Final Report

Office of Biological and Physical Sciences Research, NASA

PIs Last Name	Vekilov
First Name	Peter
Middle Initial	G.
Prefix	Prof.
Suffix	
Affiliation	University of Houston

Phone: 713-743-4315 **Fax:** 713-743-4323
Email: vekilov@uh.edu

Address:

University of Houston
Department of Chemical Engineering
Engineering Building I
Houston, TX 77204-4004

Task Research Title

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

Monitoring Center	MSFC	*NAG Number*	NAG8-1854
Research Type	Flight	*Discipline*	Biotechnology
Initiation Date	03/01/1999	*Expiration Date*	9/30/2003

Trainees	**Numbers Funded**	**Degrees Granted**
BS	6	6
MS	2	2
PhD	8	pending
PostDoctoral	5	N/A
Totals	**16**	**8**

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
"Using powers of physics to unlock biology's secrets" Dallas Morning News, August 04, 2003, p. E1
Time Warner 24 Hours News TV Channel, June 29, 2003
"Perfect Insulin Crystals", Physics News Update, 636 #2, May 7, 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
"Crystal growth starts flat", http://www.lahaaland.com/science/physics/physics255.html
"Observing the Crystallization Process" About: Composite Materials, August 2000
"First look at a crystal's "embryo"—flat, not round", naturalSCIENCE, News, August 3, 2000
"First Images Of Crystal Nucleation Are Unexpected", http://unisci.com/stories/20003/0803004.htm
"Caught on Film: Nucleating Crystals Make Their Debut", Microgravity News, vol. 7, No.3, Fall 2000, p.11
"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
"Catching Crystals at Birth", Nature, vol. 406, August 3, 2000, p. 464
"Protein Crystallization at NASA: Well Grounded", Science August 6, 1999; 285: 835

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 fro 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) $CsSO_4$, 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 [Na^+] between 0.1 and 0.15 M. The value of B_2 at the minimum of ~ 4 is equal to that expected for non-interacting hard spheres, indicating that at these [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 form 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]

$$U_{hyd} \propto \pi a f_0 \exp - (2a / L).$$

Here, f_0 and L are the parameters for the surface energy density and decay length respectively. Choosing decay length $L = 2\times0.72$ nm = 1.44 nm (twice the diameter of a hydrated sodium ion), and f_0 = 12.5 mJ/m^2, in the middle of the range determined by surface force measurements, the values of the second virial coefficient at [Na^+] > 0.15 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 ofthe 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 Mw = 450,000 Da). However, further increases of the [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 $T_{L\text{-}L}$ 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 $T \geq T_{L\text{-}L}$(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 $\phi = 3.0\ k_B T = 7.3$ kJ/mol.

The crystallization free energy, extracted from the protein solubility, is – 42 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 in to 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 $\leq$ 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 viri 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 7×10^{-3} and 4×10^{-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 mdecular-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 (striations, 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 μm across a field of view as wide as 1 mm. Such sequences can be recorded with a frequency of ~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.

Publications

Book Chapters

1. P.G. Vekilov and A.A. Chernov, *The Physics of Protein Crystallization*, in **Solid State Physics**, vol. 57, edited by H. Ehrenreich and F. Spaepen (Academic Press, New York, 2002) pp. 1-147.
2. O. Gliko and P. G. Vekilov, *Spatio-Temporal Patterns in Ferritin Crystal Growth,* in **Biological and Biomimetic Materials – Properties to Function**, edited by J. Aizenberg, J.M. McKitrick and C.A. Orme, (MRS, Warrendale, PA., 2002) pp. 141-146
3. P.G. Vekilov, *Solvent entropy effects in the formation of protein solid phases*, in **Methods in Enzymology volume 368: Macromolecular Crystallography, Part C** edited by C.W. Carter, Jr., and R.M. Sweet, (Academic Press, San Diego, 2003) pp. 84-105.
4. P.G. Vekilov, *Molecular mechanisms of defect formation,* in **Methods in Enzymology volume 368: Macromolecular Crystallography, Part C** edited by C.W. Carter, Jr., and R.M. Sweet (Academic Press, San Diego, 2003) pp. 170-188.
5. J.J. De Yoreo, P.G. Vekilov, *Principles of crystal nucleation and growth*, in **Biomineralization** edited by P.M. Dove, J.J. De Yoreo, S. Weiner (Mineral Soc. Am., Washington, DC, 2003) pp. 57-93
6. P.G. Vekilov, *Microscopic, mesoscopic, and macroscopic lengthscales in the kinetics of phase transformations with proteins*, in **Nanoscale Structure and Assembly at Solid-fluid Interfaces**, edited by J.J. De Yoreo and X.Y. Lui (Kluwer Press, New York, 2004) pp. 145-200.
7. P.G. Vekilov and O. Galkin, *Fundamental aspects of nucleation theory revealed in experiments with protein solid phases*, in **Nanoscale Structure and Assembly at Solid-fluid Interfaces**, edited by X.Y. Lui and J.J. De Yoreo (Kluwer Press, New York, 2004) pp. 105-144.
8. P.G. Vekilov, *Kinetics and mechanisms of protein crystallization at the molecular level,* in **Methods in Molecular Biology, vol. 300: Protein Nanotechnology, Protocols, Instrumentation, and Applications**, edited by T. Vo-Dinh (Humana Press, Totowa, NJ, 2005) pp. 15-52.

