Final Report
Office of Biological and Physical Sciences Research, NASA

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Task Research Title
Effects of Convective Transport of Solute and Impurities on Defect-Causing Kinetics Instabilities in Protein Crystallization

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Impact on America

Insight into the crystallization processes of biological macromolecules into crystals or aggregates can provide valuable guidelines in many fundamental and applied fields. Such insight will prompt new means to regulate protein phase transitions in-vivo, e.g., polymerization of hemoglobin S in the red cells, crystallization of crystallins in the eye lens, etc. Understanding of protein crystal nucleation will help achieve narrow crystallite size distributions, needed for sustained release of pharmaceutical protein preparations such as insulin or interferon. Traditionally, protein crystallization studies have been related to the pursuit of crystal perfection needed to improve the structure details provided by x-ray, electron or neutron diffraction methods. Crystallization trials for the purposes of structural biology carried out in space have posed an intriguing question related to the inconsistency of the effects of the microgravity growth on the quality of the crystals.

List of industry research contacts
New Century Pharmaceuticals
Abbot Laboratories
Teravance Pharmaceuticals
Astra Zeneca Pharmaceuticals

If this investigation has contributed to the development of any new technological advances, please identify each one and include a short description.

The results of the research will allow the formulation of a criterion to predict whether improvements in the quality of protein crystals grown for x-ray diffraction studies of their structure should be expected from microgravity growth. The application of this criterion to future proposals for space crystallization of proteins will allow maximization of the yield of such trials.

Who is using the results of your research?
Protein crystal growers, both in industry and academia

Where have recent graduates from your group found employment?
University of California, Berkeley
University of Alabama in Huntsville
University of Alabama at Birmingham
University of Alabama at Birmingham, Medical School
Hunter College, City University of New York
Marshal Space Flight Center, NASA
Technical University Clausthal, Germany
University of New Mexico
Baylor College of Medicine
Lawrence Livermore National Laboratory
Number of times that your work has appeared in the popular media? 19
The Slavi Trifovov Show, bTV, Sofia, July 7, 2004
Time Warner 24 Hours News TV Channel, June 29, 2003
"Could UAH become the MIT of the south" The Huntsville Times, March 9, 2003, P. A1
"Columbia goes deeper for ex-UAH prof" The Huntsville Times, February 9, 2003, P. A15
"Star UAH professor departs tired of education civil war" The Huntsville Times, June 10, 2001, P. A1
"Scientists Take First Pictures of Molecules Forming Crystals Nucleus" Technology Valley, Feb. 2001, p.16
"Observing the Crystallization Process" About: Composite Materials, August 2000
"First look at a crystal's "embryo"—flat, not round", natural SCIENCE, News, August 3, 2000
"Research Hopes Grow with Crystal Discovery", The Jackson Citizen-Patriot, August 16, 2000, p. B1
"Crystal Discovery Could Lead to Sickle Cell Cure", Seattle Times, August 12, 2000, p. A6
"UAH Breaks Crystals' Code", The Huntsville Times, August 9, 2000, p. A1
"Crystal Reveals Unexpected Beginnings", Science News, August 5, 2000, p. 84

Number of times that your work has appeared on a magazine cover? 2
Science vol. 299 Number 5609 February 14, 2003
J. Mol. Biol. Vol. 336 Number 1, Feb. 6, 2004

If you have a science website, or your work is represented on one, please include the address:

www.chee.uh.edu/faculty/vekilov/
http://www.egr.uh.edu/parameters/spring2003/?e=facultyresearch
see also items in Number of times that your work has appeared in the popular media? above

Task Objective
The general objective of the performed investigations was to understand the ways in which the presence or absence of gravity could affect the processes of protein crystallization. Our main hypothesis was that the main gravity related factor for protein crystallization is buoyancy-driven convection and that it elicits strongest response in the instabilities of the propagation of layers during the growth of the crystals.

