Chapter 3
Materials and Methods

3.1 Materials

Gelatin (Type-B, microbiology grade devoid of E. coli and liquifier presence, molecular weight \(\approx (90 \pm 10)\) kDa [Dalton with the symbol Da is defined as 12 times the ratio of the polymer molar mass and the molar mass of \(^{12}\text{C}\) i.e. \(\text{g} \text{mol}^{-1}\) determined from SDS/PAGE) and sodium chloride were bought from E. Merck, India. Gelatin (from E. Merck) samples were checked at various stages by passing these through SDS/PAGE (sodium dodecyl sulphate/polyacrylamide gel electrophoresis) to ensure absence of gelatin degradation. Gelatin samples of both Type-A (porcine skin extract, bloom = 175 and 300, nominal molecular weight \(\approx 40-50\) kDa and \(50-100\) kDa respectively) and Type-B (bovine skin extract, bloom = 75, nominal molecular weight \(\approx 20-25\) kDa) for molecular weight dependence studies, obtained from Sigma Chemicals (USA). All of the alcohols used (i.e., methanol, propanol, and tert-butanol) were obtained from Sisco Laboratories India. Ethanol was obtained from Merck, Germany. All other chemicals used were bought from Thomas Baker, India. All of the chemicals were of analytical grade. The pH of gelatin stock solution (1% w/v aqueous solution) was measured with pH titration where we added 0.1 M HCl and 0.1 M NaOH to vary the solution pH. The solvent used was deionized water (for DLS), \(\text{D}_2\text{O}\) (for SANS) for reducing the incoherent scattering and increasing the contrast factor, and the pH (using 0.1 M HCl or 0.1 M NaOH) and ionic strength of the solvent were first set as per the experimental requirement and the gelatin solutions (1% w/v) were prepared by dispersing gelatin in this medium at 60 °C. The macromolecules were allowed to hydrate completely; this took 30 min to 1h. The gelation concentration \(c_g\) of gelatin in water is \(\approx 2\%\) (w/v), the gelatin concentration chosen in these experiments was deliberately kept lower than this to avoid formation of gels. Dry gelatin \((c_g \geq 2\%\text{w/v})\) granules were allowed to swell in deionized water followed by heating for nearly 1h at 60°C. Then it was stirred well for nearly 30 min. The solutions were allowed to equilibrate for 10-12h at room temperature (25 °C) through Newtonian cooling only and no quenching was resorted to. During this period the gelatin solutions formed homogeneous and optically transparent gels.
3.2 Preparation of coacervate samples

Typically 100 ml of stock solution (1% w/v aqueous gelatin) was taken in a beaker kept on a magnetic stirrer and was stirred at moderate speed with stir bars throughout the titration process. Alcohol was taken in a calibrated burette and added in drops to the reaction beaker and the volume of alcohol added to produce the first occurrence of turbidity was measured (Vt) and the process was continued until a sharp point of inflection was noticed (Vp), where the turbidity attained its maximum value. Addition of more alcohol drove the system towards precipitation point. The values of Vt and Vp characterized the initiation of intermolecular folding and intra molecular aggregate formation of the charge neutralized gelatin molecules, and the subsequent micro coacervate droplet formation. As Vp is reached, the system follows a high centrifugation (~ 10,000 r.p.m / relative centrifugal force ~ 6637g), which allows the aggregates to form coacervate phase while the folded gelatin molecules mostly stay in the dispersed supernatant. The polymer rich phase at bottom was collected after decanting the supernatant. This was repeated at least thrice which yielded the coacervate. The concentration of gelatin present in coacervate was ≈13%w/v, which was quantified after lypholyzing the sample up to 48 hours at temp -70 °C.

3.3 Choice of Biopolymers in Simple Coacervation

The polymers used in this study are of biological origin and are thus referred to as biopolymers. Proteins belong to the biopolymer family. The biopolymers (such as gelatin) that are polyampholytes with weakly basic and acidic groups so that charge asymmetry changes with pH of the solution. The collapse of polyampholytes with zero net charge on each chain was studied in detail in a recent theoretical work by Higgs and Joanny [1]. Traditionally, complex coacervation of Gelatin-Gelatin [2], Gelatin-Gum Arabic (GA) [3-6], Gelatin-Acacia [7] were studied and used industrially. But, there is a need to study a simple complex system of gelatin in more details to understand the exact dynamics of coacervation and its microscopic structure. Gelatin was choosing because of its following special characteristics.
3.4 Characteristics of gelatin

Gelatin is proteinaceous in nature, being composed of long chains amino acids joined through peptide linkages, obtained from the denaturation of native parent protein collagen which is found in the white connective tissues of animals [8]. Gelatin is unique among all proteins owing to the absence of appreciable internal order, so that the peptide chains take up many random configurations in aqueous medium. This situation is similar to the behavior of synthetic linear chain high polymers and allows one to examine the dynamics and structural behavior of gelatin complexes from the point of view of theories and experiments developed to treat such high polymeric systems. Gelatin is produced by the degradation of large structure “collagen” [8], which results in a variety of peptide-chain species. Since the degradation is not completely random, most gelatin preparations are not homogeneous with respect to molecular weight distribution, which is again analogous to that in systems of synthetic polymers. So the molecular distribution becomes a factor of equal importance with the average molecular weight in specifying the state of a particular gelatin system. The electrically charged sites (both acidic and basic functional groups of the amino acids side chains) govern the interactions between gelatin molecules, and between the gelatin molecules, and the solvent. Hence to understand a gelatin complex system one must take into consideration the net charge of gelatin molecule and their internal distribution. Finally, in spite of its above emphasis on polymeric polyampholyte character, the specific peptide composition of gelatin molecules cannot be ignored, which controls the chain configuration of the molecule through the solvent-peptide backbone interactions and by certain preferred orientations of some of the peptide linkages.

Figure 3.1: Schematic of peptide bond (CO-NH) in gelatin.
3.4.1 Physical properties

Gelatin is a vitreous, brittle solid that is faintly yellow to white and nearly tasteless and odorless. It contains 84-90% protein, 1-2% mineral salts and 8-15% water. Gelatin is a foodstuff and not a food additive [8]. Depending on the process of recovery the gelatin molecules bear different physical characteristics. Type-A gelatin is acid processed, has an isoelectric pH, \( \text{pI} \approx 9 \) whereas the alkali processed Type-B gelatin has \( \text{pI} \approx 5 \). Various grades of gelatin with different particle sizes and molecular weight are sold commercially in the form of translucent sheets, granules, or powders. It is insoluble in acetone, chloroform, ethanol (95%), ether, and methanol. It is soluble in glycerin, acids, alkalis, although strong acids or alkalis cause its precipitation. It swells and softens in water, gradually absorbing 10 to 20 minutes its own weight in water. It solubilizes in hot water. Upon cooling to \( 27^\circ \text{C} \), it forms a gel. At temperature above \( 30^\circ \text{C} \), the system exits as sol. The overlapping concentration (\( c^* \)) and gelation concentration (\( c_g \)) of gelatin is about \( \approx 0.5\% \) to \( 2\% \). Gelatin is usually graded according to the gel strength expressed as bloom strength. The higher the molecular weight of gelatin is higher the bloom strength or gel strength. The conformational and structural properties of two states (sol and gel) of gelatin are well studied in literatures [9,10]. Is there any possibility of the coexistence of in solvent gelatin other than in sol and gel states? The new state i.e. "coacervate" is discovered and studied systematically in my thesis. This work attempts to answer this question in a systematic manner.

<table>
<thead>
<tr>
<th>Physical parameters (Type-B)</th>
<th>Experimental data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Weight (M_w)</td>
<td>( \approx (90 \pm 10)\text{kD} )</td>
</tr>
<tr>
<td>Hydrodynamic Radius (R_h)</td>
<td>( \approx 20\text{nm} )</td>
</tr>
<tr>
<td>Radius of Gyration (R_g)</td>
<td>( \approx 30\text{nm} )</td>
</tr>
<tr>
<td>Gelation Temperature (T_g)</td>
<td>( \approx 27^\circ \text{C} )</td>
</tr>
<tr>
<td>Density (( \rho ))</td>
<td>( \approx 1.44\text{gm/cm}^3 )</td>
</tr>
<tr>
<td>Melting Temperature (T_m)</td>
<td>( \approx 60^\circ \text{C} )</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Physical parameters (Type-B)</th>
<th>Experimental data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross Section of Chain (R_s)</td>
<td>( \approx 0.3\text{nm} )</td>
</tr>
<tr>
<td>Persistence length (l)</td>
<td>( \approx 2\text{nm} )</td>
</tr>
<tr>
<td>Contour length (L)</td>
<td>( \approx 520\text{nm} )</td>
</tr>
<tr>
<td>Isoelectric Point (pI)</td>
<td>( \approx 5.01 )</td>
</tr>
<tr>
<td>Degree of Polymerization (N)</td>
<td>( \approx 370 )</td>
</tr>
<tr>
<td>Monomer length (b)</td>
<td>( \approx 1.5\text{nm} )</td>
</tr>
</tbody>
</table>

Table 3.1: Physical properties of gelatin used in our studies.
3.4.2 Chemical Composition

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>g amino acids per 100 g pure protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>11.3</td>
</tr>
<tr>
<td>Arginine *</td>
<td>9.0</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>6.7</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>11.6</td>
</tr>
<tr>
<td>Glycine</td>
<td>27.2</td>
</tr>
<tr>
<td>Histidine *</td>
<td>0.7</td>
</tr>
<tr>
<td>Proline</td>
<td>15.5</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>13.3</td>
</tr>
<tr>
<td>Hydroxylysine</td>
<td>0.8</td>
</tr>
<tr>
<td>Isoleucine *</td>
<td>1.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>g amino acids per 100 g pure protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine *</td>
<td>3.5</td>
</tr>
<tr>
<td>Lysine *</td>
<td>4.4</td>
</tr>
<tr>
<td>Methionine *</td>
<td>0.6</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.5</td>
</tr>
<tr>
<td>Serine</td>
<td>3.7</td>
</tr>
<tr>
<td>Threonine *</td>
<td>2.4</td>
</tr>
<tr>
<td>Tryptophan *</td>
<td>0.0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.2</td>
</tr>
<tr>
<td>Valine</td>
<td>2.8</td>
</tr>
</tbody>
</table>

* Essential Amino Acid

Table 3.2: Chemical composition of gelatin

Gelatin contains 9 of the amino acids essential for humans. As said earlier, collagen is the prime source of gelatin. Let’s discuss the structure of a collagen as what it consists of? Collagen has an aminoacid composition that comprises mainly of hydroxiproline, and is extraordinarily rich in glycine and proline. It has very low sulphur content. Although the chemical composition of collagen is not unique, a typical sequences of collagen protein [8] is given by

\[-(\text{Gly} - \text{Pro} - X)_n - \text{or} - (\text{Gly} - X - \text{Hypro})_n -\]

where ‘X’ represents different amino-acids, mostly lysine, arginine, methionine and valine ~ 6% and ‘n’ is degree of polymerization. As per Merck technical data, One third of the chain is comprised of glycine ~33% and another one third is either proline or hydroxyproline ~33%. The rest are other residues. Molecule is ~13% positively charged (lysine and arginine), ~12% is negatively charged (glutamic and aspartic acid) and ~11% of this chain is hydrophobic in nature (comprising leucine, isoleucine, methionine and valine). Glycine, proline and hydroxyproline form rest of the chain. The +ve : -ve : hydrophobic segments are present in the approximate ratio 1:1:1,which makes this polypeptide special. The presence of a five membered ring structures in proline and hydroxyl-proline gives an enhanced localized rigidity to the chain. The side groups of the
amino-acids also play an important role in the stability of proteins. Some of these are polar groups (i.e. OH, O, NH₂, etc) which are likely to interact with water molecules and establish hydrogen bonds. There are also polar charged groups (like: NH₃⁺, COO⁻, etc.) in variable amounts. The portion of charged and uncharged groups varies with pH. NH₂ group for example, may be either in NH₃⁺ or NH₂ state. That's why gelatin is called annealed polyelectrolytes. Charged groups are also hydrophilic. Hydrophobic groups (such as proline) are also encountered. The conformation that a protein adopts when dissolved in solution (generally aqueous solution) is a direct consequence of the balance between hydrophilic and hydrophobic interaction, which in turn results from molecular composition. Gelatin is called a polyampholyte or a polypeptide, which is an ideal case for complex formation in solution.

