View on biocrystallization from Jena, 2002

Our community

Jena enjoyed the widest representation of our growing community. There were approximately 230 participants at the ICCBM7, 1998 in Granada, Spain; 320 participants at the ICCBM8, 2000 in Sandestin, USA and 420 participants at the IICBM9, 2002 in Jena, Germany.

The Jena meeting saw the culmination of the 17-year long development of our community since its first meeting at Stanford in 1985 in the creation of the International Organization for Biological Crystallization (IOBC). This decision was made by the International Advisory Board on March 25, 2002. The major goal of the IOBC is to organize future conferences and workshops, to undertake initiatives aimed at the international promotion of biocrystallization science and engineering and to provide assistance to national communities targeting the same goals. The International Advisory Board was transformed into the IOBC Council for which future election procedures will be determined by the Constitution of the IOBC. The Constitution will be refined based on discussions held by the Board during the Jena meeting.

The Chair of the ICCBM9, Professor R. Hilgenfeld will serve as the President of the IOBC, expanding the functions established by earlier chairs of the ICCBM's.

The Council approved the application of Chinese delegates to organize ICCBM10 in Beijing, China, in 2004.

Biological crystallization has developed into an interdisciplinary area in science and engineering between crystallography, biochemistry, physics and biophysics, materials, crystal growth, colloidal and polymer sciences and informatics. Production of crystals for structural biology is the primary goal. Industrial production and crystallization of some proteins presents an additional major challenge. Mutual education, collaborative efforts of experts in these fields and resulting mutual fertilization are crucial for future success in biocrystallization.

With these developments in place, it may be useful to examine biocrystallization, as seen from the ICCBM9, and attempt to highlight some directions of interest. Of course, the choice of these directions reflects the author's expertise and views.

Crystallization

It is impossible to satisfy the present overwhelming demand for perfect crystals for structural biology without improvements in expression and purification of the biomolecules. Therefore, significant attention is being paid to these processes. Protein production permitting higher productivity, perhaps using cell-free systems, with consequent control of folding, activity and solubility and application of focused mutagenesis represent some of the topics discussed in Jena. Miniaturizing the crystallization setups to tens of nanoliter droplets is being developed by several laboratories while more traditional issues are being addressed as well. Developments in high throughput structural proteomics include efforts to automate not only the crystallization screening, but also diffraction data collection and analyses. Bioinformatic studies of various classes of biomacromolecules could begin to bridge the primary sequence of amino acids and nucleotides and the protein foldings, and permit at least approximate predictions of the molecular structure and crystallization conditions.

On the other hand, the science of crystal nucleation and growth is closely related to the more general problems of macromolecular interactions and molecular recognition in solutions. A new physics and chemistry should be developed to describe crystals with solution filling intermolecular spacings and several size scales, atomic and molecular in the first place. X-ray diffraction and other forms of imaging of weakly scattering lattices built of objects subject to conformational changes is another interesting area to explore. Biomineralization with its synergism between living cells, proteins and inorganic crystalline and amorphous phases is also already a quickly developing a branch of biocrystallization.

The past decade revealed numerous phenomena supporting and specifying the now well-established conclusion that, not surprisingly, macromolecules follow the same crystallization laws as small molecules. However, there are important features making a difference in biomacromolecular crystallization, with parameter screening as its major paradigm today. This specificity is associated with versatility of the biomacromolecular surfaces and complex solution chemistry. However, the recent structural genomics and proteomics efforts suggest we can still only expect success rates variously estimated at between 10% and 50% in growing crystals of scattering to ~3Åresolution or better if synchrotron radiation is used. Many of the structures solved so far under the high-throughput programs still require refinement and deposition in the Protein Data Banks. This is the precious and growing "mineralogical collection" of the 21st century. The remaining 50% represent proteins and protein-nucleic acid complexes are piling up uncrystallized, or at least not allowing the atomic resolution, and are rapidly growing in number. In parallel, numerous laboratories continue to apply essentially intuitive screening to crystallize proteins and complexes of known biological significance. Progress is much faster in structural analysis based on a clear understanding of the diffraction physics, diffraction theory, software and instrumentation. Full automation of structure determination is rapidly becoming a reality. It will probably be achieved in the next five to ten years. Structures will routinely be determined in a matter of weeks, as is the case for some proteins now, provided perfect crystals are available. Thus, the crystallization bottleneck for structural biology is becoming even narrower. Therefore, more intensive effort to reach deeper insight into crystallization chemistry and physics and to move from art to science of growing crystals must become a high priority.