The aim of the proposed investigations was to obtain fundamental insight into the onset and development of the defect-causing instabilities that arise due to the coupling of the bulk transport and non-linear interfacial kinetics during growth in the mixed regime, utilizing the reduction of the convective contribution to the bulk transport under microgravity. These studies will build upon the data on the effects of quantitative variations of the forced convection velocity on the averaged and time-dependent kinetic behavior of protein crystal growth systems that have recently been obtained in our laboratory.
Task Description

Our NASA-supported ground-based research has revealed that protein crystallization occurs with intrinsic growth rate fluctuations that arise from the coupling of bulk transport to nonlinear interfacial kinetics. Furthermore, we established a one-to-one correspondence between these fluctuations that are due to the bunching of growth steps, and the formation of defects in the crystals. In addition, based on numerical simulations, we have developed a criterion for the improvement of crystal quality through imposed changes in the transport conditions in the solution. Depending on the specific diffusivity and kinetic coefficient of a protein and the impurities in the solution, either transport enhancement through forced flow or transport reduction under reduced gravity can result in a reduction of the step bunching and, thus, growth with higher structural perfection. Most recently, we have been able to confirm the forced flow aspects of this rationale in ground-based experiments with lysozyme utilizing flowing solutions with varying, well characterized impurity contents. The microgravity aspects of our rationale, though supported by the numerical modeling and scaling analysis of the space results of other investigators, require scrutinization through specifically designed flight experiments. Here we propose experiments to quantitatively test our system-dependent criterion for efficient selection of proteins that can be expected to benefit from crystallization under reduced gravity.

In the flight experiments, we planned to use three proteins, chosen for their different combinations of diffusivities and kinetic coefficients. These will be crystallized in six individually temperature-controlled cells, from pure as well as specifically heterogeneity-doped solutions. To put the protein and impurity choices on a quantitative basis we will first identify and quantify the impurities in these proteins by capillary and gel electrophoresis, develop chromatographic purification protocols, determine diffusivities by dynamic light scattering, and measure growth kinetics coefficients by high-resolution interferometry and atomic force microscopy. In addition, we will monitor the response of kinetics fluctuations to variations in the convective bulk transport conditions on Earth. This, together with numerical simulations of dependence of the kinetics instabilities on the diffusive-convective transport of solute and impurities, will allow us to optimize the scientific yield of the flight experiments.

All instrumentation for the ground-based characterization and control experiments are available in our laboratory. For the kinetics monitoring of crystal surfaces in space, we will design an automatic phase-shifting interferometer for in-situ surface characterization that can be operated from the ground. Based on the insight gained from the space experiments in comparison with the controls on Earth, we will chose specific growth conditions that are expected to result in a minimization or enhancement of crystal defect formation on Earth. Crystals grown under these conditions will be evaluated for their x-ray diffraction resolution. This will result in a closure of our rationale on the role of transport in crystal perfection.
Task Significance

The proposed research will extend the fundamental insight into the intrinsic and impurity-induced instabilities during protein crystal growth. Since these instabilities are caused by the coupling of interfacial kinetics to bulk transport, data obtained with significantly reduced convective flows in microgravity are a must. Equally important, the research will test the system-specific, quantifiable criteria for benefits as well as disadvantages for protein crystal growth in space, that arise from changes in growth step dynamics with reduction in convective transport. Such criteria have only recently been put forth by our group. Furthermore, the systematic studies of the effects of variation in impurity transport on the kinetic instabilities are expected to provide a new mechanism for the advantages of microgravity growth of proteins for which high (from a crystal grower’s point of view) purity is unachievable.

Main Results

The grant was awarded to allow us to prepare for the Scientific Concept Review (SCR) of a program of experiments to be carried out aboard the International Space Station with the purpose of testing the response of the defect causing kinetics instabilities to the absence or presence of gravity and buoyancy-driven convection. In preparation for the SCR, we carried out a series of experiments focused on the instabilities during layer growth, but also on other aspects of protein crystallization to ensure that as hypothesized, the instabilities during layer growth are the phenomena, which should be expected to have a strongest response to gravity-related effects.

The SCR took place in August 2002. The panel highly commended our achievements and certified the significance of the investigations proposed for space flight experiments. The Office of Biological and Physical Research at NASA approved their recommendation in November 2002. Unfortunately, the planned space flight experiments were cancelled during the redirection of the NASA’s efforts after the Columbia space shuttle disaster.

