CHAPTER 4

RESULTS AND DISCUSSION

4.0 Introduction

In this chapter the results that obtained from the experiments conducted as described in chapter 3 will be presented and discussed thoroughly.

4.1 Analysis of Area Per Molecule and Effect of Bacteriorhodopsin Suspension Concentration on Isotherm

![Graph](image)

**Figure 4.1**: Surface pressure-area isotherm for 1 mg/ml bR solution at 20° C

Figure 4.1 above shows the surface pressure ($\pi$) vs area per molecule ($A_o$) isotherm for 1mg/ml concentration of bR suspension at 20°C. The annealing isotherm is obtained by compressing the film to a target pressure of 20 mN/m and held for 20 minutes before fully decompressing it to zero pressure [59]. After leaving the film for 5 minutes at this state, the full isotherm is obtained by recompressing it till the minimum area of the
trough is achieved. Only two phases are obvious from the $\pi - A_o$ isotherm, which are the gaseous and solid phase. Somehow the liquid and condensed solid phases are indistinguishable. The area per molecule reduces at constant zero surface pressure from about 970 Å$^2$ to about 670 Å$^2$ which corresponds to the gaseous phase. Further compression causes the isotherm to rise monotonically up to around 435 Å$^2$ (22 mN/m) where the kink is observed. As we compress further, the isotherm again rises monotonically up to around 255 Å$^2$ (38 mN/m) where the second kink is observed. As with many other macro-molecule’s isotherm, there were no collapse point observed in the investigated region of the surface pressure [60]. Also no hysteresis is observed between the annealing and full isotherm.

One of the most fundamental properties that we can determine from the $\pi - A_o$ isotherm is the area per molecule – a hypothetical area occupied by a single bR molecule at zero pressure. This can be obtained by simply extrapolating the slope of the solid phase of the isotherm to the intersection of the zero surface pressure. Theoretically the area per molecule of a material should be constant at a given temperature and experimental conditions. If all the bR used to spread on the water surface stays afloat, we should expect an area per molecule of about 1150 Å$^2$ [20] which was determined by the X-ray crystallographic method. However, the $A_o$ that we obtained from our isothermal experiment at 1 mg/ml concentration and at 20°C is 620 Å$^2$. The reason for much smaller $A_o$ value is due to the fact that bR is a more hydrophilic molecule. Thus, upon spreading it onto the water surface, some molecules will dissolve into the bulk of the water[16]. We also hypothesized that some bR molecules tend to form aggregate which also caused smaller $A_o$. Furthermore, it is always difficult to determine the exact amount of bR molecules spread on the water surface. The Nima software automatically calculates the $A_o$ from the data that we input for the concentration, molecular weight
and solution volume. There are always error in the calculation due to material lost during the many stages of the experiment such as during bR suspension and spreading solution preparation and transferring the solution on to the water surface. Because of these reasons, it is difficult to conclude the actual area per molecule of bR from the isotherm. Furthermore it is not one of the objectives of this work to find the $A_o$ as more accurate values have been determined by the x-ray crystallographic method.

Figure 4.2 below shows the surface pressure vs area per molecule isotherm for different concentration of bR suspension. The initial work done on the effect of different concentrations of bR suspensions on the area per molecule obtained showed that the area per molecule reduces as the concentration of bR increases. However, as can be seen from the graph below and from our repetitive isothermal experiments, there were no relation between the area per molecule and the concentration of bR suspension.

![Surface Pressure Vs Area Per Molecule Isotherm](image)

**Figure 4.2:** Surface pressure-area isotherm of bR at various concentration at 20° C
Though the lift off area per molecule for different concentrations show different values, generally the profile of the isotherms are the same. As for the 1 mg/ml concentration, the isotherm for other concentrations also rises monotonically up to around 22 mN/m where the kink is observed. Then it rises again monotonically up to around 38 mN/m where the second kink is observed. Beyond that the isotherms are crisscrossing with each other and don’t follow any regular pattern.

**Table 4.1 Hysteresis between annealing and full isotherm at different bR concentration**

<table>
<thead>
<tr>
<th>bR Concentration (mg/ml)</th>
<th>Area Per Molecule from Annealing (Å²)</th>
<th>Area Per Molecule from Isotherm (Å²)</th>
<th>Difference (Å²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>620</td>
<td>620</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>472</td>
<td>478</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>602</td>
<td>625</td>
<td>22</td>
</tr>
<tr>
<td>7</td>
<td>572</td>
<td>598</td>
<td>26</td>
</tr>
<tr>
<td>9</td>
<td>532</td>
<td>560</td>
<td>28</td>
</tr>
</tbody>
</table>

Comparing the annealing isotherm and the full isotherm for each concentration (refer to Figures 4.14 – 4.19 in pages 62-66) shows that, as the concentration increases, the hysteresis (difference between $A_o$ obtained from the annealing isotherm and the full isotherm) increases (Table 4.1). During the annealing process the film is held at 20 mN/m surface pressure for 20 minutes before decompressing to zero mN/m pressure and held at this state for 5 minutes before recompressing to obtain the full isotherm. The hysteresis differs from 0 Å² for 1 mg/ml concentration to about 28 Å² for 9 mg/ml concentration. Mario Method et al [17] have hypothesize that when films are fully decompressed and maintained at this fully decompressed state for few minutes, bR molecules that were lost into the subphase during film spreading could be reinserted.
into the film and thus lead to a shift toward larger molecular area or the hysteresis. If this is true, we should see an increase in the area per molecule between the annealing isotherm and the full isotherm across all the concentration. However in our experiment we observed that there were no (or very minimal) hysteresis at lower concentrations of bR (1 mg/ml & 3 mg/ml).

