This chapter illustrates the influence of KCl on the interfacial surface activity and conformation of hemoglobin (Hb) using LB technique. The studies were done in absence and in presence of KCl salt, at various concentrations of KCl ($C_{KCl}$). The $\pi$-$\alpha$ study shows that the surface activity, magnitude of diffusion and rearrangement of Hb at air/water interface is a function of $C_{KCl}$. The compression of Hb monolayer was found to be involved with the free energy change of few (~5-25) Kcal/mol of Hb. The changes in area/molecule, free energy as well as other results indicate that the influence of KCl on the Hb monolayer is in line with modified DLVO theory. There is a structural/conformational change of Hb in presence of KCl salt and also there is a formation of biomineralized KCl crystal in the LB film. The overall results show that the intermolecular forces and the surface activity as well as the population of $\alpha$-helix of Hb can be tuned by KCl salt and unfolded Hb is responsible for the formation of biomineralized KCl crystal.
6.1. Introduction

The study of proteins/enzymes at the fluid/fluid and fluid/solid interfaces at molecular or sub-molecular level is a subject that has received growing interest in the advancement of both fundamental as well as potential application in various fields, such as nanobioscience, molecular biology/biotechnology etc. [1-4]. So far, for the development of biosensor or any other biomolecular device application, the immobilization of protein/enzyme onto a suitable substrate with minimum molecular aggregation is particularly important [5-7]. In addition, the immobilization on solid support without denaturation is important for chromatographic purification of different kinds of drugs, peptides, proteins as well as antibodies [8, 9]. There are several techniques to immobilize biomolecules onto solid matrix surfaces such as sol-gel process, self-assembly, Langmuir-Blodgett (LB) techniques, etc. [10-17]. Among these, the LB technique is useful not only on the formation of molecular organization onto solid substrate, but also on the study protein/enzyme at the molecular level [18-20]. On the other hand, the study of denaturation, aggregation, dissociation, as well as unfolding in different physiochemical environment has some relevance to understand the molecular mechanism of various diseases such as Thalassemia, Neurodegenerative diseases, Alzheimer's etc [21-23].

The use of KCl salt in relation with the protein covers from various fundamental to application related fields [24-31]. More precisely, in the preparation of bio-film of protein/enzyme by LB technique, KCl is frequently used to build up closely packed, organized Langmuir monolayer [24, 26]. Apart from the formation of Langmuir monolayer, KCl has contributions in various fundamental bio related processes such as cell growth regulation [27], muscles contraction [28], potassium Homeostasis, and Hypokalemia [28], as well as potential application in protein crystallization [30], and food industries [31]. In this context, it is reported that the inorganic salts can induce structural change as well as dimerization of Hb [32-34]. In addition, KCl can retain the folded intermediate with molten globule like characteristics of Lysozyme at alkaline pH [35]. However, the detail investigation about the influence of KCl on the internal conformation of Hb and its consequence to the surface activity is not well established.

In this chapter, the surface activity of Hb has been studied at an air/KCl containing water interface by monitoring in situ surface pressure (σ)-time (t) adsorption growth,
pressure ($\pi$)-area ($A$) isotherm and compressibility ($\beta$)-pressure ($\pi$) data. The molecular kinetics of protein adsorption with $C_{KCl}$ were analyzed by fitting a double exponential association equation. The $\pi$-$A$ isotherm and $\beta$-$\pi$ compressibility data reveal the squeezing out of Hb from the monolayer at different $C_{KCl}$. The changes in area/molecule of Hb with $C_{KCl}$ are explained in the context of modified DLVO theory of ion-protein interaction. The conformational evolutions of Hb with $C_{KCl}$ were characterized by CD and FTIR spectroscopy in solution and LB film. The Field emission scanning electron microscopy (FE-SEM) was employed to monitor morphological aspects as well as the structural changes of Hb in LB films at different conditions.

Apart from these, the use of high concentration of salt may also lead to another kind of phenomena such as biominalization, protein fibrillation etc [36, 37]. These studies on are important also for pathological aspect of biominalized stone formation [38-40] as well as in various material fabrication technology [41-43]. Thus, the study of the salt effect at the low as well as high concentration region has been undertaken for study in this chapter. The biominalized KCl crystal formation has been monitored by FE-SEM imaging in the LB films and characterized by EDS and diffraction technique in HR-TEM.

6.2. Experimental Section

Lyophilized human adult hemoglobin, stored at 2–8°C and KCl has been used for the studies. The details of the characterization tools, the LB technique, the Milli-Q water used and the substrate cleaning process etc have been described in chapter-1 and 2.

6.2.1 Pressure–Area ($\pi$-$A$) Isotherm Measurement

We have prepared the pure water subphase in LB trough filled with Milli-Q water with resistivity=18.2 MΩ-cm and pH=5.5. For pressure-area ($\pi$-$A$) isotherm measurement of Hb on the different subphases, an aqueous solution of Hb ($C_{Hb}$ = 0.1 mg/mL) was spread on a water subphase. The spreading was done using a micro syringe in such a manner that the surface pressure did not rise above 0.5 mN/m. The number of molecules thus spread was calculated using the average molecular weight of Hb monomer as 17 kDa [44]. The monolayer were allowed to stabilize for 20 min...
and then slowly compressed with a compression of 1Å²/(molecule-min). The computerized LB trough described in chapter-2, was used for these studies.