The main findings of our ground-based efforts are summarized below:

Thermodynamics of protein crystallization

*Intermolecular interactions in solution*

Light scattering was applied to study apoferritin solution. Apoferritin and ferritin crystals grow well in solutions buffered at pH around or above 5, in the presence of a divalent cation Mn^{2+}, Cd^{2+}, Ni^{2+}, Co^{2+}, and others. A typical set of conditions providing for high crystal quality is 0.2 M Na acetate buffer at pH 5 with 2.5 % (w/v) CsSO₄, leading 0.12 M Cd^{2+}. Since the pI of these proteins is about 4.5, at pH 5 they are negatively charged and the intermolecular interactions are strongly affected by the cations in the solution.

When plotted against the electrolyte concentration, the dimensionless second osmotic virial coefficient $B_2$ in apoferritin solutions that only contain the Na⁺ ions exhibits a
minimum at $[\text{Na}^+]$ between 0.1 and 0.15 M. The value of $B_2$ at the minimum of $\sim 4$ is 
equal to that expected for non-interacting hard spheres, indicating that at these $[\text{Na}^+]$, 
electrostatic repulsion is completely suppressed. The ascending branch of this 
dependence is a manifestation of a surprisingly strong repulsion between the molecules at 
electrolyte concentrations about and above 0.2 M, where electrostatic interactions are 
insignificant. This strong deviation from the predictions of the DLVO theory was 
attributed to the water structuring, enhanced by the accumulation of hydrophilic counter-
ions around the apoferritin molecules, giving rise to so-called hydration forces.

The interaction potential due to the hydration forces has been described as a 
function of the distance $r$ from a molecule of diameter $2a$ as [134,135]

$$U_{\text{hyd}} = -\frac{f_0}{4\pi} \frac{1}{r} \exp \left[ -\frac{a}{L} \right].$$

Here, $f_0$ and $L$ are the parameters for the surface energy density and decay length 
respectively. Choosing decay length $L = 2 \times 0.72 \text{ nm} = 1.44 \text{ nm}$ (twice the diameter of a 
hydrated sodium ion), and $f_0 = 12.5 \text{ mJ/m}^2$, in the middle of the range determined by 
surface force measurements, the values of the second virial coefficient at $[\text{Na}^+] > 0.15 \text{ M}$ 
were reproduced.

The addition of even 0.01 M Cd$^{2+}$ leads to a drop of the virial coefficient in a 
solution with 0.2 M Na$^+$ from the relatively large positive value typical of the hydration 
repulsion, to about zero. Note that even these low Cd$^{2+}$ concentrations are orders of 
magnitude higher than the apoferritin concentrations (in the micromolar range in a 1 
mg/mL solution of this protein with $M_w = 450,000 \text{ Da}$). However, further increases of 
the $[\text{Cd}^{2+}]$ up to 0.22 M = 2.5 \% (w/v) (the value typically used in crystallization trials) 
did not lower the value of $B_2$ further and it remained around zero. We interpret the action 
of Cd$^{2+}$ on the molecular interactions in the following way. The strong coordination bond 
that Cd$^{2+}$ establishes between two apoferritin molecules is reflected in the potential as a 
deep minimum limited to distances of about 0.2 nm, i.e., comparable to the size of the 
Cd$^{2+}$ ion. If, in addition to this effect, Cd$^{2+}$ would also destroy the hydration shell around 
each molecule, this deep minimum would lead to highly negative $B_2$ values. The 
closeness of the actual values to zero indicates that the repulsive hydration shells are 
present even with the Cd$^{2+}$ in the solution. This results in a potential around an 
apoferritin molecule in a crystallizing solution containing 0.2 M Na$^+$ and 0.22 M Cd$^{2+}$.

Thus, the combination of Na$^+$ and Cd$^{2+}$ in crystallizing solutions of apoferritin 
leads to the non-monotonic potential of intermolecular interactions. This shape of the 
potential significantly enhances the selectivity of the crystallization process by allowing 
only a few of the attempted collisions between the molecules, or between single 
molecules and existing clusters and crystallites, to proceed. This selectivity may be the 
factor underlying the robustness of the crystallization conditions—rather significant 
changes in the concentrations of the two ions, and not lead to decaying crystal quality.

