Chapter-2

Materials Used and Experimental Methods

This chapter focuses on the materials and various experimental techniques used for the formation and characterization of samples for different aspects, such as protein aggregation, protein-lipid/ligand interaction, ion-protein interaction etc. that has been utilized throughout the work.
2.1 Materials
The human normal Hb (M. W.= 17 kDa) and OVA (M. W.=47 kDa) lyophilized stored at 2-8°C was purchased from Sigma Chemical Co. and was used as received without further purification. The 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and D-Glucose were purchased from Aldrich. The chemical details of the proteins [1] and ligand used are presented in Table-2.1.

<table>
<thead>
<tr>
<th>Hemoglobin (Hb):</th>
<th>M. W.</th>
<th>17 kDa (Monomer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDB ID</td>
<td></td>
<td>2H35</td>
</tr>
<tr>
<td>Solubility</td>
<td></td>
<td>Aqueous Water</td>
</tr>
<tr>
<td>Isoelectric pI</td>
<td></td>
<td>6.8</td>
</tr>
<tr>
<td>Physical dimension</td>
<td></td>
<td>~4.5x4.9x4.1</td>
</tr>
<tr>
<td>Native form</td>
<td></td>
<td>Tetramer, Globular</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ovalbumin (OVA):</th>
<th>M. W.</th>
<th>45 kDa (Tetramer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDB ID</td>
<td></td>
<td>1OVA</td>
</tr>
<tr>
<td>Solubility</td>
<td></td>
<td>Aqueous Water</td>
</tr>
<tr>
<td>Isoelectric pI</td>
<td></td>
<td>4.6</td>
</tr>
<tr>
<td>Physical dimension</td>
<td></td>
<td>~7.0x4.5x5.0</td>
</tr>
<tr>
<td>Native form</td>
<td></td>
<td>Tetramer, Ellipsoidal</td>
</tr>
</tbody>
</table>

| D-Glucose : | Chemical formula | C₆H₁₂O₆ |
|-----------------------------------------------|
| Molecular weight                       | 180             |
| Solubility                             | Water           |

<table>
<thead>
<tr>
<th>DPPC:</th>
<th>Chemical formula</th>
<th>C₄₀H₈₀NO₃P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>734.0</td>
<td></td>
</tr>
<tr>
<td>Solubility</td>
<td>Chloroform</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1: Represent the molecular structure and their properties of the molecules used in the experiments.
Chapter 2: Materials used and Experimental Methods

For pH variation experiment, HCl and NaOH (Merck, India) were used. KCl and NaCl (Merck, India) were used for salt variation and biomineralization experiments.

Silver nitrate (AgNO₃) and sodium borohydrate (NaBH₄) were purchased from E. Merck (Germany). The colloidal silver nanoparticle (AgNP) were synthesized following the standard borohydride reduction method developed by Creighton et al [2].

2.2. General Description of the Major Apparatus

2.2.1. Langmuir-Blodgett (LB) technique

2.2.1.1. Historical perspective

The formation of films at air/water interface (i.e. Langmuir film) has been known for centuries. Previously people have studied the interesting properties of oil and its interaction with water. At the earliest time, Sir Benjamin Franklin in 1774 carried out the first preparation of Langmuir film. Later on, Agens Pockel reported that the movable barriers on the water surface might control the oil film. In the next two decades, Rayleigh, Devaux and Hordy studied various properties. Lord Rayleigh deduced that these films were of only one molecular thick. The pioneer work in this scientific field was done by Irving Langmuir who first developed the theoretical and experimental concepts [3] of the behavior of the molecules in insoluble monolayer. Such monolayer, floating on the interface is referred to as Langmuir monolayer. In collaboration with Kathleen Blodgett [4] Langmuir designed a process to control and transfer these monomolecular layers onto solid substrates, which they referred to as Langmuir-Blodgett (LB) film. In recent time, computerized LB film deposition systems are available, where a controlled film deposition is possible. Nowadays the researches on LB film become popular in the field of surface chemistry or surface science. Moreover, lots of LB related works are found on various kinds of amphiphilic molecules such as protein, lipid, polymer, surfactant etc. [5, 6]. For some decades, the development was not in progress, since it deals with the molecular level phenomena until the right tools were developed, such as the scanning electron microscope (SEM) and the atomic force microscope (AFM) etc. to study the LB films. It becomes now a growing field of science due to the availability of nanoscale characterization techniques.
Nowadays LB technique covers both fundamental and technologically relevant interests. It covers almost the entire area of science from surface chemistry, material science, biophysical chemistry, environmental science, geology and others [6]. The lipid monolayer at the air/water interface has been recognized to be the half portion of cell membrane and used as a model membrane to study protein-lipid interaction [7]. Apart from these, lots of literature is appearing on the assembly of nanomaterials by the LB technique [8]. The monolayer of amphiphilic molecules can serve as a template for the growth of inorganic material (i.e. biomineralization/crystallization) at the organic-inorganic 2-D interface [9]. Moreover the adsorption kinetics as well as the aggregation behavior of the molecules could be well studied by this approach [10].