6.2.2 Adsorption growth kinetics (\(\pi\)-t) at air water interface

To study the \(\pi\)-t adsorption growth kinetics measurements in different salt containing subphases, a fixed volume (10 mL) of aqueous solution of Hb of particular concentration was injected into the subphase of volume 750 mL (typical dimension 200 mm \(\times\) 100 mm \(\times\) 37.5 mm) to attain the required final concentrations of Hb in subphase of LB trough. Final \(C_{Hb}\) used were 0.0005, 0.001, 0.003 mg/mL respectively in our study. The salt (KCl) concentrations were adjusted prior to protein addition.

6.2.3 Preparations of LB Film, Cast Film and Their characterizations

For LB film preparation from the salted subphase the bare slides were dipped into the salted subphase and then proteins were spread and waited for the growth of surface pressure for film lifting. The time dependent (such as 10 min, 30 min, 1h etc) LB films were prepared by waiting for different times after spreading the proteins. The monolayer prepared at the air/water interface, were transferred very carefully through up stroke with a speed of 5 mm/min at constant surface pressure on to slides.

The cast films were prepared by drop casting the mixture of salt-protein with proper concentration onto hydrophilic substrate.

For the study of biomineralization of KCl crystal in Hb monolayer, we have lifted the LB film of Hb-KCl (with \(C_{KCl} \geq 0.5\) M).

The characterizations were made after preparing the LB films onto different substrate. For FE-SEM measurement such as surface morphology, EDS etc., the LB films were prepared on fine hydrophilic glass substrate. For FTIR measurement the LB film were lifted onto silicon substrate. The XRD measurements were performed in cast film. For CD measurement the salt-protein solution were prepared and made using quartz cuvette. The biomineralized KCl crystal in Hb monolayers were characterized by FE-SEM and HR-TEM.

6.2.4 Calculation of Fractal Dimension

The fractal dimension calculations of the FE-SEM images were carried out by using Image J program (version-1.41o). The FE-SEM images were converted in the black and white 8-bit digitized version (monochrome format) by Microsoft Paint.
The fractal dimensions were calculated using box counting method implemented within the ImageJ program [45].

6.3. Results and discussion

6.3.1. \(\pi-A\) Isotherms and \(\beta-\pi\) Compressibility Study

Figure 6.1A displays the \(\pi-A\) isotherms of Hb monolayers at air/water interface with different \(C_{KC1}\) at the subphase. The Trace-a in Figure 6.1A represents the isotherm of the pure Hb monolayer. We have estimated the area/molecule at different condition before the collapse region of isotherms [46]. In our case, the mean area/molecule estimated before the collapse at 20 mN/m is \(\sim 6.5\) nm\(^2\)/molecule.

The inset of Figure 6.1A represents the structure and dimensions of an Hb monomer as obtained from Protein Data Bank (PDB-2h35) [47] where the maximum diameter of individual monomer of Hb is \(\sim 4.5\) nm. Hence, the value of area/molecule of Hb monomer should be \(\sim 20\) nm\(^2\) depending on the orientation of Hb on the subphase. Here the observed value of molecular area (6.5 nm\(^2\)/molecule) is less than that estimated (20 nm\(^2\)/molecule), indicating that not all the Hb molecules are at the interface but a few are obviously dissolved in the bulk subphase.

With different subphases, spreading the same number of molecules in the interface and getting lesser area/molecule indicates that molecules are more soluble in that particular subphase. It is to be noted that the Hb monolayer is very much sensitive to the pH of the subphase, primarily due to change in solubility of Hb molecules into the subphase [46]. In this regard, the use of salt at the subphase is usual practice to resolve this kind of problem for protein/enzyme, for building up organized and closely packed Langmuir monolayer. Some earlier works in case of proteins/enzymes...
like Gluten, pepsin, alcohol dehydrogenase (ADH), Bovin serum albumin (BSA), Lysozyme are to be noted [12, 23, 48, 49].

In this context, it is also necessary to state that the obtained area/molecule does not correspond to the true area/molecule due to the dissolution affinity of Hb molecules into the subphase. Traces (b-g) in Figure 6.1A represent the isotherms of Hb at various $C_{KCl}$ from 5 mM to 1.5 M. Using the KCl salt at the subphase, a near perfect Langmuir monolayer of Hb is built up (trace-f, Figure 6.1A), which demonstrates the true area/molecule of Hb. This necessarily means that maximum number of molecules were at the interface and very little were in the bulk. In this regard, it necessary to mention that the increase of area/molecule does not correspond to the increment of molecular size, rather it indicates the decrease of solubility of Hb molecules at the air/water interface.