Review papers

9. P.G. Vekilov and J.I.D. Alexander, *Dynamics of layer growth in protein crystallization*, Chem. Rev. **100** (2000) 2061-2089.
10. P.G. Vekilov, *Self-Assembly of Apoferritin Molecules into Crystals: Thermodynamics of Molecular Level Processes*, Progress in Crystal Growth and Characterization of Materials **45** (2002) 175-199.
11. P.G. Vekilov, *Dense liquid precursor for the nucleation of ordered solid phases from solution*, Crystal Growth and Design, **4** (2004) 671-685.

Original papers

12. P.G. Vekilov, F. Rosenberger, H. Lin and B.R. Thomas, *Nonlinear dynamics of layer growth and consequences for protein crystal perfection*, J. Crystal Growth **196** (1999) 261-275.
13. D.C. Carter, K. Lim, J.X. Ho, B.S. Wright, P.D. Twigg, T.Y. Miller, J. Chapman, K. Keeling, J. Ruble, P.G. Vekilov, B.R. Thomas, F. Rosenberger and A.A. Chernov, *Lower dimer impurity incorporation may result in higher perfection of HEWL crystal grown in μg. A case study.* J. Crystal Growth **196** (1999) 623-637.
14. F. Rosenberger, H. Lin and P.G. Vekilov, *Finite-amplitude instability in growth step trains with overlapping step supply fields.* Phys. Rev. E **59** (1999) 3155-3164.
15. R. Feeling-Taylor, R. M. Banish, R. Elison Hirsch and P.G. Vekilov, *Miniaturized scintillation technique for protein solubility determinations*, Rev. Sci. Instr. **70** (1999) 2845-2849.
16. O. Galkin and P.G. Vekilov, *Direct determination of the nucleation rates of protein crystals* J. Phys. Chem. **103** (1999) 10965-10971.
17. M. Wang, X.-B. Yin, P.G. Vekilov, R.-W. Peng and N.B. Ming, *Intrinsic stability of concentration field in diffusion limited growth and its effects on crystallization.* Phys. Rev. **E 60** (1999) 1901-1905.
18. P.G. Vekilov, *Protein crystal growth – microgravity aspects.* Advances in Space Research **24** (1999) 1231-1240.
19. O. Galkin and P.G. Vekilov, *Are nucleation kinetics of protein crystals similar to those of liquid droplets?* J. Am. Chem. Soc. **122** (2000) 156-163.
20. D.N. Petsev and P.G. Vekilov, *Evidence for non-DLVO hydration interactions in solutions of the protein apoferritin.* Phys. Rev. Lett. **84** (2000) 1339-1342.
21. D.N. Petsev, B.R. Thomas, S.-T. Yau and P.G. Vekilov, *Interactions and aggregation of apoferritin molecules in solution: Effects of added electrolyte.* Biophysical J. **78** (2000) 2060-2069.
22. B.R. Thomas, A.A. Chernov, P.G. Vekilov and D.C. Carter, *Distribution coefficients on protein impurities in ferritin and lysozyme crystals. Self-purification in microgravity.* J. Crystal Growth **211** (2000) 149-156.
23. O. Galkin and P.G. Vekilov, *Control of protein crystal nucleation around the metastable liquid-liquid phase boundary*, Proc. Natl. Acad. Sci. USA **97** (2000) 6277–6281.
24. S.-T. Yau, B.R. Thomas and P.G. Vekilov, *Molecular mechanisms of crystallization and defect formation.* Phys. Rev. Lett. **85** (2000) 353-356.
25. S.-T. Yau and P.G. Vekilov, *Quasi-planar nucleus structure in apoferritin crystallisation.* Nature **406** (2000) 494-497.

Featured in **Nature**, vol. 406, August 3, 2000, p. 464
Science News, August 5, 2000, p. 84
naturalSCIENCE, News, August 3, 2000
Microgravity News, vol. 7 #3, Fall 2000, p.11

26. S.-T. Yau, D.N. Petsev, B.R. Thomas, and P.G. Vekilov, *Molecular-level thermodynamic and kinetic parameters for the self-assembly of apoferritin molecules into crystals.* J. Mol. Biol. **303** (2000) 667-678.
27. H. Lin, D.N. Petsev, S.-T. Yau, B.R. Thomas and P.G. Vekilov, *Lower incorporation of impurities in ferritin crystals by suppression of convection: modeling results,* Crystal Growth and Design **1** (2001) 73-79

invited paper for inaugural issue of Crystal Growth and Design

28. S.-T. Yau and P.G. Vekilov, *Direct observation of nucleus structure and nucleation pathways*, J. Am. Chem. Soc. **123** (2001) 1080-1089.
29. S.-T. Yau, B.R. Thomas, O. Galkin, O. Gliko, and P.G. Vekilov, *Molecular mechanisms of microheterogeneity-induced defect formation in ferritin crystallization,* Proteins **43** (2001) 343-352.
30. O. Galkin and P.G. Vekilov, *Nucleation of protein crystals: critical nuclei, phase behavior, and control pathways.* J. Crystal Growth **232** (2001) 63-76.
31. S.-T. Yau, B.R. Thomas, and P.G. Vekilov, *Real time, in-situ, monitoring of apoferritin crystallization and defect formation with molecular resolution.* J. Crystal Growth **232** (2001) 188-194.
32. D.N. Petsev, B.R. Thomas, S.-T. Yau, D. Tsekova, C.N. Nanev, W.W. Wilson, and P.G. Vekilov, *Temperature-independent solubility and interactions between apoferritin monomers and dimers in solution.* J. Crystal Growth **232** (2001) 27-29.
33. O. Galkin, K. Chen, R.L. Nagel, R.E Hirsch, P.G. Vekilov, *Liquid-liquid separation in solutions of normal and sickle cell hemoglobin*, Proc. Natl. Acad. Sci. USA **99** (2002) 8479–8483.
34. N. A. Booth, A. A. Chernov, P.G. Vekilov, *Characteristic lengthscales of step bunching in KDP crystal growth: in-situ differential phase-shifting interferometry study.* J. Crystal Growth **237-239** (2002), 1818-1824.
35. P.G. Vekilov, A.R. Feeling-Taylor, D.N. Petsev, O. Galkin, R.L. Nagel, R. E. Hirsch, *Intermolecular Interactions, Nucleation and Thermodynamics of Crystallization of Hemoglobin C.* Biophys. J. **83** (2002) 1147-1156.

