{-}\frac{k^2}{n} \tag{2.3} $$

Mean displacement of particle after n random steps $\langle X_n \rangle = 0$. Whereas, mean-squared displacement of a particle executing one-dimensional random walk is linear in time.

$$\langle X_n^2 \rangle = 2Dt \tag{2.4}$$

This is also known as one-dimensional diffusion law. It can also ne be written as by dicretizing the each step of length l

$$\langle (X_{n-1} + k_n l)^2 \rangle = \langle (X_{n-1})^2 \rangle + 2l\langle (X_{n-1} k_n) \rangle + l^2 \langle (k_n^2) \rangle \tag{2.5}$$

since $k_n = \pm 1$ with equal probability to move forward and backward

$$\langle X_n^2 \rangle = Nl^2 \tag{2.6}$$

where N is the total no. of steps.By using equation (2.4) & (2.6) we can write diffusion coefficient as

$$D = \frac{l^2}{2NT} \tag{2.7}$$

Similarly, since $\langle X_n^2 \rangle$ = 4Dt & $\langle X_n^2 \rangle$ = 6Dt for 2-D & 3D-random walk respectively, we get

$$D = \frac{l^2}{4NT} \quad \text{for 2-D random walk} \tag{2.8}$$

$$D = \frac{l^2}{6NT} \quad \text{for 3-D random walk} \tag{2.9}$$

2.4 Fick's Laws and Diffusion Equation

When we have substance like solute put inside a diffusing substance liquid as ink in water, then due to molecular collisions this spreads out or throughout liquid uniformly[29]. Therefore, if we have some concentration of some molecular species inside some solvent then this would typically spread out uniformly as in case of ink in water. There are certain empirical laws which tell us about how this process occurs called as Fick's laws of diffusion. These are the laws that would help us to determine conentration of diffusing species described by $\rho(\vec{r}, t)$. The diffusion equation is obeyed by this $\rho(\vec{r}, t)$ and it's derivable easily if we take two assumptions[29]

- Material(diffusing substance) is always there it never disappears.

- Empirical obeservation tells that current flows from higher concentration to lower concentration.

2.4.1 Fick's first Law

Therefore, according to first assumptions that we made $\rho(\vec{r},t)$ should follow continuity equation as

$$\frac{d\rho(\vec{r},t)}{dt} + \boldsymbol{\nabla} \cdot \vec{j} = 0 \tag{2.10}$$

where

- $\rho(\vec{r},t)$ is the concentration over space and time.
- $\vec{j}$ is the flux of the substance per unit area per unit time

2.4.2 Fick's Second Law

By following the second assumption, and since we want at the end equation of $\rho(\vec{r},t)$ alone, therefore we need to relate $\rho(\vec{r},t)$ with $\vec{j}$.

$$\vec{j}(\vec{r},t) = -D\boldsymbol{\nabla}\rho(\vec{r},t) \tag{2.11}$$

Where D is called Diffusion or more proecisely translational diffusion co-efficient. In general, equation(2.5) can be written as

$$\begin{pmatrix} j(x,t) \\ j(y,t) \\ j(z,t) \end{pmatrix} = - \begin{pmatrix} D_{xx} & D_{xy} & D_{xz} \\ D_{yx} & D_{yy} & D_{yz} \\ D_{zx} & D_{zy} & D_{zz} \end{pmatrix} \cdot \begin{pmatrix} \frac{d\rho(x,t)}{dt} \\ \frac{d\rho(y,t)}{dt} \\ \frac{d\rho(z,t)}{dt} \end{pmatrix} \tag{2.12}$$

Where D is the second rank diffusion tensor. Therefore, by combining equation (2.5) and (2.6) we get

$$\frac{d\rho(\vec{r},t)}{dt} = D\nabla^2\rho(\vec{r},t) \tag{2.13}$$

The equation (2.8) is called famous *Diffusion Equation*.

2.5 Stokes-Einstein Equation

Diffusion of spherical particles through a liquid with low Reynolds number is closely related to molecular size given by a famous Stokes-Einstein equation[13]

$$D = \frac{k_B T}{6\pi\eta r_s} \tag{2.14}$$

where

- k_B : Boltzman constant [1.38 * 10−23 m^2 kg s^{-2} K^{-1}]
- T : Temperature [K]
- η : Dynamic viscosity [Pa-sec]
- r_s : Hydrodynamic radius [m]
- D : Diffusion coefficient [$m^2 sec^{-1}$]

2.6 NMR Diffusion

Translational diffusion is the fundamental form of transport in chemical and biochemical system therefore nuclear magnetic resonance spectroscopic technique provides a unique tool to measure it experimentally and quantify its behaviour[21, 22, 23]. NMR diffusion measurements are particularly well suited to understand the processes due to its non-invasive nature and ability to measure diffusion co-efficient over a wide range of concentration[13, 17, 20, 19].

2.6.1 Origin

Since after the discovery of spin-echoes by prof. Erwin Hahn in 1950 it was first realized that this could the basic principle of spin-diffusion measurement. After troubleshooting many experimental limitations by McCall, Douglass and Anderson in 1963. Later, in 1965 first time diffusion was coefficient experimentally recorded by Stejskal and Tanner by modifying the existing spin-echo pulse sequence. This modified sequence is knows as Pulse-gradient spin-echo(PGSE) sequence. Virtually, all contemporary PFG-NMR experiments and flow experiments are solely based upon spin-echo sequence[13, 18].

Spin-Echo Pulse Sequence

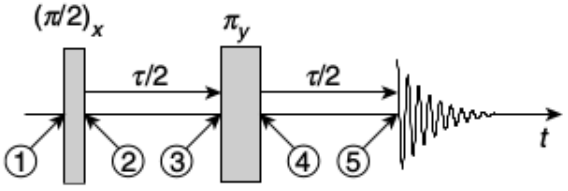


Figure 2.2: Spin-Echo Pulse Sequence[1]

Before applying to this pulse sequence, equlibrium magnetization is directed along the z-direction by applying the 90^0_x pulse to the system the magnetization in rotating frame of reference comes in the transverse plane and then due to inhomogeneities spins precess with different larmor frequencies as a result of which some spins will precess with faster angular velocity and some will slower, therefore, the system will evolve under the chemical shift during the first delay $\tau/2$. After this delay, we apply the π pulse which revert the situation happening in the first delay slowly precessing spins will be ahead of the faster ones and we allow the system to evolve under the same duration $\tau/2$ so that the magnetization refocuses. This phenomenon is known as *spin-echo*. The uniqueness of this pulse sequence is that it refocuses the interaction which are proportional to I_z in the rotating frame of reference. Mathematically, using the product operator farmalism[], the density-operator($\rho(t)$) assuming single spin-1/2 at different time point as mentioned in figure (2.2) can be calculated by using the following hamiltonian

$$\hat{\mathcal{H}}'_{\text{eff}} = -\hbar\Delta\omega I_z - \hbar\omega_1 I_x \tag{2.15}$$

wher $\Delta\omega = \omega_{\text{eff}} - \omega_{\text{rot}} \& \omega_{\text{eff}} = \omega_{\text{o}}(1-\sigma)$. Under hard-pulse limit $\Delta\omega << \omega_1$, $\hat{\mathcal{H}}_{\text{during pulse}}$ & $\hat{\mathcal{H}}_{\text{evolution}}$ can be given as

$$\hat{\mathcal{H}}_{\text{during pulse}} = -\hbar\omega_1\hat{I}_x \tag{2.16}$$

$$\hat{\mathcal{H}}_{\text{evolution}} = -\hbar\Delta\omega\hat{I}_z \tag{2.17}$$

- $\rho_1 : \rho(0) = \hat{I}_z$
- $\rho_2 : \rho(t_{\frac{\pi}{2}}) = +\hat{I}_y$
- $\rho_3 : \rho(t_{\frac{\pi}{2}} + \tau) = \hat{I}_y \cos\left(\frac{\Delta\omega\tau}{2}\right) + \hat{I}_x sin(\frac{\Delta\omega\tau}{2})$
- $\rho_4 : \rho(t_{\frac{\pi}{2}} + \tau + t_\pi) = \hat{I}_y \cos\left(\frac{\Delta\omega\tau}{2}\right) - \hat{I}_x sin(\frac{\Delta\omega\tau}{2})$

- $\hat{\rho}_5 : \rho(t_{\frac{\pi}{2}}+\tau+t_\pi+\tau) = \cos\left(\frac{\Delta\omega\tau}{2}\right)[\hat{I}_y \cos\left(\frac{\Delta\omega\tau}{2}\right)+\hat{I}_x \sin\left(\frac{\Delta\omega\tau}{2}\right)]-\sin\left(\frac{\Delta\omega\tau}{2}\right)[\hat{I}_x \cos\left(\frac{\Delta\omega\tau}{2}\right)-\hat{I}_y \sin\left(\frac{\Delta\omega\tau}{2}\right)] = +\hat{I}_y$