The $\pi-A$ isotherm of pure Hb monolayer (trace-a, Figure 6.1A) possess a number of characteristics such as, a gaseous (G) state between the surface pressure 0.5 to 2 mN/m, a liquid (L) state up to the surface pressure $\sim$ 15 mN/m as well as a bending region around 25-30 mN/m. This G to L transition upon compression is supposed to occur due to the structural rearrangement of the hydrophobic moieties of Hb molecules. Similar observations are there for other proteins/enzymes like ovalbumin (OVA), Pepsin, ADH, and apolipoprotein [12, 13, 50, 51]. Similar types of bending are observed in case of KCl containing subphases (Traces b-g, Figure 6.1A). This bending in isotherm is a characteristic of Langmuir monolayer of protein/enzyme, which is supposed to be originate due to the partial squeeze-out of protein proceedings to the full collapse [12, 13, 50]. As mentioned earlier, the use of salt at the subphase can decrease the solubility of protein/enzyme resulting into formation of organized monolayer of protein [50, 51]. In addition, by using high concentration of salt ($\geq$ 0.8 M), the isotherms almost overlap and reach the true area/molecule [12, 52]. In this regard, the change in monolayer characteristics of Hb with $C_{KCl}$ is further extracted by analyzing the $\beta-\pi$ compressibility plot as discussed below.

The compressibility coefficient ($\beta$) is calculated using the standard thermodynamic formula as described by equation-2.4 in chapter-2 [12]. Figure 6.1B shows the $\beta-\pi$ plot of Hb monolayer at different $C_{KCl}$, derived from the experimental data of $\pi-A$ isotherms. This plot may be helpful in understanding the elasticity (inverse of compressibility) as well as the squeezing out of Hb from monolayer as a function of

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$C_{\text{KCl}}$ and its consequence to the unfolding or refolding of protein/enzyme [12, 50]. In addition, it is reported theoretically and experimentally that the overall attraction between the residues of protein is ultimate necessity for the folding of protein into more globular, helical structure, which causes the hydrophobic collapse i.e. the molten globule state of protein [53].

Trace (a) in Figure 6.1B represents the $\beta$-$\pi$ curve of pure Hb monolayer shows a broad peak around 30 mN/m and the compressibility increases between $\pi \approx 22-30$ mN/m (at the bending region of the isotherm), indicates loss of elasticity. This loss of elasticity or increase of compressibility may be due to the unfolding [50], and/or partial squeeze out of protein proceedings to the full collapse [12, 13, 50] expected to be a natural process at the interface. In this regard, it is relevant to state that Rader et al had proven the loss of rigidity or increase of compressibility of protein during the unfolding process [54]. With the addition of salt at the subphase, not only the position of $\beta_{\text{max}}$ shifts towards higher pressure, but also the value of $\beta_{\text{max}}$ increases, which is expected due to unfolding and/or partial squeeze out of Hb.

The Traces (b) and (c) in Figure 6.1B represent the $\beta$-$\pi$ curves at $C_{\text{KCl}} = 0.8$M and 1.5 M respectively. The shifting of $\beta_{\text{max}}$ towards higher $\pi$ with the increase of $C_{\text{KCl}}$ implies that the film can be compressed to higher $\pi$ value. The variation of the value of $\beta_{\text{max}}$ and the $\pi$ at $\beta_{\text{max}}$ of Hb layer with different $C_{\text{KCl}}$ in the subphase is presented in the inset of Figure 6.1B. A rapid increase of $\beta_{\text{max}}$ up to certain $C_{\text{KCl}}$ followed by a slow increase with $C_{\text{KCl}}$ is evident from this Figure.

In addition, the plot of change in area/molecule with $C_{\text{KCl}}$ at different surface pressures is presented in Figure-6.2. These results indicate that the increment of $C_{\text{KCl}}$ increases the area/molecule of Hb and ultimately reaches saturation at $C_{\text{KCl}} \geq 0.8$ M. This increment of area/molecule represents a decrease of the solubility of Hb at the air/water interface with $C_{\text{KCl}}$. Ion-protein interaction that is happening at the bulk and

![Figure 6.2: Represent the plot of area/molecule of Hb with $C_{\text{KCl}}$ at different surface pressure. The inset figure represents the change in Gibbs free energy of Hb monolayer with varying concentration of KCl.](image)
at the air/water interface may be responsible for this observation and could be explained using the arguments of DLVO theory as discussed later.

We have also calculated the Gibbs free energy change ($\Delta G_c$) due to compression (shown in inset of Figure 6.2 and Table-6.1) represented as [24].

$$\Delta G_c = \int_{A_1}^{A_2} \pi dA$$  \hspace{1cm} (6.1)

This is actually the work required to compress a monolayer from a state where no molecular contacts occur (start of compression, $A_1$) to the end of compression ($A_2$). It measures the intermolecular forces in the film and depends on the interaction between the protein molecules and the ions present in the subphase. For Hb monolayers in the absence and in the presence of KCl salt (with increasing $C_{KCl}$), $\Delta G_c$ values are depicted in Table-6.1 and displays in inset of Figure 6.2.