3.5 Choice of alcohols

The relationship between liquid-liquid phase separation and microheterogeneity in water-primary alcohol mixtures was examined by analyzing the mass spectra of clusters generated through the fragmentation of liquid droplets [11]. By comparing the cluster structures of water-ethanol, 1-propanol, and tert-butanol binary mixtures at various alcohol concentrations, the differences in the molecular clusters that control phase separation was discovered. Alcohol self-association is promoted in the presence of a small amount of water (ca. 10 ~ 20 wt %), in which the water-water hydrogen-bonding network is weak and does not contribute to alcohol self-association. It has been demonstrated that alcohol self-association is also promoted by non-ideal mixing with other alcohols [11,12]. The self-association of alcohol molecules complement the loss of stabilization energy caused by the relatively weak coexisting interactions. This complementary relationship among intermolecular interactions is an inherent property of solutions, and plays a key role in the phase separation process [11,12]. The mechanism of coacervate formation in a nonaqueous system has been investigated by Ruiz [13]. The effect of various solvent compositions on the coacervation process was considered by Shively and McNickle[14]. Batzri and Korn [15] reported that single-bilayer liposomes are formed when an ethanolic solution of phospholipid is injected into water. On the other hand, although Bungenberg de Jong and Davis [16] investigated the effect of propanol and tert-butyl alcohol on the formation of phospholipid coacervates in various higher alcohols such as hexyl, octyl, decyl, dodecyl, and oleyl alcohols containing egg phosphatides. The coacervation method is a simple, reproducible method for the mass
production of liposomes as carriers for drug or gene delivery systems. Ethanol-water solvent system has also been used as a model mixed solvent to study a variety of conformational phase transitions in polyelectrolytic and micellar systems in the past [17-19]. The choice of this solvent is appropriate considering the fact that addition of ethanol reduces the polar (ionic) environment of the continuous phase, which can be tuned to one's requirement. Arscott et al. [17] studied DNA condensation induced by multivalent cations (Co$^{3+}$) in ethanol-water mixed solvent and observed B$\rightarrow$A conformational transition at ethanol concentration $\approx 40\% \ (v/v)$. Piskur and Rupprecht [20] have studied the thermal stability and structure of aggregated DNA in ethanol-water solution using a mechano-chemical method. They observed that, at a critical ethanol concentration ($\approx 40\% \ (v/v)$) aggregation in DNA sets in with a marked increase in $T_m$, the double helix melting temperature. Further increase in ethanol concentration produced B$\rightarrow$A structure transition. In an earlier work [21], they reported ethanol induced condensation of CT-DNA observed through static and dynamic light scattering, which occurred at ethanol concentration $\approx 40\%$. These observations raise a pertinent question: What happens to gelatin in ethanol-water binary solvent and specifically, in the ethanol concentration range $\approx 40-50\% \ (v/v)$ that induces conformational phase transitions in polyelectrolytes?

**Figure 3.2:** Schematic representation of the polyampholytes in the various conformations in different solvents.
3.6 Methods

3.6.1 Turbidimetry

Introduction:

Turbidity is an expression of optical property that uses light scattering properties of suspension in the sample. The Digital Turbidity Meter is a very accurate and stable instrument for measurement of turbidity up to 1000 Nephelometric Turbidity Units (NTU) for 90° scattered light measurement. The unit is Formazine Attenuation Units (FAU) for transmission measurement. This unit is only applicable if the instrument is calibrated with Formazine standards. (Conversion: 1 FNU = 1 NTU). Turbidity is the reduction of transparency of a liquid caused by the presence of undissolved matter. A sample is clear if it can be seen through. If the passage of light is hindered, the solution is turbid. Turbidimeters measure either the diffused radiation or the attenuation of a radiant flux. Optically black properties such as those of activated carbon may absorb light and effectively increases turbidity measurements. It is possible to obtain a cloud point curve as a function of composition at constant temperature.

Theory:

A novel approach to turbidimetry enabling the extraction of structural information about highly turbid systems has been developed. Turbidimetric spectra have been obtained in the wavelength region 450 nm by using a turbidity meter (Brinkmann-910, Brinkmann Instruments, USA) for detecting the transmitted light. Turbidimetry is an important application of the scattering of light by small particles, particularly the spectroturbidimetry of emulsions and similar systems which has become a very useful technique. Most of the work in this field applies to dielectric spheres, to which case we will restrict ourselves: although some of the conclusions have a wider usefulness. If the measurements of the optical density of a turbid system can be performed under ideal conditions, the total light scattering coefficient I is the only theoretical quantity needed to interpret the measurements. The turbidity will be expressed as optical density \( D \), defined as the common logarithm of the ratio between the intensities of incident and transmitted beams. Optical density is the absorbance of an optical element for a given wavelength \( \lambda \) per unit distance:
\[
OD_\lambda = \frac{A_\lambda}{l} = \frac{-1}{l} \log_{10} T = \frac{1}{l} \log_{10} \left( \frac{I_0}{I} \right)
\]  
(3.1)

where, 
\( I \) = the distance that light travels through the sample (i.e., the sample thickness), measured in cm.

\( A_\lambda \) = the absorbance at wavelength \( \lambda \).

\( T \) = the per-unit transmittance.

\( I_0 \) = the intensity of the incident light beam.

\( I \) = the intensity of the transmitted beam.

Although absorbance does not have true units, it is quite often reported in “Absorbance Units” or AU. Accordingly, optical density is measured in ODU, which are equivalent to AU cm\(^{-1}\). The higher the optical density, the lower the transmittance. Optical density times 10 is equals to a transmission loss rate expressed in decibels per cm, e.g., an optical density of 0.3 corresponds to a transmission loss of 3 dB per cm. Optical density is sometimes defined without regard to the length of the sample; in this case it is a synonym for absorbance.

### 3.6.2 Scattering techniques

**Introduction:**

In the past scattering was very exciting phenomena for characterizing large molecular systems and for studying phase transitions in complex fluids. However, the probe domain was limited to light and X-ray frequencies. All these studies are governed by the same basic scattering principle which necessitates the size of the scatterer to be of the order of the wavelength of incident electromagnetic radiation. In general, interaction of electromagnetic radiation with a molecule leads either to absorption (forms the basis of spectroscopy) or scattering of the radiation. Scattering results from the interaction of the electrons in the molecules with oscillating electric field of radiation. Thus a dipole is induced in the molecules, which oscillate with the electric field. Since an oscillating dipole is a source of electromagnetic radiation, the molecules emit light, called scattered light. The change in energy of the incident and scattered radiation determines the nature of scattering whether the phenomena is elastic (Rayleigh scattering), quasi-elastic scattering (Rayleigh-Brillouin scattering) or inelastic scattering (Raman scattering). The technique of light scattering has an advantage of being non-perturbative probe method provided the intensity is not high enough to ionize the system. To probe a wide range of
length scale, different scattering mechanisms are used i.e. typical length scale accessible in light scattering is ~ 5 to 200 nm whereas in the case of neutron scattering it is 1 to 50 nm, X-ray scattering length scale in the range of 0.1 to 0.5 nm and probe length scale smaller than this, one has to go for electron scattering.

3.6.2.1 Static and dynamic laser light scattering (SLS and DLS):
Scattering techniques do not provide structural information on biopolymers at high (atomic) resolution. However, they often provide a means to observable large scales structural heterogeneity of proteins directly in solution. As such, they are good tools in studies of proteins dynamics in solution. In static light scattering [9,22], intensity of scattered light is measured as a function of the scattering vector 'q':

\[ q = \frac{4\pi n}{\lambda} \sin \frac{\theta}{2} \] (3.2)

where \( n \) is the refractive index of the solvent, and \( \theta \) is a scattering angle. Intensity of the scattered light adjusted for background scattering and normalized to a reference solvent gives the Rayleigh ratio \( R_s(q) \), which can be expressed for dilute solutions as [23-25]

\[ \frac{1}{R_s(q)} = \frac{4\pi^2 n^2 (dn/dc)^2}{N_A \lambda^4} = \frac{1}{\langle M \rangle} + 2Bc \] (3.3)

where \( c \) is protein concentration, \( N_A \) is Avogadro’s number, \( \langle M \rangle \) is the weight averaged molecular weight of protein particles in solution, \( B \) is the second virial coefficient describing interparticle interactions in solution, and \( P(q) \) is a particle shape factor. If a particle size is small compared to the light wavelength, then it obviously acts as “point scatter,” and its shape is irrelevant for scattering (i.e \( P(q)=1 \)). Most small proteins fall into this category; hence, the only key parameter that can be obtained from the static light scattering methods is the molecular weight. Such sensitivity of static light scattering measurements to the average molecular weight of the protein particles makes it very useful in the studies of protein association and aggregation. Once the particle size becomes comparable to the wavelength used in the experiments (i.e. radius of gyration \( R_g \sim 1/q \)), \( P(q) \) can be approximated reasonably well as a quadratic function of \( q R_g \), and the scattering profiles can be used to determine the particle gyration radius. For random coil in very low scattering angle limit (Guinier Region), \( P(q) \) is given by,

\[ P(q) \equiv 1 - \frac{q^2 R_g^2}{3} \] (3.4)
**Dynamic light scattering** [23-27] is sensitive to the diffusion of scattering particles in solution, as it measures the intensity of light scattered at a fixed angle, which is then analyzed with an autocorrelator. The resulting correlation function has the particle diffusion coefficient as one of its arguments, which can be used to calculate the hydrodynamic radius of the particle through Stoke-Einstein relation. The particle sizing measurements were done by dynamic laser light scattering (DLS) technique, using a 90 plus particle size analyzer from Brookhaven Instruments Corp. (BIC), USA and a homemade goniometer. The excitation source was a He-Ne laser emitting at a wavelength of 632.8 nm in linearly polarized single frequency mode, which was focused on the sample cell and scattered light was detected by a photo-multiplier tube (Hamamatsu) and the signal was converted into intensity auto-correlation function by a digital correlator. The time dependent particle sizing measurements were done by same Brookhaven Instruments, USA, where the excitation source was a diode pumped solid state laser (Model-DPY 305-II, Adlas, Germany) emitting 50 mW of power at 532 nm in linearly polarized single frequency mode. During the course of size measurements the intensity of scattered light was monitored continuously as a function of time over a period of 12-hours. The scattering angle was fixed at 90° and the data analysis was done using CONTIN software provided by Brookhaven Instruments. This technique was used to get the size of the particles. The light source was DLS experiment measures the time correlation function \( g_2(t) \) of the scattered intensity \( I(t) \) at a given \( q \) (scattering vector) defined by [22,27]

\[
g_2(t) = \frac{\langle I(t') I(t'+t) \rangle}{\langle I(t') \rangle^2} \tag{3.5}
\]

Where \( q = \frac{(4\pi n \lambda)}{\sin (\theta/2)} \), \( n \) is the refractive index of the solution, \( \theta \) is the scattering angle, and \( \lambda \) is the wavelength of light source in medium. The intensity correlation function \( g_2(t) \) is related to the scattered field auto-correlation function, \( g_1(t) \) through the Siegert relation [22,27]

\[
g_2(t) = A + B \left| g_1(t) \right|^2 \tag{3.6}
\]

where \( A \) defines the baseline of the correlation function as,

\[
\left| g_2(t) \right|_{t \to \infty} = A \tag{3.7}
\]

and \( B \) is the spatial coherence factor. The ratio \( (B/A) \) is the signal modulation and better data quality demands \( (B/A) \geq 50 \% \). For solutions containing particles undergoing
Brownian motion (i.e. polymer or colloidal solutions), the field autocorrelation function, \( g_1(t) \) is given as,

\[
g_1(t) = \sum_i A_i \exp(-\Gamma_i t)
\]  