It can be demonstrated pictorially as

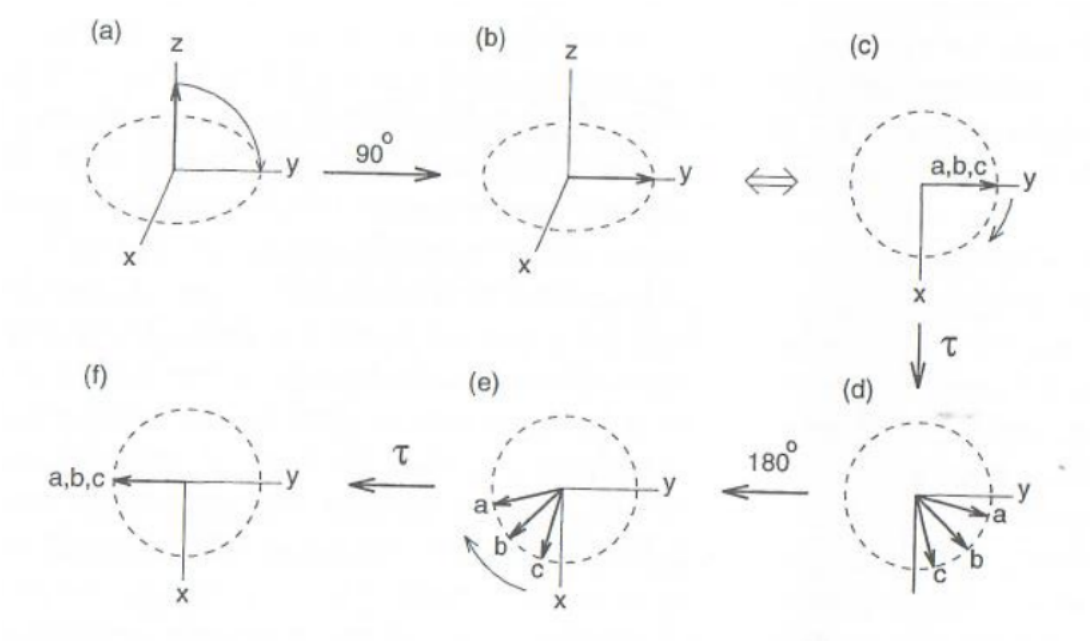


Figure 2.3: Pictorial demostration of Spin-Echo Pulse Sequence[4]

Pulsed Gradient Spin-Echo(PGSE) Pulse Sequence

In this method, the attenuation of the spin echo signal from Hahn spin-echo pulse sequence containing a magnetic field gradient pulse in each τ period of delay is used to measure the displacement of observed spins. Therefore, in PFG-NMR we deliberately apply the gradient during δ so that spins along the z-direction get additional component in frequency as

$$\omega_{\text{eff}} = (\omega_o + \gamma g_z.z) \tag{2.18}$$

The important point is that by applying a homogeneous gradient of known magnitude we are making larmor frequency as a spatial label with respect to the direction of gradient. That spatial label in larmor frequency transformed into the cumulative phase shift as

$$\phi(t) = \gamma B_0 t + \gamma \int_0^t g(t')z(t')dt' \tag{2.19}$$

In this first term representing phase shift due to static field and second term represent phase shift due to gradients. Therefore, we can say that degree of dephasing due to

gradient pulses is proportional to the type of nucleus(γ), the strength of the gradient pulse(g), the duration of the pulse(t) & displacement of spin along the direction of gradients.

Explanation : starting with the $\hat{I}_z$ magnetization first 90^0_x pulse converts the magne-

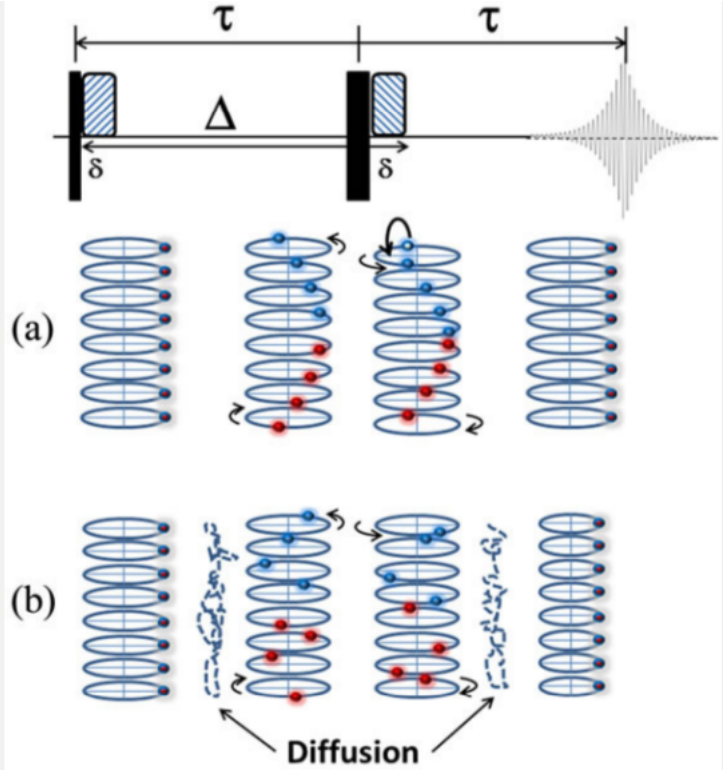


Figure 2.4: Pulsed-gradient spin echo(PGSE) sequence (a) no molecular motion (b) molecular motion

tizaton in the transverse plane after that during the first τ delay in rotating frame all tyes of spins move with their offset-frequency but in this delay a short time duration of δ is given along the z-axis in such a way that at each plane of the sample has an additional frequency due to this gradient which eventually leads to the cumulative phase shift so in the duration of Δ net movement of spins along the z-direction leads to the atenuation of the echo-signal recorded at the end of pulse sequence.This attenuation can be correlated to the famous *Stejskal-Tanner Equation* and diffusion-coefficient can be extracted.

2.7 Stejskal-Tanner Equation

The attenuation of NMR signal at the end of the PGSE sequence follows the following equation[13]

$$I = I_0 e^{[-\gamma^2 \delta^2 g^2 (\Delta - \frac{\delta}{3}) D]} \tag{2.20}$$

where

- I = Intensity
- I_0 = Maximum Intensity
- g = Gradient Strength [Gauss cm^{-1}]
- δ = Gradient Duration [sec]
- δ = Diffusion Time [sec]
- D = Diffusion Coefficient [$m^2 sec^{-1}$]

Chapter 3

Insulin : Primary Structure & its function

3.1 Introduction

Insulin is a peptide hormone which is produced by β-cells of islets of langerhans from the pancreas. Our pancreas is a double gland. It has endocrine gland and exocrine gland. Therefore,it has both endocrine functions as well as exocrine functions. The exocrine function consists of acin and aciner cells and these aciner cells basically synthesize digestive enzymes and in the structure cells in the ducts synthesize bicarbonates. These digestive enzymes along with the bicarbonates through the duct system of pancreas into duodenum, and part of the pancreas which does the following function is collectively called as Exocrime pancreas[30, 31].
The endocrine function of pancreas is done by a very small cluster of cells called *Islets of Langerhans*. These islets of langer hans make nly 1% of pancreatic mass. About 99% of pancreatic mass is concerned with exocrine pancreas.The endocrime pancreas consists of various cells like α, β, δ &pp cells, all of them secretes the peptide hormone. Inslin peptide hormone is produced by the most abundant one β cells[30, 31].

3.2 Insulin

Insulin consists of a heterodimer which has the molecular weight of around 5808 Da. Primary structure of insulin consists of 2 chains A(21 amino acids) and chain B(30 amino acid).

3.2.1 Primary Structure

Chain A made up of GLY-ILE-VAL-GLU-GLN-CYS-CYS-THR-SER-ILE-CYS-SER-LEU-TYR-GLN-LEU-GLU-ASN-TYR-CYS-ASN amino acids & chain-B consists of PHE-VAL-ASN-GLN-HIS-LEU-CYS-GLY-ASP-HIS-LEU-VAL-GLU-ALA-LEU-TYR-LEU-VAL-CYS-GLY-GLU-ARG-GLY-PHE-PHE-TIR-THR-PRO-LYS-THR amino acids. These two amino-acid chains are connected via di-sulphide linkage[31].

3.2.2 Function

When we eat our body breaks down the food and converts that into sugar via various biochemical processes and the end product contains sugar which is the main source of energy in the body or the main source of energy for cells to function properly. Glucose after its production in the pancreatic cells travels through our blood-streams working for individual cell that needs energy. For glucose to get into the cells it requires insulin. Therefore, insulin is the key the unlock the cells for glucose to enter and deliver energy. Before entering the glucose into the blood streams, the pancreas matches it with the right amount of insulin to move glucose into the cells[30, 31]. People having diabetes this process doesn't work as it should.