2.2.1.2. Basic Design of LB film deposition system

The basic principle of LB film deposition system is to prepare a water subphase and monolayer of amphiphiles and then compress the monolayer by a barrier with a desired controlled speed such that it cannot mechanically disturbs the monolayer. The basic component and design of a LB thin film deposition system is represented in Figure 2.1A. In this scheme, there is a bath with a dipper well for dipping, movable barriers, made out of Teflon to compress and expand the monolayer. The surface pressure of the monolayer is measured a Wilhelmy plate attached to using an electro balance. The substrate that can move up or down is attached to the dipper mechanism. The total control, data collection, and analysis are done using a computer.

![Figure 2.1](image.png)

**Figure 2.1:** Panel-A shows the schematic diagram of the Langmuir Blodgett film deposition technique. Panel-B shows the image of the commercially available LB instrument (model 2007DC, Apex Instruments Co. India)

The thin film deposition apparatus is a Teflon-barrier type Langmuir Blodgett (LB) trough (model 2007DC, Apex Instruments Co. India) as in Figure-2.1B is used for preparation, characterization, and deposition of film. The instrument is a double
barrier system with a dipper well for dipping. The trough width and length are 200 mm and 450 mm, respectively. The subphase volume is about 1.5 L. The LB trough is enclosed in a plexiglass box to reduce film contamination. The surface pressure is monitored using a Wilhelmy plate attached to the microbalance, to accuracy better than 0.05 mN/m. There is a temperature controller under the trough, which can be used to control the temperature of the subphase. The deposition is performed by vertically moving the substrate through the floating monolayer by means of a motor. Both the barrier and dipper speed can be controlled precisely. The whole system is computerized and the data acquisition and analysis is achieved by software.

2.2.1.3. Air/water interface

A boundary surface between two different phases is called an interface. The interface made by the air and water is called air/water interface and the thickness of this interface is only of the order of a few molecular dimension. It is approximated truly two-dimensional. The most important feature of an interface is the sudden change in density, dielectric constant, and composition that gives rise to an excess free energy at the interface.

The air/water interface is generally chosen for the LB work. The reasons for choosing water are that it has high dielectric constant, dipole moment and has ability to form hydrogen bonds, have predominant effects on the properties of this interface. The interfacial free energy is measured by the surface tension, γ that is defined as the following equation-2.1.

\[
\gamma = \left( \frac{\partial G}{\partial s} \right)_{T,P,n}
\]  

(2.1)

\(G\) is the Gibbs free energy of the system and \(s\) is the surface area at constant temperature \(T\), pressure \(P\), and composition \(n\). Water is known as universal solvent and contain a very high surface tension ~73 mN/m at 20°C, due to strong intermolecular attraction leading to pronounced ordering of the water molecules [11]. The introduction of any guest molecules can decrease the surface tension or surface energy and thus it can accommodate molecules at the interface in the monolayer form until its surface tension reduces from 73 mN/m to zero.

2.2.1.4. The Environment, Subphase & Surface Cleaning

The environment of LB Laboratory should be very much clean. Cleanliness is the first precaution in all the in situ experiments done in Langmuir trough and also for the
fabrication of LB films. The LB trough should be installed in a dust free environment.

The LB machine is placed on a vibration free table to minimize the vibration caused
by elsewhere. Removing the organic solvents and materials from the room has
ensured the cleanliness of the air. Such contaminants typically have an effect on the
order of magnitude of the measurements.

The requirement for the LB work is the deionized water, which is literally free
from ions and any other organic molecules. The standard water for LB work should
have neutral pH. In our work, the triple distilled water deionized by Milli-Q water
purification system from Millipore (U.S.A.) is used as the subphase. The resistivity of
the water is 18.2 MΩ-cm. The pH of the freshly prepared subphase is 6.8. The
temperature of the subphase is maintained constant by a temperature controller with
water circulating system.

Before spreading/injecting the material, the surface (the air/water interface) of the
subphase is cleaned thoroughly. A completely cleaned subphase should have zero
surface pressure irrespective of any position of the barrier. The zero surface pressure
is achieved by sucking the impurities from the surface of subphase by means of a
suction pump. This is conveniently done using a capillary tube attached to the water
pump. The precision of the cleaning should be at the molecular level such that the
surface pressure does not exceed 0.05 mN/m.

2.2.1.5. Langmuir Monolayer preparation

Generally the monolayer at the air/water i.e. Langmuir monolayer of amphiphilic
molecules are prepared by spreading the solution of the sample. The molecules were
dissolved in a volatile, water insoluble solvent to prepare a homogeneous solution.
For example, lipids are dissolved in chloroform or chloroform/ethanol mixture. For
water soluble molecules such as proteins, the monolayer can be prepared by spreading
or injecting the protein solution into bulk water subphase. After injecting into bulk
water subphase, some time is allowed to reach them to the air/water interface. Finally,
they form a monolayer at the air/water interface.