**Table 6.1: Different Thermodynamic Parameters of Hb Monolayer at Air/Water Interface with Varying Concentration of KCl ($C_{KCl}$) at the Subphase $^a$**

<table>
<thead>
<tr>
<th>$C_{KCl}$ \ (M)</th>
<th>$A_1$ \ ((nm$^2$))</th>
<th>$A_2$ \ ((nm$^2$))</th>
<th>$A$ \ (mN/m)(nm$^2$/molecule)</th>
<th>$\Delta G$ \ (Kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>9.30</td>
<td>6.24</td>
<td>40.50</td>
<td>5.81</td>
</tr>
<tr>
<td>0.005</td>
<td>12.80</td>
<td>8.73</td>
<td>49.80</td>
<td>7.14</td>
</tr>
<tr>
<td>0.010</td>
<td>14.50</td>
<td>9.95</td>
<td>50.50</td>
<td>7.24</td>
</tr>
<tr>
<td>0.020</td>
<td>14.50</td>
<td>9.95</td>
<td>50.50</td>
<td>7.24</td>
</tr>
<tr>
<td>0.500</td>
<td>21.80</td>
<td>14.25</td>
<td>80.20</td>
<td>11.50</td>
</tr>
<tr>
<td>0.800</td>
<td>28.90</td>
<td>19.16</td>
<td>105.40</td>
<td>15.12</td>
</tr>
<tr>
<td>1.500</td>
<td>38.00</td>
<td>23.25</td>
<td>169.30</td>
<td>24.28</td>
</tr>
</tbody>
</table>

$^a$ $A_1$ and $A_2$ are the values of the molecular area where $\pi$ starts increasing and at the end of compression where $\pi=20$ mN/m respectively. $A$ is the area under the $\pi$-$A$ curve up to the end of compression.

It is seen that the change in free energy increases with increase of $C_{KCl}$ is of the order of few (typically 5-25) Kcal/mol of Hb. This result implies that more energy is needed to compress the monolayer at higher $C_{KCl}$, also supporting the observation of shifting of $\beta_{max}$ towards higher $\pi$ with the increase of $C_{KCl}$. This change in free energy is the characteristic of any monolayer at the air/water interface and this calculation is relevant since various interfacial processes are involved with the different energetic balances at the bio-interface [55].
6.3.2. Adsorption Growth kinetics (\(\pi\)-t) of Hb at air/water and air/KCl water interface

As the interactions between ions and protein/enzyme in aqueous solutions play an important role, adding up of controlled amounts of salts in the subphase water is useful to control the solubility of protein/enzyme into subphase water.

Figure 6.3A represents the growth curves of Hb for \(C_{Hb} = 0.003\) mg/mL with varying \(C_{KCl}\) at the subphase. This is a plot of surface pressure contributed by Hb molecules at the air/water interface with time. Curve (a) is for pure water subphase, whereas curves (b) to (e) show adsorption behavior of Hb in KCl containing subphase with \(C_{KCl} = 0.01, 0.1, 0.5\) and 1M, respectively. Even at very long times, a continuous growth of \(\pi\) without any saturation in salted subphase is observed (curves (b) to (e)). In other words, the time necessary to achieve adsorption equilibrium is very long. A faster adsorption has been found in case of KCl containing subphase than a pure water subphase. The increase of \(C_{KCl}\) increases the diffusion rate of Hb, enables more Hb molecules to come to surface very quickly and thus the \(\pi\)-t curve becomes steeper.
Figure 6.3A shows that there is an initial lag time ($\pi_{lag}$) where $\pi$ values remain near zero, a significant characteristic in adsorption of protein/enzyme at the air/water interface as described also in the earlier chapter [50, 56]. In course of time, the number of Hb molecules at the interface increases and eventually come closer to each other within their interaction radius. As a result, $\pi$ starts increasing after the period of $\pi_{lag}$. We have plotted the variation of $\pi_{lag}$ with $C_{KCl}$ involved with adsorption of Hb at air/water interface in Figure 6.3B, for $C_{Hb}= 0.0005$, 0.001 and 0.003 mg/mL. In absence of salt, there is a lag time ($\pi_{lag} \approx 2600$ s) and in presence of salt, the lag time vanishes gradually with $C_{KCl}$.

After the period of $\pi_{lag}$, the curves consist of two parts; an initial faster increase followed by a comparatively slower increase, depending on $C_{KCl}$. For analyzing the kinetics of adsorption of protein at the air/water interface, the curves of Figure 6.3A are fitted after discarding the lagtime, by using the double exponential association equation as described by equation-2.6 in chapter-2 [50, 57] and the fitted results are summarized in Figure 6.C and Table 6.2. Thus by in situ $\pi$-$t$ measurements the information regarding the unfolding of protein can be found out. However, it is not as good as the in situ IR measurement.

**Table 6.2: Fitting Parameters of the ($\pi$-$t$) Adsorption Growth Kinetics of Hb ($C_{Hb}=0.003$mg/mL) with Different $C_{KCl}$ by Double exponential Association Equation**

<table>
<thead>
<tr>
<th>$C_{KCl}$ (M)</th>
<th>$A_1$</th>
<th>$t_1$ (s)</th>
<th>$A_2$</th>
<th>$t_2$ (s)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>7.88±0.04</td>
<td>1185.0±17.0</td>
<td>18.64±0.05</td>
<td>6087±40.0</td>
<td>0.999</td>
</tr>
<tr>
<td>0.01</td>
<td>11.28±0.05</td>
<td>512.0±3.0</td>
<td>12.62±0.03</td>
<td>4048±29.0</td>
<td>0.999</td>
</tr>
<tr>
<td>0.10</td>
<td>19.10±0.09</td>
<td>50.0±1.0</td>
<td>7.31±0.04</td>
<td>1276±13.0</td>
<td>0.996</td>
</tr>
<tr>
<td>0.50</td>
<td>20.00±0.10</td>
<td>74.0±0.7</td>
<td>7.2±0.04</td>
<td>1210±0.94</td>
<td>0.994</td>
</tr>
<tr>
<td>1.00</td>
<td>23.00±0.01</td>
<td>69.3±0.4</td>
<td>6.8±0.04</td>
<td>1125±0.74</td>
<td>0.993</td>
</tr>
</tbody>
</table>

$a$ $A_1$ and $A_2$ are the relative amplitudes and $t_1$ and $t_2$ are corresponding time constants of two mechanisms involving in association process. ($R^2$) is the residual square correlation coefficient.