- Type 1 diabetes suffering body's immune system mistakenly attacks and destroys the β cells in the pancreas, therefore, patient loses the ability to produce enough insulin. Till now, there is no medication available in the market to cure this[30, 31].

- Type 2 Diabetes suffering body's pancreas doesn't produce enough insulin to meet the body's requirements. As time goes along, amount of insulin becomes less and less. There is one more cause of type 2 diabetes where receptor that binds to the insulin and unlock the cells requires more amount of insulin than usual. As a result of which the amount of insulin typically becomes less and less. To cure the type 2 diabetes people regularly inject insulin in the form of proinsulin which more or less close to insulin as far as functioning is concerned[30, 31].

3.3 Role of Environment

3.3.1 Effect of EDTA

EDTA(Ethyl-ene-diamine-tetraacetic acid) is a hexadentate ligand) that binds to the moecules and forms complexes. Insulin is stored in the pancreas as an active Zn hexamer, but when it released into the blood streams due to pH change this hexamer dissociate into dimer and then subsequently into monomers which is its physiologically active form. However, monomer is less stable than hexamer therefore it tend to aggregate. Misfolded insulin monomers with exposed hydrophobic surfaces are involved in amyloidgenesis because their precursors are native state monomers their role is expected to be diminished by the formation of Zn complex.Therefore, Under physiolocal conditions,
Probability of fibril formation : Zn - Insulin $<$ Zn free insulin
due to formation of zn-insulin hexamer as

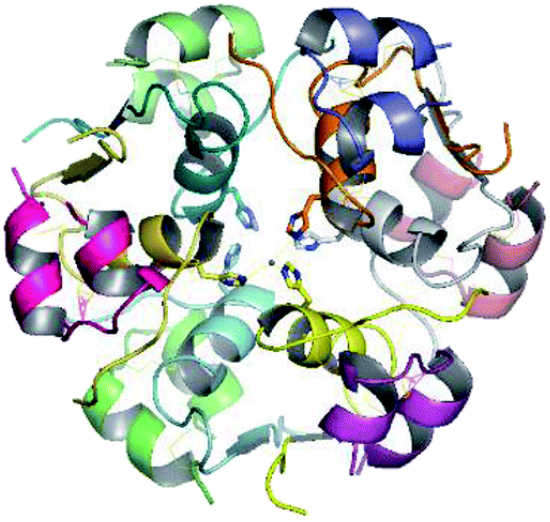


Figure 3.1: Zn-Insulin Hexamer[[10]]

Model that governs the fibril formation of insulin can be summarized as

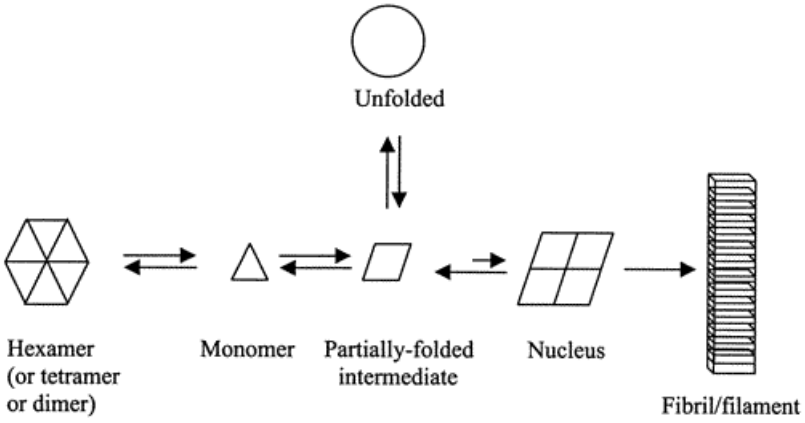


Figure 3.2: Insulin Fibril Formation Model[[12]]

3.3.2 Effect of Sucrose

It had been reported previously that cosolutes such as sugars can be used in "in vitro" protein aggregation experiments since they can mimic the crowding environment in a controlled fashion. Moreover, sugars often increase the native protein structure by intermolecular and intramolecular protein interactions[11].

Chapter 4

Experimental Techniques : Modified DOSY Pulse Sequences

As the time progresses, measuring the translational diffusion-co-efficient is very common among scientists in this field to reveal the dynamical behaviour of different system. To determine the diffusion coefficient of several systems varied from their size to properties under different conditions one Pulse-gradient spin echo sequence is not enough. Therefore, time to time as scientists encountered the problems they proposed the solutions as well. In this chapter, I would describe the modification of sequences in order to remove experimental artefacts.

4.1 DOSY Sequences

4.1.1 Pulse-Gradient Stimulated Echo(PGSTE) Sequence

In the PGSE sequence, total change in the phase angle of a particular spin at time t , after gradient has been applied. Therefore, the net phase angle can be written as

$$\phi_z(t) = \gamma B_0 t + \gamma g_z z t \tag{4.1}$$

this general phase has been accumulated in two distinct τ duration. Hence, total phase accumulation is the sum of $\phi_1 z(t) \& \phi_2 z(t)$

$$\phi_1 z(t) + \phi_2 z(t) = \phi_{tot}(t) \tag{4.2}$$

If the spins have not changed their position during the dephasing and rephasing gradient then net phase change would be zero i.e. $\phi_{tot}(t) = 0$. In order to refocus the chemical shift evolution that occurs while the magnetization is on the transverse plane 180^0 pulse between the two gradients will average out the evolution under the chemical shift[32].

- For samples with $T_1 = T_2$ the PGSE sequence is usually the preferred one.

Therefore, in PGSE sequence maximum diffusion time is limited by T_2. To get the enhanced signal for samples with $T_1 >> T_2$ which is the usual case in which 180^0 pulse is decomposed into two 90^0 pulse having crusher gradient in between them so that during the diffusion time magnetization is stored in the z-direction. Therefore, in PGSTE sequence maximum diffusion time is limited by T_1[32, 33].

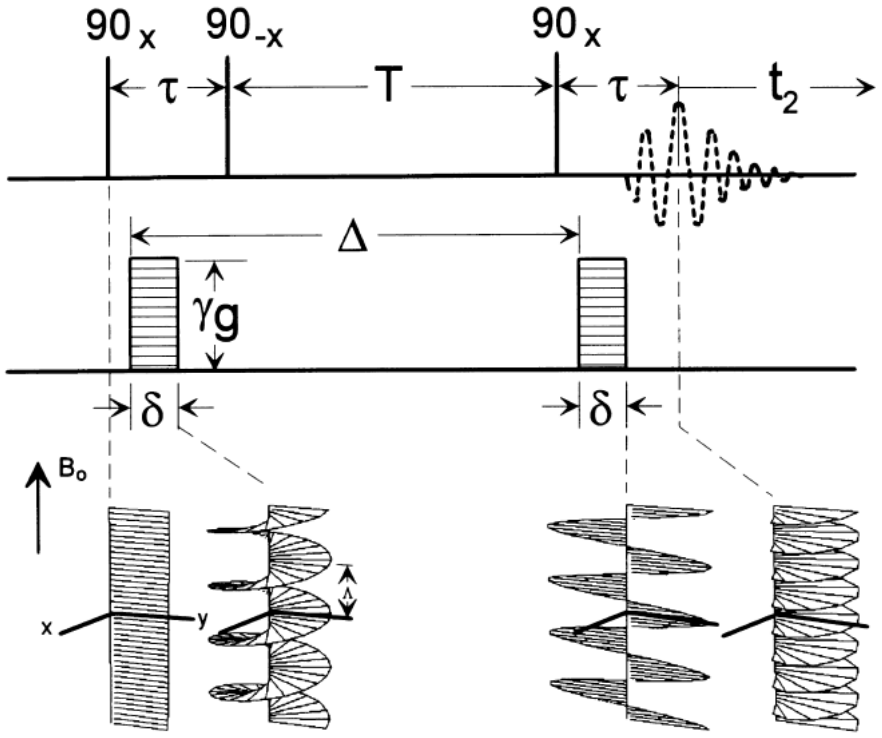


Figure 4.1: **P**ulsed **G**radient **ST**imulated **E**cho Sequence(PGSTE)[28, 33]

In PGSTE sequence, first echo after the third RF pulse is called as the Simulated echo. In the existing literature effect of diffusion on the STE with both steady and pulsed gradient has been computed.
Difference between the SE and STE sequence

- In STE sequence, the amplitude of STE sequence is reduced by a factor of two since one the second 90^0_x pulse stores the magnetization by rotating only the y components to the $\pm$ z-directions. The x- component remains in the transverse plane and contribute to the recorded echo signal[28].

