INTERFEROMETRIC STUDIES ON THE CONCENTRATION VARIATIONS AROUND A GROWING PROTEIN CRYSTAL

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ABSTRACT
The concentration fluxes in the vicinity of protein crystals growing from its aqueous solution has been investigated using laser based interferometric technique. A Mach-Zehnder interferometer has employed for the experimentation. Lysozyme (Hen-Egg-White) has used as the protein, where sodium chloride (Nacl) is used as the precipitant and Sodium acetate (CH₃COONa) as buffer. A test cell with dimensions 22.5 mm of height and 24 mm of width made up of high quality optical glass has used as the test chamber, so as to make optical measurements feasible. The Lysozyme crystals have inserted into the supersaturated solution inside the test chamber to initiate the growth process. Wedge fringe interferograms has been employed to retrieve the concentration gradients around the crystal during the growth/dissolution process. The results from the experiments are summarized in the form of interferograms during the growth/dissolution process, two dimensional concentration gradients around the crystal etc.

Keywords: Protein Crystals, Lysozyme, Interferometry, Concentration Gradients.

NOMENCLATURE
A Average intensity
B Resultant intensity
C Concentration
I Intensity
L Distance travelled by the laser beam through the test section.
n Refractive index
T Temperature

Greek symbols
\( \lambda \) Wave length
\( \phi \) Phase

Subscripts
0 References
s Solution
t Test

INTRODUCTION
Optical based measurement methods has been effectively utilized for the visualization and analysis of growth kinetics of protein crystals, which has direct application in many biological processes and to develop new drugs for illness and injuries, targeted drug delivery etc., The interest in protein analysis lies in the atomic scale to larger dimensions, where the interaction of the protein to the environment is investigated. This paper studies the concentration gradients around a Lysozyme crystal growing from its aqueous solution using interferometry to visualize the effects of concentration variations in the vicinity of crystals during their growth process.

Extensive research has been carried out to investigate the growth kinetics of protein crystals [1-3]. Still many of the phenomena involved in these processes are a point of debate. The experiments conducted by Long et al. [4] on high quality insulin protein crystals under microgravity conditions reveals that the concentration gradients setups in the vicinity of the growing crystal is one of the crucial factor that determines the size, shape and morphology of the growing protein.
Efforts are made to visualize and/or analyze these concentration gradient layers around a growing/dissolving crystal to understand the kinetics of the process. Attempts made by Komatsu [5-6] in this direction using two-beam Michelson interferometer provided a highly reliable method for the visualization of gradient layers around protein crystals during their growth process. The investigations were performed on Lysozyme protein crystals using the deformation of wedge fringe interferograms to visualize the strength of the gradients near the crystal in order to retrieve the diffusion coefficients. An experimental investigation in the similar line done by Vekilov et al [7] also demonstrate the potential of interferometric measurement methods for the real time in situ vitalization and analysis of protein crystal growth process. The experimental investigation also provides information on growth rate and step velocity on identified growth-layers. More recently Duan et al [8] utilized a phase shifting Mach-Zehnder interferometer to visualize and quantitatively analysis the concentration fluxes of Lysozyme protein crystals. The same group investigated the crystallization process of protein crystal by vapor diffusion using phase shifting interferometry. Recently Zhang et al [9] visualized the solution concentration variation during the growth process of Lysozyme crystals. The concentration gradients have been visualized using digital holographic interferometry. Investigations in this direction show the feasibility of real time observation of gradients using optical methods.

The present experiment investigates the growth kinetic of Lysozyme protein crystals, growing from its aqueous solution inside a test chamber. The real time investigation has been performed using a Mach-Zehnder interferometer in wedge fringe setting mode. The deformation of the fringes near the surface of the crystal quantifies the concentration fluxes associated in the growth kinetics. Full field phase mapping techniques are used to retrieve the two dimensional phase and/or concentration profiles in the solution and near the interfacial region. The results from the experiments are summarized in the form of interference fringes and concentration gradient maps in the vicinity of the crystal.

INSTRUMENTATION AND MATERIALS

This section discusses about the instrumentation employed, i.e., the MZI interferometric configuration and the details of the materials and preparation techniques involved in the generation of high quality protein crystals.

