Advanced methods in macromolecular crystallization IV

FEBS combined practical and lecture course PLC10 015

Academic and University Center, Nove Hrady, June 25 - July 2, 2010

Lectures - Saturday, June 26

L1

INTRODUCTION TO THE NUCLEATION AND GROWTH OF PROTEIN CRYSTALS

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In this lecture, we review the thermodynamics of protein crystallization, the basic concepts of nucleation, and the mechanisms of growth protein crystals.

To understand the thermodynamics of protein crystallization, we start with the phase diagram of the protein solution, where we define the regions of existence and coexistence of the dilute solution, the dense liquid, and the several possible crystal polymorphs. We define the lines of equilibrium between these phases and focus on the solution-crystal coexistence lines, the solidus and the liquidus since the latter is the protein solubility with respect to the chosen crystalline form. We define supersaturation as the driving force for crystallization and discuss experimental tools for its determination.

We start the discussion of nucleation by highlighting the defining role of the surface tension at the crystal-solution interface. The existence of surface tension leads to the necessity for nucleation, i.e., the appearance of just a few embryos of the new phase in a large supersaturated volume. Then we review the basic concepts of the classical nucleation theory, which is still the basic framework of understanding of all nucleation phenomena. We discuss the classical expressions for the rate of nucleation, i.e., the number of nuclei which appear in a unit volume per unit time, and how the nucleation rate could be controlled by varying the respective governing parameters.

The growth of protein crystal consists of the attachment of molecules from the solution to preformed sites on the crystal surface, called kinks. Hence, a crucial issue is the density of the kinks. Since the kinks are always located along steps on the crystal surface, we discuss three mechanisms of step generation: by two-dimensional nucleation of new crystal layers, on dislocations outcropping on the crystal surface, and by the association of dense liquid clusters existing in the solution. We also discuss three mechanisms of generation of kinks at the steps: by thermal fluctuations of the step, by "one-dimensional" nucleation of new crystal rows, and by the association of two-dimensional clusters existing on the terraces between steps. Finally, we address the role of impurities in cessation of growth and modification of crystal quality.

L2

CONVENTIONAL CRYSTALLIZATION METHODS AND THEIR MODIFICATIONS

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With the phase diagram in mind, some of the shortcomings of conventional / common crystallization vapor diffusion set-ups will be pointed out and alternative methods / modifications will be discussed.

Most important, the experimentation does not stop after the cover slide is placed over the reservoir, so to speak. Classical vapour diffusion experiments can be modified by several pre and post set-up techniques:

A selection of pre set-up, vapour diffusion experiment alterations

1. insertion of an oil barrier that will slow down the equilibration rate
2. use of gels to, among other effects, slow down convection in the droplet
3. use of capillaries in vapor diffusion mode to minimize handling of crystals
4. microseed matrix seeding to outwit nucleation

5. fluorescent dye-labelling of proteins
6. addition of proteases for *in situ,* limited proteolysis

A selection of post set-up, vapour diffusion experiment alterations

1. change reservoir precipitant concentration
2. change temperature
3. change pH
4. seeding

Also the standard microbatch trials in Terasaki plates (droplets under paraffin oil) can be manipulated by choosing different oil (mixture of silicon and paraffin oil).

Primers and Links

I. J. Drenth, Principles of Protein X-ray Crystallography (Third Edition, Chapter 16), Springer Science+Business Media LLC.

II. T.M. Bergfors, *protein crystallization strategies, techniques, and tips*, IUL Biotechnology series.

III. A. Ducruix and R. Giegé, *crystallization of nucleic acids and proteins*, Oxford University Press.

IV. A. McPherson, *crystallization of biological macromolecules*, Cold Spring Harbor Laboratory Press.

V. S. Iwata, *methods and results in crystallization of membrane proteins*, International University Line Biotechnology series.

VI. N. Chayen, Protein Crystallization Strategies for Structural Genomics, IUL Biotechnology Series.

VII. http://iobcr.org

L3

CRYSTALLIZATION OF MEMBRANE PROTEINS IN LIPIDIC MESOPHASES

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One of the primary impasses on the route that eventually leads to membrane protein structure through to activity and function is found at the crystal production stage. Diffraction quality crystals, with which an atomic resolution structure is sought, are particularly difficult to prepare currently when a membrane source is used. The reason for this lies partly in our limited ability to manipulate proteins with hydrophobic/amphipathic surfaces that are usually enveloped with membrane lipid. More often than not, the protein gets trapped as an intractable aggregate in its watery course from membrane to crystal. As a result, access to the structure and thus function of tens of thousands of membrane proteins is limited. The health consequences of this are great given the role membrane proteins play in disease; blindness and cystic fibrosis are examples. In contrast, a veritable cornucopia of soluble proteins have offered up their structure and valuable insight into function, reflecting the relative ease with which they are crystallized. There exists therefore a pressing need for new ways of producing crystals of membrane proteins. In this presentation, I will review the field of membrane protein crystallogenesis with reference to the latest update of the Membrane Protein Data Bank (http://www.lipidat.chemistry.ohio-state.edu/MPDB/index.asp). Emphasis will be placed on a crystallization approach that holds much promise and which makes use of the lipidic cubic phase. In the presentation I will describe the method and our progress in understanding how it works at a molecular level. The practicalities of implementing the method in low- and high-throughput modes will be examined. A practical demonstration of the method will be given at the Laboratory Excercises.

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Lectures - Sunday, June 27

L4

KNOWLEDGE-BASED CRYSTALLOGENESIS METHODS TO GROW BETTER CRYSTALS FOR STRUCTURAL BIOLOGY

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The multidisciplinary route to the 3D vision of biological processes by biocrystallography comprises 5 main steps [1]: (*i*) the preparation of the target macromolecules, (*ii*) the search of the crystallization conditions, (*iii*) the optimization of the crystal growth conditions, (*iv*) the characterization of the X-ray diffraction properties of the grown crystals and the data collection, and finally (*v*) the determination of the 3D structures. A crystallographic project can be stuck at each of these steps and overcoming the bottlenecks can require huge efforts and inventiveness. Here we will concentrate on strategies that can help to overcome these crystallogenesis bottlenecks with emphasis on methods to grow better crystals for X-ray analysis.