Eddy Currents: Eddy currents are the loops of electric current induced within conductors by changing magnetic field in the conductor due to faraday law of induction. Therefore, it flows in closed loops within conductors, in planes perpendicular to the magnetic field[34].

4.1.2 Longitudinal Eddy Current(LED Sequence)

Usually, Eddy current can affect the STE experiment to a non-tolerable extent and it happens when experiments are done using shaped gradient pulses since they have low slope change as compared to the rectangular gradient therefore to remove this artifacts in the existing sequence the can be modified by introducing a delay at the end of the sequence as[32, 33, 28]

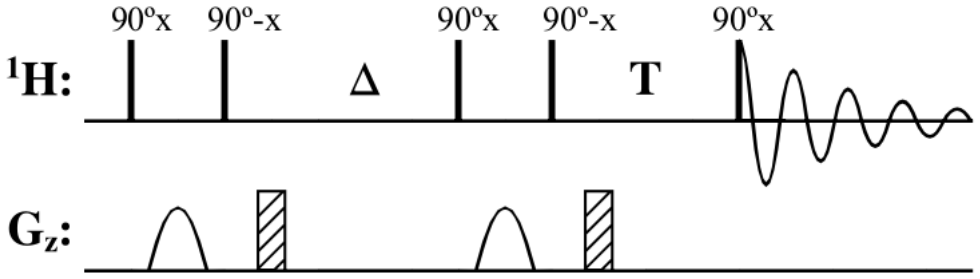


Figure 4.2: **L**ongitudinal **E**ddy Current(LED) Sequence[32, 28]

4.1.3 Bipolar-Pulse Pair Longitudinal Eddy Current(BPP-LED Sequence)

Another way to reduce the Eddy current effects is that introducing the gradient pulses which are of opposite polarity on either side of 180^0 pulse followed by insertion of π pulse in between the first and second 90^0 & third and fourth pulse 90^0. This $(Gz--180^o--(-Gz)$ composite pulse combination self-compensates for the induced eddy currents.In this sequence, τ between the two subsequent 90^0[28]. The LED experiment with the bipolar pulse pairs should help reduce most of the eddy current effects. The BPP- LED sequence is shown below:

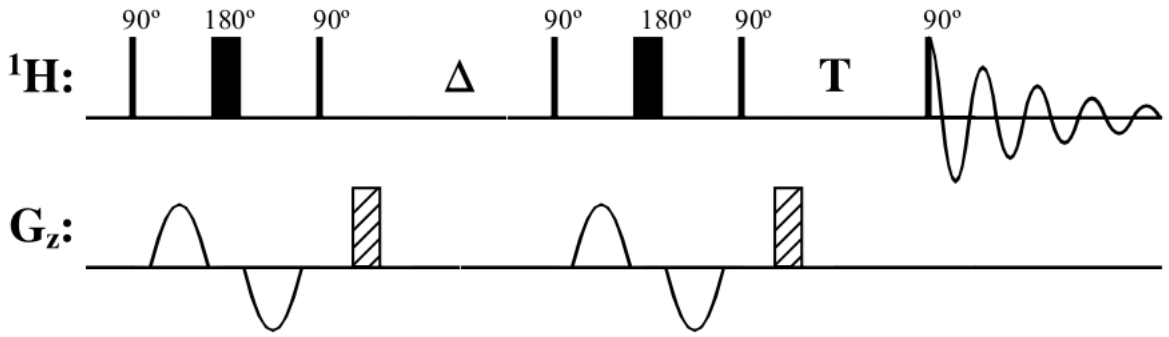


Figure 4.3: **B**ipolar-**P**ulse **P**air **L**ongitudinal **E**ddy Current(BPP-LED Sequence)[32, 28]

4.2 Solvent Supression Sequences

To deal with the unlabelled samples dissolved in some amount of D_2O create an intense signal at water resonance that appears in the spectrum at around 4.8ppm, as a result of which either sample's signal decays too fast at high gradient and disappear in the spectrum or the other signals getting suppressed in intensity even at zero gradient. Therefore, to tackle this problem some solvent suppression technique are presented in the literature.

4.2.1 PreSaturation Method

Presaturation is the most common method to suppress the solvent signal. In this method a long selective low power pulse is applied at the solvent resonance frequency after that, a hard non-selective pulse is applied[35, 36]. To get efficient water suppression we need to apply two important parameters that need to optimized properly.

- Saturation Power
- Saturation Time

Too little power in the presaturation method will result in inefficient suppressed water signal, whereas too much power results in losing the intensity of signals close to the solvent resonance and foregoes the quantitative nature of the NMR data. By using selected saturation power, the appropriate choice for the saturation time depends upon the relaxation properties on the relaxation properties as well as B_1 field homogeneity of the probe[35, 36].

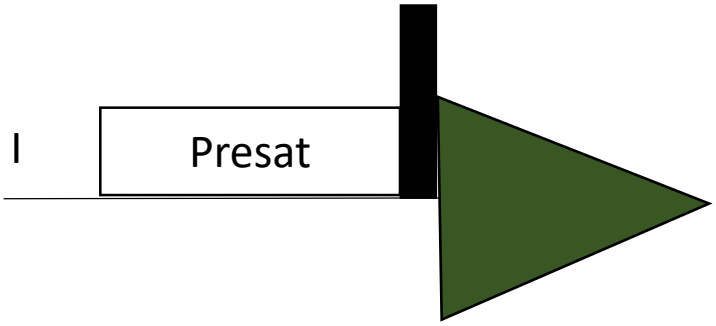


Figure 4.4: Pre Saturation Sequence, Black rectangle represents 90^0_x pulse

4.2.2 Excitation Sculpting Method

The most common pulse sequence for diffusion measurement are based on the spin echo but stimulated echo is preferred because it is less susceptible to eddy currents as well as magnetization is stored in the z-direction during the diffusion delay. In this method, solvent suppression can be done using water-selective 180^0 pulses[37]. The pulse sequence of this method is as follows

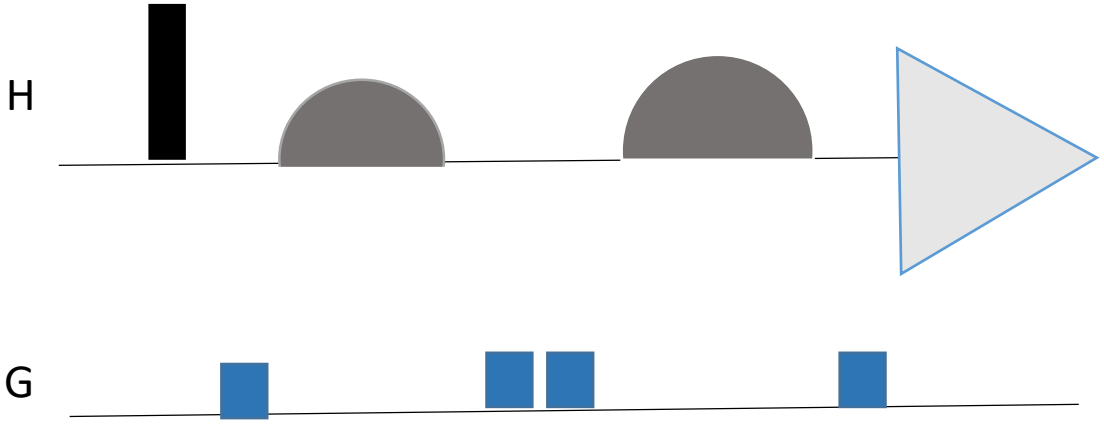


Figure 4.5: Excitation Sculpting Sequence, blue squares represents gradient pulses, shaped rectangles represents shaped gradients and filled black rectangle represents 90^0_x pulse

4.2.3 Watergate Method

This method uses a pair of gradients surrounding a composite pulse in effect invert all the solvent signal. The duration of the composite pulse should be kept long as

compared to other solvent suppression sequences so that proton exchange among the solvent and signal happen to the lesser extent[38].

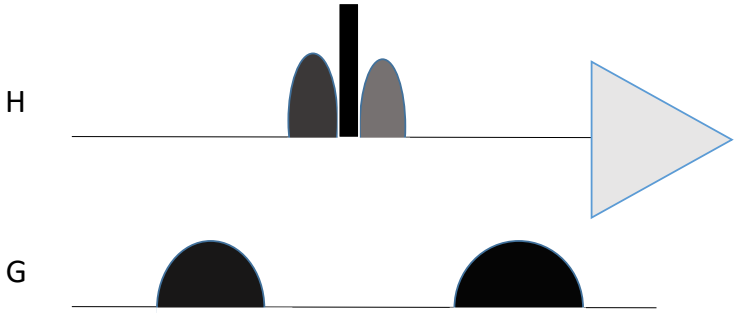


Figure 4.6: Watergate Sequence, black rectangles represents 90^0_{-x}, shaped rectangles represents shaped gradient pulses

Chapter 5

Diffusion Study of Insulin : Experimental Results

To investigate the diffusion coefficient of insulin we have used two probes namely, QXI and Diff30. We have started the experiment with combining the Bipolar Longitudinal Delay with the Excitation Sculpting(BPPLED-ES Sequence). To initiate the experiment we first calibrated the gradient diffusion coefficient(GCC).