*Solvent entropy is a main contributor to the free energy of protein crystallization*

We show with six proteins that trapping and release of the water molecules upon 
crystallization is a determinant of the crystallization thermodynamics. With HbC, a 
strong retrograde solubility dependence on temperature yields a high positive enthalpy 
of 155 kJ mol$^{-1}$, i.e., crystallization is only possible because of the huge entropy gain of
610 J mol\(^{-1}\) K\(^{-1}\), stemming from the release of up to 10 water molecules per protein intermolecular contact. With apoferritin, the enthalpy of crystallization is close to zero. The main component in the crystallization driving force is the entropy gain due to the release upon crystallization of two water molecules bound to one protein molecules in solution. With both proteins, the density of the growth sites imaged by AFM is in excellent agreement with a calculation using the crystallization free energy. With lysozyme, the entropy effect due to the restructuring of the water molecules is negative. This leads to higher solubility.

**Experimental determination of phase diagrams**

For insight into the thermodynamics and phase behavior in concentrated protein solutions, we study the liquid-liquid phase separation with lysozyme. We determine independently the binodal and spinodal lines, and the second virial coefficient of the protein at 275 < \( T < 295 \) K. From these data, we determine the protein chemical potential and osmotic pressure for concentrations as high as 320 mg mL\(^{-1}\) in the above temperature range. We find that for this protein the enthalpy of the liquid-liquid separation vanishes at the critical temperature \( T_c \), and is comparable to and may exceed the crystallization enthalpy (~65 kJ mol\(^{-1}\)) at lower \( T_s \). The enthalpy of the pair interactions averaged over all polar angles is significantly lower; this comparison suggests structuring of the dense liquid. We propose that the pair of parameters (molecular volume, second virial coefficient) may be an adequate predictor of the phase behavior of solutions of proteins with relatively simple interaction potentials.

**Monte-Carlo Simulations of phase diagrams**

We apply Monte Carlo simulation techniques to the investigation of phase diagrams associated with large biological molecules in solution. The system is modeled using short-range two-body intermolecular potentials exhibiting multiple extrema. We show that the introduction of a local repulsive maximum or a secondary attractive minimum in these potentials has dramatic effects on the phase diagram. Both stable and metastable liquid-liquid separation curves are driven to lower temperatures, the sensitivity of the solubility curve (liquidus) to temperature is significantly reduced, the enthalpy of crystallization is significantly diminished, metastable liquid-liquid separation may become stable and vice versa, and both low and high density solid phases may be observed.

**Nucleation of protein crystals and dense liquid droplets**

The nucleation mechanism of the solution—crystals phase transition. We investigated the nucleation of crystals of the protein lysozyme from aqueous solutions using a novel technique that allows direct determinations of homogeneous nucleation rates. The method is applicable to studies of crystallization, aggregation, and similar first-order phase transitions in solutions of proteins or other soluble slow-
growing materials with temperature-dependent solubility. To obtain reproducible statistical characteristics of the intrinsically random nucleation process, a large number of simultaneous trials take place under identical conditions. The nucleation theorem allows determination of the changes of the sizes of the critical cluster with increasing supersaturation as (10 or 11) → (4 or 5) → (1 or 2). Furthermore, we observe that the existence of a second liquid phase at high protein concentrations, Fig. 9, strongly affects crystal nucleation kinetics: (i) Crystal nucleation rates are lower than expected in the phase region of liquid-liquid demixing. (ii) In the immediate proximity of this region, nucleation rates vary by factors of up to two in identical experiments. Since experiments discussed in next subsection show a sharp rate maximum in this region, we attribute this kinetic instability to minor shifts of the experimental conditions toward or away from the phase boundary.