The design of such strategies requires a good understanding of the crystallization process that can be visualized on a phase diagram. Phase diagrams comprise an undersaturated region where macromolecules are soluble, and a supersaturated region, which is thermodynamically out of equilibrium, where they crystallize, both regions being delimited by a solubility curve. It is essential that crystal growers realize (*i*) that a supersaturated region contains 3 kinetically-dependent zones characterized by decreasing supersaturation levels where crystallizability differs radically and further (*ii*) that "good" crystal grow at low supersaturation.

When a first crystallization "hit" is found by conventional methods [2,3], refining the many parameters that govern the process of crystal production can be conducted on a rational basis. Important chemical parameters to be explored are the purity and homogeneity of the macromolecular samples, the nature and concentration of the crystallants, the pH and the ionic strength. Likewise physical parameters have to be considered, such as those influencing fluid properties and movement of molecules or those altering conformation of macromolecules. For instance gelled [4] and microfluidic environments [5] reduce convection thus favoring crystallization.

Tools to control parameters affecting crystal quality, experimental validation that sustains crystal optimization strategies, and ways to implement advanced crystallization methods for current practice in biology laboratories will be discussed.

1. R Giegé & C Sauter (2010) *HFSP Journal*, in press.
2. A Ducruix & R Giegé (eds) (1999) Crystallization of nucleic acids & proteins: A practical approach, IRL Press, Oxford, (2nd ed) pp. 1-435.
3. C Sauter *et al* (2010) General methods of crystallization; in *'Int. Tables Macromol. Crystallography*' vol. **F** (eds, Rossmann *et al*) John Wiley & Sons, Chichester, (2nd ed) in press.
4. B Lorber *et al* (2009) *Prog Biophys Mol Biol* **101**, 13-25.
5. K Dhouib *et al* (2009) *Lab Chip* **9**, 1412-21.

L5

CRYSTALLIZATION AND CRYSTALLOGRAPHIC ANALYSIS IN A MICROFLUIDIC CHIP

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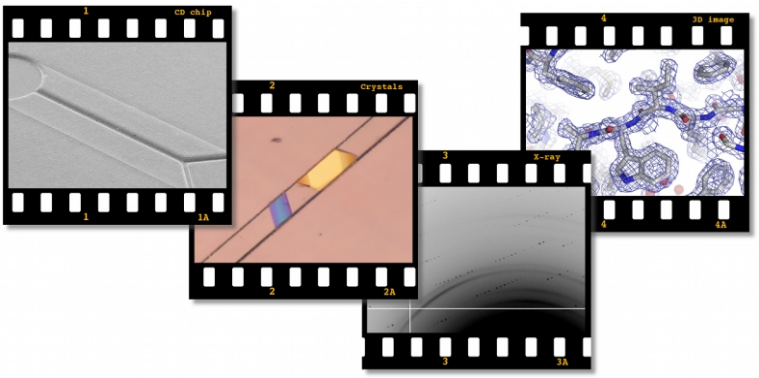
Microfluidic technology has opened new possibilities for the crystallization of biological macromolecules. Indeed, microfluidic systems offer a lot of advantages for crystal growth: they enable an easy handling of nano-volumes of solutions and, thus, extreme miniaturization and parallelization of crystallization assays. In addition they provide a convection-less environment *a priori* favorable to the growth of high quality crystals. Pioneer examples implementing free interface diffusion [1] and nano-batch [2] crystallization in microfluidic chips have already demonstrated the value of this technology, especially for high throughput screening applications in structural genomics. In the first part of the presentation, a review of current microfluidic devices will be given.



The second part will illustrate different aspects of this technology and discuss the results of a collaborative work initiated in 2004, that is focused on the design of a versatile, low cost and easy-to-use crystallization chip. Novel chips based on the counter-diffusion of solute molecules playing the role of crystallization agents will be described [3,4]. These chips are made of rigid polymers (*e.g.* PMMA, COC) that are impermeable to gases and compatible with crystal examination and monitoring in polarized light. Selected materials are also transparent to X-rays, and three-dimensional protein structures can be determined from crystals contained inside the devices using X-ray diffraction data collected on a synchrotron source. The outstanding quality of the electron-density maps demonstrates that on-chip crystal analysis is feasible. The replacement of conventional crystallization setups by inexpensive microfluidic chips for screening best crystallization agents and automated crystal diffraction analysis will be discussed.

1. C.L. Hansen, E. Skordalakes, J.M. Berger and S.R.Quake (2002). PNAS 99, 16531-6.
2. B. Zheng, J.D. Tice, L. Spencer Roach and R.F. Ismagilov (2003). Angew Chem Int Ed Engl. 43, 2508-11.
3. C. Sauter, K. Dhouib and B. Lorber (2007). Crystal Growth Des. 7, 2247-50.
4. K. Dhouib, C. Khan Malek, W. Pfleging, B. Gauthier-Manuel, R. Duffait, G. Thuillier, R. Ferrigno, L. Jacquamet, J. Ohana, J.L. Ferrer, A. Théobald-Dietrich, R. Giegé, B. Lorber & C. Sauter (2009). Lab-on-a-chip 9, 1412–1421.

L6

INTERPRETATION OF THE CRYSTALLIZATION DROP RESULTS

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The crystallization drop is full of information for the person who knows what to look for. However, for the inexperienced observer, the interpretation of the phenomena in the crystallization drop is not always a straightforward process. While it is sometimes easy to recognize a crystal, what about all those other solid phases of proteins like oils, precipitates, spherulites, and gels? Are they worth optimizing, or should one continue screening for new conditions? What does it mean when the protein "oils out"? What does phase separation look like and how does it affect the outcome of the experiment? How can you recognize a promising precipitate from a "bad" one? What are the best types of crystals to use as seeds?