From Table 6.2 it can be seen that the $A_1$ component increases with $C_{KCl}$ along with the faster decrease of $t_1$. This means adsorption is larger and the diffusion process becomes faster with increasing $C_{KCl}$. A much lesser value of $t_2$ in the salted subphase as compared to that in pure water subphase implies that diffusion of Hb is
faster in KCl containing subphase. On the other hand, there is a decrease of $A_2$ component along with the decrease of $t_2$ with increasing $C_{KCl}$. The second time constant ($t_2$) may be attributed to rearrangement with unfolding of the protein structure. At higher concentration of salt, the availability of protein molecules near the interface is more due to decreased solubility; as a result, the docking of protein at the interface is enhanced and become faster. Moreover, the decrement of $A_2$ with increasing $C_{KCl}$ suggests that the relative contribution of unfolding of Hb is small at higher $C_{KCl}$.

In this regard, another important point to note is that the charge screening effect of KCl salt to the protein may control the properties of the protein molecules, especially at pH away from its isoelectric pH [58]. The isoelectric point of Hb molecules is 6.8 [46] and at pH=5.5 it is known to have electrically positive charge ~ +13 [59]. It is documented in literature that increase of salt concentration enhances the hydrophobic interaction in a concentration dependent manner [60]. Our data (Figure-6.1, Figure-6.3 and Table-6.2) indicate the increment of the hydrophobicity of the Hb molecules with the $C_{KCl}$ due the charge screening effect of KCl.

### 6.3.3. Study of Surface Morphology of Hb Monolayer in the Absence and Presence of KCl Salt

Figure 6.4A and 4B represent high resolution FE-SEM images of Hb monolayer in LB film lifted at $\pi = 20$ mN/m in absence of KCl salt and in presence of a 0.5 M KCl salted subphase, respectively. The FE-SEM images show that the Hb LB films in the absence and in the presence of KCl salt are almost homogeneous at the nanometer scale. However, careful observation shows that the Hb monolayer consists of some globular objects with larger dimensions in absence of salt than in presence of KCl salt.

![Figure 6.4: Represents the FESEM image of Hb monolayer in LB film lifted at 20mN/m: Panel-A and B for pure water subphase and KCl containing salted (C_{KCl}= 0.5 M) subphase.](image-url)
Actually, the observed objects in absence of salt (Figure 6.4A) are the tetrameric Hb/higher aggregates, having sizes typically around 18-25 nm. Moreover, the globular objects in presence of 0.5 M KCl salt (Figure 6.4B) are the dimeric Hb/lower aggregate having dimensions ~8-12 nm. The maximum diameter of the Hb monomer obtained from PDB [47] is ~4.5 nm as presented in the inset of Figure 6.1A. Therefore, the dimer and tetramer dimension should be ~10 nm and ~20 nm, respectively. Here the FE-SEM images are consistent with the earlier literature regarding the structural change as well as dimerization of Hb by inorganic salt [33, 34]. In this regard, our prediction from FE-SEM images and other characterizations concerning the structural change of Hb by addition of KCl salt at the subphase is at least qualitatively consistent. The FE-SEM observations are well corroborated with the calculation from PDB and other studies.

We have also studied the Hb-KCl LB film higher KCl concentration and longer Hb-KCl reaction time, which leads to the biomineralization of KCl crystal as, described in the later section.

6.3.4. Study in CD spectroscopy

Figure 6.5 represents the UV-CD spectra of Hb (0.05 mg/mL) in solution with different $C_{KCl}$ (= 0, 5, 10, 100, and 1000 mM). Here CD spectroscopy in solution is employed to understand the central phenomena regarding the ion-protein interaction at the secondary structure level. The intensity change in the film sample may not be quantitative unless there is any change of peak position in CD band. This is the technical reason for doing CD spectroscopy in solution. Since the structure of Hb is predominantly $\alpha$-helical [61], we have measured the CD spectra of Hb (Figure 6.5) with increasing $C_{KCl}$ to find out the change in secondary structure of Hb with KCl. Thus, we have tried to estimate the extent of KCl-salt induced conformational change of Hb. The amount of $\alpha$-helix and other conformations are calculated from solution CD data using SELCON3 (CD-pro)
program. It is found that the amount of α-helix of pure Hb is ~79.9%, which is in agreement with the earlier data with Hb [62].

In this regard the 222 nm CD band is frequently used to assess the change in helical conformation of a protein/enzyme as well as to find out the kinetics of protein folding [63, 64]. The inset of Figure-6.5 is a plot of change in ellipticity at 222 nm with $C_{KCl}$. It shows that the ellipticity becomes more negative with increase of $C_{KCl}$, implying the increase of helix conformation, although the change is not very significant up to $C_{KCl} = 0.01$ M. Recently Jain et al has controlled and stabilized the folded population of a surface-active amphiphilic peptide by electrolyte concentration [65]. The increase in helix ultimately stabilizes the folded conformation followed by the breaking of hydrogen-bonded network of water molecules at the interior of protein [66, 67]. Since according to Chen et al [66] the interfacial water structure are more affected in presence of salt, so our prediction is that stabilization of folded conformation in a monolayer will be more in the presence of salt.