_Dense liquid precursor for the nucleation of ordered solid phases from solution._

We showed that the presence of a liquid-liquid (L-L) phase boundary hidden below the solubility line in the phase diagram of the protein solution, has a profound effect on the rate of homogeneous nucleation $J$. In the vicinity of the L-L boundary (the respective $T_{L-L}$ are marked with vertical lines) $J$ passes through a maximum, similar to predictions of some of the simulations and theory. These and other recent results on the kinetics of homogenous nucleation of protein crystals indicate that under a very broad range of conditions the nucleation of lysozyme crystals occurs as a superposition of fluctuations along the order parameters density and structure. Depending on whether the system is above or below its liquid-liquid coexistence line, a density fluctuation may never or may selectively lead to the formation of a dense liquid droplet; in the former case the high density region, the “quasi-droplet”, is metastable also with respect to the dilute solution. In both cases, the molecules contained in the high-density region may attain an ordered arrangement, i.e., a structure fluctuation is superimposed on the density fluctuation and a crystalline nucleus obtains. This outlook on the nucleation of ordered solids from dilute phases suggests that the rate of nucleation can be controlled either by shifting the phase region of the dense liquid phase, or by facilitating the structure fluctuations within a dense liquid droplet or quasi-droplet. Results from literature indicate that the proposed two-step nucleation mechanism and the related tools for nucleation control may be applicable to the formation of crystalline and non-crystalline ordered solid phases of other, protein and non-protein materials, from solution.

_Control of the nucleation rate by controlling the concentration fluctuations._

Comparing data in the same supersaturation ranges recorded away from the L-L boundary, we find that the rate of nucleation is enhanced by factors of 6 to 20. The found correlation allows control of the nucleation rate of protein crystals by using additives that enhance or suppress the concentration fluctuation in protein solutions supersaturated with respect to an ordered solid phase. Glycerol and polyethylene glycol (PEG) (these reagents that shift this phase boundary, and do not specifically bind to proteins), significantly suppress (glycerol) or enhance (PEG) the crystal nucleation rates at $T \geq T_{L-L}$ (no additive), i.e., in the region of coexistence of the dilute solution and solid
phases, where control of nucleation of the solid phase is sought. Determinations of the phase diagrams in the presence of these additives and of their effects of the protein-protein interrelations in solution indicate that glycerol enhances the repulsion between the molecules, while PEG brings about stronger attraction. Correspondingly, glycerol suppresses the concentration fluctuations, while PEG enhances them. The effects of the two additives on the concentration fluctuations correlate with their effects on the nucleation rate. This correlation supports the suggested two-step mechanism of nucleation of protein crystals, and provides for a direct control mechanism of the nucleation rate. The control mechanism does not require changes in the protein concentration, the acidity and ionicity of the solution, temperature or protein concentration. The effects of the two additives on the phase diagram depend on their concentration and this provides for further tuning of nucleation rates.

Smooth transition from nucleation to spinodal decomposition in phase separating protein solutions

For insight into the structure and dynamics of phases emerging upon crossing the metastability/instability boundary we monitor with optical microscopy, in real time and in real space, the generation of a dense liquid phase in high-concentration solutions of the protein lysozyme after temperature quenches into thermodynamically defined metastable and unstable regions. We show with this system, which is a poor fit to mean-field assumptions, that the evolution of the structure factor during nucleation is similar to that during spinodal decomposition and reveals no singularity predicted upon crossing the metastability boundary. We introduce two new kinetic definitions of the metastability/instability boundary that yield values within ~ 1.5 K, i.e., the boundary appears as an area rather than a line, which is near and above the thermodynamic prediction. Delay times for the appearance of the new phase in the unstable regime are significant, i.e., new-phase growth is hindered by kinetic barriers. While our results agree with predictions of the non-mean-field theories of phase transformations, the experimentally observed behavior is richer than the one envisioned by theory.

