Session XII - Theory and Practice of Crystallization

Thursday, July 7 - morning

S12-L1

ARE THE PROTEIN PRE-NUCLEATION CLUSTERS EQUILIBRIUM STRUCTURES OR IRREVERSIBLE AGGREGATES?

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Protein-rich clusters of steady submicron size and narrow size distribution exist in protein solutions in apparent violation of the classical laws of phase equilibrium. Even though they contain a minor fraction of the total protein, evidence suggests that they may serve as essential precursors for the nucleation of ordered solids such as crystals, sickle-cell hemoglobin polymers, and amyloid fibrils. The cluster formation mechanism remains elusive. We use the highly basic protein lysozyme at nearly neutral and lower pH as a model and explore the response of the cluster population to the electrostatic forces, which govern numerous biophysical phenomena, including crystallization and fibrillization. We tune the strength of intermolecular electrostatic forces by varying the solution ionic strength *I* and pH and find that despite the weaker repulsion at higher *I* and pH, the cluster size remains constant. Cluster responses to the presence of urea and ethanol demonstrate that cluster formation is controlled by hydrophobic interactions between the peptide backbones, exposed to the solvent after partial protein unfolding that may lead to transient protein oligomers. These findings reveal that the mechanism of the mesoscopic clusters is fundamentally different from those underlying the two main classes of ordered protein solid phases, crystals and amyloid fibrils, and partial unfolding of the protein chain may play a significant role.

The role of partial protein unfolding in cluster formation suggests that the clusters may represent irreversible aggregates of denatured protein. We determine the enzyme activity of lysozyme in cluster-containing solutions and demonstrate that it is equal to that of native lysozyme. Fluorescence spectroscopy reveals that the structures of the and lysozyme domain are intact, i.e., the partial unfolding that underlies cluster formation is constrained to the interdomain contacts. Upon solution dilution, the ratio of the cluster concentrations to that of protein monomers decreases exponentially (it should be constant for irreversible aggregates), in agreement with an equilibrium model of cluster formation. In their sum, these results demonstrate that the clusters are not irreversible aggregates, but represent equilibrium high-concentration protein domains.

1. M.A. Vorontsova, H.Y. Chan, V. Lubchenko, P G. Vekilov, *Biophysical Journal,* **109**, (2015), 1959-1968.
2. A.B. Kolomeisky, *Biophysical Journal*, **109**, (2015), 1759-1760.

S12-L2

ANTIBODIES AS CHAPERONES IN CRYSTALLISATION: PARAMETERS FOR SUCCESS

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Antibodies as Chaperones in Crystallisation: Parameters for Success. Crystallization Chaperones including Antibodies, Nanobodies and Aptamers have been shown effective in promoting the crystallisation of otherwise uncrystallisable targets. A prominent example is the nanobody used to stabilize the GPCR-G-protein complex in the 2012 Nobel Prize-winning work Nature 2011).

Here at AstraZeneca we have pursued the use of Antibody Fragments though a bioinformatics driven approach to enable crystal systems for Drug Discovery. Recent work has been successful for the Oncology target Mcl1 Myeloid Cell Leukemia 1; an anti-apoptotic protein from the Bcl-2 enabling Structure Based Design for a DNA encoded library hit. This talk describes the process by which the chaperone molecules were obtained and, using examples from this and other projects, how their biophysical properties such affinity, epitope and thermal stability correlate with their propensity to crystallise. The talk will also cover a comparison of our ligand bound Mcl1-Antibody complex with examples of Mcl1 ligand bound structures from the literature.

S12-L3

PROTEIN CRYSTALLIZATION IN HYDROGELS, CURRENT STATUS AND FUTURE PROSPECT

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Although crystallization in hydrogels is not a frequent practice in bio-crystallography, the benefits of using a polymeric media for the crystallization of macromolecules are multiple - i.e. prevents convection and crystal sedimentation, acts as impurity filter, etc. - and have been proven to produce crystals of higher quality [1]. The used of hydrogels also avoids the manipulation of the crystals and therefore the osmotic and mechanical stress exerted on the crystals during handling preserving the integrity of the macromolecule crystals even when exposed to organic solvents.