There are at least three crucial features differentiating biomacromolecular crystallization from that of small molecules: 1. One or two orders of magnitude larger size with nonsymmetrical complex shapes of the species to be packed into the lattice; 2. Macromolecular surfaces with mosaic patterns consisting of hydrophilic, neutral and hydrophobic groups with patterns specific for each protein but varying with solution conditions; 3. Conformational versatility of the surface, including "dangling" polypeptide loops and ends.

The large molecular size results in a high entropy barrier for ordering, and thus results in slow growth and requires use of high supersaturation at nucleation and growth. The high supersaturation, in turn, may practically exclude the trial-and-error attachment/detachment process of growth typically ensuring proper composition and perfection of conventional small molecule crystals. Enhanced by small conformational differences between different states of the same molecule in the crystal, this lack of "quality control" may result in the lattice disorder. It is not clear at this point if there is another mechanism replacing the trial-and-error "natural selection" of proper molecules in the correct orientation. Under slow growth conditions, interstep terraces on the growing faces of a crystal are exposed to solution for much longer periods of time, resulting in consequent poisoning of the surface with impurities typically present in the biosolution in amounts at least 10^2- 10^3 times larger than that in contemporary inorganic solution crystallization, not saying on semiconductors.

Chemical mosaic properties of a molecular surface are unknown ab-initio and, even more importantly, they are a function of solution

composition. This versatility of molecular surfaces often results in several crystalline polymorphs. All patches on the molecular surface providing intermolecular contacts, as revealed from these polymorphic modifications, are covering typically less than 50% of the surface - less than 10% per patch. The patches are different for different polymorphs, so that practically every area on the molecular surface may be capable of forming intermolecular contacts if the proper solution composition is found. In other words, the potential contacts "light up" on the molecular surfaces at these proper compositions. Determination of these conditions is the major goal of screening. One may not exclude, however, the cases when the surface chemical mosaicity is muted in terms of the intermolecular affinity so that the potential contacts do not "light up," for the solution compositions being tested do not exist, or at least do not present patches ready to form sufficiently strong specific contacts. Numerous examples of single mutations leading to crystallization or to better crystal quality are probably examples in which more contrast patches are made, lifting the surface degeneracy mentioned above.

Crystals that exhibit faceting and optical birefringence, yet do not diffract X-rays well or at all (e.g.., thermolysin) are probably characterized by good translational but poor rotational order. They seem to be the result of a weak contrast in contact patches. Predicting and engineering the contacts, solution chemistry and solubility remains a major challenge. It requires new techniques and approaches, e.g. nuclear magnetic resonance. The second virial coefficient, measured either by light or by X-ray scattering, remains a major parameter to characterize intermolecular interactions in solution, though it should be complemented by further approaches accounting for the molecular surface anisotropy. The transition from a given model's patchy surface to its solution behavior should be complemented by the understanding of the very challenging reverse problem. In attempts to predict chemical mosaicity of the macromolecular surface, exclusion of the backbone chain portions inclined to form hydrophobic α helices and β sheets might help. Formation of the contacts includes dehydration of both the molecule to be attached to a crystal (at the kink on the surface step) and the attachment site (the kink) itself. The dehydration barrier increases kinetic coefficients for the step and face growth and also contributes to the dissolution entropy and enthalpy. Such associations between thermodynamics and kinetics require further studies.

As a result of previous developments, further clarification of general principals and strategies to crystallize membrane proteins have been addressed. The lack of the protein-detergent contacts in the lattice and (not surprising) the applicability of the second virial coefficient test allows combinations of the non-specific detergent induced attraction with the specific protein attraction in the crystallization pathway. Further work is needed to successfully apply principles that lead to specific chemical recipes for crystallization.

Crystal perfection

Finding conditions to grow crystals is no less important than the next stage: achieving high structural resolution, i.e., growing the highly perfect crystal. Purification, especially from homologous, microheterogeneous impurities appears to be very important. It appears to be closely linked to the convection vs. diffusion transport, and may provide a rationale for the experiments in microgravity. The scientific component of the microgravity programs in different countries brought about major progress in the general understanding of biomacromolecular crystallization. Empirically, microgravity experiments provided important evidence that ~ 20% of crystallization experiments in space produce better crystals and that, therefore, diffusion and convection in solution might influence crystal quality. Unfortunately, we do not understand why and when this improvement happens. Further study to follow up on this evidence and achieved understanding by means of rational design and rigorous execution of experiments in space, with lesser emphasis on the "let us see" approach, would increase productivity of the microgravity experiments.