We hypothesize that as the concentration of bR suspension increases, the amount of water available for association with the polar group of the bR decreases. As a result, the degree of hydration of the bR polar group decreases. The reduced hydration of the polar groups leads to a decrease in the effective head group area [65]. This causes the bR molecules to aggregate and results in smaller area per molecule. When holding the film at fully decompressed state for 5 minutes, the bR molecules tend to dissociate from the aggregate and get reinserted into film. This explains the increase in the $A_w$ between the annealing and full isotherm. However, still there could be some bR molecules that might have not fully dissociated from the aggregate. Given sufficient time, they might fully dissociate from the aggregate. This hypothesis is depicted in Figure 4.3 below.
Figure 4.3: Schematic that explains the hysteresis (increase in area per molecule) between the annealing isotherm and full isotherm at higher concentration. A) Clusters of aggregates between monolayer film. B) Molecules start to dissociate from aggregate during decompression. C) At fully decompressed state more molecules have dissociated from the aggregate. D) Upon compressing again, still there are some aggregates that have not fully dissociated.
4.2 Identification of Phase Transition from Mechanical Properties of bR Langmuir Film

An important characteristic of a monolayer phase is its compressibility $C$ and is defined by the formula:

$$ C = -\frac{1}{A} \left( \frac{dA}{d\pi} \right) $$

It’s a measure of the rigidity of a monolayer film. If the monolayer film is highly rigid it will deform elastically in a similar manner as solid. The inverse of $C$ (or $1/C$) is the compressibility modulus $K$. It is a useful parameter to understand the phase behavior and the molecular interactions at the air water interphase. It can be directly calculated using the isotherm data from the formula:

$$ K = -A \left( \frac{d\pi}{dA} \right) $$

Figure 4.4: Surface Pressure and Compressibility Modulus Vs Area per molecule for 1mg/ml bR Solution at 20° C.
Figure 4.4 above shows the compression modulus vs area per molecule on the same graph with the isotherm. The graph can be divided into several distinct phases. The bR molecules exist in the gaseous phase (G) up to an area of 690 Å² which can be observed as almost a horizontal line with $K = 0$ mN/m. Further compression causes a phase transition to the liquid expanded phases (L1). The compression modulus increases almost vertically up to a value of 35 mN/m (from 670 - 650 Å²) in this phase, corresponds to high compressibility. This is also where the lift-off (the isothermal line starts to lift from its horizontal position) of the isotherm occurs. Upon continuous compression, the film undergoes further transition to Liquid-condensed state (L2) from 650 - 575 Å². Further compression leads to the film-forming molecules becoming closer to each other and the intermolecular interactions becoming stronger. As a result, the elasticity increases distinctively until it maximizes at a K value of 67 mN/m and an area per molecule of 520 Å². Here the monolayer reaches the solid state (S). If the monolayer is further compressed, the compression modulus start to reduce gradually up to 450 Å² (22 mN/m). This is the condensed solid phase (CS) where the molecules are compactly packed. Beyond this the $K$, falls sharply from this until it reaches a minimum at around 340 Å² (32 mN/m)

Beyond the first minimum point, the compression modulus starts to increase slightly and then tapers off in a gradual manner compared to the earlier steep drop. This occurs at surface pressure range of about 32 to 38 mN/m. We hypothesize that due to high surface pressure at the solid condensed phase, the bR molecules start to slip onto one another. The slipped molecules create voids which upon further compression causes the slight increase and the tapering of the compressibility modulus. Beyond this range the $K$ starts to reduce again with higher rate as more and more molecules start to slip on each other and forming multi-layers and bulky aggregates. Since the molecules are now more
than monolayer thickness, the rigidity of the films is much higher and shown by the lower compressibility modulus.

4.3 Thermodynamic Properties of bR-LB Film from $\pi$-$A$ Isotherm.

To calculate the thermodynamic properties for bR, first, the $\pi$-$A$ isotherm is obtained at temperatures 15, 20 and 25°C. The isotherms are graphed in the figure 4.5 below.