(3.8)

Where \( \Gamma_i \) is the relaxation frequency, which characterizes various relaxation modes that include relaxations due to the translational diffusion, rotational diffusion and bending modes etc. The relative mode strength (amplitude) of the \( i^{th} \) relaxation mode is \( A_i \). For the present case, center of mass diffusion is the dominant process and \( \Gamma_i \) has been identified as \( \Gamma_i = D_i \Omega^2 \). Where the translational diffusion coefficient of the \( i^{th} \) particle is \( D_i \) The expression for \( g_1(t) \) remains valid for polydisperse samples and for situation where the relaxation frequency distribution has several peaks. Polydispersity \( P \) can be defined as,

\[
P = \frac{\left\langle (D - \overline{D})^2 \right\rangle}{\langle D \rangle^2}
\]  

(3.9)

Further details of this discussion can be found elsewhere [22,23,27]. According to Einstein relation, the \( \overline{D}_o \) (the z-average diffusion coefficient at infinite dilution) is inversely proportional to the translational frictional coefficient, \( f_i \) at infinite dilution by the relation,

\[
\overline{D}_o = \frac{k_B T}{f_i}
\]  

(3.10)

where \( k_B \) is Boltzmann constant and \( T \) is absolute temperature. The value of \( f_i \) obtained via Eq. 3.7 can be used for a direct estimation of hydrodynamic radius, \( R_h \) of the particles provided they have a spherical shape using the relation \( f_i = 6\pi\eta R_h \) as per the Stokes law

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

(3.11)

where \( \eta \) is the solvent viscosity at temperature \( T \). Since, the size measurements were done at a finite concentration of biopolymers, it will be appropriate to refer to \( R_h \) as \( R_{\text{eff}} \). The intensity correlation function is related to diffusion coefficient of the particles and hence to the hydrodynamic radius by Stoke-Einstein’s equation through Eqs. 3.5-3.11.

3.6.2.2 Small angle neutron scattering (SANS):

Small Angle Neutron Scattering (SANS) [28-29] is an emerging technology (as far as application to biological systems is concerned). It utilizes the same principle as SAXS [29], although the scattering centers are predominately nuclei (as opposed to electrons in
SAXS). Analysis of the SANS profiles can reveal important information related to the mass and the overall shape of the scattering particles, as well as the distribution of interatomic vectors within a molecule. Unfortunately, such experiments remain a rarity, as access to the research facilities possessing neutron beam generators remain limited. SANS is a diffraction technique, which involves scattering of a monochromatic beam of neutrons (thermal neutrons having energy ~25 meV are now routinely used for studying the microscopic structure and dynamics of the condensed matter) from the sample and measuring the scattered neutron intensity as a function of the scattering angle. Unlike, a conventional diffraction experiment, where the structure of the materials is examined at atomic resolution (~ 0.1nm), SANS is used for studying the structure of materials with spatial resolution of ~10 nm.

Scattering Vector:

The quantity colloquially referred to as “the scattering vector” (q) is the modulus of the resultant between the incident, $K_i$ and scattered, $K_i$, wavevectors, (see Figure 3.3) and is given by [28-34]

$$q = |q| = |K_f - K_i| = \frac{4\pi n}{\lambda} \sin \theta$$  \hspace{1cm} (3.12)

Although, as has already been shown, in neutron scattering $n \sim 1.$ q has dimensions of (length)$^{-1}$; normally quoted in nm$^{-1}$ and $A$. Substituting Eq. 3.12 into Bragg’s law of Diffraction,

$$\lambda = 2d \sin \theta$$  \hspace{1cm} (3.13)

yields a very useful expression, $d = \frac{2\pi}{q}$  \hspace{1cm} (3.14)

where d is the distance between the sample and detector. Eqs. 3.12, 3.13 and 3.14 are central to SANS experiments because through their combined use it is possible to both configure an instrument (i.e., ensure that its “q-range” allows to see what we expect) and to quickly and rapidly “size” the scattering bodies in a sample from the position of any diffraction peak in q-space. For example, the SANS instrument at the BARC Dhruva nuclear reactor source, the wave vector transfer $q = \frac{4\pi \sin \theta}{\lambda}$, where $2\theta$ is the scattering angle and $\lambda$ is the wavelength of neutron) in SANS experiments is small, typically in the range of $10^{-2}$ to 10 nm$^{-1}$. The wavelength of the neutrons used for these experiments
usually being $\approx 0.4$ to $1$ nm. To obtain low $q$ values ($\approx 0.3$ nm$^{-1}$) SANS spectrometers use large wavelength ($\sim 0.8$ nm) neutrons and small scattering angles ($\sim 0.05 - 1$ nm). The technique is therefore called small-angle neutron scattering.

![Figure 3.3: Schematic representation of SANS spectrometer.](image)

**Neutron Source:**

Neutron source is a general term referring to a variety devices that emit neutrons [9], irrespective of the mechanism used to produce the neutrons. Both natural and artificial sources of neutron exist. The ones used in the SANS are produced artificially. There are five types of artificial neutron source are generally used in laboratories. They are: 1. radioisotope sources; 2. photoneutron sources; 3. accelerator sources; 4. nuclear reactors; 5. spallation sources. The first two described above, are small and can be easily accommodated in a standard laboratory. The remaining three are large and need expensive facilities with each having its own particular features. For carrying out precise measurements on the properties of the neutron itself, and for for radioactive isotopes, the nuclear reactor has been the far most important source till date. However, spallation sources are likely to become more significant in future for studies related to condensed matter. In Bhaba Atomic Research Center, the neutron source is a reactor and Paul Scherrer Institute (Villigen, Switzerland), the neutron source is a spallation source. We have used both these facilities for our work [30,31].

**SANS spectrometer:**

A SANS instrument mainly consists of three parts [30] (i) monochromator, (ii) collimator and (iii) detector. A white beam (Maxwellian distribution) of neutrons from the nuclear
reactor is monochromatized by the monochromator. The typical flux of the neutrons produced from the nuclear reactors is $\sim 10^{14}/\text{cm}^2$ and at sample position is $\sim 10^7/\text{cm}^2$. In case of spallation source, the peak neutron flux $\sim$ reactor and the time averaged neutron flux $\sim$ reactor/50. The monochromator used for SANS instrument is velocity selector or double crystal monochromator or filter monochromator. The monochromator for SANS do not require very good wavelength resolution. The wavelength resolution ($\Delta \lambda / \lambda$) used is about 5-15%. To achieve the small scattering angles, SANS instruments require high collimation, less than 0.5°. The collimators used for this purpose are about few meters long. The neutrons are neutral and hence are detected by indirect method. The charged particles are first produced in the detecting medium by the neutrons through a nuclear reaction and then are detected. He$^3$ Position Sensitive Detectors (PSDs) are generally used for SANS measurements. The use of PSD allows simultaneous recording of data over a large angular range.

**Reactor Source at BARC:**

SANS experiments were performed on the spectrometer at the G.T laboratory, Dhruva reactor (Bhaba Atomic Research Centre, India). The wavelength of the neutrons used covered the scattering vector ($q$) range \[30\]

$$0.18 \leq q \leq 3 \text{ nm}^{-1}$$

given by $q = (4\pi / \lambda) \sin \theta$, $\lambda$ being the wavelength of neutron and $\theta$ is the scattering angle. The dense polymer phase, coacervate was transformed to a quartz cell (Hellma, Germany) of thickness 2mm and scattered intensity was measured as a function scattering vector. The measured intensity was corrected for the background and the empty cell contribution and the data were normalized to get the structure factors.

**Swiss Spallation Source:**

The small angle neutron scattering (SANS) experiments were performed at the Swiss neutron source at Paul Scherrer Institute, Switzerland. The measurements [31] were performed with a mean neutron wavelength of 1.68nm at a sample-to-detector distance 2m, 6m and 15m. An extended range for the magnitude of the scattering vector $q = 4\pi \sin \theta / \lambda$ (where $\theta$ is the scattering angle and $\lambda$ is the neutron wavelength) from 0.03 nm$^{-1}$ $\leq q \leq 3.4$ nm$^{-1}$ was covered by three combinations of the above-mentioned sample-to-detector distances. The scattered neutron intensity was recorded with a two-dimensional detector. The samples were kept in stoppered quartz cells (Hellma, Germany) with a path.
length of 1mm. The neutron spectra of water were also measured in a 1-mm path length quartz cell. The raw spectra were corrected for background from the solvent, sample cell, and electronic noise by conventional procedures. Furthermore, the two dimensional isotropic scattering spectra were azimuthally averaged, converted to absolute scale, and corrected for detector efficiency by dividing with the incoherent scattering spectra of pure water [31].

**Scattering Cross Section:**

Scattering cross-section is an important parameter that characterizes the physical properties i.e., shape and size of the scatters in medium. This is an experimentally measurable quantity obtained from the direct normalization of the scattered intensity data [28-30].

![Figure 3.4: Scattering cross sections versus wave vector (q) for intraparticle P(q) and interparticle scattering S(q).](image)
The differential scattering cross section [28-29] from a collection of particles consists of two terms, the first of which depends on the intraparticle (denoted as $P(q)$, depends on the shape and size of the particle and in principle can be calculated for any geometry) scattering and the second on the interparticle scattering (denoted as $S(q)$, depends on the correlation between the spacing of the particles and their sizes and orientations), which can be written as

$$\frac{d\Sigma}{d\Omega(q)} = n (\rho_p - \rho_m)^2 V^2 P(q) S(q) \quad (3.16)$$

Here, $(\rho_p - \rho_m)^2$ is referred to as the contrast factor depends on the square of the difference between the average scattering length density of the article and the average scattering length density of the solvent, $n$ is the number of particles per unit volume of the sample, $V$ is the average volume of the single particle, $P(q)$ is the intraparticle structure factor for a spherical particle and $S(q)$ is the interparticle structure factor.

**Determination of Intraparticle and Interparticle structure factor:**

Dilute systems are ideally suited for studying the shapes and sizes of the particles. In these systems, particles concentration is very low. As the interparticle distances are much larger than the particle size in these systems, the interparticle interference is negligible and $S(q) \sim 1$. Thus the scattering distribution depends on the functionality of the $P(q)$. The Eq. 3.16 for diluted system becomes

$$\frac{d\Sigma}{d\Omega(q)} = n (\rho_p - \rho_m)^2 V^2 P(q) \quad (3.17)$$

In the small $q$ region ($qR_g < 1$), $P(q)$ decreases exponentially and is known as Guinier approximation [28,29].

$$P(q) = \exp(-q^2R_g^2/3) \quad (3.18)$$

which is equivalent to Eq. 3.4. $R_g$ is the radius of gyration of the particle and is defined by

$$R_g^2 = \int r^2 \, dr/V \quad (3.19)$$

With this approximation the intensity of scattered neutrons, $I(q)$ can be written as

$$I(q) = n (\rho_p - \rho_m)^2 V^2 \exp(-q^2R_g^2/3) \quad (3.20)$$

Thus a plot of logarithm of scattering intensity versus $q^2$ will be a straight line in the small $q$-region and the slope gives the radius of gyration of the particle.