This lecture will give present pictorial examples of the most commonly encountered results in crystallization drops and discuss 1) how to recognize the different phenomena, and 2) what to do with them.

The pictorial library of crystallizatiaon drop phenomena can be accessed at:
http://xray.bmc.uu.se/terese.

L7

THE USE OF MICROSEEDING IN PROTEIN CRYSTALLIZATION: PRACTICAL VARIATIONS ON THE MICROSEED MATRIX SCREENING (MMS) METHOD

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Traditionally, microseeding has been used as an optimization step, i.e. seed crystals are dispensed or transferred into variations of the original crystallization hit solution [e.g. 1, 2]. A novel, systematic approach, referred to as the Microseed Matrix Screening (MMS) method, was introduced by Ireton and Stoddard [3]. This method was automated and further improved by D'Arcy et al.[4], who first used seeding with commercial random screening kits. Experience has shown that MMS used in this way not only produces more hits, it also generates better-diffracting crystals because crystals are more likely to grow in the metastable zone [5]. Systematic users of the method report that it gives a useful improvement in about 75% of cases [6].

Douglas Instruments - as a member of the Opticryst consortium [7] - has investigated several variations in the method. In real-life projects, new crystallization conditions are often sought in an effort to reduce twinning, improve diffraction resolution, find crystals with new space groups etc. Moreover, it is desirable to reduce the number of salt crystals that typically arise from combining the hit solution with all of the various solutions in a screen. We therefore investigated suspending the seed crystals in alternative solutions (i.e. replacing of the original hit solution, which D'Arcy et al. used). Solutions investigated included protein solution ("preseeding the protein stock"), ammonium sulphate, PEG, NaCl and ethanol. The original hit solution was found to be slightly more effective, but several other solutions also worked well. Note that these alternative solutions reduce the likelihood of crystallization by an additive effect, so they can be used when the original conditions give poor diffraction (especially PEG or NaCl, which are less likely to give salt crystals). Throughout the project, the statistical significance of experiments carried out was increased by focusing on "pregnant" conditions, which were defined as conditions that reliably gave crystals when seeds were present, but which otherwise did not support crystallization.

The stability of seeds in various solutions at room temperature was also investigated. When using the original hit solution (see above), up to 75% of seeds were lost after 3 hours, and 99% after 24 hours.

Obviously the MMS method has the disadvantage that it cannot be used until at least one hit has been obtained. We therefore investigated nucleation with micro-porous glass [8] and zeolites [9]. These materials were less effective than microseeding, but may nevertheless be useful - because they can of course be used in the absence of previous hits.

1. Terese Bergfors. 'Seeds to Crystals'. Journal of Structural Biology, 142 (2003), 66-76.
2. Enrico A. Stura (1999). 'Protein Crystallization', edited by T.M. Bergfors, 139-153.
3. Gregory Ireton and Barry Stoddard. 'Microseed matrix screening to improve crystals of yeast cytosine deaminase". Acta Crystallographica section D60 (2004), 601–605. Available on-line at http://scripts.iucr.org/cgi-bin/paper?S0907444903029664
4. Allan D'Arcy, Frederic Villarda, May Marsh. 'An automated microseed matrix-screening method for protein crystallization'. Acta Crystallographica section D63 (2007), 550–554. On-line at http://scripts.iucr.org/cgi-bin/paper?S0907444907007652
5. Further information on the theory and practice of the MMS method is available at the Douglas Instruments web-site, http://www.douglas.co.uk/mms.htm
6. Personal communication, Paris Ward, Thomas Malia, Galina Obmolova, Allan D'Arcy and others.
7. See http://www.opticryst.org. Opticryst is a consortium funded by the European VI Framework Programme for Research and Development. The consortium aims to develop and implement new techniques for protein crystal optimization.
8. N.E. Chayen; E. Saridakis; R.P. Sear. 'Experiment and theory for heterogeneous nucleation of protein crystals in a porous medium'. P NATL ACAD SCI USA. 103:597-601.
9. M. Sugahara, Y. Asada, Y. Morikawa, Y. Kageyama and N. Kunishima. 'Nucleant-mediated protein crystallization with the application of microporous synthetic zeolites'. Acta Cryst. (2008). D64, 686-695. Available on-line at http://scripts.iucr.org/cgi-bin/paper?bw52

Lectures - Monday, June 28

L8

COUNTER DIFFUSION METHODS FOR PROTEIN CRYSTALLIZATION AND SCREENING: GELS, CAPILLARY VOLUMES AND MICROGRAVITY

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Vapor diffusion and micro-bath under-oil are the most used techniques in Structural Biology laboratories for protein crystallization. In vapor diffusion technique the slow evaporation of a drop with a mixture of protein and precipitant brings the system towards the supersaturated region at certain rate while in batch methodologies protein and precipitating solutions are mixed to immediate reach a particular supersaturation value. Both techniques have inherent buoyancy driven convection and consequently crystals are grown in a heterogeneous environment compromising uniform crystal growth and quality. Chaotic mixing and convection can be reduced when the crystallization process proceed by diffusively mixing the protein and precipitant solutions. This effect can be achieve with liquid-liquid diffusion (free-interface diffusion) techniques in which protein and precipitant are allowed to diffuse one against each other in any media permitting diffusive mass transport (gels, capillaries, microfluidic devices or microgravity).

There are different ways to implement this technique. Among them, the most effective configuration proven to be useful for growing macromolecules crystals is the counter-diffusion (CD) technique. Unlike other techniques aimed at finding initial conditions close to equilibrium, counterdiffusion looks for initial high values of supersaturation thus provoking even the formation of amorphous precipitates at the earliest stages of the experiment. Then, by using a long protein chamber the technique exploits the simultaneous event of diffusion and crystallization giving rise to a supersaturation gradient along the length of the crystallization chamber.