Molecular processes of growth and defect formation of protein crystals

Molecular-level thermodynamic and kinetic parameters for the self-assembly of apoferritin molecules into crystals

The self-assembly of apoferritin molecules into crystals is a suitable model for protein crystallization and aggregation; these processes underlie several biological and biomedical phenomena, as well as for protein and virus self-assembly. We use the atomic force microscope in-situ, during the crystallization of apoferritin to visualize and quantify at the molecular-level the processes responsible for crystal growth. To evaluate the governing thermodynamic parameters, we image the configuration of the incorporation sites, “kinks”, on the surface of a growing crystal. We show that the kinks are due to thermal fluctuations of the molecules at the crystal-solution interface. This allows evaluation of the free energy of the intermolecular bond $\phi = 3.0 \ k_B T = 7.3 \ \text{kJ/mol}$. 
The crystallization free energy, extracted from the protein solubility, is \(-42 \text{ kJ/mol}\). Published determinations of the second virial coefficient and the protein solubility between 0 and 40 °C revealed that the enthalpy of crystallization is close to zero. Analyses based on these three values suggest that the main component in the crystallization driving force is the entropy gain of the waters bound to the protein molecules in solution and released upon crystallization. Furthermore, monitoring the incorporation of individual molecules into the kinks, we determine the characteristic frequency of attachment of individual molecules at one set of conditions. This allows a correlation between the mesoscopic kinetic coefficient for growth and the molecular-level thermodynamic and kinetic parameters determined here. We found that step growth velocity, scaled by the molecular size, equals the product of the kink density and attachment frequency, i.e., the latter pair are the molecular-level parameters for self-assembly of the molecules into crystals.

**Diffusion-limited kinetics of the solution-solid phase transition of molecular substances**

For critical tests of whether diffusion-limited kinetics is an option for the solution-solid phase transition of molecular substances, or they are exclusively determined by a transition-state, we performed crystallization experiments with ferritin and apoferritin, a unique pair of proteins with identical shells but different molecular masses. We find that the kinetic coefficient for crystallization is identical (accuracy \(\pm 7\%\)) for the pair, indicating diffusion-limited kinetics of crystallization. Data on the kinetics of this phase transition in systems ranging from small-molecule ionic to protein and virus suggest that the kinetics of solution phase transitions for broad classes of small-molecule and protein materials are diffusion-limited.

**Capillarity effects on crystallization kinetics: insulin**

During layerwise growth of crystals, capillarity governs the generation of new crystal layers. Theory predicts that the line tension of the layer edge determines, via the characteristic two-dimensional capillary length \(L_c\), the rates of generation and initial growth of the new layers. To test the correlation between \(L_c\) and the rate of layer generation, we used *in-situ* Tapping Mode Atomic Force Microscopy (TM-AFM) to study the generation and spreading of layers during crystallization of rhombohedral, R3, porcine insulin. We show that crystallization of this insulin form is uniquely suitable for such an investigation due to the linear kinetics of step growth it exhibits. This linear kinetics reflects the abundance of the incorporation sites along the rough steps, the lack of long-range step-step interactions, and the transport control of the growth kinetics. The kinetic coefficients are \(7 \times 10^{-3}\) and \(4 \times 10^{-2}\) cm s\(^{-1}\), respectively, in the absence and presence of the co-solvent acetone—somewhat high for proteins and comparable to values for inorganic systems. We show that (i) the relevant capillary length, the size of a critical quadrangular 2D nucleus \(L_c\), is the main scaling factor for the density of growth steps, while (ii) all steps longer than \(L_c\) grow with a rate determined only by the supersaturation and independent on their length. We explain the divergence of (ii) from
theoretical predictions with the high supersaturations typical of the growth of this protein system.

Evidence for the surface diffusion mechanism of solution crystallization from molecular-level observations with ferritin

We employ atomic force microscopy to monitor in situ, in real time, the molecular processes of crystallization of ferritin, a protein which has an inorganic single-crystalline core that can be varied. We determine the statistics of molecular attachment and detachment at the growth sites and find that the ratio of the fluxes in and out of the kinks is significantly lower than expected assuming direct incorporation of the molecules from the solution. Determinations of the energy barrier for incorporation yield ~ 30 kJ mol⁻¹, significantly higher than expected for this mechanism. We conclude that attachment of molecules occurs via the surface adsorption layer. The surface coverage resulting from this mechanism is ~ 0.9, suggesting a growth mode different from the classical surface diffusion mechanism.