Instrumentation- Mach-Zehnder Interferometer

Laser based optical interferometer is employed. A Mach-Zehnder interferometer with a low magnification objective (4x/0.13 Olympus Co.) has been used for the visualization of protein kinetics. The interferometer configuration employs a high coherent He-Ne laser source (13mW, wavelength =632nm) which is expanded and collimated using the optical configuration of spatial filter aperture and a Plano convex lens. The collimated beam directed to the first beam splitter (BS-1) where the beam splits into a reference beam and a test beam. These beams traces their respective paths and strikes on plane mirrors M1 and M2, having 99% reflectivity. These reflected beams from the mirrors coincide on the second beam splitter where interference patterns will be produced as a result of the superposition of beams with a constant phase difference. The objective lenses are used in the optical configuration to magnify the sample with a dimension as small 500 micron. The interference pattern produce in BS-2 is captured using a CCD camera (25 frames/ second, Throlabs Co.) and fed into the PC. The optical configuration of the MZI interferometer has been shown in Figure 1.

![FIGURE 1. Schematic of the optical configuration of Mach-Zehnder interferometer employed for visualizing the convective field in the proposed experiment. SP: Spatial Filter, L: Collimating Lens, M: Mirrors, BS: Beam Splitters, OB: Objectives, RC: Reference Chamber, TC: Test Chamber CCD: CCD Camera, PC: Personal Computer](image)

The test section consists of a test chamber made up of high quality optical glass. The chamber is filled with the super saturated protein solution. The protein crystal has been inserted into chamber to initiate the growth process. A chamber of same dimensions and same solution concentration has been kept in the reference arm of the interferometer to compensate the additional path length variations owing to the extra path travelled by the light through the chamber and due to the density gradients in the medium. The interference fringes produced from lights through the test and reference chambers contains information about the gradient fluxes, produced due to the growth kinetics of the crystal. Figure 2 shows the schematic of the test chamber assembly.
The preparation of Lysozyme protein crystals are as follows: First the buffer solution is prepared by dissolving sodium acetate (concentration 50mM) in distilled water. The PH of the solution is maintained to the desired level (PH=4.5 in the present experiments) by adding commercially obtained concentrated HCL solution into the buffer in drop wise manner. The Lysozyme solution is prepared by dissolving the Lysozyme, procured from Sigma Aldrich, without any further purification into the CH₃COONa buffer to produce a final solution concentration of 80mg/ml. The precipitant solution is prepared by dissolving 5% of Nacl in the buffer solution. An equal amount of the protein and precipitant solution are mixed together. This solution is kept for crystallization under 20-22°C. Small crystals of size 400-800 microns will produce after 4 days of crystallization. The table below shows the summary of different parameters used to produce high quality Lysozyme protein crystals.

**Table1: SUMMERY OF MATERIALS AND EXPERIMENTAL CONDITIONS USED TO GENERATE PROTEIN CRYSTALS**

<table>
<thead>
<tr>
<th>Material</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>Sodium Acetate 50mM</td>
</tr>
<tr>
<td>Protein</td>
<td>Lysozyme 80mg/ml</td>
</tr>
<tr>
<td>Precipitant</td>
<td>Sodium Chloride 5% (w/v)</td>
</tr>
</tbody>
</table>

**Experimental Parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gravity</td>
<td>Normal</td>
</tr>
<tr>
<td>Temperature</td>
<td>20-22°C</td>
</tr>
<tr>
<td>PH</td>
<td>4.54-4.52</td>
</tr>
</tbody>
</table>

The produced crystals are used to insert into the super saturated solution of Lysozyme. A glass road of diameter 2 mm is used to hold the crystal in position and the glass road along with crystal has been inserted into the test chamber containing super saturated protein solution maintained at a similar environment as the crystal. Since the variation in PH and temperature are the key parameters which affects the growth kinetics of the protein, enough care has been taken to maintain these parameters in their optimised range.

**DATA REDUCTION**

The experimentally recorded wedge fringe interferograms have been quantitatively analysed to obtain the two dimensional concentration fields around the growing Lysozyme crystal. The wedge fringes, alternate dark and bright bands represent the lines of constant phase difference. These recorded interferograms can be mathematically expressed as an intensity distribution $I(x,y)$ in a $(x,y)$ plane.

$$I(x,y,t) = A(x,y) + B(x,y) \cos \left[ 2\pi f_0 r + \Delta \phi(x,y,t) \right]$$

(1)

Here $f_0$ is a constant vector corresponding to the tilt of reference mirror of the interferometer and $\Delta \phi$ is the phase difference induced due to the difference in the optical path lengths of the two arms of the interferometer. In the context of the present experiment the optical path difference produced between the beams from the two arms of the interferometer is owing to the generation of gradient fields in the solution due to the growth kinetics of the protein crystal. The refractive index gradient and the optical path difference thus the phase of the recorded interferograms can be connected to the concentration fluxes generated in the medium.