2.2.1.6. Pressure-Area (π-A) Isotherm Study

It is a two dimensional analogue of the thermodynamic isothermal pressure-
volume (p-V) diagram. Since the molecules of the spread monolayer are confined to
the air/water interface, this system is most suitable for studies of two dimensional
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association processes. The surface tension, \( \gamma \), is a measure of the free energy at the surface and the presence of other substances at the interface will normally lead to a lowering of \( \gamma \). Therefore, the surface pressure, \( \pi \), can be defined as the difference between the surface tension \( \gamma_0 \) of the "clean" surface and the surface tension \( \gamma \) in the presence of spreading molecule. The surface pressure can be represented by equation-2.2.

\[
\pi = (\gamma_0 - \gamma) \tag{2.2}
\]

Using a Wilhelmy plate arrangement, \( \pi \) can be measured as a function of monolayer area that in turn easily may be converted to the mean area available for a single molecule (area/molecule) of the monolayer. The compression isotherm is achieved by moving the barrier very slowly with a compression speed of \( 1 \text{Å}^2/\text{(molecule min)} \) to decrease the trough area. The barrier is then allowed to return to the maximum position at the same speed to get the expansion isotherm. The difference in compression and expansion isotherm is the hysteresis, which is a measure of stability of the monolayer. Thus the \( \pi-A \) isotherm could be experimentally plotted for a particular molecule. It is almost a molecular characteristic of a particular molecule. Hence, it is useful study to understand the molecular interaction, molecular dimension, orientation of the molecules forming Langmuir monolayer at the air/water interface [12]. There are various thermodynamic phases such as gaseous (G), liquid expanded (LE), liquid condensed (LC), solid (S) and collapse may be observed during the compression of the Langmuir monolayer. The G state in \( \pi-A \) isotherm is represented by the gaseous feature of the molecules at the air/water interface where no interaction between the molecules. For an ideal 2D G phase, inter molecular distance should be large enough in compare with the size of the molecules and thus follow the 2D gas equation as represented by equation-2.3.

\[
\pi A = kT \tag{2.3}
\]

Where, \( A = \text{area/molecule}, T = \text{absolute temperature} \) and \( k = \text{Boltzman constant}. \) Here, \( A \) is equivalent to \( V \) and \( \pi \) is equivalent to \( P \) in the ideal gas equation.

When the intermolecular distance is gradually decreasing by a movable barrier then other several phases will appear and the transition of the phases. The first phase transition is assigned to G to LE state and subsequently LC, S, and collapse phase. The LE-LC phase transition is very much significant since it is associated with the

physics of disordered to ordered transition. Several phenomena are occurring in this (LE-LC) transition region e.g. the domain formation in phospholipid molecule [13]. Upon further compression, the condensed state or solid state of the Langmuir monolayer could be achieved which has closely packed and uniformly oriented structure. This phase is suitable for preparing the LB film for practical purpose. The next phase transition is solid to collapse which is significant for the formation of monolayer to multilayer or three-dimensional structures due to mechanical stability [12, 14]. Figure-2.2 shows the different phases and the transition of phases of Hb during compression of Langmuir monolayer.

2.2.1.7. Compressibility (β-π) Study

The compressibility study is an important tool to characterize interfacial phenomena in Langmuir monolayer. It clearly demonstrates the phase transition, squeeze out of molecules from the monolayer etc [15, 16]. The compressibility coefficient (β) is calculated by taking first derivative of the π-A isotherm according to the following equation-2.4 [16].

\[
\beta = \frac{1}{A} \left( \frac{\partial A}{\partial \pi} \right)_T
\]  

(2.4)

Any phase transition is reflected as a positive peak in the β-π curve indicates the maximum compressibility (β_{max}) of monolayer, represents the greatest intermolecular cooperativeness. The asymmetry of the peak indicates that phase transition may consist of several steps [15].

2.2.1.8. Pressure-Time (π-t) Kinetics Study

The interfacial surface activity or the adsorption kinetics could be studied by monitoring the change in surface pressure (π) with time (t) in a LB trough. The π-t curve represents the surface activity of the molecule, injected at the bulk water subphase. The kinetics data may contain several features such as lag time followed by
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the growth of surface pressure. Generally, for the case of protein, the \(\pi-t\) kinetics is found to be three-step process. In the initial lag phase where the value of \(\pi\) remains nearly zero. This initial lag phase is the diffusion-limited regime, a significant characteristic of protein/enzyme adsorption process where the interface is lacking sufficient quantity of protein/enzyme for noticeable change in \(\pi\).

Thus, the lag time is the time required for attaining the minimum monolayer coverage for an effectual and measurable surface pressure. After the lag phase, an initial faster increase followed by a slower increase of \(\pi\) is observed. The first step is the diffusion of the molecule to just below the interface after which the adsorption is in effect at the air/water interface. In the second step, rearrangement among the adsorbed molecules takes place. Most of the cases, the kinetics data could be fitted by double exponential association equation after discarding the initial lag time as represented by equation-2.5 [17, 18].

\[
\pi_t = \pi_0 + A_1 \left[ 1 - \exp\left(-\frac{t}{\tau_1}\right) \right] + A_2 \left[ 1 - \exp\left(-\frac{t}{\tau_2}\right) \right] 
\]

Where \(\pi_t\) and \(\pi_0\) in equations (1) are the surface pressures at time \(t=t\) and \(t=0\) respectively. Constants \(A_1\) and \(A_2\) are the relative contributions and \(\tau_1\) and \(\tau_2\) are corresponding time constants of two mechanisms (diffusion with adsorption and rearrangement with unfolding) involving in adsorption process [16]. The different processes involved are shown in Figure-2.3.