### 6.3.5. Study in FTIR Spectroscopy

The FTIR spectroscopic technique is widely recognized to be extremely sensitive, especially to the amide bands (amide-I ~1600 to 1700 cm$^{-1}$ and amide-II ~1500 to 1600 cm$^{-1}$) of protein/enzyme [12, 68, 69].

![Figure 6.6](image)

**Figure 6.6**: Panel-A represent the FTIR spectra of Hb-cast film in the amide-I and amide-II region. Panel B, C and D represents the deconvoluted normalized FTIR spectra of amide-I bands in Hb-cast film, Hb in LB film lifted at in pure water subphase and Hb in LB film lifted at 1 M KCl subphase respectively. In both cases, LB film is lifted at 20 mN/m surface pressure.
The panel A of Figure 6.6 shows the FTIR spectra of Hb-cast film in amide-I and amide-II region without any normalization. The panels B, C, and D of Figure 6.6 describe the deconvoluted FTIR spectra of the normalized amide-I band of Hb-cast film, Hb-LB film lifted at 20mN/m, and Hb-LB film lifted at 1M KCl subphase, respectively. Here the FTIR data of Hb at $C_{\text{KCl}} = 0.01$, and 0.1 M are not shown. Since the LB films were lifted on hydrophilic Si (100) substrate via upstroke technique, it contains bilayer.

We have deconvoluted the amide-I band that spans different conformational components, such as $\alpha$-helix, $\beta$-sheet, turns, unordered structures, or random coil, intra- and intermolecular aggregates [70, 72]. The fitting and area under each deconvoluted peaks gives the relative contribution of the components.

The results as obtained from the deconvolution of amide-I band of Hb at different conditions are summarized in Table 6.3 and Figure 6.7. The amount of $\alpha$-helix, $\beta$-sheet and other conformational elements of Hb (in %) are presented in Table 6.3. In addition, the relative amount of $\alpha$-helix over $\beta$-sheet of Hb at different condition are described by bar diagram in Figure 6.7. It is seen that the amount of $\beta$-sheet in cast film is largest which necessarily mean that the aggregation of protein is great in cast film. In the LB film, the helicity in comparison to $\beta$-sheet is improved compared to the cast film and subsequent to the addition of KCl salt, the relative amount of $\alpha$-helix increases. In this regard, it is to be noted that the calculated amount of $\alpha$-helix of pure Hb LB film is $\sim$39% whereas in pure Hb solution it is 79% [15].

Here this discrepancy in the result of secondary structure is due to different calculations, one from the solution CD data, and the other from the thin film FTIR data. This is primarily due to the presence and absence of aqueous water medium in
solution phase CD spectra and thin LB film FTIR spectra, respectively [15]. In reality, the hydrophobic air phase is present in the case of a thin film.

Moreover, in case a LB film, the surface tension of the air/water interface before lifting the LB film, may also be the reason for a lesser amount of $\alpha$-helix and a greater amount of $\beta$-sheet, whereas the surface tension is not present at the bulk solution of Hb [73]. However, both the measurements show the trend of increasing $\alpha$-helix with $C_{KCl}$. Overall, the FTIR data show that the amount of $\beta$-sheet of Hb decreases and $\alpha$-helix increases in presence of KCl salt. Thus, the FTIR results are also well corroborated with the earlier studies in FE-SEM, CD, and in LB measurements.

**Table 6.3: Fitting Parameters Obtained From the Deconvolution of Amide-I Band of Hb at Different Conditions**

<table>
<thead>
<tr>
<th>Conformers</th>
<th>Cast film</th>
<th>$C_{KCl}$=0.0M</th>
<th>$C_{KCl}$=0.01M</th>
<th>$C_{KCl}$=0.1M</th>
<th>$C_{KCl}$=1M</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_1$</td>
<td>3.13</td>
<td>5.26</td>
<td>1.74</td>
<td>3.18</td>
<td></td>
</tr>
<tr>
<td>$\beta$</td>
<td>45.19</td>
<td>32.51</td>
<td>25.23</td>
<td>23.57</td>
<td></td>
</tr>
<tr>
<td>$\alpha$</td>
<td>30.00</td>
<td>39.50</td>
<td>44.84</td>
<td>42.86</td>
<td></td>
</tr>
<tr>
<td>$T$</td>
<td>10.94</td>
<td>18.67</td>
<td>26.43</td>
<td>26.62</td>
<td></td>
</tr>
<tr>
<td>$A_2$</td>
<td>10.74</td>
<td>4.06</td>
<td>1.76</td>
<td>3.77</td>
<td></td>
</tr>
</tbody>
</table>

$R^2$ | 0.9992 | 0.9992 | 0.9998 | 0.9992 |
$\chi^2$ ($\times 10^{-5}$) | 10.0 | 6.0 | 10.0 | 2.0 | 10.0 |