For SANS studies one should follow mean field theory [35] which reveals that the polymers in a good solvent at equilibrium has led to a form of structure factor of
concentration fluctuations at low wave vector, known as the Ornstein-Zernike (O-Z) or Lorentzian function given by

\[ S_L(q) = S_L(0)/(1+q^2\xi^2) \]; \( q\xi << 1 \) \hspace{1cm} (3.21)

where \( S_L(0) \) is the extrapolated structure factor at zero wave number and \( \xi \) is the correlation length associated with the size of the entangled network. Physically, \( S_L(0) \) is related to the cross-link density and longitudinal osmotic modulus. An "excess scattering" has been reported at low wave numbers from polymeric solutions. This is caused by the enhanced long wavelength concentration fluctuations in the system. It is not clear so far as to what causes this excess scattering. If the spatial scale of density fluctuations due to the presence of inhomogeneities is large compared to the correlation length \( \xi \), then the two contributions can be treated separately and added to give the total structure factor as [35]

\[ S(q) = S_L(q) + S_{\text{ex}}(q) \] \hspace{1cm} (3.22)

where \( S_L(q) \) is the Ornstein-Zernike function, and the Debye-Buche structure factor has the form \( S_{\text{ex}}(q) \) given by

\[ S_{\text{ex}}(q) = S_{\text{ex}}(0)/(1+q^2\zeta^2)^2 \] \hspace{1cm} (3.23)

where \( S_{\text{ex}}(0) \) is the extrapolated structure factor at zero wave vector and \( \zeta \) is the inhomogeneities in the system. Often it is impossible to probe low \( q \)-domain of the structure factor because of the instrumental limitations of SANS spectrometers.

**Notion of Contrast:**

Scattered neutron intensity in a SANS experiment depends on \((\rho_p - \rho_m)^2\) - the square of the difference between the average scattering length density of the particle and the average scattering length density of the solvent. \((\rho_p - \rho_m)^2\) is referred to as the contrast factor [28-30]. The contrast factor in SANS experiments is somewhat equivalent to the contrast term in optics, where it is decided by the difference in the refractive indices of the particle and the solvent. The scattering length of the hydrogen is negative \((-0.3723 \times 10^{-12} \text{ cm})\) and that for deuterium is positive \((0.6674 \times 10^{-12} \text{ cm})\). It is thus possible to have a very good contrast between the hydrogenous particle and the solvent by deutering either the particle or the solvent. SANS signal from gelatin solutions, for example, increases by three orders when the solution is prepared in \( \text{D}_2\text{O} \) instead of \( \text{H}_2\text{O} \).
contrast between the particle and the solvent can in fact be varied continuously by using mixed hydrogenated and deuterated solvents. This possibility of contrast variation in SANS experiments is particularly useful for examining the structure of complex particles.

3.6.3 Transmission electron microscopy (TEM)

Introduction:

The conventional electron microscopy is now a day called TEM (transmission electron microscopy). Transmission electron microscopy (TEM) is an imaging technique whereby a beam of electrons is focused onto a specimen causing an enlarged version to appear on a fluorescent screen or layer of photographic film, or to be detected by a CCD camera [36, 37]. The first practical transmission electron microscope was built by Albert Prebus and James Hillier at the University of Toronto in 1938 using concepts developed earlier by Max Knoll and Ernst Ruska. In the past, light microscopes have been used mostly for imaging due to their relative ease of use. However, the maximum resolution that one can image is determined by the wavelength of the photons that are being used to probe the sample; nothing smaller than the wavelength being used can be resolved. Visible light has wavelengths of 400-700 nanometers; larger than many objects of interest. Ultraviolet could be used, but soon runs into problems of absorption. Even shorter wavelengths, such as X-rays, exhibit a lack of interaction: both in focussing (nothing interacts strongly enough to act as a lens) and actually interacting with the sample. Like all matter, electrons have both wave and particle properties (as theorized by Louis-Victor de Broglie), and their wave-like properties mean that a beam of electrons can in some circumstances be made to behave like a beam of radiation. The wavelength is dependent on their energy, and so can be tuned by adjustment of accelerating fields, and can be much smaller than that of light, yet they can still interact with the sample due to their electrical charge. Electrons are generated by a process known as thermionic discharge in the same manner as the at the cathode in a cathode ray tube, or by field emission; they are then accelerated by an electric field and focussed by electrical and magnetic fields onto the sample. The electrons can be focused onto the sample providing a resolution far better than is possible with light microscopes, and with improved depth of vision.

The TEM is used heavily in both material science/metallurgy and the biological sciences. In both cases the specimens must be very thin and able to withstand the high vacuum present inside the instrument. For biological specimens, the maximum specimen thickness
is roughly 1 micrometer. To withstand the instrument vacuum, biological specimens are typically held at liquid nitrogen temperatures after embedding in vitreous ice, or fixated using a negative staining material such as uranyl acetate or by plastic embedding. Average particle size, size distribution and morphology were examined by Fei-Philips Morgagni 268D transmission electron microscope (Digital TEM with image analysis system and Maximum Magnification = 2,80,000 × ) at a voltage of 100kV. The non-aqueous dispersion of the gelatin nanoparticles in supernatant phase was drop-cast onto a carbon coated copper grid with negative staining material such as uranyl acetate and grid was air dried at room temperature (25 °C) before loading on the microscope. Another type of TEM is the scanning transmission electron microscope (STEM), where the beam can be rastered across the sample to form the image. In analytical TEMs the elemental composition of the specimen can be determined by analysing its X-ray spectrum or the energy-loss spectrum of the transmitted electrons. Modern research TEMs may include aberration correctors, to reduce the amount of distortion in the image, allowing information on features on the scale of 0.1nm to be obtained (resolutions down to 0.08nm have been demonstrated, so far). Monochromators may also be used which reduce the energy spread of the incident electron beam to less than 0.15eV.

**Principle of Operation:**

In transmission electron microscope (TEM), the ray of electrons is produced by a pin-shaped cathode heated up by current. The electrons are vacuumed up by a high voltage at the anode. The acceleration voltage is between 50 and 150 kV. The higher it is, the shorter are the electron waves and the higher is the power of resolution. But this factor is hardly ever limiting. The power of resolution of electron microscopy is usually restrained by the quality of the lens-systems and especially by the technique with which the preparation has been achieved. Modern gadgets have powers of resolution that range from 0.2 - 0.3 nm. The useful resolution is therefore around 2, 80,000 ×. The accelerated ray of electrons passes a drill-hole at the bottom of the anode. Its following way is analogous to that of a ray of light in a light microscope. The lens-systems consist of electronic coils generating an electromagnetic field. The ray is first focused by a condenser. It then passes through the object, where it is partially deflected. The degree of deflection depends on the electron density of the object. The greater the mass of the atoms, the greater is the degree of deflection. Biological objects have only weak contrasts since they consist mainly of
atoms with low atomic numbers (C, H, N, O). Consequently it is necessary to treat the preparations with special contrast enhancing chemicals (heavy metals) to get at least some contrast. Additionally they are not to be thicker than 100 nm, because the temperature is rising due to electron absorption. This again can lead to destruction of the preparation. It is generally impossible to examine living objects. After passing the object the scattered electrons are collected by an objective. Thereby an image is formed, that is subsequently enlarged by an additional lens-system (called projective with electron microscopes). The thus formed image is made visible on a fluorescent screen or it is documented on photographic material. Photos taken with electron microscopes are always black and white. The degree of darkness corresponds to the electron density (= differences in atom masses) of the candled preparation.

**Imaging in the TEM:**

The contrast in a TEM image is not like the contrast in a light microscope image [36,37]. A crystalline material interacts with the electron beam mostly by diffraction rather than absorption, although the intensity of the transmitted beam is still affected by the volume and density of the material through which it passes. The intensity of the diffraction depends on the orientation of the planes of atoms in a crystal relative to the electron beam – at certain angles the electron beam is diffracted strongly, sending electrons away from the axis of the incoming beam, while at other angles the beam is largely transmitted. Modern TEMs are often equipped with specimen holders that allow the user to tilt the specimen to a range of angles in order to obtain specific diffraction conditions, and apertures placed below the specimen allow the user to select electrons diffracted in a particular direction. A high contrast image can therefore be formed by blocking electrons deflected away from the optical axis of the microscope by placing the aperture to allow only unscattered electrons through. This produces a variation in the electron intensity that reveals information on the crystal structure, and can be viewed on a fluorescent screen, or recorded on photographic film or captured electronically. This technique (known as Bright Field or Light Field) is particularly sensitive to extended crystal lattice defects in an otherwise ordered crystal. As the local distortion of the crystal around the defect changes the angle of the crystal plane, the intensity of the scattering will vary around the defect. As the image is formed by the distortion of the crystal planes around the defect, the contrast in these images does not normally coincide exactly with the defect, but is
slightly to one side. It is also possible to produce an image from electrons deflected by a particular crystal plane. By either moving the aperture to the position of the deflected electrons, or tilting the electron beam so that the deflected electrons pass through the centred aperture, an image can be formed of only deflected electrons, known as a Dark Field image.

**Limitations:**

There are a number of drawbacks to the TEM technique. Many materials require extensive sample preparation to produce a sample thin enough to be electron transparent, which makes TEM analysis a relatively time consuming process with a low throughput of samples. The structure of the sample may also be changed during the preparation process. Also the field of view is relatively small, raising the possibility that the region analysed may not be characteristic of the whole sample. There is potential that the sample may be damaged by the electron beam, particularly in the case of biological materials.

### 3.6.4 Atomic force microscopy (AFM)

**Introduction:**

In the early 1980s, after being introduced into the world of microscopy, scanning probe microscopes (SPM) have emerged as powerful techniques capable of characterizing surface morphological features at ambient conditions and generating 3-dimensional images of the surface topography with nanometer resolution [38,39]. So, they are extensively used for imaging in most of the disciplines including biological, chemical, molecular and materials science, medicine, micro-circuitry, semiconductor industry, information storage systems, and so forth. SPMs are not only used for surface morphological characterization, but also for proximity measurements of magnetic, electrical, chemical, optical, thermal, spectroscopy, friction, wear, and other mechanical properties. The family of SPMs include: scanning tunneling microscopes (STM), atomic force microscopes (AFM), friction force microscopes (FFM), scanning magnetic microscopes (SMM) (or magnetic force microscopes, MFM), scanning electrostatic force microscopes (SEFM), scanning near-field optical microscopes (SNOM), scanning thermal microscopes (SThM), scanning chemical force microscopes (SCFM), scanning electrochemical microscopes (SEcM), scanning Kelvin probe microscopes (SKPM), scanning chemical potential microscopes (SCPM), scanning ion conductance microscopes...
(SICM), and scanning capacitance microscopes (SCM) [40]. The family of instruments which measures forces (e.g., AFM, FFM, SMM, and SEFM) is also referred to as scanning force microscopes (SFM).

Although Transmission electron microscopes (TEMs) and Scanning electron microscopes (SEMs) provide a size distribution of individual particles or dispersed particles in a matrix, they can not provide the three-dimensional structure of a particle. Also, the samples for SEM should be conductive; while for imaging dispersed nanoparticles in a matrix, the sample preparation for TEM is very cumbersome. Further, when using TEMs, questions arise on the stability of fine features to be imaged because of the high energy electron beam usage. Atomic force microscope has an added advantage for the high resolution profiling of non-conducting surfaces. Also, AFM is a non-destructive technique and it does not require any specific sample preparations. Furthermore, the resolution capabilities of the AFM are near or equal to those of electron microscopes (an example illustrating the capability of AFM imaging is it can image as small as a carbon atom (0.25 nm in diameter) and as large as the cross section of a human hair (80 μm in diameter) [41]; nonetheless, AFM differs from electron microscopes in that it does not have a lens, does not require coating or staining, and can be operated at atmospheric pressure, in fluids, under vacuum, low temperatures, and high temperatures. The vertical resolution of AFM images is generally dictated by the interaction between the tip and the surface and the lateral resolution is determined by the size of the tip. Imaging in liquid allows the study of live biological samples, and it also eliminates water capillary forces present in ambient air present at the tip-sample interface [40]. Low-temperature (liquid helium temperatures) imaging is useful for the study of biological and organic materials and the study of low-temperature phenomena such as superconductivity or charge density waves. Low-temperature operation is also advantageous for high-sensitivity force mapping due to the reduction in thermal vibration. Thus, AFM operation is relatively simple, artifacts are reduced and materials can be examined in their native state. In my experiment, Atomic Force Microscope (AFM) pictures were taken using a Autoprobe CP Research AFM system, model AP-2001 (Thermomicroscopes, USA) using a 90μm scanner and tapping mode. During the experiment, a drop of the solution was removed from the reaction beaker and allowed to spread out uniformly (shearing by a cover-slip plate) on a degreased glass cover-slip plate over a period of one hour. These experiments
were initially studied with gelatin (Type- B, microbiology grade devoid of *E. coli* and liquifier presence, molecular weight $\approx 90\pm10$ kDa, E. Merck, India) and repeated with the gelatin sample (Type-B, bloom = 75, resourced from Sigma chemicals, USA) and for each of these two samples, four different substrates were chosen namely; glass, quartz, silicon and mica.