Featured on the **cover of Science** vol. 299 #5609 February 14, 2003

36. K. Chen and P.G. Vekilov, *Evidence for the surface diffusion mechanism of solution crystallization from molecular-level observations.* Phys. Rev. E **66** (2002) 021606.
37. O. Gliko, N. A. Booth, E. Rosenbach, P. G. Vekilov, *Phase-shifting interferometry for the study of the step dynamics during crystallization of proteins.* Crystal Growth and Design **2** (2002) 381-385.
38. P. G. Vekilov, A. R. Feeling-Taylor, S.-T. Yau, and D. Petsev, *Solvent entropy contribution to the free energy of protein crystallization*, Acta Crystallogr. Section D. **58** (2002) 1611-1616.
39. O. Gliko, N. A. Booth, P. G. Vekilov, *Step bunching in a diffusion-controlled system: phase-shifting interferometry investigation of ferritin.* Acta Crystallogr. Section D. **58** (2002) 1622-1627.
40. N.A. Booth, B. Stanojev, A.A. Chernov, P.G. Vekilov, *Differential phase-shifting interferometry for in situ surface characterization during solution growth of crystals*, Rev. Sci. Intsr. **73** (2002) 3540-3545.
41. O. Gliko and P. G. Vekilov, *Spatio-temporal step patterns during crystal growth in a transport controlled system*, J. Phys. Chem., **106** (2002) 11800 – 11804.
42. D.N. Petsev, K. Chen, O. Gliko, and P.G. Vekilov, *Diffusion-limited kinetics of the solution-solid phase transition of molecular substances*, Proc. Natl. Acad. Sci. USA **100** (2003) 792-796.
43. P.G. Vekilov and O. Galkin, *On the methods of determination of homogeneous nucleation rates of protein crystals*, Colloids and Surfaces A, **215** (2003) 125-130.
44. H. Lin, S.-T. Yau and P.G. Vekilov, *Dissipating step bunches during crystallization under transport control*, Phys. Rev. E, **67** (2003) 0031606.
45. D.N. Petsev, X. Wu, O. Galkin and P.G. Vekilov, *Thermodynamic functions of concentrated protein solutions from phase equilibria*, J. Phys. Chem. B **107** (2003) 3921-3926.
46. O. Gliko, I. Reviakine, P.G. Vekilov, *Stable equidistant step trains during crystallization of insulin*, Phys. Rev. Lett. **90** (2003) 225503.

Featured in **Physics News Update** 636 #2, May 7, 2003

47. I. Reviakine, D.K. Georgiou, P.G. Vekilov *Capillarity effects on crystallization kinetics: insulin.* J. Am. Chem. Soc. **125** (2003) 11684-11693.
48. .L. Bergeron, L. Filobelo, O. Galkin, P.G. Vekilov, *Thermodynamics of the hydrophobicity in crystallization of insulin*, Biophys. J. **85** (2003) 3935–3942.
49. O. Galkin and P.G. Vekilov, *Mechanisms of homogeneous nucleation of polymers of sickle cell anemia hemoglobin in deoxy-state*, J. Mol. Biol. **336** (2004) 43–59.

Featured on **cover of J. Mol. Biol.** Vol. 336 Number 1, Feb. 6, 2004

50. N.A. Booth, A.A. Chernov, P.G. Vekilov, *Interplay of impurities and solution flow as determinants of step patterns dynamics*, Phys. Rev. E **69** (2004) 011604.

51. A.R. Feeling-Taylor, S.-T. Yau, D.N. Petsev, R.L. Nagel, R. E. Hirsch, P.G. Vekilov, *Crystallization mechanisms hemoglobin C in the R-state.* Biophys. J., **87** (2004) 2621-2629.
52. M. Shah, O. Galkin, and P.G. Vekilov, *Smooth transition from metastability to instability in phase separating protein solutions.* J. Chem. Phys. **121** (2004) 7505-7512.
53. Y. Qutub, I. Reviakine, C. Maxwell, J. Navarro, E. Landau, and P.G. Vekilov, *Mechanisms of in cubo of growth and defect formation of three-dimensional bacteriorhodopsin crystals*, J. Mol. Biol. **343**, (2004) 1243-1254.
54. A.A. Chernov, J.J. De Yoreo, L.N. Rashkovich and P.G. Vekilov, *Step and kink dynamics in inorganic and protein crystallization,* MRS Bulletin **29** (2004) 927-934.