*The symbols represent different conformers of protein: $A_1$=inter-molecular aggregates, $\beta$=$\beta$-sheet structure, $\alpha$=$\alpha$-helical structure, $T$=$\beta$-turns structures, and $A_2$=intra-molecular aggregates respectively. $(R^2)$ is the residual square correlation coefficient. $\chi^2$ value is a test statistics that is calculated as the sum of the squares of observed values minus expected values divided by the expected values.*

6.3.6. Ion-Protein Interaction and Relevance to DLVO Theory

In this chapter, the experiments on Hb were conducted in the absence and in the presence of KCl salt. Therefore, ion-protein interaction may be the central phenomena behind all the observations. In this regard, the DLVO theory of ion-protein interaction that was done completely on theoretical basis may be relevant to our experiment. This theory deals with the inter particle interaction and colloidal stability in terms of equilibrium of repulsive electrostatic and attractive van der Waals forces due to so called double layer of counter ions [74, 75].
Chapter 6 Influence of KCl on the Hemoglobin and Biommerahzed Crystal of KCl

In the lower concentration region of salt \( (C_{\text{KCl}} \leq 0.01 \text{ M}) \), not much significant changes are observed in the area/molecule (Figure 6.2), the lag time (Figure 6.3B) during adsorption of Hb and the free energy during compression of Hb monolayer with \( C_{\text{KCl}} \), however more significant changes of the same entities are observed at \( C_{\text{KCl}} \geq 0.1 \text{M} \).

According to the DLVO theory, the energy barrier due repulsive forces prevents two particles approaching one another and adhering together. However, due to Brownian motion, at low salt concentration, the particles can tunnel the energy barrier and the attractive van der Waals force will then pull them into contact where they adhere together and the proteins/enzymes will be more soluble in the subphase. This arguments can explain our results in the lower concentration region of salt \( (C_{\text{KCl}} \leq 0.01 \text{ M}) \). However, at high salt concentration, the barrier height is so high that the particle cannot be overcome to adhere together and the theory loses its validity \([12, 74, 75]\). According to Ninham et al and Bostrom et al inclusion of many body ion-protein dispersion potential originated from the polarizabilities of ions and proteins along with the electrostatic interactions is needed at high salt concentration \([76, 77]\). We believe that the observed sudden and rapid changes at \( C_{\text{KCl}} \geq 0.1 \text{M} \) may be due to many body ion-protein dispersion potentials.

Thus by using salt in subphase, one can manipulate the weak intermolecular forces (such as hydrogen bonding, steric interaction, hydrophobic interaction and van der Waals interaction) which in effect modify the property of the protein \([24, 78]\). At very least the interfacial properties of protein/enzyme can easily be tuned by salt concentration. Balla et al had demonstrated the salt effect on interfacial properties of a protein complex, namely gluten \([24]\). Jain et al has controlled the folding of a surface-active amphiphilic peptide by electrolyte concentration \([65]\).

The ion-protein interactions modify the water structure by perturbing the hydrogen-bonding network \([66, 67, 75]\) and different kinds of ions interact following the well-known Hoffmaister series \([75]\). In FE-SEM study, it is seen that the LB film in presence of deionized water contained mostly tetrameric Hb with some aggregates, but in the presence of KCl salt it contained lower aggregate of Hb. Also, the CD and FTIR studies show that the helix of Hb increases with KCl concentration. Such an observation may be due to the breaking of water structures as well as manipulation of intermolecular forces by KCl salt. However, in our earlier studies with pepsin and...
ADH [12, 50], these enzymes have a larger sheet and aggregated structure in presence of salt. This may be due to the difference between the structure and conformation of protein and enzyme in question, or may be a speciality of Hb protein. It has been reported by Lakshmanan et al in the case of BSA, that the percentage of helicity, which is a measure of compactness and folding of protein, increases in presence of different electrolytes [79]. It has been reported that Hb dissociated from tetramer to dimer in the presence of inorganic salt, as revealed from light scattering experiment as well as sedimentation velocity measurements [34]. The dissociation of Glucose Oxidase (GOX) is also reported in presence of ionic strength by neutron reflectivity experiments [55]. In our experiments the structural changes of Hb as well as the increase of helicity than the \( \beta \)-sheet in presence of KCl salt are the main outcome of ion-protein interaction. The observed changes in area/molecule, free energy, and lag time all are correlated with ion protein interaction.

Apart from the effect of KCl salt on the Hb protein molecules, the biomineralization of the KCl salt at the Hb protein interface may sometimes happen. We have studied this Hb-KCl biomineralization by LB technique as described in the next section.

6.3.7 Formation of Biomineralized KCl Crystal in Hb LB film

We have studied the formation and growth of biomineralized KCl crystal in the Hb monolayer at air/water interface as a function of the time and concentration of KCl, below its super saturation concentration in water (~4 M) [80]. The time and \( C_{\text{KCl}} \) dependent growth of the KCl crystal in the LB films were monitored using the FE-SEM imaging. The low-resolution FE-SEM image of LB film lifted from subphase containing KCl (\( C_{\text{KCl}} \geq 0.5 \text{ M} \)) shows the biomineralized KCl crystal along with the unfolded Hb.