**Principle of Operation:**
In simple terms, the underlying operational principle of AFM imaging is based on the interatomic force-distance concept which is schematically illustrated in Figure 3.5. As the AFM tip atom approaches the atoms on the surface being studied, a variety of forces are sensed depending on the interatomic distance, which include van der Waals, electrostatic, magnetic, capillary, or ionic repulsion forces. These forces cause a deflection of the lever on which the tip is mounted following even a minor change of topography, which is utilized to produce images of topography [42-44].

![Figure 3.5: Schematic illustration of interatomic force - distance approach used in AFM.](image)

More descriptive information on the operation of AFM is as follows. The first AFM used a scanning tunneling microscope at the end of the cantilever to detect the bending of the lever, but now most AFMs employ an optical lever technique. The Figure 3.6 illustrates
how this works; as the cantilever flexes, the light from the laser is reflected onto the split photo-diode. By measuring the difference signal (A–B), changes in the bending of the cantilever can be measured. Since the cantilever obeys Hooke’s law for small displacements, the interaction force between the tip and the sample can be found. The movement of the tip or sample is performed by an extremely precise positioning device made from piezo-electric ceramics, most often in the form of a tube scanner. The scanner is capable of sub-angstrom resolution in x, y and z-directions. The z-axis is conventionally perpendicular to the sample. By following a raster pattern, the sensor data forms an image of the probe-surface interaction [42-44]. The feedback from the sensor (for atomic force microscopy the sensor is a position-sensitive photodetector that records the angle of reflection from a laser beam focused on the top of the cantilever) in AFM can be operated in two principal modes, (1) with feedback control and (2) without feedback control.

If the electronic feedback is switched on, then the positioning piezo which is moving the sample (or tip) up and down can respond to any changes in force which are detected, and alter the tip-sample separation to restore the force to a pre-determined value. This mode of operation is known as constant force, and usually enables a fairly faithful topographical image to be obtained (hence the alternative name, height mode). If the feedback electronics are switched off, then the microscope is said to be operating in constant height or deflection mode. This is particularly useful for imaging very flat samples at high resolution. Often it is best to have a small amount of feedback-loop gain, to avoid problems with thermal drift or the possibility of a rough sample damaging the tip and/or cantilever. Strictly, this should then be called error signal mode. The vertical resolution of the AFM is very high; the lateral resolution is somewhat lower because of its dependence on the parameters such as of tip diameter and sample shape. Additionally, surface analysis with AFM is confined to very limited areas. This is due to the inherent inability of the AFM to scan areas larger than approximately 90x90 μm². Furthermore, the performance, precision, and accuracy in imaging are dependent on many inherent (such as artifacts arising because of the electronics of the control system) and external factors.
Interaction force:

The z-axis (vertical) component of the force of interaction is calculated from the z-displacement of the cantilever and the spring constant of the cantilever. From Hooke's Law, \( F = -kz \), where \( k \) is the spring constant. The spring constant for a cantilever is provided by the cantilever supplier. A constant force on the probe tip is maintained by feedback from measurement of the interaction force. The probe is moved up and down to maintain the measured constant force. Typical spring constants are between 0.001 to 100 N/m and motions from microns to \( \sim 0.1\text{Å} \) are measured by the deflection sensor. Typical forces between tip and sample range from \( 10^{-11} \) to \( 10^{-6} \) N. For comparison the interaction between two covalently bonded atoms is of the order of \( 10^9 \) N at separations of \( \sim 1\text{Å} \). Therefore, non-destructive imaging is possible with these small forces [45].

Tip Selection:

AFM tips are generally made of silicon or silicon nitride. For most applications, pyramidal silicon nitride tips are used. They are relatively durable and present a hydrophobic surface to the sample. Conical silicon tips are often used for bio-molecular applications because they are very sharp and present a hydrophilic surface. However,
they are relatively less durable. For the ultimate sharpness, tips of carbon nanotubes have been made. The tip would ideally consist of only one atom, which is brought in the vicinity of the sample surface. However, generally, the radius of curvature of most commercially available tips is around 100 nm. Improved fabrication processes have also made it possible to produce tips with a radius of curvature of 2 nm, but at an expensive price. The tip is held at the apex of a miniature cantilever. In addition to this, the sharpness of the tip also plays a dominant role in determining the lateral resolution of AFM when imaging surfaces with higher relief. If the surface features to be imaged are deeper than the probe length, it can result in artifacts (Figure 3.7). A common artifact is the resulting image, which will be a combination of the actual sample surface and the shape of the cantilever. On the contrary, the vertical resolution in an AFM is established by relative vibrations of the probe above the surface. Sources for vibrations are acoustic noise, floor vibrations, and thermal vibrations. To obtain the maximum vertical resolution, it is necessary to minimize the vibrations of the instrument [42-44].

**Figure: 3.7:** Comparison of AFM imaging of a surface structure with higher surface relief: (a) blunt probe and (b) sharp probe. Arrows indicate the artifacts.

**Tip-surface interactions:**

Mechanical forces that occur when the atoms of the probe physically interact with the atoms on the sample surface are the strongest forces between the probe and the surface. However, other forces between the probe and surface can have an impact on an AFM image. These include

(a) **Surface contamination:** In ambient air, all surfaces are covered with a very thin layer, < 50 nm, of contamination. This contamination comprises water and hydrocarbons and depends on the environment the microscope is located in. When the AFM probe comes into contact with the surface contamination, capillary forces can pull the probe towards the surface, ultimately leading to a distorted image.
(b) Electrostatic forces: It is well known that insulating surfaces can store charges on their surfaces. When these charges interact with charges on the AFM probe or cantilever, the forces can be so strong that they even bend the cantilever when scanning a surface.

(c) Surface material properties: When imaging heterogeneous surfaces, which generally have regions of different hardness and friction, the interaction of the probe with the surface can change when moving from one region to another. Such changes in forces on one hand are advantageous giving a contrast that is useful for differentiating between materials on a heterogeneous surface. On the other hand, they also can be disadvantageous if the changes in hardness, adhesion or frictional properties of the surface are drastic.

Mode of operation:
Depending on the AFM tip and sample surface interaction, three conventional scanning modes have been used for imaging: contact mode, non-contact mode, and tapping mode [42-44].

1. Contact mode: In contact mode AFM, the tip and sample remain in close contact as the scanning proceeds (repulsive region of the interatomic force curve) (Figure 3.8 (a)), creating large forces on the sample. This mode is generally used for high resolution imaging. Since the maximum vertical force is also controlled, the compression of the sample can be limited. In this mode although the lateral forces as the tip moves over the surface can be a problem, in some situations they can actually be an advantage. For example, the lateral deflection can give information about the friction between the tip and the sample, and show areas that may have the same height, but different chemical properties. Also, it is important to consider here that the set point value is the deflection of the cantilever, so a lower value of the set point gives a lower imaging force.

2. Non-contact mode: In the non-contact operation, the probe is held at a small distance away from the surface and the cantilever is oscillated above the surface of the sample (Figure 3.8 (b)). However, due to the attractive forces there is a possibility of the tip coming into contact with the surface. So, this method is not widely used. The capillary force makes this even difficult to control in ambient conditions. Very stiff cantilevers are needed so that the attraction does not overcome the spring constant of the cantilever.
Despite these disadvantages, the lack of contact with the sample makes this mode of imaging beneficial in that it causes a minimum damage to the surface.

3. Intermittent contact or tapping mode: To obtain quality images, in general, the tip should not damage the surface being scanned (so the tip should not be in continuous contact with the sample surface to be imaged, as in contact mode) but that it contacts the surface to obtain high resolution topographic imaging of the sample surfaces. This is possible in tapping mode and so is the most commonly used mode of imaging in AFM. In this mode, the cantilever oscillates and the tip makes repulsive contact with the surface of the sample at the lowest point of the oscillation (Figure 3.8 (c)). In other words, the cantilever is oscillated at its resonant frequency (often hundreds of kilohertz) and positioned above the surface of the sample, so that it only taps the surface for a very small fraction of its oscillation period. This is still contact with the sample in the sense defined earlier, but the very short time over which this contact occurs means that lateral forces are dramatically reduced as the tip scans over the surface. When imaging poorly immobilized or soft samples, tapping mode may be a far better choice than contact mode for imaging. This method has the advantage that the tip does not damage the surface of the sample being scanned, and there is also a large reduction in the lateral forces, since the proportion of the time where the tip and sample are in contact is quite low. Also, the phase of the cantilever oscillation can give information about the sample properties, such as stiffness and mechanical information or adhesion. The resonant frequency of the cantilever depends on its mass and spring constant; normally, stiffer cantilevers have higher resonant frequencies. In this mode, the set point value is the amplitude of the oscillation, so a higher set point value means less damping by the sample and hence lower imaging forces. Other (more interesting) methods of obtaining image contrast are also possible with tapping mode. In constant force mode, the feedback loop adjusts so that the amplitude of the cantilever oscillation remains (nearly) constant. An image can be formed from this amplitude signal, as there will be small variations in this oscillation amplitude due to the control electronics not responding instantaneously to changes on the specimen surface. More recently, there has been much interest in phase imaging. This works by measuring the phase difference between the oscillations of the cantilever driving piezo and the detected oscillations. It is thought that image contrast is derived from image properties such as stiffness and viscoelasticity.
3.6.5 Rheology

Introduction:
Rheology has been properly defined as the study of flow and deformation of matter. In practice, rheology is principally concerned with extending the "classical" disciplines of elasticity and (Newtonian) fluid mechanics to materials whose mechanical behavior cannot be described with the classical theories [46,47]. It is also concerned with establishing predictions for mechanical behavior (on the continuum mechanical scale) based on the micro or nanostructures of the materials, e.g. the molecular size and architecture of polymers in solution or the particle size distribution in a solid suspension. Rheology unites the seemingly unrelated fields of plasticity and non-Newtonian fluids by recognizing that both these types of materials are unable to support a shear stress in static equilibrium. In this sense, a plastic solid is a fluid. Granular rheology refers to the continuum mechanical description of granular materials. One of the tasks of rheology is to empirically establish the relationship between deformations and stresses, respectively their derivatives by adequate measurements. These experimental techniques are known as rheometry. Such relationships are then amenable to mathematical treatment by the established methods of continuum mechanics. The classification of continuum mechanics can be described as follows.
The rheology experiments were performed using a AR-500 stress controlled rheometer (T. A. Instruments, UK). Controlled stress rheology [48] is a powerful technique for characterizing the flow properties of materials. The schematic for a typical controlled stress rheometer is shown in Figure 3.9. Mechanically, a controlled stress rheometer is fairly simple in that there are only a few important components. One of these critical components is the bearing, which supports the drive shaft and measurement geometry. Generally, floating the drive shaft on an air cushion (air bearing) is preferable to a mechanical bearing because the former almost entirely eliminates friction so that small sample deformations at low stress levels can be measured with minimal losses.