The phase distribution in the interferograms is directly connected to the refractive index gradient in the medium. Since the thickness of the test cell (approximately around 0.24cm) is very small and hence the variation in refractive index along this direction can be assumed constant. Then the change in the phase can be attributed to the two dimensional refractive index gradients $n(x,y)$ and the pathway the light travels through the test section $L(x,y)$. This can be expressed as

$$\Delta \phi(x,y) = \frac{2\pi}{\lambda} \left[ n_{\text{test}}(x,y) - n_{\text{reference}} \right] L(x,y)$$

(2)

The above equation can be simplified as

$$\Delta \phi(x,y) = \frac{2\pi}{\lambda} \Delta n(x,y) L(x,y)$$

(3)
The relation between refractive index and solute concentration can be obtained from the below expression

\[
\Delta n(x, y) = \left[ \frac{\partial n}{\partial T} \right]_T \left[ T_r(x, y) - T_s(x, y) \right] \\
+ \left[ \frac{\partial n}{\partial C} \right]_T \left[ C_r(x, y) - C_s(x, y) \right]
\]  

(4)

The above relation can be simplified to conditions with constant temperature as

\[
\Delta n(x, y) = \left[ \frac{\partial n}{\partial C} \right]_T \left[ C_r(x, y) - C_s(x, y) \right]
\]  

(5)

The solution concentration distribution can be derived by combining Equation (3) and Equation (5), the final equation takes the form

\[
C_r(x, y) = C_s(x, y) + \left( \frac{\lambda}{2\pi d(x, y)} \Delta \phi(x, y) \right) \frac{1}{\partial n/\partial C}
\]  

(6)

The two dimensional phase field in Equation (4) can be obtained using phase extraction algorithms; the present study uses Hilbert Transform techniques. The extracted wrapped phase is unwrapped using phase unwrapping algorithms after proper filtering of the wrapped phase. The details phase extraction methods used in this analysis has been described in detail in literature [10].

RESULTS AND DISCUSSION

The present section discusses about the result of the experimental investigation carried out to visualize and/or analysis the concentration fluxes in the medium during the process of protein crystal growth. Interferometric fringes in wedge fringe mode have been used for the analysis of concentration gradients around the crystal. Wedge fringes sets normal to the crystal surface has been used as the initial fringe pattern as shown in Figure 3. As the protein crystal is inserted into the supersaturated solution the convection currents sets up which will manifest as bending of fringes near the surface of the crystal where the gradient of concentration exists. Figure 3 (a) and (b) shows the wedge fringe interferograms after the crystal is inserted to the solution in different time frames. The fringe deformation near the surface can be attributed to the density gradients and thereby the refractive index gradients in the medium.

![Wedge fringe interferograms during protein crystal growth process (a) 5 hrs (b) 10 hrs after initiation of growth process](image)

The growth of protein crystals is relatively a slower process; hence the development of gradient currents due to the solution concentration variation will be very small in the initial time frames. The same effect can be seen in terms of the degree of bending of fringes in the near surface of the crystal in Figure 3(b). In the initial stages the growth process is much similar to the nucleation process, hence the development of concentration gradients are much lower which can be seen in Figure 5.

Figure 4 shows the wedge fringe interferograms taken at different time frames during growth process of the Lysozyme protein crystal. The crystal is inserted into the supersaturated solution using a glass rod as mentioned earlier. Since the temperature is a crucial parameter which determines the growth rate and morphology of growing crystals enough precautions are taken to maintain the solution temperatures to the desire values.

![Wedge fringe interferograms during growth process of Lysozyme protein crystal](image)
Figure 4(a) shows the interferograms recorded after 3 hours of initiation of crystal growth and Figure 4(b) is the interferograms after 6 hours of growth process of Lysozyme crystals. As can be seen as the time marches the gradients near the surface of the crystal increases and leads to higher degree of deformation in the interferometric fringes.

The whole field concentration distribution can be retrieved from the recorded wedge fringe interferograms as shown in the above diagram. As stated earlier full field measurement techniques are used to retrieve the phase information/maps from the interference pattern. The region of protein crystal is properly masked using image processing and the reference is taken in the in the region away from the crystal surface, devoid of concentration gradients. In order to retrieve the true phase from the wrapped phase suitable unwrapping procedures are used. The unwrapped phase maps are used to obtain the value of phase to extract the concentration gradient as in Eqn (6).

Figure 5 shows the retrieved concentration gradient field from the wedge fringe interferograms (Figure 4). The figure shows the concentration gradients near the surface of a protein crystal during different time laps.