Molecular mechanisms of microheterogeneity-induced defect formation in ferritin crystallization

We apply in-situ atomic force microscopy to the crystallization of ferritins from solutions containing ~5% (w/w) of their inherent molecular dimers. Molecular resolution imaging shows that the dimers consist of two bound monomers. The constituent monomers are likely partially denatured resulting in increased hydrophobicity of the dimer surface. Correspondingly, the dimers strongly adsorb on the crystal surface. The adsorbed dimers hinder step growth and upon incorporation by the crystal initiate stacks of up to 10 triple and single vacancies in the subsequent crystal layers. The molecules around the vacancies are shifted by ~ 0.1 molecular dimensions from their crystallographic positions. The shifts strain the lattice and, as a consequence, at crystal sizes > 200 μm, the accumulated strain is resolved by a plastic deformation whereupon the crystal breaks into mosaic blocks 20 to 50 μm in size. The critical size for the onset of mosaicity is the similar for ferritin and apoferritin and close to the value for a third protein, lysozyme; it also agrees with theoretical predictions. Trapped microcrystals in ferritin and apoferritin induce strain with a characteristic lengthscale equal to that of a single point defect, and, as a consequence, trapping does not contribute to the mosaicity. The sequence of undesired phenomena that include heterogeneity generation, adsorption, incorporation and arising lattice strain and mosaicity in this and other proteins systems could be avoided by improved methods to separate similar proteins species (microheterogeneity), or by increasing the biochemical stability of the macromolecules against oligomerization.

Defects, strain, mosaicity and diffraction resolution.

The growth of protein crystals, as well as any other crystal, occurs by the ordered addition of molecules. For a perfect crystal, a huge number of such additions (of the
order of $10^{15}$ and higher) must occur in a strictly identical fashion. This large number awards many opportunities for misaligned attachment of single molecules, molecular aggregates (amorphous and crystalline), or other species present in the nutrient medium, as well as for short- and long-term variability of the growth process. As a result, defects ranging in scale from the molecular (mutated and conformationally-different molecules, misaligned molecules and single vacancies) through trapped impurities, clusters and oligomers, dislocations, and twinning planes, to the macroscopic (striaations, occlusions, twins, blocks and grains and zones) are formed.

How dangerous are the defects? One may argue that even if the crystal contains 1 % of misplaced molecules, this will only result in 1 % decrease in diffraction intensity, or 1 % increase in the background noise. Unfortunately, this is not so. Even for the smallest, molecular level, “point” defects, it has been shown that 1) they replicate in subsequent layers during growth; 2) they cause strain with the strain field extending to 5 to 10 molecular diameters; and 3) the accumulation of strain leads to mosaicity and block structure.

Are all defects dangerous? Arguments have been put forth that diffraction resolution is only affected by short-scale molecular disorder and not by mosaicity, striae, zoning, and block structures. There are examples in which heavily mosaic crystals diffract to high resolution. For insight, we note that the diffraction resolution is determined by the signal-to-noise ratio of high-index reflections. Since high-index crystal planes have low molecular density, larger areas of rotationally and translationally aligned molecules are needed to enhance the intensity of the reflections from these planes. Hence, crystal imperfections on the scale of microns, e.g., striae, and even tens and hundreds of microns, e.g., block structures, twins, etc., should affect the diffraction resolution obtainable from a crystal. Mosaicity, striae and block structures often lead to broader or split diffraction spots, and, hence, lower accuracy of the structure determination [58,59]. However, if the crystal consists of a few large blocks, the beam in a X-ray diffraction experiments can be focused on only one of these blocks, and high resolution structure determinations can still be achieved.