Protein crystals differ from small molecule crystals in their essential liquid content, a result of the large size of biomolecules and their hydrophilicity. This liquid is connected with the mother solution and is not a passive medium. Cryoprotection induced contraction or expansion of this liquid, along with that of the molecules, occurring due to (inhomogeneous) amorphization or freezing are known to enhance crystal mosaicity and deteriorate diffraction resolution. Cryoannealing, on the other hand, sometimes leads to perfection improvement. Neither process is well understood and merits further study.

Intermolecular cavities in crystals of biomolecules are of the nanometer size and the solution filling these cavities cannot be considered as a bulk liquid. In particular, the hydration and electrostatic forces in thin films are known to induce an additional, so called disjoining, pressure within the film so that the total pressure in the film exceeds the pressure in the surrounding bulk solution (by $10 - 10^2$ MPa between two parallel charged membranes, a quantity comparable with the strength of biocrystals). This disjoining pressure tends to separate the membranes bordering the film. In the intermolecular spacings, the liquid is confined in three dimensions as opposed to one, as in thin films. Therefore one may expect even larger intracrystalline disjoining pressure. The contribution from attractive pressure caused by opposing electrostatic charges located outside contact areas between macromolecules also causes an increase in this pressure. Being multiplied by the specific molecular volume, the pressure mentioned above provides the work in the kcal/mol range, i.e. comparable with the crystallization enthalpy. These are additional unknown factors that may affect crystallization.

Inorganic solute in the intracrystalline liquid responsible for the osmotic pressure, comparable with crystal strength, depends on the solute concentration. Transfer of a crystal from solution of one salt concentration to that with another induces additional differences between internal and external pressures and may cause cracking.

Internal inhomogeneous stress induced by homologous and, to a lesser extent, foreign impurities and molecular misalignment, appears to be the major contributor to crystal mosaicity during crystal growth. Energies of point defects, dislocations, grain boundaries, stacking faults and the conditions necessary for their creation are still unknown, as well as their role in low structural resolution. In summary, our understanding of general protein crystal mechanics, freezing damage and annealing in particular, are at an initial stage of development.

Crystallization in gel provides convection free environment and has been proven to be efficient in a number of experiments yielding higher crystal quality and strength. The strengthening probably occurs because the gel fills the intermolecular spacings. Excluding convection, the gel makes possible diffusion purification. This technique also allows for the screening in one experiment. Further quantitative development of this approach may bring about both fundamental and practical benefits.

It is hardly possible to solve the problem of producing perfect macromolecular crystals without methods to characterize these crystals. Overall diffraction resolution is considered the final product, and detailed information on the defects responsible for poor resolution is needed. We were witnesses to large-scale development of such methods for semiconductors, but no such development has yet occured for biocrystals. The same may be said of methods for

achieving high levels of purification. Crystal mosaicity is a well-known reason for loss of higher orders of diffraction. Rocking curve analysis combined with X-ray topography suggests that the mosaicity is due to lattice misorientation in the mosaic blocks rather than internal stress and the resulting variation of the lattice spacings between these blocks. Recently addressed mosaic statistics in several reflections suggest a correlation between the misorientation and crystal habit. It is also a function of impurity content. However, much more needs to be done to correlate mosaicity with growth conditions. The role of conformational and rotational disorder is also not clear, though the latter seems to be the natural reason for lack of diffraction in faceted and optically birefigent crystals.

X-ray topography is an important tool, though the images obtained so far are much less clear than that for inorganic materials. It may be related to the weak signal and the fact that extinction length is larger than the crystal size (opposite to conventional crystals), so that no dynamic contribution to the orientational contrast is expected. Phase contrast imaging, or defocusing, may be an option to better visualize inhomogenieties in a low absorbing crystal. Though weak, diffuse scattering might also be explored to search for confirmations and point defects. All of this makes it evident that further development of X-ray characterization techniques in combination with the others is important. Laser tomography was not discussed in Jena, though it may be of significance.

Only a few of the numerous challenges discussed in Jena this March have been mentioned in this brief overview. It confirms, however, that while a lot more science is needed , we are successfully accumulating more and better knowledge of biomolecular crystallization. The already recognized breakthroughs in many different directions leads one to hope that progress will continue at an ever accelerating pace and result in a robust scientific framework for efficient production of perfect crystals of biological macromolecules.

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Co-Chairmen, International Advisory Board, 2000-2002