5.0.4 Diffusion Calibration

The most common way to calibrate the gradient diffusion coefficient by using the diffusion coefficient of known sample. We have used to distilled water to calibrate the diffusion coefficient.

The gradient calibration co-efficient of the probe: $g_{app} = 5.3500094$ G mm^{-1}

$$g = g_{\text{app}}\sqrt{\frac{D_{app}}{D_0}} \tag{5.1}$$

after doing the calculation we got log D = -8.554 of peak at 4.6929 ppm which leads to 2.911 * 10^{-9} m^2 sec^{-1}.At the end we have calculated $g_{calculated} = 5.30$ G mm^{-1}

5.1 Sample Preparation

- We have first prepared a phosphate buffer solution of the desired pH.

- Adding insulin to the buffer in such a way that final solutions contains 2mg/ml of insulin.
- Further, We add 10% D_2O solution to it and recorded the spectra.

5.2 Insulin Behaviour under different pH

Condition	Behaviour
Acidic	Dimers
Basic	Monomers
Neutral	Tetramers with hexamers as well
Neutral + EDTA	Hexamers

Insulin with 7.4pH at 298K

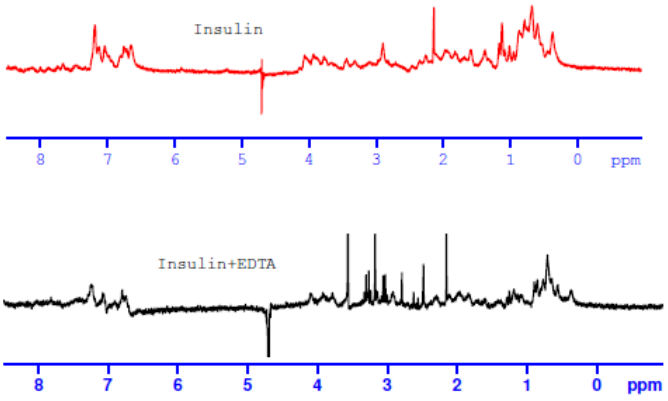


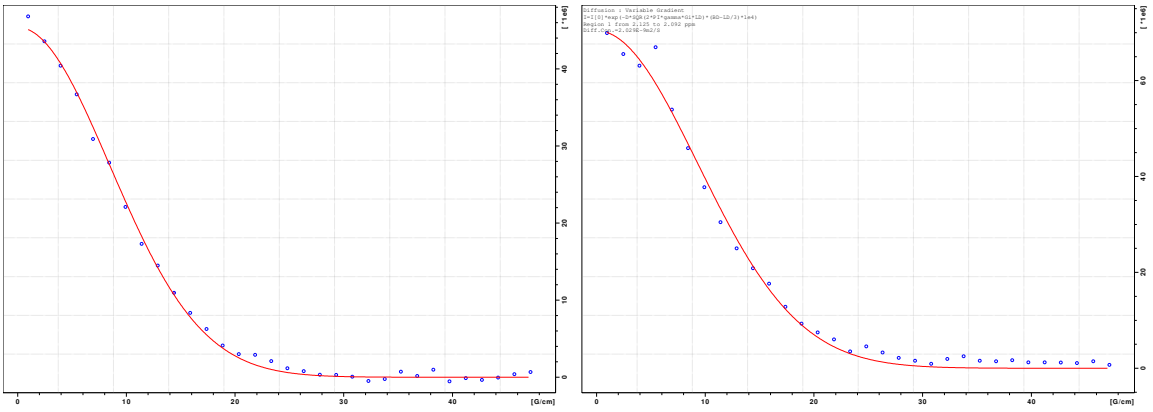
Figure 5.1: Insulin in presence and absence of EDTA

The average distance between the adjacent insulin molecules[13]

The average spacing between insulin molecules in a 0.3 mM solution is of the order of 0.1nm. Yet, taking the monomer diffusion coefficient at 298 K to be of the order of $1*10^{-11}$ m^2 sec^{-1} , $\Delta = 100$ ms, the RMSD is about 1000 μ m. Thus, during Δ there is a high probability for the insulin molecules to collide numerous time and aggregate.

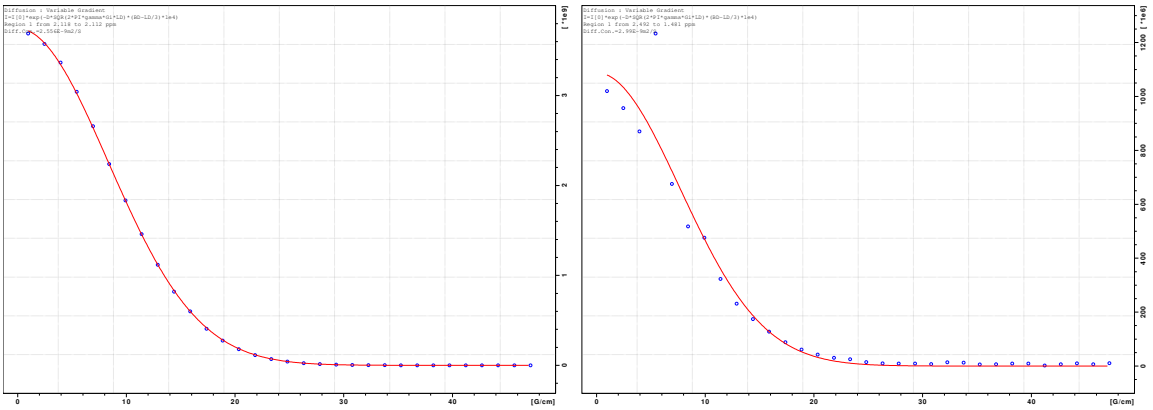
5.3 Results : LED-ES on QXI

Diffusion Coefficient(D) determined by BPPLED-ES Sequence with QXI probe at defined pH and Temperature



(a) pH = 10.8 at Temperature - 295K (b) pH = 10.8 at Temperature - 292K

Figure 5.2: Insulin at pH 10.4 with temp 292K & 295K



(a) pH = 2.4 at Temperature - 295K (b) pH = 2.4 at Temperature - 292K

Figure 5.3: Insulin at pH 2.4 with temp 292K & 295K

S.No.	pH	Temp.(K)	Peak Position(ppm)	D(m^2/s)±St.Dev.
1.	2.8	292	2.0856	$2.990*10^{-09}\pm 6.062*10^{-02}$
2.	2.8	295	2.1148	$2.556*10^{-09}\pm 2.445*10^{-03}$
3.	10.8	292	2.1072	$2.029*10^{-09}\pm 2.846*10^{-02}$
4.	10.8	295	2.1417	$2.452*10^{-09}\pm 1.253*10^{-02}$

5.4 Results : DiffSTe on Diff30

According to insulin's molecular weight i.e. 5808 Da its diiffusion coefficient should be around $10^{-11}m^2sec^{-1}$. Therefore to correct the above results we have used diff30

probe which has gradient magnitude of around 1200 Gcm^{-1}.