2.2.1.9. Area-time Relaxation Study: Stability of the monolayer

The monolayer should be stable for the preparation of the LB film. The monolayer stability curve can be measured by monitoring the change in monolayer area with

Figure 2.3: Represents the \(\pi-t\) kinetics of a representative data of a protein molecule injected in the subphase, showing different regions following the double exponential association equation.
time for a constant surface pressure. This relaxation of monolayer area evolve with time as a single exponential decay as represented by the following equation-2.6 [19].

\[
\frac{A(t)}{A(0)} = a \cdot \exp \left( -\frac{t}{\tau} \right) + c
\]  

Where, \( A(t) \) and \( A(0) \) = area of the monolayer at time \( t \) and \( t=0 \) respectively, \( a, c= \) constant or parameter of the decay kinetics, \( \tau= \) time constant of the decay. Figure-2.4 shows a representative relaxation curve for Langmuir monolayer of protein.

Area relaxation measurements are very useful since the monolayer pass through several phase changes during compression of the monolayer. The mechanism responsible for the instability can be determined from the shape of the relaxation curve. It is important to track the evolution monolayer at the air/water interface at the very basic level.

2.2.1.10. Transfer of Monolayer onto Solid Substrate

The monolayer of amphiphiles is generally transferred onto solid substrate (hydrophilic) by vertical upstroke technique at constant surface pressure corresponding to the solid phase of the isotherm. The substrates are immersed into the subphase before the monolayer preparation. The monolayer on the air/water interface slowly compresses (~5 mm/min or less) to a desired surface pressure and then transfer onto the solid substrate. The schematic in Figure-2.5 illustrates the lifting of monolayer onto substrates. Sometime liquid condensed (LC) state is chosen for the LB film lifting. This transfer is accompanied with the molecular surface forces. The successful deposition (transfer ratio ~ 1) of the LB films depends on several factors e.g. material forming monolayer,
surface pressure, barrier compression rate, temperature and pH of the subphase, substrate preparation and dipping speed etc.

However, for the deposition of multilayer the dipping process are repeated after allowing some drying time between each deposition. Also for the deposition of monolayer onto hydrophobic substrate it is downward dipping for the first layer so that hydrophobic alkyl part of amphiphile attached to the substrate. There are three types of vertical depositions, such as Y, Z, and X type depending on the tail-tail, tail-head, or head-tail configuration of deposition (Figure-2.6A). Also sometime Langmuir Shafer (LS) technique has been employed to transfer the monolayer onto solid substrate (Figure-2.6B) by just the monolayer is taken on spoon like manner and placed on the substrate.

In this regard it is to be noted that the substrates (generally glass, quartz, and silicon wafer etc) were cleaned by ultrasonication method [18]. Substrates are first kept in freshly prepared chromic acid to remove any organic matter present in the substrate. Then it is washed out thoroughly with water from Milli-Q system. After that, the substrates are cleaned in a liquid soap ultrasonic bath followed by repeated rinsing with Millipore water before use. They are then immersed in acetone in an ultrasonic bath. Finally, they are cleaned using Millipore water in the ultrasonic bath. Uniform layer of water indicates the hydrophillicity of the slide.

Here it is to be noted that there are several advantages of LB films to prepare ultrathin protein or protein-lipid film such as (i) the low amount of protein/enzyme needed for membrane preparation, (ii) the ability to prepare a bioactive layer in a one-step procedure, (iii) the possibility to work at ambient temperature and pressure for monolayer design avoiding thermal treatments that can denature the biological compounds [20, 21].

*Figure 2.6: Panel-A shows the schematic illustration of LB film deposition technique. Panel-B shows the schematic illustration of LS film deposition technique.*
2.2.1.11. Protein molecules at air/water interface

Generally, protein/enzyme monolayer (Figure 2.7) is formed at air/water interface by spreading the aqueous solution of proteins/enzymes on the water subphase. Alternatively, the protein solution is injected at the bulk water subphase and then time is allowed to adsorb at the air/water interface.

The main problem in preparing the Langmuir monolayer of protein is the dissolution affinity of protein/enzyme molecules into water subphase. As result, the surface pressure and molecular area of the obtained monolayer of protein may decrease with time. One may not get actual area/molecule of the monolayer since the monolayer is not perfectly a Langmuir monolayer [10, 22, 23]. This problem sometimes could be solved by setting the appropriate pH and ionic strength of the subphase [10, 24].

2.2.2 Field Emission Scanning Electron Microscope (FE-SEM)

An electron microscope basically uses electron beams to illuminate the sample and thus it has much better resolution than an optical microscope. Since the wavelength of electron depends on the applied voltage, so increasing the voltage decreases the wavelength of electron. Thus the resolution of an FE-SEM is ~1-2 nm (for 1-20 KV SEM and it is ~0.3 nm for 30 KV).

The sample under study is exposed to the focused electron beam. As result of the energy loss by bombardment of different types of electrons are emitted such as secondary electron (low energy), back scattered electron (high energy), X-ray, diffracted electron and in elastically scattered electron. Figure-2.8A shows the different kinds of signals generated due to the interaction of electron with the sample.