Controlled stress rheology [48] has become the most popular approach for making rheological measurements for a variety of reasons including the ability to nondestructively probe the structure of delicate materials such as colloids and dispersions. The ability to evaluate these difficult-to-measure materials is directly dependent on the rheometer’s ability to reproducibly apply small stresses and to detect the small responses produced by those stresses. The former capability is determined by the characteristics of the rheometer torque motor and associated electronics. The latter capability is determined by the rheometer displacement measuring sensor. The high resolution optical encoder provides excellent displacement (strain) resolution (2.5 μradians) which is adequate for even the most demanding material evaluations. This capability is beneficial in flow
(where lower shear rates can be measured), creep (where smaller strains allow the linear portion of the curve to be detected more quickly), and oscillation (where smaller strains allow materials with very small linear viscoelastic regions to be more accurately evaluated and more frequencies to be used in multiwave oscillation without exceeding the materials linear limits).

**Figure 3.9:** Schematic picture for a typical controlled stress rheometer.

In controlled stress rheometers the angular deflection under a controlled stress is the measured dependent variable. To be able to evaluate the widest range of materials, an optical encoder system to measure this angular deflection is ideal. It is able to detect movements as small as 2.5 μradians and angular velocities up to 50 radians/second. An optical encoder is generally used since it is accurate, mechanically simple, and relatively low cost. In addition, an optical encoder needs no calibration, being an absolute measure of angular movement as well as being free from the drift present in analogue displacement measuring systems. Furthermore, an optical encoder can measure a wide dynamic range of displacement.
The two common approaches used in rotational rheometers are shown in Figure 3.10 below. In the controlled rate approach, the material being studied is placed between two plates. One of the plates is rotated at a fixed speed and the torsional force produced at the other plate is measured. Hence, speed (strain rate) is the independent variable and torque (stress) is the dependent variable. In the controlled stress approach, the situation is reversed. A torque (stress) is applied to one plate and the displacement or rotational speed (strain rate) of that same plate is measured. This latter approach (controlled stress) is the better approach for determining apparent yield stress because the variable of primary interest can be more carefully controlled.

Figure 3.10: Schematic representation of controlled stressed and controlled rate rotational rheometer.

That is, in the controlled stress approach it is possible to gradually increase the stress applied to the material and detect the point at which movement (yield) first occurs. Conversely, in the controlled rate approach, movement (yielding) actually has to be occurring before measurement can even occur. Hence, apparent yield stress can only be measured by back extrapolation from a finite level of motion to the point of zero motion.
or yielding. The remainder of this discussion considers only controlled stress measurements. The controlled stress approach provides three alternative experimental methods for determining apparent yield stress: (1) Stress can be ramped slowly from zero to some higher value. The stress level at which motion first occurs is the apparent yield stress. As indicated previously, this value may be affected by the stress ramp rate (rate of stress increase). (2) After initially shearing the material at a stress above the yield stress, the stress can be decreased in a slow ramp and the point where motion stops is the apparent yield point. Again as indicated previously, this value may be affected by the decreasing ramp rate and the time-dependent ability of the material to rebuild structure. (3) A creep experiment can be used where stress is applied to the material and strain (displacement) is monitored with time to establish an equilibrium yield stress.

**Peltier Plate:**

There is one option Peltier plate options for the AR-500 i.e. the Standard Peltier System (SPS) has a top temperature of 99.9°C. It both serves as the lower plate of the cone & plate and parallel plate geometries. The principle of operation is based on the Peltier effect which is essentially the reverse of the effect produced by a thermocouple. In the Peltier effect, a current is introduced to create a temperature change at the junction, which is subsequently transferred to the sample. Water continually circulating through the Peltier plate acts as a heat sink or heat source to facilitate this Peltier effect. With narrow gaps, temperature changes from the plate are rapidly conducted through the sample. Hence, the Peltier plate is capable of heating/cooling rheology samples at up to 50°C/minute. Furthermore, the open design of the Peltier plate facilitates sample loading and cleaning.

**Geometries:**

Controlled stress rheometers (like the TA Instruments), can measure a vast array of materials using a wide variety of measurement geometries including cone & plate, parallel plate, and concentric cylinders. In each of these geometries, the material being evaluated is either placed directly on a fixed surface or placed inside a cylindrical container. Then the measurement geometry is brought into contact with the material and a known stress or strain profile is applied. The final results (e.g. viscosity) are calculated from the raw experimental data using an equation which depends on the geometry used (such as that shown in Figure 3.11(1) and (2). All of these geometric equations assume
that the space (gap) between the moveable probe and the fixed surface or container is precisely set and that the sample completely fills this gap. In the controlled stress rheometers, a high precision micrometer assembly is used to adjust the gap (see Figure 3.9). To avoid person-to-person variation and potential artifacts, automated software control of this micrometer is preferred. Auto gap set capability for this rheometer is designed specifically to meet that need by addressing the two primary concerns associated with proper setting of the initial gap, namely setting zero gap and closing the gap.

\[
\sigma \text{ (Stress)} = \frac{3M}{2\pi R^3} \quad \dot{\gamma} \text{ (Shear rate)} = \frac{\omega}{\alpha}
\]

Figure 3.11(1): (the cone and plate geometry) and Figure 3.11(2): (parallel geometry) shows the geometric factors used in converting torque and displacement into stress and strain. The stress factor \(\sigma\) and the shear rate factor \(\dot{\gamma}\) are defined based on the torque \(M\), the radius \(R\), the angular velocity \(\omega\) and the cone angle \(\alpha\).
Mode of operations:

1. Flow mode: In flow mode, rheological measurements are normally performed in kinematic instruments in order to get quantitative results useful for design and development of products and process equipment. For design of products, e.g. in the food, cosmetic or paint industry, rheometric measurements are often performed to establish the elastic properties, such as gel strength and yield value, both important parameters affecting e.g. particle carrying ability and spreadability. For design of process equipment the properties during shearing of the product is of prime interest. Those properties are established in a normal viscosity measurement. A rheometric measurement normally consists of a strain (deformation) or a stress analysis at a constant frequency (normally 1Hz) combined with a frequency analysis, e.g. between 0.1 and 100 Hz. The fluid products are normally divided into three different groups according to their flow behavior.

(a) Newtonian Fluid:

In Newtonian Fluid, \( \sigma \) is only stress generated in simple shear flow (no normal stress difference). Shear viscosity (\( \eta \)) does not vary with shear rate. It is constant with time of shearing. \( \sigma \) in fluid falls immediately to zero when shearing is stopped. When sheared again, the \( \eta \) is as was previously measured (regardless of delay between measurements). Shear viscosity (\( \eta \)) can be measured in different types of deformation are in proportion to one another. A Liquid showing any deviation from Newtonian is said to be non-Newtonian.

(b) Non-Newtonian Fluids, time independent, \( \eta = \eta(\gamma) \):

In Non-Newtonian Fluids, time independent case, the viscosity of fluid is dependent on shear rate but independent of the time of shearing. The viscosity is presented at a specific shear rate and referred to as the “apparent viscosity”, “shear viscosity” or “shear dependent viscosity”. Shear-thinning (a decrease in viscosity with increasing shear rate, also referred to as Pseudoplasticity) and Shear-thickening (an increase in viscosity with increasing shear rate, also referred to as Dilatancy) are the two properties of Non-Newtonian, time independent fluid.
(c) Non-Newtonian Fluids, time dependent, $\eta = \eta(\gamma, t)$:

In Non-Newtonian Fluids, time dependent case, the viscosity of fluid is dependent on shear rate and the time during which shear rate is applied. Thixotropy (a decrease in apparent viscosity with time under constant shear rate or shear stress, followed by a gradual recovery, when the stress or shear rate is removed) and Rheopexy (an increase in apparent viscosity with time under constant shear rate or shear stress, followed by a gradual recovery when the stress or shear rate is removed, also called anti-thixotropy or negative thixotropy) are the two properties of Non-Newtonian, time dependent fluids.

2. Oscillation mode:

(a) Frequency sweep measurement:

The material response to increasing frequency (rate of deformation) is monitored at a constant amplitude (stress or strain) and temperature. The uses of frequency sweep are (1) viscosity information - zero shear $\eta$, shear thinning, (2) Elasticity (reversible deformation) in materials, (3) molecular weight ($M_w$) & molecular weight distributions differences polymer melts and polymer solutions, (4) finding yield in gelled dispersions, (5) high and low rate (short and long time) modulus properties, (5) extend time or frequency range with time temperature superposition (TTS). The frequency sweep gives information of the elastic modulus $G'$, the viscous modulus $G''$ and the phase angle $\delta$. A large value of $G'$ in comparison of $G''$ indicates pronounced elastic (gel) properties of the product being analyzed. For such a product the phase angle is also small, e.g. 20° (a phase angle of 0° means a perfectly elastic material and a phase angle of 90° means a perfectly viscous material) shown in Figure 3.12. The frequency sweep gives information about the gel strength where a large slope of the $G'$ curve indicates low strength and a small slope indicates high strength.

![Figure 3.12: Schematic picture of the phase angle between stress and strain.](image-url)
The stress in a dynamic experiment is referred to as the complex stress $\sigma^*$. The complex stress can be separated into two components: an elastic stress in phase with the strain, $\sigma' = \sigma^* \cos \delta$ (where $\delta$ is the degree to which material behaves like an elastic solid) and a viscous stress in phase with the strain rate, $\sigma'' = \sigma^* \sin \delta$ (where $\delta$ is the degree to which material behaves like an ideal liquid). The complex stress and strain is shown in Figure 3.13.

$$\sigma^* = \sigma' + i\sigma''$$

**Figure 3.13**: Schematic picture of phase angle between complex stress and strain.

The complex modulus, $G^* = G' + iG'' = \text{(stress*strain)}$ is the measure of materials overall resistance to deformation. The elastic (storage) modulus, $G' = \text{(stress*strain)} \cos \delta$ is the measure of elasticity of material. The ability of the material to store energy. The viscous (loss) modulus, $G'' = \text{(stress*strain)} \sin \delta$ is the ability of the material to dissipate energy. Energy lost as heat. Tan delta ($\tan \delta = G''/G'$) is the measure of material damping - such as vibration or sound damping. The viscosity measured in an oscillatory experiment is a complex viscosity much the way the modulus can be expressed as the complex modulus. The complex viscosity contains an elastic component and a term similar to the steady state viscosity. The complex viscosity is defined as: $\eta^* = \eta' - i\eta''$ or $\eta^*(\omega) = G^*/\omega$

$$= \left[ (G'(\omega))^2 + (G''(\omega))^2 \right]^{1/2} / \omega.$$  For viscoelastic gels, the complex viscosity is given by

$$\eta^*(\omega) = \eta_0 / \sqrt{1 + \omega^2 \tau_m^2}$$

where, for the Maxwell model, $\eta_0$, the zero-shear viscosity and $\tau_m$ is the relaxation time. The dynamic viscosity $\eta'$ is the part of the complex viscosity related to the steady state viscosity and is the part of that measures the rate of energy dissipation. Dynamic viscosity ($\eta'$) for a viscoelastic liquid approaches the steady flow viscosity ($\eta_0$) as the frequency approaches zero which is defined as: $\eta' = G''/\omega$. The imaginary viscosity $\eta''$ measures the elasticity or stored energy and is related to the shear storage modulus which is defined as: $\eta'' = G'/\omega$. The material response in frequency sweep is given in Figure 3.14.
(b) **Dynamic temperature ramp:**

In dynamic temperature ramp, a linear heating rate is applied and the material response is monitored at a constant frequency and constant amplitude of deformation. Data is taken at user defined time intervals. The uses of temperature ramp are cure studies and transition of materials. A step and hold temperature profile is applied. The material response is monitored at one, or over a range of frequencies, at constant amplitude of deformation. An example of dynamic temperature ramp or step and hold and the corresponding material response is given in the Figure 3.15.

![Figure 3.14: The material response in frequency sweep](image)

![Figure 3.15: Dynamic temperature ramp or step and hold and the corresponding material response.](image)
In my rheology experiments, the steady state shear flow and dynamic rheology (frequency sweep) of the solutions were studied using a cone plate geometry of radius 60mm and angle 2°. Thixotropic measurements were performed (in flow mode) on water-ethanol and water-ethanol-gelatin system at ethanol concentration fixed at 45 ± 2 % (v/v).