Insulin with pH = 10.4 at 289K

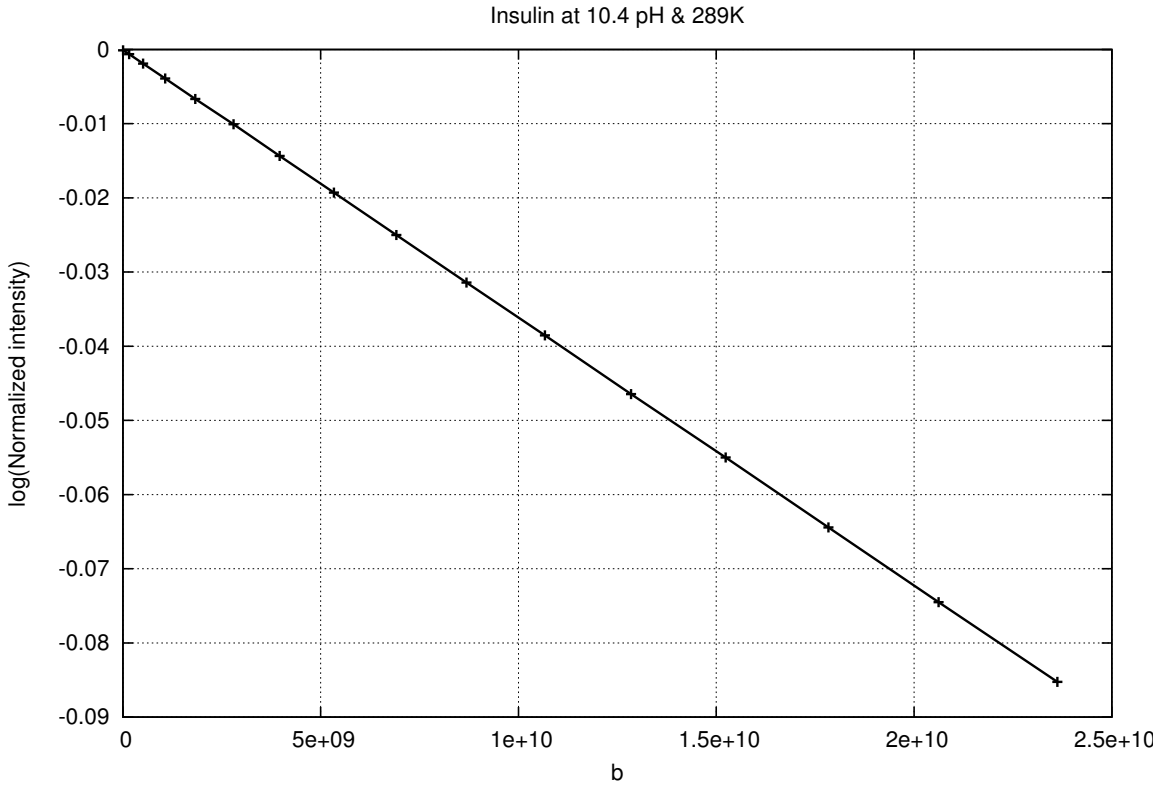


Figure 5.4: NS = 128, Δ = 900ms, δ=1ms with DiffSte sequence yields D$[m^2sec^{-1}]$ = 9.45422*10^{-11}

System	Crowding Agent(Conc.)	pH	D(m^2sec^{-1})
Insulin	No	2.4	1.94*10^{-13}
Insulin	No	10.4	9.45422*10^{-11}

System	Crowding Agent(Conc.)	pH	D(m^2sec^{-1})
Insulin	EDTA(1mM)	2.4	1.28*10^{-13}
Insulin	EDTA(1mM)	7.4	4.31*10^{-13}
Insulin	EDTA(1mM)	10.4	5.83*10^{-13}

System	Crowding Agent(Conc.)	pH	D(m^2sec^{-1})
Insulin	Sucrose(0.75mg)	2.4	$1.14*10^{-13}$
Insulin	Sucrose(0.75mg)	10.4	$1.69*10^{-14}$

System	Crowding Agent(Conc.)	pH	D(m^2sec^{-1})
Insulin	Sucrose(0.5mg)	10.4	$4.82*10^{-15}$
Insulin	Sucrose(1.5mg)	10.4	$1.07*10^{-13}$

5.5 Conclusions and Future Direction

As a preliminary diffusion study of insulin, our results as diffusion coefficient clearly resonates with the existing results as far as insulin alone in acidic & basic pH is concerned. Insulin in the neutral pH range with the same parameters couldn't be able to show any peak. Therefore, the parameters need to be further optimized to see the result of insulin at neutral pH.

In order to investigate its behaviour under presence crowding agent as EDTA(Ethylene-diamine-tetraacetic acid- a hexadentate ligand). The diffusion coefficients in presence of EDTA reduce down to the factor of 100, it was reported previously that insulin in neutral pH range with EDTA exists as a tetramer or hexamer. Therefore, according to our data resembles the argument to such extent and irrespective of pH it tries to exist as the same form.

Insulin injections are the key things for diabetic patients and diabetes is the result of increase in blood glucose level. Therefore, it looks an interesting problem to see the behaviour of insulin diffusion in presence of glucose itself. According to the previous study[11] that sucrose delays the insulin aggregation. Therefore, we have recorded diffusion spectra in presence of different conc. of sucrose at different pH. We found that till sucrose is around 1.5mg there is not any trace of monomeric diffusion coefficient that implies that in solution different oligomeric species coexists except low amount of monomers, therefore, we hypothesize that there should be a minimum threshold

of sucrose conc. after which a significant amount of monomers are present in the solution.

To see the aggregation behaviour one should recored the spectra at different time points after sample preparation as well as with different concentrations and tempreature & get the proportion of different oligomeric species which can be computed by fitting experimental data into existing theoretical models.

Appendix A

Diffusion Experiments Results

Note : This appendix might contains few results that are not clearly fitted to the linear decreasing function due to inappropriate application to the gradient unit.

Insulin with 2.4pH at 289K

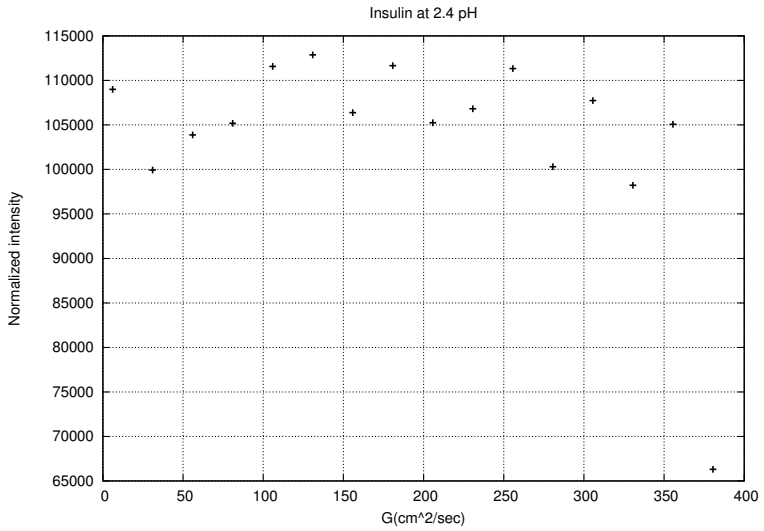


Figure A.1: NS = 128, Δ = 900ms, δ=1ms with DiffSte sequence yields D$[m^2 sec^{-1}]$ = 1.94*10^{-13}

Insulin with EDTA at 2.4 pH & 289K

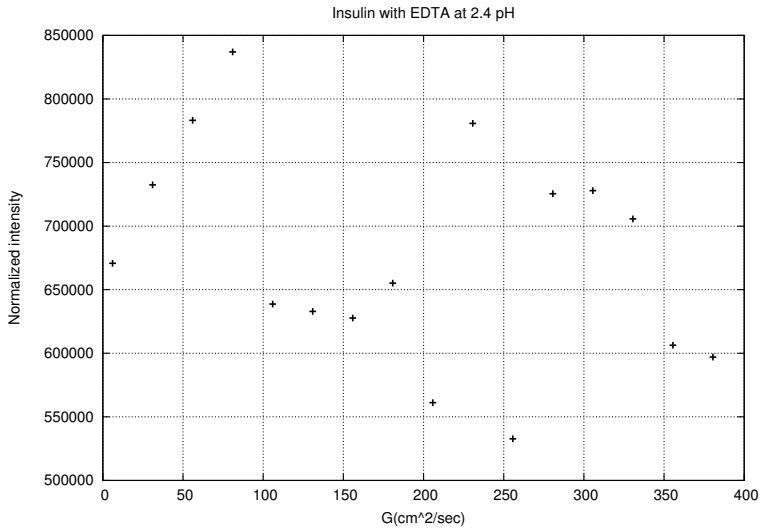


Figure A.2: NS = 128, Δ = 900ms, δ=1ms with DiffSte sequence yields D$[m^2 sec^{-1}]$ = 1.28*10^{-13}

Insulin with EDTA at 7.4pH & 289K

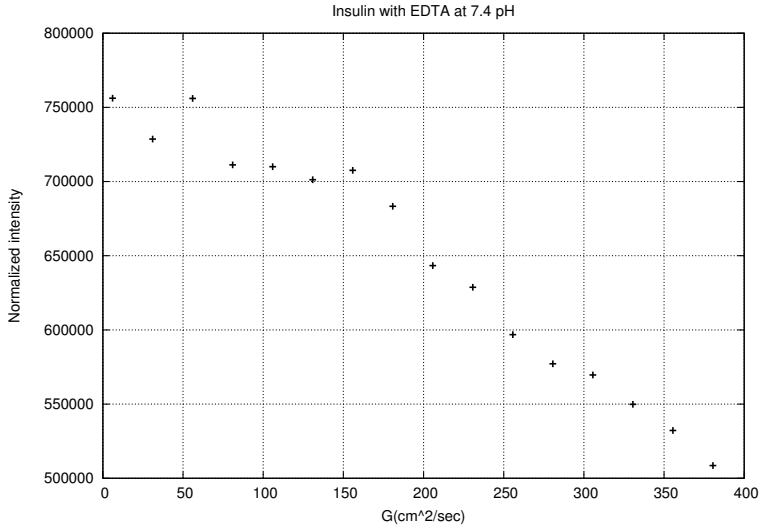


Figure A.3: NS = 128, Δ = 900ms, δ=1ms with DiffSte sequence yields D$[m^2 sec^{-1}]$ = 4.31*10^{-13}