There are many types of hydrogels (agarose, silica, PEGs base hydrogel, sephadex, etc) that are compatible with protein crystallization. Among them, agarose is probably the most widely tested and can be used at concentrations below its critical gelling point, thus facilitating its mix with the protein solution. Hydrogel gel media should also be taken into account not only for improving crystal quality but also to exert control over the nucleation and growth processes. Even more, gel-grown protein crystals have been recently shown to be excellent candidates to produce crystals of bigger size for neutron diffraction studies using crystals grown in agarose as seeds [2] or even be used as media for the continuous delivery of nano/micro-metre size crystals needed for serial femtosecond crystallography. It is therefore expected that protein crystals grown *in situ* in hydrogel will be used in XFEL experiments to minimize sample consumption [3]. In this work we will revise the current tends in the use of hydrogels focusing on our most recent results obtained with dipeptides-based hydrogels for the production of high quality protein crystal [4] and the formation of new polymorphs [5]. The preservation of crystal integrity will be explained on the basis of the incorporation of the gel matrix within the crystals. The amount of incorporated hydrogel is quantified using thermogravimetrical analysis (TGA) of silica grown crystals.

1. B. Lorber, C. Sauter, A. Theobald-Dietrich, A. Moreno, P. Schellenberger, M.-C. Robert, B. Capelle, S. Sanglier, N. Potier, R. Giege, Progress in biophysics and molecular biology, **101**, (2009), 13.
2. S. Sugiyama, N. Shimizu, K. Kakinouchi, O. Hiraoka, H. Matsumura, H. Y. Yoshikawa, Y. Takahashi, M. Maruyama, M. Yoshimura, H. Adachi, K. Takano, S. Murakami, T. Inoue, M. Murata, Y. Mori, CrystEngComm, **17**, (2015), 8064.
3. C. E. Conrad, S. Basu, D. James, D. Wang, A. Schaffer, S. Roy-Chowdhury, N. A. Zatsepin, A. Aquila, J. Coe, C. Gati, M. S. Hunter, J. E. Koglin, C. Kupitz, G. Nelson, G. Subramanian, T. A. White, Y. Zhao, J. Zook, S. Boutet, V. Cherezov, J. C. H. Spence, R. Fromme, U. Weierstall, P. Fromme, *IUCrJ,* **2**, (2015), 421.
4. M. Conejero-Muriel, J. A. Gavira, E. Pineda-Molina, A. Belsom, M. Bradley, M. Moral, J. d. D. G.-L. Durán, A. Luque González, J. J. Díaz-Mochón, R. Contreras-Montoya, Á. Martínez-Peragón, J. M. Cuerva, L. Álvarez de Cienfuegos, *Chem. Commun*. **51**, (2015), 3862.
5. M. Conejero-Muriel, R. Contreras-Montoya, J. J. Díaz-Mochón, L. Álvarez de Cienfuegos, J. A. Gavira, *CrystEngComm*, **17**, (2015), 8072

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S12-L4

FLYING THROUGH OPTIMIZATION SCREENING WITH dragonfly

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Protein crystal optimization is vital to ensure high quality X-ray diffraction data for the solving of high resolution structures. This process involves the set-up of a series of complex screening combinations where the ratios of the individual components identified from primary crystallization studies are varied.

In order to reduce the effort and tedium of this time consuming process, TTP Labtech have designed dragonfly® for crystallization screening as an addition to their successful mosquito® liquid handling portfolio.

dragonfly is a liquid handler for simple, fast and accurate crystal screen optimization with unsurpassed reproducibility. dragonfly's positive displacement technology ensures highly accurate dispensing, from 0.5 µL up to 4 mL, across a wide range of viscosities. Its rapid plate preparation is uniquely combined with non-contact dispensing to ensure zero cross-contamination. Dispense resolution is 0.1 µL, allowing very fine gradients to be created.

The partner software – dragonfly designer has been designed from the ground up to be an easy-to-use gradient design tool which is stand alone and provided without restriction on user numbers to significantly reduce ongoing costs.

In this talk we will review both the technology behind the dragonfly and real world examples of speeding up successful crystallization projects.

S12-L5

PROTEIN CRYSTALLISATION - TRICKS AND PRACTISE

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Preparation of highly ordered protein crystals is essential for using X-ray crystallography technique, which is still the most powerful tool for determining the three-dimensional structure. At high technical level of data collection at synchrotron sources and software developments for data processing and structure determination and refinement, protein crystallization still remains more art than science and represents one of the major limiting steps. Conditions at which a protein might produce highly ordered crystals cannot be computed, they must be identified empirically. Crystallization is influenced by extremely large number of parameters from which the most important is protein itself. It is well known that subtle changes in conditions as well as protein modifications may have a dramatic effect on crystallization behaviour.