The primary components of an electron microscope are electron gun, electromagnetic lens and the detector. The details schematic diagram of the different components of a FE-SEM is presented in Figure-2.8B. Since the microscope deals with the electron beam, it needs electromagnetic lens (optical analogue for electron)
for converging and focusing the beam. A scintillation photomultiplier detector receives the secondary electrons and an image of the sample surface is constructed based on the intensity variation with respect to scanning primary electron beam. In commercial SEM, it is called SEI image or secondary electron image. So there are separate detectors to receive the various signals. A detector is dedicated for X-ray giving the energy dispersive X-ray spectra (EDS). Thus from FE-SEM various kind of information of the sample could be extracted such as topography, surface characteristics, and specimen composition etc.

![Diagram](image)

**Figure 2.8:** Panel-A shows the different signal generated due to the electron-sample interaction. Panel-B shows the schematic diagram of the basic design, components and the working principle of FE-SEM.

In our experiment, the FE-SEM (JSM-6700F, JEOL, Japan) has been used to determine the surface morphology of the samples in LB film, drop cast film etc. The accelerating voltage was in the range 1 to 20 kV. The magnification could be varied up to 650,000 X. Probe-current was 10 μA. The instrument can be operated in two types of image modes e.g. Secondary Electron Image (SEI) and Backscattered Electron Image (BEI). The operating voltage was 0.5 - 30 kV with a lateral resolution of 1.2 nm at 15 kV and 2.2 nm at 1 kV. The images are taken at 5 kV, the lateral resolution, was 2 nm.

### 2.2.3 High Resolution Transmission Electron Microscope (HR-TEM)

The HR-TEM is very much popular for atomic resolution imaging as well as for diffraction pattern, fringe pattern, and EDS analysis. It utilizes the transmitted part of
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the incident electron beam and thus the limitation in resolution due to secondary electrons in SEM could be achieved.

![Diagram of basic components of HR-TEM instrument]

**Figure 2.9:** Shows the schematic diagram of the basic components of HR-TEM instrument. The image has been taken from ref-[25].

The basic components of TEM are very much similar to a SEM. It needs electron beam optics for higher current density in compare to SEM. It utilizes several magnifying lens for the transmitted beam before reaching the CCD camera. HR-TEM employed the phase contrast imaging technique for high resolution imaging (HR-image and FFT) of atomic layer [26]. The maximum resolution still achieved in TEM is ~0.08 nm. Figure-2.9 shows the basic diagram of a HR-TEM.

In our experiment the high-resolution transmission electron microscopy (HR-TEM, model No.: JEOL-TEM-2011) with an operating voltage of 200 kV, were used for high-resolution imaging and material characterization. Carbon coated copper grid having 300 square meshes were used as substrate. Dispersive X-ray Spectroscopy (EDX) was also performed in this instrument.

**2.2.4 Atomic force microscope (AFM)**

The AFM is one of the most useful tools for imaging, measuring, and manipulating matter at the atomic and molecular resolution. AFM has resolution of fractions of a nanometer and it is more than 1000 times better than the optical diffraction limit. The
basic principle of an AFM is based on sensing the surface with a mechanical probe. The mechanical probe used is a piezoelectric element that is capable of precise scanning with computer controlled command enable. The schematic illustration of working principle of AFM is illustrated in Figure 2.10.

The AFM consists of a cantilever with a sharp tip (probe) at its end that is used to scan the sample surface. The cantilever is typically made of silicon or silicon nitride with a tip radius of curvature on the order of nanometers. When the tip is scanning a sample surface, the forces between the tip and the sample lead to a deflection of the cantilever according to Hooke's law. In general, the force acting between the cantilever and the sample is a sum of Van der Waals, electrostatic, magnetic, electrodynamic and capillary forces [27]. Typically, the deflection is measured using a laser spot reflected from the top surface of the cantilever into an array of photodiodes. Thus from the obtained signal the topography of the sample surface is generated.

In generally the AFM is operating by the principle of maintaining constant force between the tip-sample not with constant height. If the tip maintained at a constant height during scanning there would be a risk to collide with the surface, causing damage. Hence, in most cases a feedback mechanism is employed to adjust the tip-to-sample distance to maintain a constant force between the tip and the sample. Traditionally, the sample is mounted on a piezoelectric tube (PZT), which allows the sample to move in the z direction for maintaining a constant force, and the x and y directions for scanning the sample. In newer designs, the tip is mounted on a vertical piezo scanner while the sample is being scanned in X and Y using another piezo block. The resulting map of the area $s = f (x, y)$ represents the topography of the sample. The AFM can be operated in a number of modes, depending on the application. In general, possible imaging modes are divided into static (also called

Figure 2.10: Schematic diagram of working principle of an Atomic Force Microscope.
contact) modes and a variety of dynamic (or non-contact) modes where the cantilever is vibrated. Apart from these AFM is capable of measuring force spectroscopy as well as magnetic domains by MFM technique.