In this talk we will discussed the effectiveness of counterdiffusion technique not only for improving crystal quality but also for the search of initial crystallization conditions when compared with traditional crystallization technique.

1. J.M. Garcia-Ruiz, Counterdiffusion methods for protein crystallization. *Methods in Enzymology*, **368** (2003) 130-154.
2. M.C. Robert, J.M. García-Ruiz, O. Vidal and F. Otálora. Crystallization in Gels. In Crystallization of nucleaic acids and proteins: a practical approach. A. Ducruis and R. Giegé, Eds. Oxford: IRL Press. 331pp. Ch 6 (1999) 149-175.
3. F. Otálora, J.A. Gavira, J.D. Ng and J.M. García-Ruiz. Counterdiffusion methods applied to protein crystallization. *Progress in Biophysics and Molecular Biology,* **101** (2009) 26-37.

L9

"WHAT TO DO IF EVERYTHING HAS FAILED"

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Protein crystallization projects usually have two stages. The initial one involves screening parameters to find promising lead conditions. Useable crystals may already appear at this stage, but the most typical scenario is that a second round of experiments is required to optimize the potential leads. This lecture will present some of the major considerations in choosing particular strategies or "routes" for screening and optimization. However, since the pathway in a crystallization project often contains dead-ends, the protein crystallizer also needs to be equipped with a plan for dealing with the "detours". When it seems that everything has failed, what are the options left to try?

To address this problem, the following questions will be discussed:

- Can pre-screening the protein buffer improve the protein behavior in the crystallization drops?
- How many conditions should the initial screen contain: 150 or 1500?
- Are all crystallization kits equally successful or are some better than others?
- Which is more critical: the choice of precipitant or the kinetic pathway?
- How does one recognize the kind of leads that are worth optimizing? For example, should one try optimizing drops with phase separation or keep screening for new conditions?
- What kind of tools exist for predicting if a protein is going to crystallize? How reliable are they?

This lecture will answer these questions from the perspective of an academic laboratory with little automation and which works on a limited, but very focused, group of targets from *Mycobacterium tuberculosis.*

1. Bergfors, T. M., ed. Protein Crystallization, 2nd Edition, 2009, International University Press, La Jolla California.
2. Bergfors, T.M. *Screening and optimization methods for nonautomated crystallization laboratories.* 2007, Methods in Molecular Biology, **vol. 363,** 131-152.

L10

ON THE USE OF ADDITIVES IN PROTEIN CRYSTALLIZATION

Rolf Hilgenfeld

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The addition of certain small molecules to the protein crystallization experiment can have dramatic effects. Such small molecules may be cofactors required by the protein under study, for example nucleotides. These often change the conformation of the protein by binding to specific sites. The same is true for metal ions, for which specific binding is frequently observed in the active sites of proteins, or at their surface. For relatively few of these additives, their effects can be rationally explained today. We have studied the effect of the addition of zinc ions to a number of proteins and found several cases where zinc binding to glutamate or histidine residues at the surface of the protein had the effect of cross-linking the protein molecules in a crystal lattice [1]. A very popular additive is 2-methyl-2,4-pentanediol (MPD) or 1,4-Dithiothreitol (DTT) [2]. MPD is frequently used as the major precipitant in crystallization of proteins and, in particular, nucleic acids. We have studied the structural consequences of MPD binding to proteins in a statistical analysis [3]. While these studies provided rational explanations for some individual additives, McPherson and Cudney have recently shown that the broad use of mixtures of additives can improve the success rates of protein crystallization trials dramatically [4]. These and other ideas will be discussed.

1. Riboldi-Tunnicliffe, A., König, B., Jessen, S., Weiss, M.S., Rahfeld, J., Hacker, J., Fischer, G. & Hilgenfeld, R.: Crystal structure of Mip, a prolylisomerase from *Legionella pneumophila*. *Nature Struct. Biol.* **8** (2001) 779-783.
2. Ponnusamy R., Moll R., Weimar T., Mesters J.R. & Hilgenfeld R.: *J. Mol. Biol*., in press (2008).
3. Anand, K., Pal, D. & Hilgenfeld, R.: An overview on 2-methyl-2,4-pentanediol in crystallization and in crystals of biological macromolecules. *Acta Cryst.* **D58** (2002) 1722-1728.
4. McPherson, A. & Cudney, B.: Searching for silver bullets: Alternative strategies for crystallizing biological macromolecules. *J. Struct. Biol*., (2006) in press.

L11

UNCONVENTIONAL CRYSTALLIZATION TECHNIQUES FOR SCREENING AND OPTIMISATION

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The past six years have seen momentous progress in the miniaturisation, automation and analysis of crystallisation experiments. However, high-throughput has not yet resulted in high-output and producing high quality crystals still presents a major barrier to structure determination.

There are no 'magic bullets' that will guarantee the production of good crystals, hence the development of new and improved technologies for obtaining high quality crystals is of crucial importance to progress.

This talk will present strategies for increasing the chances of success and highlight several practical methods that can be used in cases where standard screening and optimization procedures have failed. The methods involve active influence and control of the crystallization environment while the trial takes place, in order to lead crystal growth in the direction that will give the best results. Most of the techniques can be automated and adapted to high throughput mode.

1. Chayen, N.E. and Saridakis, E. (2008) "Protein crystallization: From Purified Protein to Diffraction-quality Crystal" *Nature Methods* 5, 147-153.
2. Saridakis, E. and Chayen, N.E. (2009) "Towards a 'Universal' Nucleant for Protein Crystallization *"Trends in Biotechnology* **27**, 99-106.
3. Protein Crystallization Strategies for Structural Genomics" N.E. Chayen ed. International University Line, Ca, USA 2007.