The figure depicts the concentration gradients around a growing protein crystal. As seen the gradient fluxes increases near the surface of the crystal when the time span increases. As mentioned already the growth of protein crystals is a very slow process and hence the time variation of solute concentration also gives a slow trend which can be seen in the form of fringe bending also where the bending is relatively low as compared to the undisturbed solution.

Figure 6 shows the variation of concentration normal to the crystal surface. The concentration profile shown is corresponds to a particular section of the concentration plot in Figure 5. Figure 6 (a) and (b) are the variation of concentration at a particular section (corresponds to 150th pixel along the X axis) in both the images i.e., figure 5(a) and 5(b)
The graphs shown in Figure 6(a) and (b) show a reduction in the concentration of protein solution near the surface of the crystal as compared to the supersaturated bulk solution. A closer look to the values of concentrations reveals that the variation in actual values is very small during these initial hours of the growth process. This is exactly coherent with the fringe deformation seen in the wedge fringe interferograms in Figure 4(a) and Figure 4(b). The gradients are increasing as the time marches forward implies the growth kinetics of the crystal. As the growth process stabilizes, the gradients are increasing as seen in Figure 6. This indicates the effect of increasing convection currents in the medium owing to the growth kinetics of the protein crystals. A simultaneous reduction in the bulk concentration can also be observed from these figures.

COMPARISON OF ORGANIC AND INORGANIC CRYSTAL GROWTH PROCESS

Attempts are made to compare the growth kinetics of organic and inorganic crystals using interferometric measurements. The inorganic crystals used for this study is sodium chlorate with 4% super saturation. Wedge fringe interferograms has been used to retrieve the two dimensional concentration fields in sodium chlorate crystals as employed in Lysozyme protein crystals. Figure 7 shows the wedge fringe interferograms during the growth process of sodium chlorate crystal. The image corresponds to a super saturation level of 4% and the time frame is 3 hours after the initiation of the growth process.

The figure shows a clear bending of wedge fringes near the surface of the crystal as compared to the supersaturated bulk solution. The bending in the surface of the crystal indicates the generation of concentration fluxes in the region. As compared to the wedge fringe interferograms of Figure 4(a), the bending near the surface of inorganic crystals during their growth process is much higher indicating a higher growth rate and concentration gradients in inorganic crystals as compared to protein crystals. Hence the growth of protein crystals is relatively a slower process as compared to that of inorganic crystals.

Figure 8 shows the variation of concentration at a particular section normal to the crystal surface (sodium chlorate, in figure 7). A comparison of Figure 6(a) and Figure 8 shows the variation of concentration gradients and

![Figure 6](image_url)

**FIGURE 6.** Variation of concentration of protein solution at a particular section normal to the crystal surface (a) after 3 hrs (b) after 6 hrs of initiation of growth process

![Figure 7](image_url)

**FIGURE 7.** Wedge fringe interferograms during Sodium Chlorate crystal growth process [11]

![Figure 8](image_url)

**FIGURE 8.** Variation of concentration of protein solution at a particular section normal to the Sodium Chlorate crystal surface

growth kinetics of Sodium chlorate (inorganic crystal) and Lysozyme crystals (organic crystals) in the same instant of time after the commencement of growth process. From the graphs it can be seen that the gradient fields and concentration fluxes are much higher in inorganic crystals as compared to the growth kinetic of protein crystals
indicating a much slower process of growth in protein crystals.

Since the growth of protein crystals is a tricky process and many parameters directly affect the process, a perfect optimization of experimental parameters has yet to be achieved in the present manuscript. The surface morphology of the growing crystal is again highly depends upon these parameters, experimental investigations on improving the surface morphology and/or optimizing the experimental parameters are still in progress.

CONCLUSION

The experimental investigation reported in the present work presents the optical imaging based measurement technique to study the growth kinetics of growing protein crystals. Mach-Zehnder interferometer has been used. The deformation of initial wedge fringes during the protein crystal growth has been used to visualize and quantitatively analysis the concentration variation of the solution as a result of the growth of protein crystals from its aqueous solution. The primary results obtained from these experiments shows the growing kinetics of protein crystals and the phase extraction based fringe analysis qualitatively conclude concentration fluxes associated with the process. Since the protein crystal growth is a relatively small process the variation of concentration in the solution is very small and can be as close to the nucleation process for around 7-8 hours. The experimental parameters to obtain more precise conclusions have to be optimised through further experimentation. But the results shows that interferometric methods coupled with phase extraction based two dimensional concentration retrieval algorithm provides a feasible method to the problem of protein crystal growth and the fluxes associated with the above process.

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REFERENCES