The atomic force microscope VECCO diCP-II (Model No AP-0100) has been used to determine the surface roughness and morphology of the thin films. Besides its better-quality resolution, the AFM provides extraordinary topographic contrast, direct height measurements, and un-obscured views of surface features (no coating is necessary) compared to Scanning Electron Microscopes (SEM). Three-dimensional AFM images can be achieved without expensive sample preparation and yield far more complete information than the 2-dimensional profiles available from cross-sectioned samples. The tapping mode is used in air to minimize any kind of force exerted on the films from the scanning tip. Thin phosphorous-doped silicon cantilever (with no coating on front side and 50±10 nm aluminum coating at the back side) of resistance 1-10 Ω-cm is used for scanning. The thickness of the cantilever ranges from 3.5-4.5 μm with a length of 115-135 μm as well as width of 30-40 μm. The processed images are subsequently analyzed by software Proscan 1.8 image analysis.

2.2.5 Phase Contrast Inverted Microscope (PCIM)

The electromagnetic signal carries both amplitude and phase part. The phase part along with the amplitude contains important information that has been utilized in phase contrast inverted microscope (PCIM) to enhance the contrast of the sample images. This methodology is widely known as phase contrast technique. During 1930-40's Zernike discovered the phase contrast method and thus made a significant contribution in Optics. Zernike was awarded the Nobel Prize in Physics in 1953 for his demonstration of the phase contrast method, especially for his invention of the phase contrast microscope.

PCIM is mainly used for biological samples to enhance the contrast since the bio samples, which are not so conducting to give much contrast. Figure-2.11A shows the schematic basic components of a standard phase contrast inverted microscope and Figure-2.11B shows the PCIM camera from Motic Company. In some cases, we have used the phase contrast inverted microscope (Motic model AE31) fitted with MINI-LB2006C LB Trough of Apex Instruments Co. India for the study the surface morphology of monolayer at the air/water interface.
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Figure 2.11: Panel-A shows the basic schematic components of phase contrast inverted microscope (PCIM). Panel-B shows the image of commercially available PCIM (Image taken from Ref. [28]).

2.2.6 UV-Visible Absorption spectroscopy

The UV-visible spectrometer measures the absorbed light from UV region to visible region (IR region by recent spectrometer) following the well known Beer-Lambert law. For this purpose, the lamp used is mercury or hydrogen gas lamp for UV signal; the tungsten lamps for visible signal; and carborundum or Silicon carbide (SiC) for IR signal.

The steady-state electronic UV-Vis absorption spectra of protein solution and in films were recorded using a quartz cuvette having a path length of 1 cm. We used the commercial absorption spectrophotometer with model No Shimadzu UV-Vis 2401PC.
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The basic schematic diagram of a double window absorption spectrometer is presented in Figure-2.12.

2.2.7 Steady State Excitation and Emission spectroscopy

Emission spectroscopy is also a powerful spectroscopic tool to monitor the intrinsic fluorescence of proteins such as Tryptophan fluorescence ~335 nm. Since emission spectrum gives the excited state spectroscopy, thus it is very much sensitive to the environmental conditions. These environmental effects alter the relative energy of ground and excited states, and this alteration causes spectral shifts. The steady state fluorescence spectra were recorded by a Hitachi F-4500 fluorescence spectrophotometer using 1 cm quartz cell.

Measurements were carried out at room temperature. For solution spectra, the emissions are collected at right angle to the excitation beam. For LB films, the emission spectra were collected by placing the films on quartz plate at an angle 45° to the excitation beam so that the direct beam could be avoided. The schematic diagram of the optical system of an emission spectrophotometer is shown in Figure 2.13. The excitation light source is a 150 Watt high-pressure Xenon lamp. The beam from the source incidents on the excitation monochromator and wavelength selected light illuminates the sample. Part of this beam is directed to the monitor detector by means of a beam splitter. The emitted light goes to the output differentiator via emission monochromator and sample PMT. The resolution of the spectrometer is 1.0 nm. An online computer processes the signal.

On the other hand, the excitation spectra were recorded by the same spectrometer. It is basically gives the information about the excited states responsible for the emission. In recording the excitation spectra, the emission monochromator is set at the desired peak of emission spectra. Subsequently, the exciting monochromator is
scanned from the shorter to longer wavelength near emission band of the molecule of interest.

2.2.8 Time Resolved Emission Spectroscopy

Time resolved emission spectroscopy deals with the dynamic molecular processes in materials/molecules in the excited state. One can monitor these processes by using pulsed signal in time domain measurement system [29]. It can monitor the process at very shorter timescale such as in picoseconds ($10^{-12}$ s). Using a pulsed signal, molecules are excited and allows for relaxation to emit their photon before coming to the lower energy state. Generally the relaxation in excited state is in exponential in nature with lifetime ~ ps or ns. This time resolved data is important to understand the molecular processes. Each individual molecule emits randomly after excitation and we get an average lifetime. Fluorescence lifetimes especially for proteins are generally on the order of 1-10 ns. The basic schematic diagram for time resolved spectrometer is shown in Figure-2.14.