![Surface Pressure and Compressibility Modulus VS Area per molecule for 1mg/ml bR Suspension at 20°C](image)

**Figure 4.5:** Surface Pressure and Compressibility Modulus VS Area per molecule for 1mg/ml bR Suspension at 20°C.

Generally the isotherms follow the typical profile as discussed earlier and as such, will not be discussed in detail in this section. However, the isotherms shift towards higher area per molecule as the temperature increases. Based on the isotherm above, the Gibbs free energy is calculated using equation (4) and depicted in the graph below (Figure 4.6). It can be seen that the Gibbs free energy increases monotonically up to a maximum value of 63 kJ/mole at surface pressure value of 28 mN/m and starts to
decrease beyond that. Also, it is evident that the $G_c(\pi)$ increases as the temperature is raised from 15 to 25° C.

**Figure 4.6: Gibbs Free Energy VS Surface pressure.**

From the $G_c(\pi)$ values obtained, the $H_c(\pi)$ and $S_c(\pi)$ values were calculated over the temperature range of 15 – 25° C by the use of Equation (1) from page 33. These values are plotted in Figures 4.7 and 4.8, respectively. Both the entropy and the enthalpy of compression decrease gradually with increasing surface pressure until reaching a minimum at surface pressure of 22mN/m. This decrease corresponds to the molecular ordering that occurs as the floating bR molecules are pushed closer and closer by the moving barriers. Best molecular ordering takes place at a surface pressure of 22mN/m which coincides with the minimum point of $S_c(\pi)$. From this minimum point the $H_c(\pi)$ and $S_c(\pi)$ increases again until it reaches a maximum at surface pressure of 28 mN/m. Again both $H_c(\pi)$ and $S_c(\pi)$ decrease gradually until they reach second minimum at around 38 mN/m. Then the $H_c(\pi)$ and $S_c(\pi)$ again increases abruptly. This second
increase and decrease and abrupt increase again in the \( H_c(\pi) \) and \( S_c(\pi) \) agrees well with the molecular slippage and hypothesis that discussed for the inverse compressibility \( K \).

**Figure 4.7:** Entropy of Compression VS Surface Pressure.

**Figure 4.8:** Enthalpy of Compression VS Surface Pressure.
4.4 Spectroscopic Characterization

The absorption spectrum obtained for the deposited bR monolayer film on an ITO slide at surface pressure 22mN/m is shown in Figure. The light adapted absorption spectrum with peak absorption wavelength at around 568nm confirms the reconstituted bR molecules are able to generate photocycle.

*Figure 5.9: Absorption UV-Vis spectroscopy of bR monolayer film deposited on ITO slide.*
4.5 Structural Characterization

Figures 4.10 to 4.12 shows the SEM micrographs of bR monolayer that deposited at 25 mN/m at different magnification. Since 25 mN/m surface pressure is beyond the optimal deposition pressure of 22mN/m, we can clearly observe the slipped molecules as aggregates. Since we did not do any gold or carbon coating on our deposited monolayer film we could not obtain any SEM micrographs for films that deposited at 22mN/m and below as the high powered electron beam of SEM burn the monolayer molecules and left behind nothing to visualize. As such SEM may not be a suitable characterization method for uncoated monolayer bR film.

![Figure 4.10: 10 X SEM Micrograph of bR monolayer film deposited at surface pressure of 25 mNm](image)

*Figure 4.10: 10 X SEM Micrograph of bR monolayer film deposited at surface pressure of 25 mNm*
**Figure 4.11**: 3000 X SEM Micrograph of bR monolayer film deposited at surface pressure of 25 mNm

**Figure 4.12**: 8000 X SEM Micrograph of bR monolayer film deposited at surface pressure of 25 mNm
4.6 bR Monolayer Film Thickness Measurement

A typical surface analysis AES graph of bR LB film is shown below (Figure 4.13) highlighting the presence of Nitrogen (N) molecules. Though detected as weak peak, the presence of N confirms the presence of protein molecule on the ITO substrate.

![Surface analysis results of bR monolayer film](image)

**Figure 4.13: Surface analysis results of bR monolayer film**

The depth profile combines the sputtering technique to reveal the elements present in the buried layers. Figure 4.14 below shows the typical depth profile of the bR film. The absence of Nitrogen molecule will indicate that the ITO substrate is not coated with bR molecules at all. However one should understand that the long escape depth of Auger electron is 6nm. Therefore any film that is thinner than 6 nm will not be detected using this method. Therefore we can confidently conclude that the thickness of the deposited bR film is less than 6 nm.
The results obtained from the surface profiler, showed that the average thickness of the deposited film is 4.5nm. This with the AES analysis confirms that the deposited film is indeed, monomolecular thick.
Figure 4.15: Surface Pressure Vs Area permolecule for 1mg/ml bR Solution at 20° C.
Figure 4.16: Surface Pressure Vs Area per molecule for 3mg/ml bR Solution at 20° C.
Figure 4.17: Surface Pressure Vs Area per molecule for 5mg/ml bR Solution at 20° C.
Figure 4.18: Surface Pressure Vs Area permolecule for 7mg/ml bR Solution at 20° C.
Figure 4.19: Surface Pressure Vs Area per molecule for 9mg/ml bR Solution at 20° C.