Insulin with EDTA at 10.4 pH & 289K

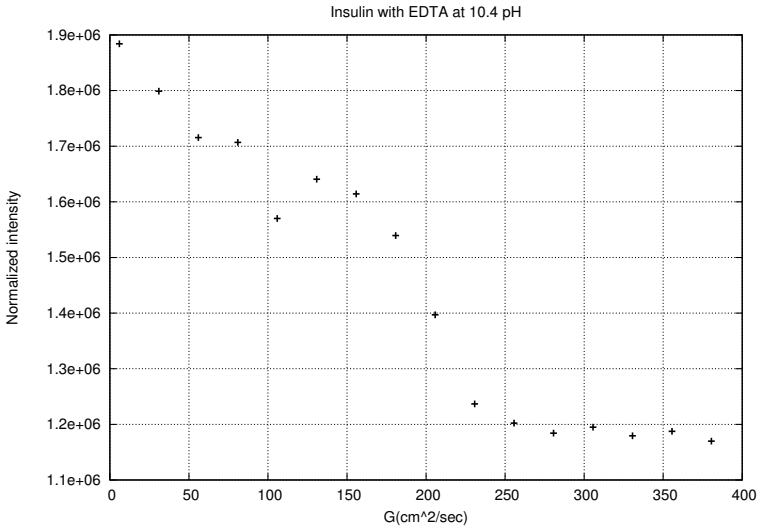


Figure A.4: NS = 128, Δ = 900ms, δ=1ms with DiffSte sequence yields D$[m^2 sec^{-1}]$ = 5.83*10^{-13}

Insulin with sucrose(0.75mg) at 2.4 pH & 289K

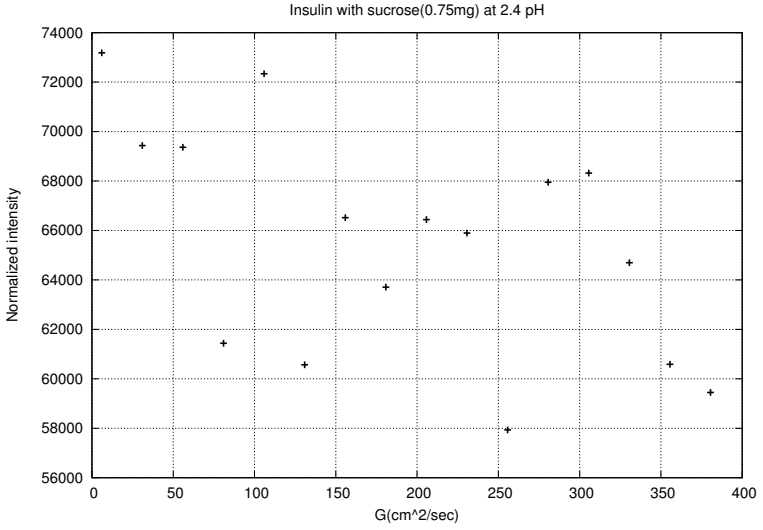


Figure A.5: NS = 128, Δ = 900ms, δ=1ms with DiffSte sequence yields D$[m^2 sec^{-1}]$ = 1.14*10^{-13}

Insulin with sucrose(0.75mg) at 10.4 pH & 289K

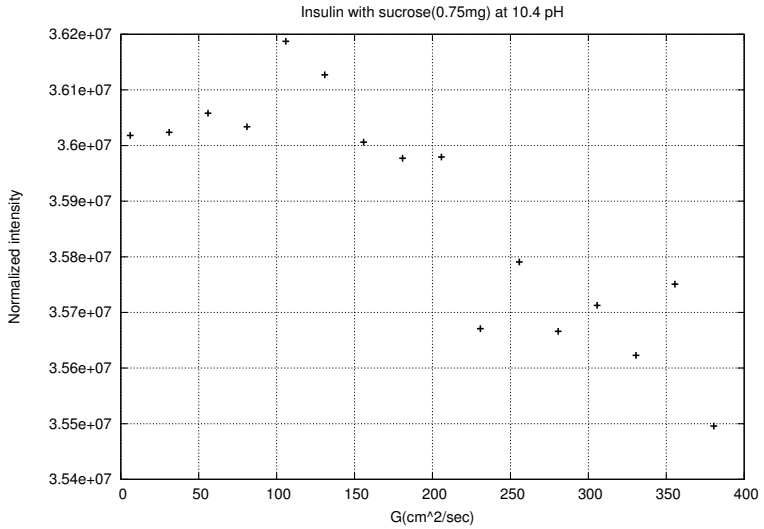


Figure A.6: NS = 128, Δ = 900ms, δ=1ms with DiffSte sequence yields D$[m^2 sec^{-1}]$ = 1.69*10^{-14}

Insulin with sucrose(0.5mg) at 10.4 pH & 289K

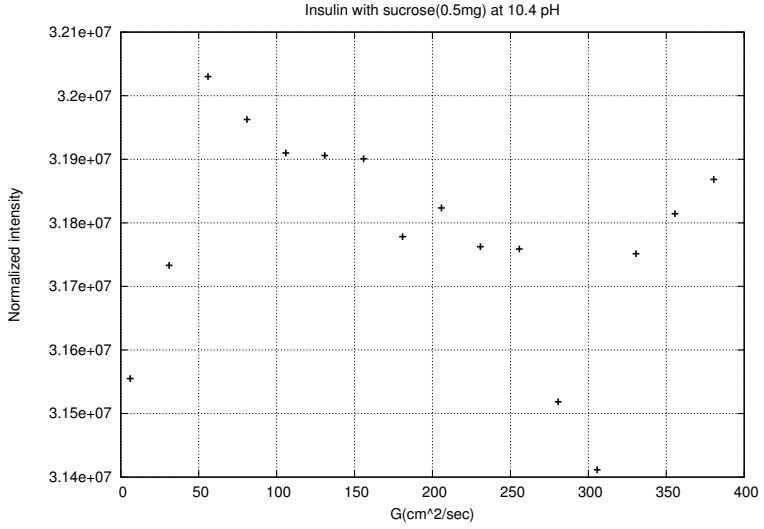


Figure A.7: NS = 128, Δ = 900ms, δ=1ms with DiffSte sequence yields D$[m^2 sec^{-1}]$ = 4.82*10^{-15}

Insulin with Sucrose(1.5mg) at 10.4pH & 289K

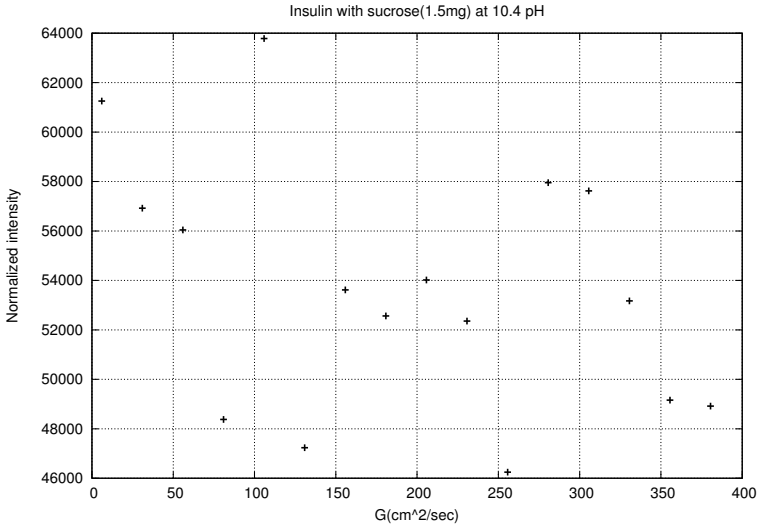


Figure A.8: NS = 128, Δ = 900ms, δ=1ms with DiffSte sequence yields D$[m^2 sec^{-1}]$ = 1.07*10^{-13}

Appendix B

Three Letter Codes of Amino Acids

Amino Acids	Three Letter Code
Alanine	Ala
Arginine	Arg
Asparagine	Asn
Aspartic Acid	Asp
Cysteine	Cys
Glutamine	Gln
Glutamic Acid	Glu
Glycine	Gly
Histidine	His
Isoleucine	Ile
Leucine	Leu
Lysine	Lys
Methionine	Met
Phenylalanine	Phe
Proline	Pro
Serine	Ser
Threonine	Thr
Tryptophan	Trp
Tyrosine	Tyr
Valine	Val

Appendix C

Topspin Commands

-edc : To create a directory.
-rsh : To read the shim file.
-rga : To set receiver gain.
-zg : To start Acquisition.
-atmm: To tune and match manually.
-atma: To tune and match automatically.
-getprosol: To set the default value in the experiment.
-i : To copy the experiment.
-wrpa : To copy the experiement at desired location.
-pulsecal : To caliberate pulse length automatically.
-efp: Perform fourier processing.
-apk: Automatic hase correction.
-abs: Automatic base line correction.