The panels A, B and C of Figure-6.8 represent the FE-SEM images (low resolution image 10 \( \mu \text{m} \) scale and 2300 magnification) of LB film of Hb from KCl (\( C_{\text{KCl}}=0.5\text{M} \)) containing sub phase at different stabilization time, \( t=10 \text{ min}, 30 \text{ min}, \) and 1 hour respectively. It is found, that the films are associated with the biomineralized KCl crystals (as identified and confirmed by the HR-TEM and EDS characterization of the films), with varying dimensions/sizes from nm to \( \mu \text{m} \) depending on the growth time. Panel-A exhibits the growth of KCl crystals at \( t=10 \text{ min} \) having dimension at the nm
scale and the individual crystals are being aligned to build up a linear kind of structure. Similar appearance of linear array-like alignment due to self assembly in LB films, was reported in earlier literatures [81, 82].

Figure 6.8: Panel-A, B, C represent the KCl crystal formation (with $C_{KCl} = 500$ mM) in Hb LB film, with different reaction time ($t=10$ min, 30 min and 1h respectively). Panel-D represents the data of fractal dimension calculation by box counting method of the images in panel-A, B, and C.

The panels B and C present the crystal growth at $t=30$ min and 1 h respectively. It is found that the dimension of the individual crystal increases from nm to $\mu$m scale and the alignment of the crystals are branched in nature. It is established from the previous reports that this form of branching arrangement, widespread in nature, appears due to the fractal type of growth [83]. This branched structure depends on the crystal growing time as it increases the interaction time with their neighbors during mineralization (as depicted in Figure-6.8). We have analyzed the fractal dimension by box counting method implemented in Image-J software [45] and found that the fractal dimensions are 1.66, 1.44, and 1.51 for $t=10$ min, 30 min, and 1h respectively, as shown in panel D of Figure-6.8. The fractal morphology of KCl crystal growth was also observed by Szabo et al but in different scale and technique [36]. The self-assembly during evaporation of the Hb-KCl LB films [84] and the diffusion limited aggregation (DLA) process may be the driving factors for such fractal alignment of KCl crystal [85]. Moreover, the biomineralization of KCl via ion-protein interaction
may lead to alignment of the crystals in a particular fashion, such as network, fractal, etc. [37].

6.3.8 HR-TEM Study of the Biomineralized KCI Crystal

Panel-A of Figure-6.9 shows HR-TEM images of the biomineralized KCI crystal in LB film lifted on TEM grid from the subphase containing 0.5 M KCl. Panel B shows the selected area electron diffraction (SAED) pattern of the same crystal.

Panel C displays the HR-TEM image with distinct lattice fringes of the KCI crystal having lattice spacing of 2.15 Å. The SAED pattern of the KCl crystal having (222), (200), (111) diffraction spots correspond to the interplaner spacing (d-values) of 0.181, 0.314, and 0.363 nm of the bulk cubic KCl crystal (JCPDS 75-0296). However, the d-values from HR-TEM image for KCl crystal (0.215 nm) do not correspond to the preferred d-values and this may be due to that the preferred orientation (200) may differ with the diffraction condition [16]. Moreover, the EDX spectra of the crystal show the clear presence of the KCl (Figure-6.9D) crystal at the organic (C, O) environment.
6.3.9 Role of unfolding of Hb in the biomineralization of KCl crystal

With the increase of mineral concentration, the protein-mineral interaction enhances the unfolding of protein and changes the anisotropy of the surface tension of air/water interface, which later on favors to induce the crystallization of KCl in LB film [37]. Thus, the measured surface pressure data in combination with DLVO theory as discussed earlier further suggest that the electrostatic as well as the Van der Waals forces are contributed here for the Hb-KCl interaction for further crystallization processes [75].

The increment of α-helix and decrement of β-sheet in Hb-KCl system as derived from FTIR studies, indicates the unfolding of Hb at the biomineralized KCl crystal [37]. Thus, the unfolding of Hb may be responsible for the nucleation of KCl crystal.

6.4. Conclusion

In this chapter we have studied the interfacial activity, monolayer characteristics of Hb as a function of KCl concentration at the subphase by monitoring in situ π-A isotherm, β-π compressibility, and π-σ adsorption kinetics. In addition, it has been demonstrated the salt induced tuning of the intermolecular forces as well as the interfacial surface activity. Here the ion-protein interaction may be the central phenomena, which are well correlated with the modified DLVO theory. The increase of α-helix as characterized from CD and FTIR spectroscopy is revealed to be the reason of structural change of Hb in presence of KCl salt. The β-π compressibility study shows the loss of elasticity or rigidity of Hb in presence of KCl salt. The adsorption kinetics study shows that the diffusion and rearrangement mechanism involved in the adsorption process, can be controlled by introduction of salt (KCl) at the subphase. The FE-SEM study indicates the structural change accompanying with the internal conformational change of Hb in presence of KCl salt.

We observed the biomineralized KCl crystal formation in the high salt concentration region. The biomineralized crystal formation is due to the unfolding of the protein in presence of salt. The growth of KCl crystal is a slow process (minute to hour) having temporal and KCl concentration dependency. This kind of study may find potential in the area of effect of salt on the protein and the effect of proteins on the formation of biomineralized crystal of the minerals.
REFERENCES


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[47] Hemoglobin, Protein Data Bank (PDB ID-2DN2).


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