Lectures - Tuesday, June 29

L12

IN SITU DYNAMIC LIGHT SCATTERING FOR ANALYSIS AND OPTIMIZATION OF CRYSTALLIZATION PROCESSES

K. Dierks[1], A. Meyer[1], D. Oberthuer[2], H. Einspahr[3], Ch. Betzel[2]

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We have developed a combined imaging and dynamic light scattering (DLS) system for routine measurements in droplets in the multi-well plates used in protein crystallization. The system can be used to analyse and image Geltube crystallisation experiments and is also able to investigate crystallization experiments kept under oil. The system was tested with several standard proteins and found to be of high value for rapid identification of good crystallization conditions. A relationship between the rate of protein-aggregate-size increase and the probability of crystal formation was observed and results will be presented accordingly.

Today automated methods to crystallize macromolecules are widely used and can easily generate thousands of crystallization droplets. Nevertheless, evaluation of crystallization experiments to find optimal growth conditions remains a bottleneck. Therefore we have investigated methods to improve the process of evaluating results and finding crystal growth conditions. Besides imaging drops, two methods have emerged as most promising. One is dynamic light scattering (DLS), which already has many applications, but which we have found useful for detection of aggregation and nucleation in droplets. The other is the use of combined white/UV illumination for microscopic determination of whether crystal-like objects are biomolecular and identification of crystals in crystallisation set-ups. DLS is a widely accepted method to determine the size and mode of aggregation of proteins and other biomolecules in solution. Up to now, it has been impossible to determine the particle size directly in protein solution droplets because of size and configuration constraints. We have developed a CCD camera-based imaging instrument and combined a laser source and a detector to perform DLS measurements in protein solution droplets of crystallization trials *in situ*, in a configuration in which source and detector are entirely external to the trial and without cuvette [1]. The plate-screening system (Spectro-Imager 501, Molecular Dimensions, UK) allows one to monitor and evaluate the entire process of crystallisation in an automated way. The application of this external DLS device allows one to investigate the stages of nucleation and the progress of crystal growth in an individual protein droplet without disrupting the course of equilibration. We will present results obtained from experiments performed with various proteins and different scenarious.

The data provide information to understand in greater detail the process of crystal initiation and growth and will allow further optimisation, thereby leading to better crystals. Finally we will also describe a method to support the identification of protein crystals, exploiting the fact that most proteins and other biomolecules fluoresce when illuminated with UV light [2]. The Spectro-Imager is a completely new instrumental design incorporating all of the techniques described above in one device. The system allows the experimenter to assess the probability of obtaining macromolecular crystals a long time before the crystallization actually takes place. We will present results showing that the rate of the particle size growth is a good predictor of the outcome of crystallization experiments.

1. Dierks, K., Meyer, A., Einspahr, H., Betzel, C., *Cryst. Growth Des*., 2008, **8**, 1628-1634.
2. Dierks, K., Meyer, A., Oberthuer, D., Rapp, G., Einspahr, H., Betzel Ch., *Acta Crystallogr*. F, in press, 2010.

L13

E. coli - A FACTORY FOR RECOMBINANT PROTEINS

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Rapid development in recombinant DNA techniques has made it possible to overproduce selected proteins that are normally expressed only at low concentrations, as well as those that have been proven to be difficult to purify to homogeneity from natural sources.

Several alternative systems for the expression of foreign genes have been developed including mammalian cells, insect cells, fungal cells, bacterial cells, and transgenic animals or plants. However, the most widely used and convenient system for the production of foreign proteins remains that based on the simple Gram negative prokaryote – *Escherichia coli*. The final requirements for the desired product will govern the initial choice of cloning and expression system. Generally, there is no ideal host and no expression system that meets all requirements.

The purpose of this lecture, therefore, will be to provide background information and at the same time address practical solutions to the most common problems in expression of heterologous proteins. This should enable you to design a competent strategy in your own future research. Some of the major factors that must be considered in selecting an *E. coli* expression strategy will be reviewed in this lecture (for example: cloning strategy, functional activity of the proteins, medium engineering, difficulties in removing fusion tags). Furthermore, tips and tricks on how to provide enough functional material for biological studies will be discussed.

L14

THE ROAD FROM PROTEIN EXPRESSION AND PURIFICATION TO PROTEIN CRYSTALLIZATION

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Many efforts have been performed to understand the relationship between the structure of proteins and their biological function. In addition, the huge number of candidate proteins generated by genomics programs has increased the interest in all aspects of protein expression and purification. Proper expression and purification techniques are essential for the large-scale production of pure proteins needed for 3-D structure determination. One can obtain high levels of expression by choosing good host strains, vectors, and growth conditions. However the purification flowchart that leads you into a successful crystallization experiment will have to be established by yourself for every single new protein you might get. Commonly questions one should answer before starting a new protein crystallization project include: should my protein(s) be expressed in bacteria, in yeast, in insect cells or in human cells? Which expression vector should I use? If I choose bacterial expression, which strain(s) should I use? Should I express the full-length protein or a fragment? Should my protein be tagged, and which affinity tag is the best? Do I need to remove the tag? Which buffer should I use? What is a good purification strategy for my protein?

Unfortunately, because every protein is different, you will not be able to answer these questions *a priori*, and you will have to work out the purification protocols and strategies for each individual protein. The better the protocol is established the higher is the chance you will get good crystallization hits. In this talk, you will get an illustration of some of the most commonly used strategies helping the researchers in such a complicated task.

L15

PROTEIN AS THE MAIN VARIABLE IN CRYSTALLIZATION

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Preparation of high quality protein crystals is essential for the structure determination using X-ray techniques. Statistics from the projects of structural genomics shows that the success rate of high-throughput crystallization is only 10-30 % and thus preparation of protein crystals becomes the rate limiting step.