Appendix D

Stokes-Einstein Equation

Newton's equation of motion for a prticle which is moving under a constant external force F_{ext} in x-direction

$$\frac{dv_x}{dt} = \frac{F_{\text{ext}}}{m} \tag{D.1}$$

where v_x : velocity of particle in x-direction. & m : mass of particle.

$$v_x(t) = v_{0,x} + \frac{F_{\text{ext}}}{m} \tag{D.2}$$

$$\Delta x = v_{0,x} + \frac{F_{\text{ext}}(\Delta t)^2}{m} \tag{D.3}$$

Taking average at both sides of equation

$$< \Delta x >= \frac{F_{\text{ext}}(\Delta t)^2}{2m} \tag{D.4}$$

Since ¡$v_{0,x}$¿ is zero on an average due to equal probability. Therefore due to friction particle acquire a terminal velocity v_{ter} as

$$v_{\text{ter}} = \frac{F_{\text{ext}}}{f} \tag{D.5}$$

where the viscous friction coefficient as

$$f = \frac{2m}{\Delta t} \tag{D.6}$$

f for a spherical object can be defined as the stokes's formula as

$$f = 6\pi\eta R \tag{D.7}$$

where
η : Viscosity[Pa.sec]
R: Hydrodynamics Radius
As derived in the chapter 2 1D diffusion law can be written as

$$D = \frac{L^2}{2\Delta t} \tag{D.8}$$

Using above two equations we can write as

$$fD = \frac{mL^2}{\Delta t^2} \tag{D.9}$$

$$fD = mv_{0,x}^2 \tag{D.10}$$

Using equipartition theorem we can write as

$$< v_{0,x}^2 >= \frac{k_B T}{m} \tag{D.11}$$

$\Rightarrow$ fD =k_B T
i.e. D = $\frac{k_B T}{f}$
The above equation is known as *Stokes-Einstein Equation.*

Bibliography

[1] Levitt Malcolm H., Spin Dynamics : Basics of Nuclear Magnetic Resonance-2nd edition, ISBN 978-0-470-51117-6, John Wiley & Sons Inc.

[2] James Edward Keeler, Understanding NMR Spectroscopy, 2nd Edition James Keeler,ISBN: 978-0-470-74608-0, John Wiley & Sons Inc.

[3] Hore P.J., J.A. Jones and S Wimpris, NMR : The Toolkit, Oxford Chemistry Primers(OCP), ISBN:0-19-56704-9.

[4] Hore P.J., Nuclear Magnetic Resonance, Oxford Science Publications, ISBN-978-0-19-855682-4.

[5] Steven M. Pascal, NMR Primer : An HSQC-Based Approach with vector animations.

[6] William S. Price, NMR Studies of Translational Motion, ISBN-978-0-521-80696-1, Cambridge University Press.

[7] Ivan S. Oliveira, Tito J. Bonagamba, Roberto S. Sarthour, Jair C.C. Freitas, Eduardo R. deAzevedo, NMR Quantum Information Processing, ISBN: 978-0-444-52782-0. ELSEVIER.

[8] Smith, Palke, Gerig,Concepts of Magnetic Resonance, 1992, 4, 107-144

[9] Smith, Palke, Gerig,Concepts of Magnetic Resonance, 1992, 4, 181-204

[10] Sklepari M., Rodger A., Reason A., Jamshidi S., Prokes I. & Blindauer C.A., Biophysical charecterizationof a protein for structural comparison : methods for identifying insulin structural changes, Anal. Methods, 2016, 8, 7460

[11] Carlotta Marasini,Vito Foderb & Bente Vestergaard, Sucrose modulates insulin amyloid-like fibril formation: effect on the aggregation mechanism and fibril morphology, RSC Adv., 2017, 7, 10487.

[12] Liza Nielsen, Ritu Khurana, Alisa Coats, Sven Frokjaer, Jens Brange,Sandip Vyas, Vladimir N. Uversky and Anthony L. Fink, 'Effect of Environmental Factors on the Kinetics of Insulin Fibril Formation: Elucidation of the Molecular Mechanism',Biochemistry 2001, 40, 6036-6046.

[13] William S. Price, Fumihiko Tsuchiya, and Yoji Arata,Lysozyme Aggregation and Solution Properties Studied Using PGSE-NMR Diffusion Measurements, J. Am. Chem. Soc. 1999, 121, 11503-11512

[14] GAng Xheng, Allen M Torres, William S Price,Solvent Supression using phase-modulated binomial-like sequences ad applications to diffusion measurement, Journal of magnetic resonance 1994(2008)108-114.

[15] Stephane Balayssac, MArc-Andre Delsuc, Veronique Gilard, Yann Prigent, Mariam Malet Martino, Two-Dimensional DOSY experiment with excitation scultpting water supression for the analysis of natural and biological media, Journal of Magnetic Resonance 196(2009)78-83

[16] Davy Sinnaeve, The Stejskal-Tanner Equation Generalized for any gradient shape-An overview of most Pulse Sequences Measuring Free Diffusion.

[17] Mengfen Lin, Cynthia K. Larive, Detection of Insulin Aggregates with Pulsed-Field Gradient Nuclear Magnetic Resonance Spectroscopy, Analytical Biochemistry 229,214-220(1995)

[18] William S. Price, Pulsed-Field Fradient Nuclear Magnetic Resonance Spectroscopy as a tool for studying translational diffusion: Part:1. Basic Theory.

[19] A.Gaemers, C.J. Elsevier, and Ad Bax, NMR of Biomolecules in low-viscosity,liquid CO2, chem. Phys. Lett. 301(1999)-138-44

[20] J.P. Mackay, G.L. Shaw and G.F. King, Backbone Dynamics of c-Jun Leucine Zipper:15N NMR Relaxation Studies.Biochemistry 35(1996)4867-77

[21] C. H. Everhart and C. S. Johnson, Jr., The Determination of Tracer Diffusion Coefficients for Proteins by Means of Pulsed Field Gradient NMR with Applications to Hemoglobin. J. Magn. Reson. 48 (1982), 46674.

[22] I. V. Nesmelova and V. D. Fedotov, Self-Diffusion of Myoglobin and Water Molecules in Solutions. Polym. Sci. A 39 (1997), 3615

[23] A. J. Dingley, J. P. Mackay, B. E. Chapman, M. B. Morris, P. W. Kuchel, B. D. Hambly, and G. F. King, Measuring Protein Self-Association Using Pulsed-Field-Gradient NMR Spectroscopy: Application to Myosin Light Chain 2. J. Biomol. NMR 6 (1995), 3218.

[24] S. J. Gibbs, A. S. Chu, E. N. Lightfoot, and T. W. Root, Ovalbumin Diffusion at Low Ionic Strength. J. Phys. Chem. 95 (1991), 46771

[25] A. Dehner and H. Kessler, Diffusion NMR Spectroscopy: Folding and Aggregation of Domains in P53. ChemBioChem 6 (2005), 155065.

[26] W. S. Price, M. Nara, and Y. Arata, A Pulsed Field Gradient NMR Study of the Aggregation and Hydration of Parvalbumin. Biophys. Chem. 65 (1997), 17987.

[27] Y. Aso, S. Yoshioka, and S. Kojima, Determination of the Diffusion Coefficient of Insulin and Lysozyme in Crosslinked Dextran Hydrogels by Pulsed-Field-Gradient NMR. Chem. Pharm. Bull. 46 (1998), 18369.

[28] C.S. Johnson Jr.,Diffusion ordered nuclear magnetic resonance spectroscopy: principles and applications, Progress in Nuclear Magnetic Resonance Spectroscopy 34 (1999) 203256.

[29] v.Balakrishnan, lecure notes, mathematical physics.

[30] https://www.youtube.com/watch?v=qGaTj8Semrk&t=190s

[31] http://www.biotopics.co.uk/as/insulinproteinstructure.html

[32] MEASURING DIFFUSION BY NMR, Bruker Instrument Inc.Sophie Kazanis Application Scientist August 2000

[33] DOSY and Diffusion by NMR, bruker, biospin manual.

[34] Princeton review article on eddy currents.

[35] http://u-of-o-nmr-facility.blogspot.in/

[36] http://u-of-o-nmr-facility.blogspot.in/search?q=presaturation

[37] http://triton.iqfr.csic.es/guide/tutorials/solvent/zgesgp.html

[38] http://triton.iqfr.csic.es/guide/eNMR/eNMRsolv/wg.html