Presentations Invited

1. P.G. Vekilov, *Step pattern evolution and protein crystallization.* Laboratory of Enzymology and Structural Biology, CNRS, Gif-sur-Yvette, France, December 11, 1998.
2. P.G. Vekilov, *Crystallization processes on three lengthscales*, Institute of Theoretical Chemistry, Technical University of Munich, Munich, Germany, December 17, 1998.
3. P.G. Vekilov, *Nonlinear step dynamics in protein crystal growth.* 1999 Centennial Meeting of the American Physical Society, Atlanta, GA, USA, March 20-26, 1999.
4. P.G. Vekilov, *Protein crystallization processes on various lengthscales,* 1999 Tricampus Conference on Materials Science, University of Alabama in Huntsville, Huntsville, AL, April 23, 1999
5. P.G. Vekilov, *Protein crystallization beyond the needs of structure studies*, 1999 American Crystallographic Association Annual Meeting, Buffalo, NY, USA, May 22-26, 1999.
6. S.-T. Yau and P.G. Vekilov, *Molecular mechanisms of crystallization and defects formation*, 11 American Conference on Crystal Growth and Epitaxy (ACCGE-11), Tucson, AZ, August 1-6, 1999.
7. P.G. Vekilov, S.-T. Yau, B.R. Thomas, *Real time* in-situ *monitoring of ferritin crystal growth with molecular resolution*, Department of Chemistry, University of Alabama in Huntsville, Colloquium, Huntsville, AL, USA, November 12, 1999.
8. P.G. Vekilov, S.-T. Yau, B.R. Thomas, *Molecular processes of protein crystallization: why should crystallographers care*, University of Texas Southwestern Medical Center, Colloquium, Dallas, TX, USA, December 3, 1999.
9. P.G. Vekilov, *Optical Interferometry: Part I of Tutorial M: Experimental methods for Investigating Crystal Fluid interfaces*, 2000 Materials Research Society Spring Meeting, San Francisco, California, USA, April 16, 2000
10. S.-T. Yau, B.R. Thomas, D.N. Petsev, and P.G. Vekilov, *Real time in-situ monitoring of molecular processes during growth of protein crystals.* 2000 Materials Research Society Spring Meeting, San Francisco, California, USA, April 24-28, 2000.
11. P.G. Vekilov and S.-T. Yau, *Real time in-situ monitoring of ferritin crystallization with molecular resolution.* 8-th International Conference on Crystallization of Biological Macromolecules, Destin, Florida, USA, May 20-26, 2000 ***(plenary lecture)***
12. P.G. Vekilov, S.-T. Yau, D.N. Petsev, and B.R. Thomas, *Real time in-situ monitoring of molecular processes during protein crystallization.* Colloquium, Institute of Physical Chemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria, June 20, 2000.
13. P.G. Vekilov, O. Galkin, and S.-T. Yau, *Nucleation of protein crystals: structures, dynamics and control pathways*, 19 European Crystallographic Meeting, Nancy, France, August 25-31, 2000
14. P.G. Vekilov, O. Galkin, and S.-T. Yau, *Phase transitions is protein solutions: structures, dynamics and control pathways.* 2000 Biology Retreat, Guntersville, Alabama, USA, September 29-30, 2000.
15. P.G. Vekilov, *Protein crystallization processes at three length scales: molecular, capillary and transport.* Texas Christian University, Dallas, Texas, November 2, 2000.
16. P.G. Vekilov, O. Galkin, and S.-T. Yau, *Structures, dynamics and control pathways of protein crystal nucleation.* Southern Methodist University, Fort Worth, Texas, November 3, 2000.
17. P.G. Vekilov, S.-T. Yau, D.N. Petsev and B.R. Thomas, *What do we learn about biological molecules from watching them partake in phase transitions?* Seminar, Department of Biological Sciences, University of Alabama in Huntsville, Huntsville, Alabama, USA, November 29, 2000.