We have crystallized a number of proteins add determined their tertiary structures. The main objects were mutants of ribonuclease Sa from bacteria *Streptomyces aureofaciens*, glucoamylases and their mutants from the yeast *Saccharomycopsis fibuligera*, actin-binding domain of cytoskeletal protein plectin from *Mus musculus*, CE16 acetylesterase from fungi *Trichoderma reesei*, GH30 xylanase A from bacteria *Dickeya chrysanthemi* and other enzymes. Successful crystallization was conditioned by the use of special approach tailored to each protein or mutant. Similarly, the choice of cryoprotectant and optimisation of the crystal flash cooling procedure was crucial for collecting good sets of data. The tested tricks and successfully used approaches will be described, illustrated and discussed.

The work was supported by the Slovak Academy Research Grant Agency VEGA grant No. 2/0190/14 and Slovak Research and Development Agency grant APVV-0602-12. We acknowledge the EMB c/o DESY, Hamburg and MFPL Vienna University for providing us with synchrotron source and laboratory X-ray source facilities.

S12-L6

TRANSMISSION ELECTRON MICROSCOPY FOR THE EVALUATION AND OPTIMIZATION OF CRYSTAL GROWTH

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Crystallization of protein samples remains the most significant challenge in structure determination by X-ray crystallography. Here we demonstrate the effectiveness of transmission electron microscopy (TEM) analysis to aid in the crystallization of biological macromolecules. We found the presence of well-order lattices with higher order Bragg spots, revealed by Fourier analysis of TEM images, as a good predictor of diffraction-quality crystals. Moreover use of TEM allowed 1) comparison of lattice quality among crystals from different conditions in crystallization screens; 2) detection of crystal pathologies that could contribute to poor X-ray diffraction, including crystal lattice defects, anisotropic diffraction and crystal contamination by heavy protein aggregates and nanocrystal nuclei; 3) qualitative estimation of crystal solvent content to explore the effect of lattice dehydration on diffraction; and 4) selection of high quality crystal fragments for microseeding experiments to generate reproducibly larger size crystals. Applications for X-ray free electron laser (XFEL) and micro electron diffraction (MicroED) experiments are also discussed.

Session XIII - Crystallization in Industry and Biomedicine

Thursday, July 7 - morning

S13-L1

A RATIONAL APPROACH TO CRYSTALLISING PROTEINS IN THE PHARMACEUTICAL INDUSTRY, THE IMPACT OF MICRO SEED MATRIX SEEDING

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Crystallization is often referred to as a bottleneck in protein structure determination. However applying rational, knowledge based strategies and attention to detail, has proved to be very successful in obtaining crystallization systems for studying protein/inhibitor complexes in drug discovery.

This seminar will describe the rationale and the methods that have been successfully used to crystallize medically relevant proteins [1] in four different pharmaceutical companies (Hoffmann-la Roche, Morphochem, Novartis and Actelion). The first part will deal with protein characterization prior to initiating crystallization trials [2], the different methods and strategies of screening and examples of protein modification for crystallization.

The second part will focus on seeding and describe general seeding methods as an introduction to the most important subject of the talk, Microseed Matrix Seeding (MMS) [3-4]. One of the most powerful methods introduced into protein crystallization in the past 10 years in particular for establishing suitable cocrystallisation or soaking systems for obtaining X-ray structures of inhibitors complexes.

Examples will be given of increased hit rates, elimination of twinning, improved diffraction and promoting different space groups.

1. Crystallizing proteins - a rational approach? D'Arcy A. *Acta Crystallogr D* Biol Crystallogr. 1994 July.
2. The protein as a variable in protein crystallization. D'Arcy A, Mac Sweeney A, Haber A. *Acta Crystallogr D* Biol Crystallogr. 2003 Jul;59(Pt 7):1343-6. Dale GE, Oefner C, D'Arcy A. *J. Struct Biol. 2003* Apr;142(1):88-97. Review.1;50(Pt 4):469-71.1.
3. An automated microseed matrix-screening method for protein crystallization. D'Arcy A, Villard F, Marsh M. *Acta Crystallogr D* Biol Crystallogr. 2007 Apr;63(Pt 4):550-4.
4. Microseed matrix screening for optimization in protein crystallization: what have we learned? D'Arcy A, Bergfors T, Cowan-Jacob SW, Marsh M. *Acta Crystallogr F* Struct Biol Commun. 2014 Sep;70(Pt 9):1117-26.

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