In this procedure, starting at lowest shear rate (\( \gamma \)) available, one obtains the shear stress (\( \sigma \)) developed in the system. After a given time, the shear rate is increased to its next higher value and shear stress measured again. This is repeated until the highest shear rate is reached and the system is sheared to its equilibrium shear stress. The process is reversed in the next step. The shear rate is reduced stepwise and stress measured continuously until the minimum shear rate is reached. A plot of shear stress as function of shear rate reveals the thixotropic behavior of the system. The dynamic rheology (frequency and temperature sweep) of gel and coacervates were studied by using steel parallel plate geometry of radius 20mm and angle 0° with truncation gap 500\( \mu \)m. The truncation gap used in the parallel plate geometry was kept this large intentionally to avoid the breaking of structures inside the coacervate samples. Appropriate inertial corrections were made prior to accepting data. Sponges and silicon oil were used as solvent trap to prevent loss of solvent due to evaporation. The solvent trap system is normally able to minimize drying such that an hour or more work can be performed without artifacts due to drying of the solutions.

### 3.6.6 Differential scanning calorimetry (DSC)

DSC experiments were performed by using a Q10 -V8.1- Build 261 differential scanning calorimeter (Exo up - TA Instruments, UK). Here the objective was to determine the melting temperature of coacervates and to correlate the same with the results obtained from rheology. In a DSC experiment, typically 8.2 mg samples were taken on a stainless steel pan and the temperature sweep was performed with the heating rate maintained at 0.5 °C / min. The differential scanning calorimeter measures the heat absorption of a sample as a function of temperature. It measures the amount of energy absorbed or released by a sample as it is heated, cooled, or held at a constant temperature [50,53]. Typical applications include determination of melting point temperature and the heat of melting; measurement of the glass transition temperature; curing and crystallization studies; and identification of phase transformations. A pair of cells is placed in a
thermostated chamber. The sample cell is filled with a protein solution. The reference cell is filled with an identical volume of solvent. The two cells are heated with a constant power input to their heaters during a scan. Any temperature difference between the two cells is monitored with a feedback system so as to increase (or decrease) the sample cell’s power input. Since the masses and volumes of the two cells are matched, the power added or subtracted by the cell feedback system is a direct measure of the difference between the heat capacity of the sample and reference solutions. The cell feedback power is the raw data, expressed in units of cal/min. By knowing the scan rate (typically 1 K/min) and the sample concentration, these units are converted to cal/mol-K (or cal/g-K).

In practice, the sample and reference cells can be slightly mismatched. The usual practice is to record a reference baseline for the experimental scan; this is subtracted from the experimental data to yield $C_p$ vs. $T$. The heat capacity ($C_p$) is the temperature derivative of the enthalpy function [51-53]:

$$C_p = \left(\frac{d\Delta H}{dT}\right)_p$$  \hspace{1cm} (3.24)

The enthalpy is obtained from a DSC experiment by integration of the heat capacity curve between two temperatures (initial and final):

$$\Delta H_{cal} = \int C_p dT - \text{baseline(s)}$$  \hspace{1cm} (3.25)

If we take an experimental (DSC) scan for protein folding and unfolding studies, the peak occurs near $T_m$ (in $C_p$ vs. $T$ plot, figure not shown) is called the transition midpoint inhibits the protein unfolding. For all-or-none transitions between the native and unfolded states, it is necessary (but not sufficient) that

$$\Delta H_{cal} = \Delta H_{vH}$$  \hspace{1cm} (3.26)

where $\Delta H_{vH}$ relates the Van’t Hoff enthalpy change relates the change in temperature to the change in the equilibrium constant.

We used differential scanning calorimetry technique to study what happens to polymers when they are heated. We use it to study what we call the thermal transitions (melting point) of polymer complexes. And what are thermal transitions? They are the changes that
take place in a polymer when we heat it. The melting of a crystalline polymer is one example. The glass transition is also a thermal transition. So how do we study what happens to a polymer when we heat it? The first step would be to heat it, obviously. Differential Scanning Calorimeter was used to run the gel and coacervate sample where the heating rate was fixed at 1°C/min. Specifically the plot has made in between temperature versus the difference in heat output of the two heaters at a given temperature [51] shown in Figure 3.16.

Figure 3.16: Conventional DSC thermodynamical curve showing a glass transition (with enthalpic relaxation ($T_g$), a cold crystallization exotherm ($T_c$) and a melting endotherm ($T_m$)).

Figure 3.16 represents a plot of heat flow per gram of material, versus temperature. Heat flow is heat given off per second, so the area of the peak is given in units of heat × temperature × time⁻¹ × mass⁻¹. Usually, the unit would put in such as joules × kelvins × (seconds)⁻¹ × (grams)⁻¹: \[ \text{area} = \frac{\text{heat} \times \text{temperature}}{\text{time} \times \text{mass}} = \frac{\text{JK}}{\text{g}} \]. The heating rate is in units of K/s. After dividing the area by the heating rate of the DSC experiment, the expression becomes: \[ \frac{\text{area}}{\text{heat/time}} = \frac{\text{JK/g}}{\text{K/s}} = \frac{\text{J}}{\text{g}} \]. The unit become more simple when it is just multiplied by the mass of the sample: \[ \left( \frac{\text{J}}{\text{g}} \right) \times \text{g} = \text{J} \]. For the glass transition, there is no dip, and there is no peak, either. This is because there is no latent heat given off, or absorbed, by the polymer during the glass transition. Both melting and crystallization involve giving off or absorbing heat. The only thing it shows at the glass transition
temperature is a change in the heat capacity of the polymer. Because there is a change in heat capacity, but there is no latent heat involved with the glass transition, the glass transitions a second order transition. Transitions like melting and crystallization, which do have latent heats, are called first order transitions. The latent heat of melting can be measured by measuring the area of this peak. The polymer gives off heat when it crystallizes that call crystallization an exothermic transition. And of course, the polymer adds energy to make it melt that call melting an endothermic transition.

3.6.7 Circular dichroism (CD)

Circular Dichroism experiments were carried out with a JASCO-810 instrument and the far-UV absorption spectrum was measured with a scanned Shimadzu JV-2401 PC model spectrophotometer to estimate the degree of helicity in gelatin coacervates. The CDNN program, which is Windows 95 based, will read in a JASCO ASCII file only if the HT [V] values are stripped. The HT [V] values can be removed by using the spectral analysis program, “move channel”, and by selecting the CD data only. This will open only the CD data in a new window that can be saved as “ASCII” file. The CDNN program needs data between 210 and 260 nm and is reasonably self-explanatory.

CD is a technique based upon the interactions of proteins with circularly-polarized light (usually within the 170-700 nm range). Absorbance of circularly-polarized light consists of two components $\varepsilon_L$ (left circularly polarized light) and $\varepsilon_R$ (right circularly polarized light) components. These components interact differentially with amino acids resulting in an ellipse of polarization. This is characterized as the molar ellipticity of a protein [54-57]:

$$\theta = 3300(\varepsilon_L - \varepsilon_R)$$

(3.27)

where $\theta$ is in degrees cm$^{-2}$ mole$^{-1}$. The molar ellipticity varies with wavelength to describe the CD spectrum in the Figure 3.17.
Since most biological molecules possess multiple chiral chromophores, their CD spectra may provide important insights on the three dimensional arrangement of such macromolecules with varying specificity. Specifically, CD spectroscopy is often used to measure overall secondary structural content of peptides and proteins (the far-UV region, 190-250 nm), chirality due to unique arrangement of the aromatic side chains and/or disulphide bridges within the protein (250-300nm), as well as protein interaction with chiral ligands and cofactors (i.e., Soret band). Although the assessment of protein conformations afforded by CD spectroscopy typically yields a low-resolution picture, the experiments are relatively simple and are not very demanding in terms of sample workup and consumption. As a result, CD measurements are often used to complement measurements by other techniques. The other techniques such as X-ray scattering or NMR spectroscopy, have the ability to determine molecular structures with much greater specificity than CD spectroscopy; those techniques have very limited time resolutions. These techniques are invaluable in providing equilibrium molecular structures. However, in many systems, such as biomolecules, the function of the system depends on structural changes, and to truly understand the mechanism by which these systems function one must understand the time evolution of their structures. As a technique based on optical
measurements, CD spectroscopy thus provides a structurally sensitive method that has the inherently potential for high time-resolution measurements.

In practice, the quantum mechanical calculation of the CD of most biopolymers is still a formidable task. Chromophores such as the nucleic acid bases and aromatic aminoacids are far more complex than the peptide group. Many more electronic states are involved, and transmission moment directions and intensities are rarely known for enough of them with sufficient precision. However, a large body of experimental optical activity data exists for polypeptides, proteins, and nucleic acids with known three-dimensional structures. A number of ways have been developed for using such data to predict or analyze the optical properties of less-well-characterized systems. The discussion of CD method for protein and nucleic acids are different as the two classes of compounds are somewhat different. For proteins, the major objective has been to deduce the average secondary structures of the peptide chain from measured CD or Optical Rotary Dispersion (ORD) spectra. Unless an unusual fraction of aromatic aminoacids is present, optical activity in the region of the spectrum between 190 nm and 230 nm is dominated by the peptide backbone. Numerous experiments have shown that, at least qualitatively, the nature of particular aliphatic side chains does not markedly affect CD spectrum in this region [57]. Therefore, as an approximation, one considers a protein simply as a linear combination of backbone region with \( \alpha \)-helical, \( \beta \)-sheet, or random coil structures. Estimate of the spectra of each type of region can be obtained by using the measured CD of homopolypeptides known to be pure \( \alpha \) helices, \( \beta \) sheets, or random coils. If the actual fractional composition of secondary structure types (\( \chi_\alpha \), \( \chi_\beta \), \( \chi_r \)) were known for a particular protein, one could reside CD at each wavelength [54,57]:

\[
[\theta] = \chi_r [\theta]_r + \chi_\alpha [\theta]_\alpha + \chi_\beta [\theta]_\beta
\]

(3.28)

where the fractional contributions of the individual structures corresponding to random coil, \( \alpha \)-helix and \( \beta \)-turns are \( \chi_r \), \( \chi_\alpha \) and \( \chi_\beta \) respectively. The corresponding molar ellipticity are \( [\theta]_r \), \( [\theta]_\alpha \) and \( [\theta]_\beta \) respectively. In the early days of optical activity measurements on proteins and nucleic acids, CD instrumentations was unavailable, and ORD could be measured only at wavelengths far away from any absorption bands. Therefore, experimental data had to be fit to the Drude equation,
where \( A_0 \) is a constant related to the intensity of the corresponding CD spectrum, and \( \lambda_0 \) is the crossover wavelength. Conclusion about molecular structures and conformation were derived from the values of the parameters \( A_0 \) and \( \lambda_0 \).