Crystallization is influenced by many parameters, from which the most important one is the protein itself, its purity, homogeneity and specific properties, namely its propensity to form crystals. Protein crystallizability may be enhanced by the methods of molecular biology. This may involve the preparation of proteins with various kinds of fusion partners or tags, removal of their most flexible parts (N- and C-termini or flexible loops), increasing the homogeneity by modifications of free cysteines or potential sites of glycosylation, replacement of unfavourable amino-acid residues at the surface of the molecule, etc.

Requirement of protein purity and homogeneity will be discussed and stressed. The influence of protein modifications on its crystallizability and/or crystal packing and quality will be documented on results obtained in our laboratory and examples from literature. Some rational approaches and strategies oriented on enhancing the protein crystallizability as well as the possibility of its computational prediction will be presented.

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Lectures - Wednesday, June 30

L16

PREPARATION OF PROTEIN SAMPLES FOR CRYSTALLIZATION EXPERIMENTS

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Protein crystallization process is influenced by a large number of various factors and one of the most important is the property of the protein sample to be crystallized. Preparation and characterization of the protein sample plays a crucial role in protein crystallization.

In the lecture, the most widely used techniques to judge evaluate protein sample purity and quality before crystallization experiments will be reviewed and discussed. For crystallization trials highly pure and homogeneous protein sample is usually recommended, however, if larger amount of protein is available 'impure' protein sample can be also screened. Guidelines and tips for protein handling before crystallization trials will be addressed.

Common methods to analyze protein purity and stability will be described: (a) SDS polyacrylamide gel electrophoresis (PAGE), (b) native PAGE, (c) isoelectric focusing, (d) size exclusion chromatography (gel filtration), (e) mass spectrometry, (f) dynamic light scattering (DLS), and (g) differential scanning fluorimetry (DSF).

Recommended protein concentration for initial crystallization screening is in range of 5 - 20 mg. The higher protein concentration provides more opportunity for crystal nucleation to occur but, on the other hand, also can cause protein aggregation. The best concentration is usually tuned as one of the variables during optimization procedure. All components of the protein buffer should be carefully considered since they might influence crystallization. Storage conditions have to be checked experimentally for each protein, but most protein can be stored at -70 C or 4 C. Lyofilization should be avoided and if inevitable, extensive dialysis before crystallization is recommended.

For more general reading further references are recommended [1, 2].

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2. Bergfors T.M. (1999). Protein Crystallization Techniques, Strategies and Tips, Chapter 3. Protein Samples, pp. 19 – 25 International University Line, La Jolla, CA, USA.

L17

RATIONAL BIOCHEMICAL APPROACHES TO IMPROVE CRYSTALLOGENESIS

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Biochemistry offers a number of simple strategies that can lead to improved crystallogenesis of essentially every protein. These strategies represent good general guidelines that should be followed even in cases of facile crystallogenesis, and they also share characteristics of desperation measures to try when everything else has failed. All of them have in common that they depend on biochemical knowledge about the protein, reflecting the fact that the more biochemical understanding one has, the more avenues one can pursue to achieve crystallization. Published and unpublished examples will be used to illustrate the underlying principles. For specific, one-on-one advice, students are invited to meet the speaker in the evening discussion sessions, and should be prepared to present a summary of the available biochemical knowledge about their proteins.

L18

TIPS AND TRICKS FOR PROTEIN CRYSTAL MANIPULATION AND HANDLING

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The possibility to solve any protein structure depends on the ability to obtain a crystal suitable for x-ray diffraction. The observation of new crystals is the best starting point for a way that sometimes can be very tedious. The next steps will include; i) proof that the crystal is a protein and in some cases it is also necessary to demonstrate that the protein keeps its activity/function, ii) initial diffraction at room temperature, iii) low temperature data collection and iv) derivatization in some cases. This manipulation can put at risk your crystal quality and therefore the quality of your structure. In this talk we will try to fill the gap between the microscopy and the X-ray "observation" of your crystals with some tips and tricks. We will also see how to include new additives, i.e. cryoprotectant, scatter atoms, etc, into your crystal avoiding the loss of quality and how to perform *in situ* cryo-crystallography from crystals grown by the capillary counterdiffusion method.

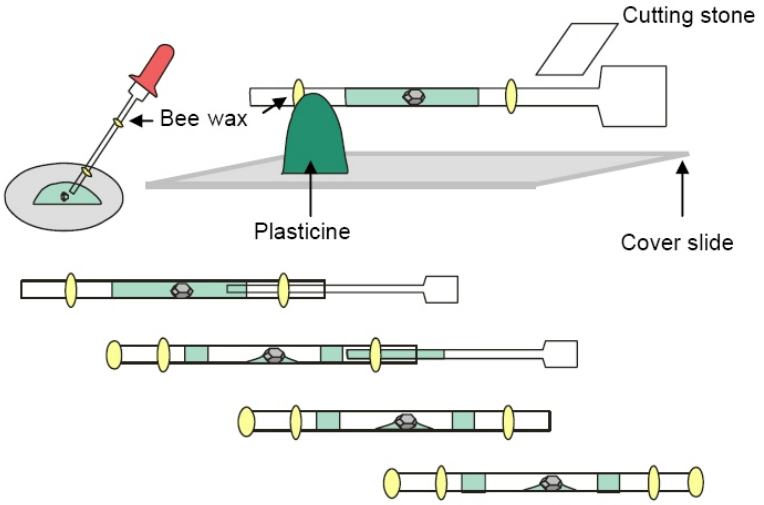

Figure 1. Schematic representation of how to prepare your crystal for room temperature x-ray diffraction test or data collection.

L19

PUBLICATION OF SCIENTIFIC RESULTS WITH EMPHASIS ON CRYSTALLIZATION COMMUNICATIONS

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The crystallization communication is often the beginning scientist's first encounter with scientific publication. The fundamental principles of scientific publication will be presented with special emphasis on crystallization results either as a crystallization communication or as part of a structure report. Included will be an introduction to ***publBIO***, a collection of novel web-based tools for authors developed by IUCr Journals to facilitate drafting of crystallographic publications and speed editorial processing after submission.