18. P.G. Vekilov, S.-T. Yau, O. Galkin, D. Petsev, B. Thomas, *How do molecules arrange themselves into crystals?* Seminar, Hokaido National Industrial Research Institute, Sapporo, Japan, December 8, 2000.
19. P.G. Vekilov, S.-T. Yau, H. Lin, D. Petsev, B. Thomas *Characteristic lengthscales of the protein crystallization processes: where can gravity affect growth,* Japan Space Utilization Promotion Center Tokyo, Japan, December 12, 2000.
20. P.G. Vekilov, S.-T. Yau, O. Galkin, D. Petsev, B. Thomas, *How do molecules arrange themselves into protein crystals?* Seminar, Tohoku University, Sendai, Japan, December 13, 2000.
21. P.G. Vekilov, O. Galkin, S.-T. Yau, M. Wu, D.N Petsev, *Phase transitions in protein solutions: dynamics, structures and control strategies.* Department of Chemical Engineering, University of Illinois, Champaign, IL, February 1, 2001
22. S.-T. Yau, D.N. Petsev, B.R. Thomas, and P.G. Vekilov, *Tracking individual molecules as they attach themselves to crystals: statistics, dynamics and mechanisms.* Physics Colloquium, University of Alabama in Huntsville, Huntsville, Alabama, February 7, 2001.
23. P.G. Vekilov, S.-T. Yau, O. Galkin, D.N Petsev, *Phase Transitions in Protein Solutions: Dynamics, Structures and Control Strategies.* Department of Chemical Engineering, University of Houston, February 16, 2001
24. P.G. Vekilov, *Molecular mechanisms of crystallization of proteins.* Marshal Space Flight Center, Material and Crystal Growth Seminar, Huntsville, Alabama, USA, February28, 2001.
25. P.G. Vekilov, S.-T. Yau, O. Galkin, D.N. Petsev, B.R. Thomas, *Phase Transitions in Protein Solutions: Dynamics, Structures and Control Strategies*, University of Alabama in Huntsville, Research Council Meeting, Huntsville, Alabama, April 2, 2001. P G. Vekilov, D.N. Petsev, S.-T. Yau, and K. Chen, *Crystallization of Small and Large Molecules*, 9th Inhalation Technology Seminar, Orion Pharma, Espoo, Finland, June 6, 2001
27. P.G. Vekilov, *Mechanisms of crystallization form solutions: a short course.* VTT (Technology Research Center of Finland) Helsinki, Finland, June 7-8, 2001.
28. P.G. Vekilov, O. Galkin, M. Wu, K. Chen, *Phase transition in protein solutions: dynamics and control strategies*; Colloquium, Institute of Physical Chemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria, June 12, 2001.
29. P.G. Vekilov, O. Galkin, D.N. Petsev, M. Wu, *Dynamics of phase transition in proteins solutions*, Albert Einstein College of Medicine, Department of Medicine, Division of Hematology, The Bronx, NY, June 27, 2001.
30. S.-T. Yau, D.N. Petsev, P.G. Vekilov, *Molecular-level parameters for the self assembly of biological macromolecules into crystals*, Gordon Conference on Thin Films and Crystal Growth, Williams College, Williamstown, Massachusetts, USA, July 1-6, 2001
31. O. Galkin and P.G. Vekilov, *Liquid-liquid separation in solutions of proteins: implications for the formation of condensed phases.* 13th International Conference on Crystal Growth, Kyoto, Japan, July 30 – August 4, 2001.
32. S.-T. Yau, D.N. Petsev, and P.G. Vekilov, *Molecular-resolution atomic force microscopy movies of step propagation around surface defects and impurities.* 13th International Conference on Crystal Growth, Kyoto, Japan, July 30 – August 4, 2001.
33. S.-T. Yau, D.N. Petsev, and P.G. Vekilov, *Direct visualization of nucleus structure and nucleation pathways in apoferritin crystallization.* 13th International Conference on Crystal Growth, Kyoto, Japan, July 30 – August 4, 2001.
34. P.G. Vekilov, S.-T. Yau, and H. Lin, *Characteristic lengthscales of the protein crystallization processes: where can* gravity *affect growth.* 13th International Conference on Crystal Growth, Kyoto, Japan, July 30 – August 4, 2001.P.G. Vekilov, *Phase transitions in protein solutions: dynamics, structures and control strategies,* Department of Chemical Engineering, University of Houston, Industrial Advisory Board Meeting, October 12, 2001, Houston
36. P.G. Vekilov, *Phase transitions in protein solutions: structures, dynamics, and control strategies,* School of Chemical Engineering, Cornell University, November 12, 2001, Ithaca, New York.
37. P.G. Vekilov, *Is mass a parameter for phase transitions in solutions, or transition-state or diffusion-limited kinetics?* 4th East-west Surface Science Workshop "Nanostructures on Surfaces", Pamporovo, Bulgaria, February 23-March 1, 2002.

38. P.G. Vekilov, D.N. Petsev, S.-T. Yau, A. Feeling-Taylor, *Solvent entropy contribution to the free energy of protein crystallization,* 9-th International Conference on Crystallization of Biological Macromolecules (ICCBM-9), Jena, Germany, March 21-26, 2002.
39. P.G. Vekilov, *Fundamental aspects of nucleation theory in the formation of protein condensed phases*, Department of Chemical Engineering, University of California – Berkeley, April 2, 2002.
40. O. Galkin, P.G. Vekilov, *Nucleation dynamics of protein solid phases*, 223 National Meeting of the American Chemical Society, Orlando, Florida, April 7-11, 2002.
41. P.G. Vekilov, S.-T. Yau, H. Lin, O. Gliko, *Nonlinear Dynamics and Pattern Formation on the Growth Interfaces of Protein Crystals*, 223 National Meeting of the American Chemical Society, Orlando, Florida, April 7-11, 2002.
42. P.G. Vekilov, O. Galkin, *Control strategy for nucleation of protein solid phases*, International Meeting Particles 2002, Orlando, Florida, April 20-23, 2002.
43. P.G. Vekilov, *Fundamental aspects of nucleation theory in the formation of protein condensed phases*, Department of Chemical and Nuclear Engineering, University of New Mexico, May 3, 2002.
44. S.-T. Yau, B.R. Thomas, D.N. Petsev, O. Galkin, O. Gliko, and P.G. Vekilov, *Defect formation during crystallization of ferritins: molecular mechanisms.* 2002 American Crystallographic Association Meeting, San Antonio, TX, May 25-30, 2002.
45. P.G. Vekilov, *Diffusion-limited kinetics of phase transitions in solutions*, Seminar, Institute of Physical Chemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria, July 16, 2002.
46. M. Shah, O. Galkin, D.N. Petsev, M. Wu and P.G. Vekilov, *Atto- and femto-litter droplets of concentrated protein solutions: liquid-liquid phase separation.* Texas Nano-vivo Summit, Houston, TX, August 1, 2002.
47. P.G. Vekilov, *Fundamental aspects of nucleation theory in the formation of protein condensed phases*, Materials Science Laboratory, National Institute of Standards and Technology, Gaithersburg, Maryland, September 17, 2002.
48. P.G. Vekilov, *Diffusion-limited kinetics of phase transitions in solutions*, Department of Chemistry, Rice University, Houston, Texas, October 17, 2002.
49. P.G. Vekilov, *Fundamental aspects of nucleation theory in the formation of protein condensed phases*, Department of Chemistry, Iowa State University, Ames, Iowa, November 1, 2002.
50. P.G. Vekilov, *Fundamental aspects of nucleation theory in the formation of protein condensed phases*, Faculty of Pharmacy, University of Paris V, Paris, France, January 28, 2003.
51. O. Gliko, H. Lin, S.-T. Yau, I. Reviakine, P.G. Vekilov, *Dynamics of Pattern Formation on Protein Crystal Surfaces,* 2003 Surfaces and Interfaces Conference, Lille, France, January 29-31, 2003.
52. P.G. Vekilov, *Fundamental aspects of nucleation theory in the formation of protein condensed phases*, Department of Chemistry, University of Houston, Houston, Texas, February 4, 2003.
53. P.G. Vekilov, *Fundamental aspects of nucleation theory in the formation of protein condensed phases*, Biophysical Seminar, University of Texas – Medical Branch, Galveston, Texas, February 19, 2003.
54. P.G. Vekilov, D.N. Petsev, S. Brandon, P. Katsonis, *Hydration interactions between apoferritin molecules and the phase behavior of the solution*, 225 National Meeting of the American Chemical Society, New Orleans, Louisiana, March 22-27, 2003.
55. P.G. Vekilov, O. Galkin, and S.-T. Yau, *What would Gibbs do if he were thinking of nucleation of protein solid phases.* Center for Study of Gene Structure & Function, Hunter College, City University of New York, New York, New York, May 14, 2003.
56. P.G. Vekilov, D.N. Petsev, K. Chen, *Diffusion-limited kinetics of the solution solid phase transition of molecular substances.* Marshal Space Flight Center, Material and Crystal Growth Seminar, Huntsville, Alabama, May 16, 2003.
57. P.G. Vekilov, O. Galkin, S.-T. Yau, and D.N. Petsev, *Fundamental aspects of nucleation theory in the formation of protein crystals*, AstraZeneca Central Research, Göteborg, Sweden, May 20, 2003.
58. P.G. Vekilov, O. Galkin, S.-T. Yau, and D.N. Petsev, *Fundamental aspects of nucleation theory in the formation of protein condensed phases*, Department of Physics, Université Libre de Bruxelles, Brussels, Belgium, May 22, 2003.