In our experiment, we have carried out the fluorescence lifetime in time correlation single photon counting (TCSPC) system. The samples were excited at 280 nm using a picoseconds diode laser (IBH Nanoled-07) in an IBH Fluorocube apparatus. The repetition rate is 1 MHz and the resolution of the instrument is ~40 psec. The fluorescence decays were collected on a Hamamatsu MCP photomultiplier (C487802). The fluorescence decays were analyzed using IBH DAS6 software.

2.2.9 Fourier Transform Infrared Spectroscopy (FTIR)

An FTIR spectrometer deals with the infrared region (from NIR to FIR) of electromagnetic spectrum. The basic components of an FTIR are shown schematically in Figure 2.15. It consists of a fixed mirror, a moving mirror, and a beam splitter. The beam splitter is a partially silvered that reflects and transmits light equally. The collimated IR beam from the source is partially transmitted to the moving mirror and partially reflected to the fixed mirror by the beam splitter. The two IR beams are then
reflected back to the beam splitter by the mirrors. The detector then sees the transmitted beam from the fixed mirror and reflected beam from the moving mirror simultaneously.

The two combined beams interfere constructively or destructively depending on the wavelength of the light and the optical path difference, \( \delta \) (cm) introduced by the moving mirror. To obtain an interferogram, \( I(\delta) \), the detector signal is digitized and recorded as a function of \( \delta \). The interferogram is a simple sinusoidal wave when a monochromatic source is used. For a continuum (or polychromatic) source, \( I(\delta) \) is a superposition of sinusoidal waves for IR light at all wavenumbers. At zero path difference (ZPD) or \( \delta=0 \), all the sinusoidal waves are totally constructive, producing a center burst on the interferogram.

The amide linkages between amino acid residues in proteins give rise to well-known signatures in the infrared region of the electromagnetic spectrum. In this regard, FTIR is a very sensitive tool to study the proteins amide bands. The position of the amide I (the C=O band in amide linkage at 1600-1700 cm\(^{-1}\)) and amide II band (the N-H stretch mode of vibration in the polypeptide linkage at 1500-1600 cm\(^{-1}\)) in the FTIR spectra of proteins are sensitive markers for conformational transitions in the secondary structure of protein [30]. The deconvolution of amide I band gives the percentage of helix, sheet, turns, intra, and intermolecular aggregates of protein in different protein lipid thin film. The FTIR characterization of the films are carried out on a Magna IR-750 spectrometer (series-II), Nicollet, U.S.A, operated in the absorbance mode at a resolution of 4 cm\(^{-1}\). A total of 300 scans are adapted for a good signal to noise ratio of the IR spectra. The spectra are recorded from 400 to 4000 cm\(^{-1}\). The films are deposited on silicon wafers, which are chemically very stable and not have strong lattice absorption bands in the useful infrared region. The absorption correction was done using a reference silicon wafer.
2.2.10 Raman Spectroscopy

The necessary components for the observation of Raman spectra are: (i) source of monochromatic radiation, (ii) an appropriate device to mount the sample illuminated optimally and efficient scattered light gathering mirrors, (iii) a dispersive system, and (iv) a detection device. Raman scattering being a weak process as one out of a million incident photons in normal case, one needs an effective dispersive device with high light gathering optics and a very sensitive detection device. Single, double, double-pass tandem and triple monochromators are now available as good dispersing system. Both photographic and photoelectric technique are used, PMT (photomultiplier tube) is still in common use. A very sensitive detection device, which has come up in last few years, is the CCD (charge-coupled device).

![Figure 2.16: A schematic diagram of the Laser Raman set up](image)

In the present work, however, we have used a Spex double monochromator (Model 1403) fitted with a holographic grating of 1800 grooves/mm along with a cooled photomultiplier tube (Model R 928/5, Hamamatsu Photonics, Japan) and photon counting system. Samples were illuminated by different laser radiation (514.5, 488.0, and 476.5 nm) available from a Spectra Physics Ar⁺ ion laser (Model 2020-05) at a power of 100 mW. Raman scattering was collected at a right angle to the excitation. Spex Datamate 1B was used for monochromator control, data acquisition, and analysis. A schematic diagram of the Raman set up is shown in Figure 2.16. Brief description of the instruments used is given below. The Raman measurements were done by solution mixture of the sample with silver nano colloid.

2.2.11 Circular dichroism (CD) spectroscopy

Circular dichroism (CD) is an important tool to characterize the protein secondary structure [31]. It utilize the polarized light and measures the differential absorption of...
left- (LCP) and right-(RCP) handed circularly polarized light as represented by equation-2.8.

\[ \text{CD (mDeg)} = \Delta A(\lambda) = A(\lambda)_{\text{LCP}} - A(\lambda)_{\text{RCP}}. \tag{2.8} \]

Where, \( A \) is the absorbance of light and \( \lambda \) is the wavelength. In a standard CD spectrometer, the differential absorption of polarized light is recorded as a function of wavelength. There appears positive or negative peaks depending on which among the left or right polarized light absorbed more. This absorption involves with the chiral chromophores such as proteins, peptide etc. CD can be used in analyzing the secondary structure or conformation of macromolecules, particularly proteins. Since the secondary structural elements of proteins such as the \( \alpha \)-helix and the \( \beta \) sheet are sensitive to its environment, e.g. temperature or pH, CD can be used to observe how secondary structure changes with environmental conditions or on interaction with other ligands, ions, nanoparticle etc. For quantitative analysis of the different conformation of proteins could be extracted by fitting the CD data by CD-Pro software [32].