Lectures - Thursday, July 1

L20

NUCLEATION OF PROTEIN CRYSTALS: NOVEL INSIGHTS

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There are several fundamental aspects with crucial practical implications, related to the nucleation of protein crystals. The first one is, assuming that a crystal form with a sufficiently low free energy exists, how does one make sure that nuclei of this form appear within reasonable time in a tested solution? Another one is how does one ensure that just a few, and not numerous interfering crystals reproducibly form in a crystallization experiment? Furthermore, if several crystal polymorphs with different structures are possible, a crystallographer may want to select the one providing better detail of the protein atomic structure.

Experiments, simulations, and theoretical work, carried out in our and other laboratories in recent years, have revealed intimate details about the nucleation mechanisms of protein crystals. It was shown that crystals of many proteins follow a two-step mechanism of nucleation, whereby the formation of an ordered nucleus is preceded by a metastable dense liquid droplet. It was found that such droplets exist in proteins solutions, even if they are undersaturated with respect to any solid phase. It was shown that the formation of the metastable dense liquid droplets is an intrinsic property of the solution, which is a consequence of the structuring of the water molecules at the protein molecular surface. A kinetic theory, linking the properties and the volume occupied by the dense liquid droplets to the crystal nucleation rate has been developed.

The most significant practical consequence related to these theoretical insights is that by controlling the formation and the properties of the dense liquid droplets, several aspects of the nucleation protein crystals: number of crystals, reproducibility of nucleation, polymorph selection, can be controlled. These and other issues of interest to the practicing crystallographers will be discussed.

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L21

THE GROWTH OF LARGE CRYSTALS FOR NEUTRON DIFFRACTION: THERMAL CONTROL

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In contrast to X-rays that are scattered from the electron clouds of atoms – X-ray scattering lengths (also called scattering factors) hence being proportional to the number of electrons – neutron scattering is a purely nuclear process. The scattering lengths are different for each nucleus, *i.e.* each isotope of each element, and they are completely unrelated to the nuclear mass. Therefore the scattering length of hydrogen is of the same order as those of heavier atoms found in proteins, such as carbon, nitrogen and oxygen. This means that neutron crystallography (NC) is the most unambiguous method for determining the protonation states of proteins and their bound ligands or water molecules. Knowing exactly where hydrogen atoms are and how they are transferred between biomacromolecules, solvent molecules and substrates is important for understanding many biological processes. The big disadvantage of NC is the relatively low flux of available neutron beams. Even the brightest neutron sources are very weak, so the data collection times are measured in days or weeks. Even then the crystal size required to collect data is about of 1 mm^3: roughly a thousand times that used routinely for X-ray crystallography [1].

While the objective of NC is to locate hydrogen atoms, one of the biggest technical problems is related to hydrogen. As the neutrons are scattered by the nuclei, each isotope of the same element behaves differently. The light isotope of hydrogen, ^1H, is particularly problematic, because it has, due to its spin, a very high incoherent scattering cross section. The incoherent scattering does not contribute to the Bragg reflections, it only adds to the background in crystallographic data collection. In practical terms this means that all the hydrogen atoms in the sample have to be replaced with deuterium (^2H), which has a comparable coherent scattering cross-section but significantly weaker incoherent scattering. Therefore all the crystals used for neutron data collection have to be grown under deuterated conditions and in many cases it is necessary to produce the protein material in D_2O with deuterated carbon source (known as perdeuteration) in order to replace also the non-exchangeable aliphatic hydrogens with deuterium [2]. Growing large crystals of proteins in deuterated conditions is not easy as it may sound, because the protein solubility is different in D_2O [3].

Whether the sample is hydrogenated or perdeuterated, optimisation of crystal volumes remains a critical step towards the success of neutron protein crystallographic studies. The knowledge of the phase diagram becomes crucial and a method allowing for manipulation of the kinetics of the crystallization process, taking advantage of generic features of the phase diagram is needed. Although large crystals can sometimes be grown by vapour diffusion by repeated microseeding or dialysis, temperature is the only variable where the rate of change can be reliably controlled. This has led to the development of sophisticated methods of controlled crystal growth [4] that make use of the temperature dependence of protein solubility.

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L22

ILLUMINATING THE SCREENING PROCESS WITH FLUORESCENCE

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In an effort to provide feedback and bring more rational methods to what has heretofore been a random trial and error process, we are developing fluorescence for use in screening for macromolecule crystallization conditions. Two approaches are under development: First is the use of fluorescence anisotropy (FA) as a means of determining likely crystallization conditions. Second is the use of fluorescence in the finding of crystals in crystallization plates.

The FA approach determines anisotropy, the rotational rate of fluorescently labeled protein molecules, as a dilute solution measure of the macromolecules response to the imposed screen solution. The FA data is collected as a function of protein concentration. Pipetting difficulties in the initial experiments led to the incorporation of fluorescence intensity data as a means of tracking pipetting errors, and the subsequent discovery that the change in intensity was also a strong indicator of likely crystallization conditions. The anisotropy and intensity vs. concentration data are analyzed to determine likely lead conditions, which are then tested using capillary counter diffusion. The strength of the approach comes from being able to find those conditions sufficiently close to crystallization that would have otherwise shown up as clear or precipitated solution in standard plate assays. Preliminary studies with a series of model proteins indicated that ~twice as many crystallization conditions were found using the FA method when compared to plate assays. To date, we have found crystallization conditions for all cases using test proteins, where the FA approach was the sole screening method.