59. P.G. Vekilov, O. Galkin, S.-T. Yau, and D.N. Petsev, *Fundamental aspects of nucleation theory in the formation of protein condensed phases*, Department of Physics, Université Joseph Fourier, Grenoble, France, May 26, 2003.
60. M. Shah, O. Galkin, X. Wu, D.N. Petsev and P.G. Vekilov, *Dynamics of liquid-liquid separation in protein solutions,* European Synchrotron Radiation Facility, Grenoble, France, May 27, 2003.
61. M. Shah, O. Galkin, X. Wu, D.N. Petsev and P.G. Vekilov, *Dynamics of liquid-liquid separation in protein solutions,* Institute of Physical Chemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria, June 2, 2003.
62. P.G. Vekilov, O. Galkin, S.-T. Yau, and D.N. Petsev, *Fundamental aspects of nucleation theory in the formation of protein condensed phases*, 77th ACS Colloid and Surface Science Conference, Atlanta Georgia, June 15-18, 2003.
63. P.G. Vekilov, D.N. Petsev, S. Brandon, P. Katsonis, *Intermolecular interactions and the thermodynamics and kinetics of phase transitions in protein solutions*, 2003 Annual Meeting of the American Crystallographic Association, Covington, Kentucky, July 26-31, 2003
64. P.G. Vekilov, D.N. Petsev, K. Chen, *What drives and what delays the attachment of a molecule to a growing aggregate in solution,* 2003 Nano Summit Conference, Houston, Texas, July 31, 2003.
65. O. Galkin, P.G. Vekilov, *Mechanisms of nucleation of the deoxy-HbS polymers.* Laboratory of Chemical Physics National Institute of Diabetes & Digestive & Kidney Diseases, NIH, Bethesda, Maryland, September 12, 2003.
66. S.-T. Yau, O. Galkin, L. Filobelo, D. Petsev, P.G. Vekilov, *Fundamentals and control strategies for nucleation of protein crystals in solution.* Association for Crystallization Technology, 12th Larson Workshop, Groton, Connecticut, September 15-17, 2003
67. P.G. Vekilov, *Thermodynamic and Kinetic Controls for the Nucleation of Crystals in Solution*, Abbott Laboratories, North Chicago, Illinois, January 12, 2004
68. P.G. Vekilov, *The Physical chemistry of sickle cell anemia,* Cullen College of Engineering Leadership Board, University of Houston, Houston, January 23, 2004,
69. P.G. Vekilov, *Fundamental aspects of nucleation theory in the formation of protein condensed phases*, Department of Chemical Engineering, North Carolina State University, Raleigh, North Carolina, February 2, 2004.
70. P.G. Vekilov, *Why do protein crystal grow slowly?* Joint Annual Conference of the German Crystallographic Association and the German Association for Crystal Growth, March 15-19, 2004, Jena, Germany. ***(plenary lecture)***
71. M. Shah, O. Galkin, D.N. Petsev, and P.G. Vekilov, *Dynamics of the Liquid-Liquid Phase Separation in Protein Solutions*, 225th Meeting of the American Chemical Society, March 27 - April 1, 2004, Anaheim, California.
72. P.G. Vekilov, O. Galkin, L. Filobelo, P. Katsonis, W. Pan, S.-T. Yau, A. Kolomeisky, *Fundamental aspects of nucleation theory in the formation of protein condensed phases,* University of California – Los Angeles, Department of Chemical Engineering, April 16, 2004, Los Angeles, California.
73. P.G. Vekilov, *Water Structuring and the Dynamics of Phase Transitions with Proteins*, 12th Texas Protein Folders' Meeting, Navasota, TX, May 28-30, 2004.
74. P.G. Vekilov, *Why do protein crystal grow slowly?* 10th International Conference on Crystallization of Biological Macromolecules, June 4-10, 2004, Beijing, China.
75. P.G. Vekilov, *Fundamental aspects of nucleation theory in the formation of protein condensed phases* FOM Institute for Atomic and Molecular Physics, Amsterdam, Netherlands, June 18, 2004
76. P.G. Vekilov, *Phase transitions in protein solutions*, Protein-Protein Interactions in Vitro and in Vivo Workshop, Isaac Newton Institute for Mathematical Sciences, Cambridge, UK, June 21-23, 2004
77. D.N. Petsev, M. Shah, O. Galkin, X. Wu, P.G. Vekilov, *Does the anisotropy of the intermolecular interactions determine the protein crystal symmetry?* 2004 Annual Meeting of the American Crystallographic Association, Chicago, Illinois, July 17-22, 2004.
78. P.G. Vekilov, O. Galkin, L. Filobelo, W. Pan, A. Kolomeisky, *Two-step mechanism for the nucleation of crystals from solution*, 14th International Conference on Crystal Growth, Grenoble, France, August 9-14, 2004.