The various defects that may be present in a protein crystal have been classified as:

I. Sub-molecular level defects: mutated molecules, conformational changes;

II. Rotational and translational lattice defects:
   - variability of the rotational orientation of the molecules in the crystals;
   - vacancies or molecules out of their lattice positions;

III. Impurity-related defects, caused by other species present in the solution;

IV. Linear and planar defects: dislocations, twins, sector, grain and block boundaries;

V. Striations, occlusions;

VI. Incorporation of microcrystals.

Step pattern instabilities

Phase-shifting interferometry for the study of the step dynamics during crystallization of proteins—a prototype for space-flight apparatus
We have developed a novel phase-shifting interferometry technique for high-resolution in-situ investigations of the unsteady dynamics of growth steps during the crystallization of proteins. The phase-shifting algorithm employs five-image sequences captured with a phase shift of $\pi/2$; digital processing of the sequence allows reconstruction of the surface morphology with a depth resolution $< 5$ nm and a lateral resolutions of $0.5 \mu$m across a field of view as wide as 1 mm. Such sequences can be recorded with a frequency of $\sim 1$ s$^{-1}$ and allow monitoring of the appearance and evolution of local morphology features, such as step bunches. Time traces of the variations of the growth rate and local slope (proportional to the density of the growth steps) at up to 10 select locations on a studied crystals facet are recorded with time resolution that can be as low as 0.2 s. Application of this technique to the ferritin crystals shows extensive fluctuations of growth rate and local slope as a result of step bunching.

**Spatio-temporal step patterns during crystal growth in a transport-controlled system**

We aim at insight into the unsteady kinetics and the formation of spatio-temporal patterns of steps during the crystal growth in systems, in which the growth rate is controlled by the rate of supply of material. For this, we apply phase-shifting interferometry to the crystallization of the protein ferritin. We find that the locally measured growth rate, step density and step velocity fluctuate by up to 80-100% of their average values. The fluctuations are due to passage of step bunches generated at the facet edges due to unsteady surface nucleation. The fluctuation amplitudes decrease with higher supersaturation and larger crystal size, as well as with increasing distance from the step sources, even while the average value of local slope, a destabilizing factor, increases. Since size and supersaturation are parameters affecting the solute supply field, we conclude that fluctuations are rooted in the coupling of the interfacial processes of growth to the bulk transport in the solution. To understand the counterintuitive suppression of the instability, we analyzed the step velocity dependence on local slope and found only a very weak interaction between the steps, likely due to competition for supply from the solution. Accordingly, the step bunches propagate with the same velocity as elementary steps. We conclude that in transport-controlled systems with non-interacting or weakly interacting steps the stable growth mode is that via equidistant step trains, and randomly arising step bunches decay. Stronger step interactions may reverse this conclusion, or slow down the rate, at which step bunches decay and stability is reached.

**Dissipating step bunches during crystallization under transport control**

In studies of crystal formation by the generation and spreading of layers, equidistant step trains are considered unstable – bunches and other spatio-temporal patterns of the growth steps are viewed as ubiquitous. We provide an example to the opposite. We monitor the spatio-temporal dynamics of steps and the resulting step patterns during crystallization of the proteins ferritin and apoferritin using the atomic force microscope. The variations in step velocity and density are not correlated, indicating the lack of a long-range attraction between the steps. We show that (i) because of its coupling to bulk transport, nucleation
of new layers is chaotic and occurs at the facet edges, where the interfacial
supersaturation is higher; (ii) step bunches self-organize via the competition for supply
from the solution; and, (iii) bunches of weakly interacting steps decay as they move along
the face. Tests by numerical modeling support the conclusions about the mechanisms
underlying our observations. The results from these systems suggest that during
crystallization controlled by transport, with weakly or non-interacting growth steps, the
stable kinetic state of the surface is an equidistant step train, and step bunches only arise
during nucleation of new layers. Since nucleation only occurs at a few sites on the
surface, the surface morphology may be controllably patterned or smoothened by locally
controlling nucleation.

Stable equidistant step trains during crystallization of insulin

Bunching of growth steps plagues layerwise crystallization of materials in laboratory,
industrial, and geological environments, and theory predicts that equidistant step trains
are unstable under a variety of conditions. Searching for an example of stable equidistant
step trains, we monitored the generation and spatio-temporal evolution of step trains on
lengthscales from 100 nm to 1 mm during the crystallization of insulin, using atomic
force microscopy and phase-shifting interferometry. We show that near-equidistant step
trains are generated by single and cooperating screw dislocations. The lack of step-step
interactions and the overall transport-controlled growth regime further regularize the step
train and insure the stability of the obtained equidistant arrangement.

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**Presentations Contributed**