Several groups have now shown that fluorescence can be a powerful means to finding crystals in screening plates. The fluorescence can come from native tryptophan[1], from free probe added to the solution[2], or from covalently binding the probe to the protein[3], the method of choice in this laboratory. When covalently bound the fluorescent signal gives the location of the protein in the crystallization drop. As the emitted intensity is proportional to the local probe concentration, and the crystalline form is the most densely packed solid, then the intensity is substantially greater from crystals than from amorphous precipitate. Intensity is a more easily implemented search parameter than straight lines, and fluorescence-based screening plates can be rapidly scanned by eye, with crystals readily apparent. Alternatively, image analysis software, currently under development, can rapidly find crystals in a drop. We have shown that in some instances there are 'bright spots' in amorphous precipitate, the presence of which is a strong indicator of likely crystallization conditions.

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L23

READY, SET, SCREEN: X8 PROSPECTOR

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The current bottleneck in protein crystallography is to get well diffracting crystals. The process of screening crystals is consequently vital, not only to monitor improvement in the crystallization trials but also to select the best crystals for the data collection at the synchrotron.

Thus, when screening a large number of potential candidates, it is important having a dedicated system, which is easy to use, has low maintenance costs, and has an excellent DCE [1] to enable the scientist to judge the scattering power of the crystal.

The only way to figure out if and how well your crystal diffracts is a direct check using a sufficiently strong X-ray beam. Why waste your valuable synchrotron time screening crystals there when you could easily screen in the lab? The X8 PROSPECTOR (Fig. 1) is a system designed to fulfil the demand for efficient screening, economical and reliable. With its compact design and small footprint, it can be placed in any laboratory. Moreover it gives you all the information needed prior to go to the beamline.

The source is completely air-cooled and therefore requires no external plumbing. Furthermore it uses regular,

single-phase power without the need of special electrical wiring. A microfocus I S sealed tube coupled with the QUAZAR optics delivers a highly stable X-rays beam with intensity equivalent to the one of a traditional sources, such as rotating anode generator with a 300 micron focus combined with 6 cm mulilayer mirrors. The I S delivers the smallest beam – focussing the X-rays where you need them and minimising background. The rock-solid fourth generation APEX II CCD detector is the most sensitive available, and when coupled with this powerful I?S source it enables you to measure diffraction from the most weakly diffracting crystals.

The X8 PROSPECTOR not only speeds up your screening work, it also leads the user through the whole process. The PROTEUM2 software controls the hardware and analyzes the results to give quick and reliable answers to your questions.

Examples will be presented showing how easily the X8 PROSPECTOR finds the jewels in your crystallization plates.

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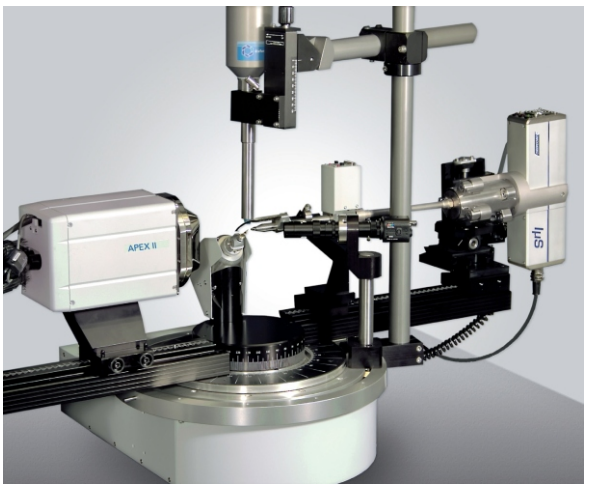

Figure 1. X8 PROSPECTOR with I S microfocus source, QUAZAR optics, three circles goniometer and APEXII CCD detector: the ideal system for screening crystals.

Laboratory exercises

INTRODUCTION TO LAB EXERCISES - MACROMOLECULAR CRYSTALLIZATION

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Crystallization of macromolecules used to be rather empirical procedure, and because of its unpredictability and frequent irreproducibility, it has long been considered as an "art" rather than science. It is only in the last 20 years that a real need has emerged to better understand and rationalize the crystallization of biological macromolecules. As the molecules involved in crystallization exhibited such contrarious behavior and were poorly characterized, conducting serious research into their crystallization was conceded as hopeless. Only when the demand for crystals by crystallographers and later molecular biologists could no longer be ignored, researchers pursued in earnest the study of macromolecular crystal growth (McPherson 1999).

Researchers working in the protein X-ray crystallography laboratory understand that the first requirement for protein structure determination is to grow suitable crystals.

Without crystals there is no X-ray structure determination of a protein!

The characteristics of crystals and biological macromolecules crystallization data have been compiled in the Biological Macromolecule Crystallization Database (BMCD) (Gilliland *et al*. 1994, Gilliland 1998). The BMCD contains crystal data and the crystallization conditions, which have been collected from the literature. The current version of the BMCD contains 43406 crystal entries (Tung and Gallagher, 2009) from macromolecules for which diffraction quality crystals have been obtained. These include proteins, protein-protein complexes, nucleic acid, nucleic acid:nucleic acid complexes, protein:nucleic acid complexes, and viruses. All crystallographic information about particular macromolecule including X-ray diffraction data and statistics information are available online in the Protein DataBank (RCSB PDB) database (Berman *et al*. 2000). The RCSB PDB provides a variety of tools and resources for studying the structures of biological macromolecules and their relationships to sequence, function, and disease.

CRYSTALLOGENESIS KEYWORDS

Macromolecules

All of the macromolecules are polymers of one of the precursor classes that include the amino acids, the ribonucleotides and deoxyribonucleotides, sugars of various sorts, fatty acids, *etc.* These small molecules are linked together in a sequence by complicated series of chemical reactions in the cell to form the macromolecules such as proteins, nucleic acids (RNA and DNA), polysaccharides and lipids. The structural complexity and physiological role of macromolecules are a function of the diversity of the precursors, the sequence in which they are joined together, the number of precursors in the polymer, and finally, the 3D form after polymer synthesis.