79. P.G. Vekilov, *Why do protein crystals grow slowly?* 14th International Conference on Crystal Growth, Grenoble, France, August 9-14, 2004.
80. P.G. Vekilov, *Water Structuring and the Dynamics of Phase Transitions with Proteins*, Department of Biochemistry and Biology, University of Houston, Houston, Texas, September 10, 2004
81. P.G. Vekilov, *Water Structuring and the Dynamics of Protein Crystallization Processes*, Department of Molecular Physiology and Biological Physics, University of Virginia School of Medicine, Charlottesville, Virginia, October 1, 2004
82. P.G. Vekilov, *Phase transitions in protein solutions*, Department of Chemical Engineering, California Institute of Technology, Pasadena, California, October 7, 2004.
83. P.G. Vekilov, *The Role of Water Structuring in the Thermodynamics and Kinetics of Phase Transitions with Proteins*, 2004 MRS Fall Meeting, Boston, November 29 – December 3, 2004
84. P.G. Vekilov, *Phase Transitions in Protein Solutions*, 21 New England Workshop on Complex Fluids, Harvard University, Cambridge, MA, December 3, 2004
85. P.G. Vekilov, *Phase Transitions in Protein Solutions,* University of Maryland, Informal Statistical Physics Seminar, College Park, Maryland, December 7, 2004.

Presentations Contributed

1. P.G. Vekilov, S.-T. Yau and H. Lin, *Variations of impurity incorporation and step bunching dynamics in response to changes in transport conditions.* Gordon Conference on Gravitational Effects in Physicochemical Systems, Henniker, New Hampshire, USA, June 26 – July 2, 1999
2. S.-T. Yau, H. Lin and P.G. Vekilov, *Surface structures in protein crystallization resulting from molecular, capillary and transport processes.* Gordon Conference on Thin Films and Crystal Growth Mechanisms, Plymouth, New Hampshire, USA, June 20 - 25, 1999
3. P.G. Vekilov and S.-T. Yau, *Self-assembly of biological macromolecules into crystals: real time, in-situ monitoring with molecular resolution*, 3-rd International Conference on Molecular Structural Biology, Vienna, Austria, Sept 5-11, 1999.
4. M.D. Serrano, O. Galkin, S.-T. Yau, B.R. Thomas, R.L. Nagel, R. E. Hirsch, and P.G. Vekilov, *Phase transitions in protein solutions and kinetics of HbS polymerization,* 24the Annual Meeting of the National Sickle cell Disease Program, Philadelphia, Pennsylvania, USA, April 8-12, 2000.
5. S.-T. Yau and P.G. Vekilov, *Protein crystallization processes at three length scales: molecular, capillary and transport,* First International Symposium on Microgravity Research and Applications, Sorrento, Italy, September 10-15, 2000.
6. P.G. Vekilov, S.-T. Yau, D.N. Petsev and B.R. Thomas, *Real-time in situ monitoring with molecular resolution of the elementary processes of crystallization of apoferritin.* 2000 Annual Meeting of the American Institute of Chemical Engineers, Los Angeles, California, November 12-17, 2000.
7. A.R. Feeling-Taylor, S.-T. Yau, D.N. Petsev, O. Galkin, R. Nagel, R.E. Hirsch, and P.G. Vekilov, *Molecular Mechanisms of Crystallization of HbC,* 25th Annual Meeting National Sickle Cell Disease Program, New York, NY, April 13 - 17, 2001.
8. H. Lin, S.-T. Yau, O. Gliko, and P.G. Vekilov, *Dynamics of Trains of Non-interacting Steps Growing under Diffusion Control*, 2001 Spring Materials Research Society Meeting, San Francisco, CA, April 16-21, 2001
9. S.-T. Yau, P.G. Vekilov, *Direct visualization of nucleus structure and nucleation pathways in apoferritin* crystallization, Gordon Conference on Thin Films and Crystal Growth, Williams College, Williamstown, Massachusetts, USA, July 1-6, 2001
10. S.-T. Yau, D.N. Petsev, P.G. Vekilov, *Phase transition in protein solutions: dynamics and control strategies*, Gordon Conference on Thin Films and Crystal Growth, Williams College, Williamstown, Massachusetts, USA, July 1-6, 2001
11. S.-T. Yau, D.N. Petsev, P.G. Vekilov, *Molecular-level thermodynamic and kinetic parameters for crystallization*, Gordon Conference on Gravitational Effects in Physicochemical Systems, Colby Sawyer College, New London, New Hampshire, USA, July 8-13, 2001