### 3.6.8 UV-spectroscopy

Many molecules absorb ultraviolet (UV) or visible (Vis.) light. The absorbance of a solution increases as attenuation of the beam increases. Absorbance is directly proportional to the path length, \( b \), and the concentration, \( c \), of the absorbing species. Beer's law states that \( A = \varepsilon b c \), where \( \varepsilon \) is a constant of proportionality, called the absorbivity. Different molecules absorb radiation of different wavelengths. An absorption spectrum will show a number of absorption bands corresponding to structural groups within the molecule. For example, the absorption that is observed in the UV region for the carbonyl group in acetone is of the same wavelength as the absorption from the carbonyl group in diethyl ketone. If a beam of monochromatic radiation of radiant power \( P_0 \), directed at a sample solution, absorption takes place and the beam of radiation leaving the sample has radiant power \( P \). The amount of radiation absorbed may be measured in a number of ways:

\[
A = \log_{10} \frac{P_0}{P} \\
A = \log_{10} \frac{1}{T} \\
A = \log_{10} \frac{100}{\%T} \\
A = 2 \log_{10} \%T
\]

The last equation, \( A = 2 - \log_{10} \%T \) (% Transmittance, \( \%T = 100 \% \)), allows one to easily calculate absorbance from percentage transmittance data. So, if all the light passes through a solution without any absorption, then absorbance is zero, and percent transmittance is 100%. If all the light is absorbed, then percent transmittance is zero, and absorption is infinite. Proteins contain several chromophores that absorb light in the ultraviolet and infra red regions. Many also display fluorescence. The most important chromophores are the aromatic rings of Phenylalanine (Phe), Tyrosine (Tyr) and Tryptophan (Trp). UV absorbance and fluorescence are useful probes of structure and structural changes. This is due to the fact that chromophores display shifted spectra upon increasing or decreasing polarity of their environment, with changes in wavelength of
efficient (ε) possible
scribed in Table 3.3.

efficient (ε) increase
able group becomes
histidine imidazole

<table>
<thead>
<tr>
<th>w</th>
<th>h</th>
<th>o</th>
<th>l</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

maximum wavelength

amino acids that are
changes occurring in
acids in active site
ence spectroscopy” in
contains the protein
a perturbant such as
to the absorbance of
e relative amount of
ured with a scanned
gree of helicity in the

101
3.6.9 Electrophoresis

Introduction:

Electrophoresis is the phenomenon of the movement of a charged particle relative to the liquid suspended in, under the influence of an applied electric field [60-62]. This is the technique to understand and control colloidal suspensions. Examples include complex biological systems such as blood and functional ones like paint. Surface forces at the interface of the particle and the liquid are very important because of the microscopic size of the colloids. One of the major surface effects is electrokinetic. Each colloid carries a like electrical charge which produces a force of mutual electrostatic repulsion between adjacent particles. If the charge is high enough, the colloids will remain discrete, disperse and in suspension. Reducing or eliminating the charge has the opposite effect - the colloids will steadily agglomerate and settle out of the suspension or form an interconnected matrix. This agglomeration causes the characteristics of the suspension to change. Particle charge can be controlled by modifying the suspending liquid. Modifications include changing the liquid’s pH or changing the ionic species in solution. Another, more direct techniques is to use surface active agents which directly absorb to the surface of the colloid and change its characteristics. Therefore, zeta potential is a very good index of the magnitude of the interaction between colloidal particles and this measurement is an important parameter to characterize colloidal dispersions. It can be defined as the measure of overall charge, a particle acquires in a specific medium. It is proportional to the difference in the electrical charge between the dense layer of ions surrounding the particles and the charge of the bulk of the suspended fluid surrounding this particle. In other words, it is the electrostatic potential of a molecule or particle. It is usually measured by electrophoretic mobility and is related to the surface potential and a measure of the electrostatic forces or repulsion the particle or molecule is likely to meet when encountering another of the same sign of charge.

Theory:

Development of a net charge at the particle surface affects the distribution of ions in the surrounding interfacial region, resulting in an increased concentration of counter-ions (ions of opposite charge to that of the particle) close to the surface and the ions with a small amount of the same polarity distributed diffusely. Thus, an electrical double layer
exists round each particle and this is a model used to visualize the ionic environment in the vicinity of a charged colloid and explains how electrical repulsion forces occur. The double layered model is understood as a sequence of steps that would take place around a single highly negative colloids (charged macro-ion) if it’s neutralizing ions were suddenly stripped away. The effect of the colloid on the positive ions (often called counter-ions) in solution is described as follows. Initially, attraction from the negative colloid causes some of the positive ions to form a firmly attached layer around the surface of the colloid; this layer of counter-ions is known as the Stern layer. In other words, it is an inner region that exists at a distance almost equal to the radius of the hydrated ion from the interface where the counter-ions are strongly bound. Additional positive ions are still attracted by the negative colloid, but now they are repelled by the Stern layer as well as by the positive ions that are also trying to approach the colloid. This dynamic equilibrium results in the formation of a diffusive layer of counter-ions. They have high concentration near the surface which gradually decreases with distance, until it reaches equilibrium with the counter-ion concentration in the solution. So there is a lack of negative ions in the neighborhood of the surface, because they are repelled by the negative colloid. Negative ions are called co-ions because they have the same charge as the colloid. Their concentration will gradually increase with distance as the repulsive forces of the colloid are screened out by the positive ions, until equilibrium is again reached.

The diffusive layer can be visualized as a charged atmosphere surrounding the liquid. The charge density at any distance from the surface is equal to the difference in concentration of positive and negative ions at that point. Charged density is greatest near the colloid and gradually diminishes towards zero as the concentration of positive and negative ions merge together. The attached counter-ions in the Stern layer and the charged atmosphere in the diffusive layer are altogether referring as the double layer. The thickness of this layer depends upon the type and concentration of ions in solution. Within the diffuse layer is an imaginary boundary called slipping plane or shear plane within which the particle acts as single entity. The potential at this boundary is called Zeta potential (See Figure 3.18). That is to say that, the potential at the surface of shear of a charged particle i.e. for macro-ions it is the potential at the surface of the hydrodynamic particles. The conventional symbol used for zeta potential is ‘ζ’ and it is usually measured in millivolts (mV). The double layer is formed in order to neutralize the charged colloid and in turn, causes an electrokinetic potential between the surface of the colloid and any point in
the mass of the suspending liquid which is referred to as the surface potential. Consider a
dilute suspension of spherical colloidal negatively charged particles of radius ‘R’ with
surface charge density σ or the total surface charge \( Q = 4\pi R^2\sigma \) in a salt free medium
containing only counterions. It is assumed that each sphere is surrounded by a spherical
free volume of radius ‘a’ (Figure 3. 18), within which counterions are distributed so that
electrical neutrality as a whole satisfied. The region (I) and (II) are the charge free region
where the surface potential satisfies Laplace equation [61-65]:

\[
\nabla^2 \psi (r) = 0 \tag{3.31}
\]

and the region with mobile charge (III) satisfies Poisson equation [61-65] :

\[
\nabla^2 \psi (r) = -4\rho \pi /\varepsilon \tag{3.32}
\]

where \( \rho \) is the time average charge density (continuous distribution of charge which will
in general be a function of position) and \( \varepsilon \) is the dielectric constant of the medium. The
magnitude of the surface potential \( \psi (r) \) is related to the surface charge and the thickness
of the double layer. The surface potential in the region-I and on the surface is constant i.e.

\[
\psi (r \leq R) = \left( \frac{Q}{\varepsilon R} \right) \left( 1 - \frac{\kappa R}{1 + \kappa a} \right). \quad \text{As we leave the surface, the potential drops off roughly}
\]

linearly in the stern layer (region-II), \( \psi (R < r < a) = \left( \frac{Q}{\sigma r} \right) \left( 1 - \frac{\kappa R}{1 + \kappa a} \right) \) and then

exponentially (region-III), \( \psi (r > a) = \left( \frac{Q}{\varepsilon} \right) \left( \frac{e^{\kappa a}}{1 + \kappa a} \right) \left( \frac{e^{-\kappa r}}{r} \right) \) through the diffusive layer,

approaching zero at the imaginary boundary of the double layer. The units of \( \kappa \)

\[
= \left( \frac{8\pi Z^2 e^2}{\varepsilon K_B T} \right)^{1/2} N^{1/2}
\]

is reciprocal length and \( 1/\kappa = “\text{thickness”} \) of the electrical double
layer i.e. Debye screening length \( \approx 1\text{nm} \) (0.1M NaCl). Here, \( Z \) is the valence of counter-
ions, \( e \) is the elementary electric charge, \( K_B \) is the Boltzmann constant, \( N \) is the
concentration of mobile counterions and \( T \) is the temperature. This assumption is justified
for the central ions of sufficiently low charge density and for external solutions with
sufficiently low concentration of mobile counter-ions. Note that counter-ions
condensation occurs in the vicinity of the particle surface because of the very strong
electric field there.
Figure 3.18: The picture on the top shows a negatively charged molecule. Due to electrostatic attraction the counter-ions make a layer around it to neutralize the interfacial potential, called stern layer or fixed layer and the ions with a small amount of the same polarity are distributed diffusely, called diffuse layer. The picture below shows the behavior of surface potential $\psi(r)/mV$ versus distance from surface 'r'.

A zeta potential value quoted without a definition of its environment (pH, ionic strength, concentration of any additives) is a meaningless number. The magnitude of zeta potential gives an indication of the potential stability of the colloidal system. As the zeta rises, the repulsion between the particles becomes stronger. The stability of the dispersion gets higher while on the other hand as zeta approaches zero, the electrostatic build up is relaxed allowing easier aggregation. A dividing line between the stable and unstable aqueous dispersion is generally taken at either +30 mV or -30 mV. Particles with zeta potentials more negative than -30mV or more positive than +30mV are normally considered stable (See Figure 3.19). The most important factor that affects zeta potential is pH. The pH at which the net surface charge on the molecules become zero (or at which zeta potential becomes zero) is known as isoelectric point of the solution. It can be found by adjusting the pH to an approximate value at which the net surface charge on the molecules goes to zero. Imagine a particle in suspension with a positive zeta potential if more acid is added to this suspension then the particles tend to acquire more positive charge ($H^+$-ions from the solution). If alkali is added to this suspension then a point will
be reached where the charge will be neutralized. Further addition of alkali will cause a build up of negative charge (because of release of $H^+$-ions that bind to $OH^-$ groups present in the solution). In general, a zeta potential versus pH curve will be positive at low pH and lower or negative at high pH. And the point where the curve passes through zero zeta potential is the iso-electric point. It is normally the point where the colloidal system is least stable.

![Zeta Potential versus pH Curve](image)

**Figure 3.19:** The system responds to pH by becoming more positively charged at lower pH (positive zeta potential) and more negatively charged at higher pH (negative zeta potential). At some intermediate pH, zeta potential is zero called the iso-electric point.

The particles move with a characteristic velocity, which is dependent on the strength of the electric field ($E$), the dielectric constant and the viscosity of the medium and the zeta potential. The velocity ($v$) of a particle in a unit electric field is referred to as its electrophoretic mobility. Under equilibrium, Frictional force ($QE$) = Force by electric field ($6\pi\eta v$). So, $v = QE/6\pi\eta R$ (by using the relation of electrophoretic mobility, $\mu = v/E = Q/6\pi\eta R$). These assumptions are considered by neglecting interactions terms in a much diluted solutions. Zeta potential is related to the electrophoretic mobility by the Henry equation [63-65],

$$\mu = \frac{2\varepsilon f(\kappa R)}{3\eta}$$  \hspace{1cm} (3.33)

where, $\mu =$ electrophoretic mobility, $\zeta =$ zeta potential, $\varepsilon =$ dielectric constant, $\eta =$ viscosity of solvent, $f(\kappa R) =$ Henry's function, $R =$ radius of the particle. For particles in
polar media the maximum value of \( f(\kappa R) \) is 1.5 (Smoluchowski approximation) [62] while for particles in non-polar media the minimum value of \( f(\kappa R) \) is 1.0 (Huckel approximation) [63,64]. The schematic representation of Smoluchowski approximation and Huckel approximation is shown in Figure 3.20.

**Figure 3.20:** Particles, for which \( f(\kappa R)=1.0 \), obey Hückel approximation and particles, for which \( f(\kappa R)=1.5 \), obey Smouchowski’s approximation.

In my electrophoresis experiments, zeta potential measurement was performed on a instrument (ZC-2000, Microtec, Japan) due to the surface charges on gelatin nanoparticle via coacervation (described in Chapter 8). The sample solution is always necessary to dilute with deionized water. Dilution was done to isolate all individual particles from the aggregates and to know the distribution of charges on the surfaces of the single particle. In order to minimize the influence of electrolysis to the measurements, molybdenum (+) and platinum (-) were used for electrodes. Also, during the measurements, the cell chamber tap on molybdenum electrode was kept open to release the air bubbles, for the purpose of reducing their effects on the particle movements. During the measurements, the molybdenum anode was cleaned each time as it turned from a metallic to blue-black color.
3.7 References


[50] www.tainstruments.com