**Figure 2.17: Schematic block diagram of a Circular Dichroism (CD) spectropolarimeter.**

Figure 2.17 shows a schematic diagram of CD spectrometer, where two beams of monochromatic left and right circularly polarized light are passes through modulator and sample. These polarized beams are absorbed following the Lambert-Beer’s law from both components to a different extend. Subsequently, both beams are detected by a photomultiplier, amplified, and recorded by a computer.

In this work, we have recorded the far-UV CD spectra by a Jasco J-815 CD spectropolarimeter (model no J-815-150S). The scan speed was 100 nm/min and the response time was 0.125 sec with a band width of 1 nm. Quartz cells with an optical path of 0.1 cm are used for room temperature measurements of solutions of protein.
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We have measured the range of spectra from 180 to 250 nm with a spectral resolution of 0.2 nm. Typically, three scans are accumulated and subsequently averaged. The CD spectra of LB films are collected by placing the films on quartz plate at the positions of quartz cells.

2.2.12 Dynamic Light Scattering (DLS) and Zeta Potential Measurement

DLS is basically a photon correlation spectroscopy used to determine the size distribution of small sized particle in colloidal form. On the other hand, the zeta potential is an electrostatic property of the particle surface and can be measured by DLS technique.

We measured all the size distribution and Zeta potential measurements were performed using a Malvern Zetasizer Nano ZS (Part no: ZEN3600) instrument at 25°C at scattering angle (175°) and detection angle (12.8°) with a He–Ne laser (laser power 4 mW, wavelength 632.8 nm and beam diameter 0.63 nm (l/e2) respectively). The machine has size measurement range 3.8 nm to 100 µm and having no practical range of zeta potential measurement. The samples were placed in standard Malvern zeta potential disposable capillary cells and polystyrene cuvettes for zeta potential and size measurements, respectively [45]. All measurements were repeated three times. The schematic diagram for DLS measurement system is presented in Figure-2.18.

2.2.13 X-ray Diffraction (XRD)

X-rays are electromagnetic radiation with a much shorter wavelength than light. They are produced when electrically charged particles of sufficient energy are decelerated. In an X-ray tube, the high voltage maintained across the electrodes draws electrons toward a metal target (the anode). X-rays are produced at the point of impact, and radiate in all directions. Tubes with copper targets, which produce their strongest characteristic radiation (Kα) at a wavelength of about 1.5Å, are commonly used for geological applications. If an incident X-ray beam encounters a crystal...
lattice, general scattering occurs. Although most scattering interferes with itself and is
eliminated (destructive interference), diffraction occurs when scattering in a certain
direction is in phase with scattered rays from other atomic planes. Under this
condition, the reflections combine to form new enhanced wave fronts that mutually
reinforce each other (constructive interference). The schematic diagram for X-ray
diffraction is shown in Figure-2.19.

The relation by which diffraction occurs is known as the Bragg law or equation.
Because each crystalline material has a characteristic atomic structure, it will diffract
X-rays in a unique characteristic pattern. The basic geometry of an X-ray
diffractometer involves a source of monochromatic radiation and an X-ray detector
situated on the circumference of a graduated circle centered on the powder specimen.

Figure 2.19: Panel-A shows the schematic diagram for X-ray diffraction technique.
Panel-B shows the basic XRD arrangement.

In contemporary with X-ray diffraction, the grazing angle XRD technique (GIXD)
is important for very thin material layer characterization. We have carried out grazing
XRD of the biomineralized crystals on glass substrate by X-ray diffraction (XRD)
machine (model No.-PW-1710) using CuKα radiation of wavelength 0.154 nm.

2.2.14 Protein-Ligand Docking Simulation

To understand the protein-ligand complexation, we have carried out the docking of
the macromolecule and the ligand by Auto Dock 4.2 [33]. For docking purpose, the
pdb file of the proteins was taken from protein data bank (RCSB-PDB). Sometimes
the sdf file of the ligand was converted into pdb file by PYMOL-v1.1 [34]. The
structures of the proteins were validated using Procheck-v.3.0 [35], which measures
the stereochemical quality of the protein structure by measuring the allowed region of
Ramachandran plot. The Auto Dock Tool (ADT-version 1.5.4) [33] was used to read
and correct the pdb file, such as removing water molecules, adding charges and
hydrogen etc. The energy-based Auto Dock includes the interaction terms for short-range van der Waals and electrostatic interactions, loss of entropy upon ligand binding, and hydrogen bonding. In generally at first blind docking of the proteins was performed with larger grid spacing using Auto Grid and after that the specific docking at the specific site position was carried out with spacing 0.375 Å and dimension of (65 × 65 × 65) points using Auto Grid. Here the Lamarkian genetic algorithm (LGA) [33] was used with genetic algorithm population size and maximum number of energy evaluations could be set according to purpose. During the docking, the different parameters could be chosen as default or set according to choice. All the output could be viewed by ADT tool.

REFERENCES


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