12. O. Galkin, D.N. Petsev, P.G. Vekilov, *Phase transition in protein solutions: dynamics and control strategies*, Gordon Conference on Gravitational Effects in Physicochemical Systems, Colby Sawyer College, New London, New Hampshire, USA, July 8-13, 2001
13. S.-T. Yau, P.G. Vekilov, *Direct visualization of nucleus structure and nucleation pathways in apoferritin crystallization*, Gordon Conference on Gravitational Effects in Physicochemical Systems, Colby Sawyer College, New London, New Hampshire, USA, July 8-13, 2001
14. P.G. Vekilov, S.-T. Yau, B.R. Thomas, O. Galkin, and O. Gliko, *Molecular mechanisms of microheterogeneity-induced defect formation in ferritin crystallization* 13th International Conference on Crystal Growth, Kyoto, Japan, July 30 – August 4, 2001.
15. S.-T. Yau, D.N. Petsev, P.G. Vekilov, *Molecular-level Thermodynamic and Kinetic Parameters for Crystallization.* 13th International Conference on Crystal Growth, Kyoto, Japan, July 30 – August 4, 2001.
16. P.G. Vekilov and O. Galkin, *Phase transition in protein solutions: dynamics and control strategies*, Keck Center 2001 Annual Research Conference, Galveston, TX, USA, September 21, 2001.
17. P.G. Vekilov, D.N. Petsev, S.-T. Yau, A. Feeling Taylor, *Solvent structuring around protein molecules and dynamics of the molecular self-assembly.* 2001 Annual Meeting of the American Institute of Chemical Engineers, Reno, Nevada, November 4 – 9 , 2001
18. P.G. Vekilov, D.N. Petsev, S.-T. Yau, B.R. Thomas, O. Galkin, O. Gliko, *Molecular Mechanisms of Microheterogeneity-induced Defect Formation in Ferritin Crystallization.* 2001 Annual Meeting of the American Institute of Chemical Engineers, Reno, Nevada, November 4 – 9 , 2001
19. O. Galkin, D.N. Petsev, and P.G. Vekilov, *Control of protein crystal nucleation around the metastable liquid-liquid phase boundary.* 2001 Annual Meeting of the American Institute of Chemical Engineers, Reno, Nevada, November 4 – 9 , 2001
20. P.G. Vekilov, D.N. Petsev, S.-T. Yau, *Direct visualization of nucleus structure and nucleation pathways in apoferritin crystallization.* 2001 Annual Meeting of the American Institute of Chemical Engineers, Reno, Nevada, November 4 – 9 , 2001.
21. O. Gliko and P.G. Vekilov, *Step bunching in a diffusion-controlled system: phase-shifting interferometry investigation of ferritin*, 9-th International Conference on Crystallization of Biological Macromolecules (ICCBM-9), Jena, Germany, March 21-26, 2002.
22. P.G. Vekilov, *Solvent entropy effects in the formation of protein solid phases.* 2002 Spring Materials Research Society Meeting, San Francisco, CA, April 1-5, 2002.
23. D.N. Petsev, K. Chen, O. Gliko and P.G. Vekilov, *Diffusion-limited kinetics of the solution-solid phase transition of molecular substances*, 19th Conference on Crystal Growth and Epitaxy, Stanford Sierra Camp, Fallen Leaf Lake, CA, June 2-5, 2002
24. M. Shah, O. Galkin, D.N. Petsev, M. Wu and P.G. Vekilov, *Control of the size and distribution of atto- and femto-litter protein droplets: liquid-liquid phase separation.* Bio-fuel Cells Workshop, Washington, DC, June 30- July 2, 2002.
25. P.G. Vekilov, S.-T. Yau, H. Lin, O. Gliko, *Nonlinear Dynamics and Pattern Formation on the Growth Interfaces of Protein Crystals*, 2002 Annual Meeting of the American Institute of Chemical Engineers, Indianapolis, Indiana, November 3 – 8 , 2002.
26. D.N. Petsev. K. Chen, O. Gliko, P.G. Vekilov, *Diffusion-limited kinetics of phase transitions in solutions*, 2002 Annual Meeting of the American Institute of Chemical Engineers, Indianapolis, Indiana, November 3 – 8 , 2002.
27. O. Gliko, P.G. Vekilov, *Phase-shifting interferometry for the study of the spatio-temporal evolution of crystal-solution interfaces,* 2002 Annual Meeting of the American Institute of Chemical Engineers, Indianapolis, Indiana, November 3 – 8 , 2002.
28. D.N. Petsev, K. Chen, and P.G. Vekilov, *Diffusion limited kinetics of the solution-solid phase transition of molecular substances,* 77th ACS Colloid and Surface Science Conference, Atlanta Georgia, June 15-18, 2003.
29. P.G. Vekilov, D.N. Petsev, K. Chen, *Diffusion-limited kinetics of the solution-solid phase transition of* molecular *substances*, 15th American Conference on Crystal Growth and Epitaxy, Keystone, Colorado, July 20-25, 2003.

30. P. G. Vekilov, L. Bergeron, L.F. Filobelo, O. Galkin, *Thermodynamics of the hydrophobicity in crystallization of insulin*, 2003 Annual Meeting of the American Institute of Chemical Engineers, San Francisco, California, November 16-22, 2003.
31. O. Gliko, N. Neumaier, M. Fischer, I. Haase., A. Bacher, S. Weinkauf, P.G. Vekilov, *Dense liquid droplets as a step source for the crystallization of lumazine synthase,* 14th International Conference on Crystal Growth, Grenoble, France, August 9-14, 2004.
32. O. Gliko, I. Reviakine., H. Lin., S.-T. Yau, P.G. Vekilov, *Stability of Step Trains in Protein Crystallization*, 14th International Conference on Crystal Growth, Grenoble, France, August 9-14, 2004.