Advanced methods in macromolecular crystallization

(FEBS 2024 Advanced Course)

Faculty of Science University of South Bohemia České Budějovice, Czech Republic, June 09 - July 15, 2024

Lectures - Monday, June 10

L1

INTRODUCTION TO FEBS, THE FEDERATION OF EUROPEAN BIOCHEMICAL SOCIETIES

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I shall introduce to all participants the main activities of FEBS, The Federation of European Biochemical Societies. FEBS is a registered charitable organization advancing research in the molecular life sciences across Europe and beyond. It was founded in 1964, is self-funded and is made of 39 constituent Societies (i.e., national biochemistry and molecular biology societies) that account for >35,000 members. Through its various programmes FEBS warrants innovative and extensive support for research training, education, interaction and communication. For instance, it grants FEBS Fellowships – stipends supporting training and mobility, and a FEBS Excellence Award – funds for early-career group leaders. Of note, FEBS supports every year Lecture Courses (like this FEBS-EMBO Advanced Lecture Course "Lipids, proteins and their interactions in organelle biology"), Practical Courses, Workshops and Special Meetings, in order to stimulate on a regular basis education, training and networking among young European (and non-European) biochemists. Finally, I shall remark that a online forum for the molecular life science community is offered by the FEBS Network, and that FEBS wholly ownes high-quality, peer-reviewed journals whose income funds FEBS programmes. As a general reference, the FEBS official website (www.febs.org) should be visited.

Mutay Aslan MD., PhD. completed her medical residency training at Akdeniz University Faculty of Medicine, in Turkey. She received her Ph.D. in Biochemistry and Molecular Genetics from the University of Alabama at Birmingham, in the United States. She is currently working as General Director of the Clinical Lab. at Akdeniz University Hospital and is also a lecturer at Akdeniz University Faculty of Medicine. Prof. Aslan currently serves as a member and was on the Management Committee of EU-COST. Dr. Aslan has been an independent expert/evaluator for the European Commission in selection of research proposals and has also been elected to the Advanced Courses Committee of FEBS for a four-year term. Aslan's research focuses on lipoproteins, fatty acids and sphingolipids in diabetes and cancer.

https://avesis.akdeniz.edu.tr/mutayaslan

L2

TACKLING THE REPRODUCIBILITY CRISIS IN SCIENTIFIC RESEARCH

Sara Fuentes

Managing Editor of FEBS Open Bio (on-line)

L3

ALPHAFOLD AND BIOCHEMICAL CONSIDERATIONS FOR PROTEIN CRYSTALLIZATION

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AlphaFold [1] is an artificial intelligence program by *DeepMind* that can be used to predict a protein's three-dimensional structure from its known primary sequence. In many cases, the *AlphaFold* neural network system provides some impressive accuracy with small protein molecules [2]. Even though *AlphaFold* is showing great promise for reliable protein structure prediction, it still falls short of predicting the structural arrangements of ligands, cofactors, metal ions, solvent interactions and any types of post-translational modifications. Therefore, experimental methods using X-ray crystallography, Cryo-EM or NMR are still important necessities.

Since *AlphaFold* concerns only the arrangements of amino acid sequences, the program can also be used to predict the crystallizability of proteins suitable for X-ray diffraction. For example, basic structural symmetry arrangements, low-energy states, random loops, flexibility [3] and inter- and intra-molecular contacts can be predicted using *AlphaFold* to provide assessments of crystallization probability. In this workshop, we will predict the protein crystallizability by computational methods combining AlphaFold/AlphaFold2, XtalPred and biochemical considerations. A pipeline of crystallization approaches will be discussed starting with bioinformatics strategies to practical set-ups [4].

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3. Ma, P., Li, D.W. and Bru¨schweiler, R. (2023). Predicting protein flexibility with AlphaFold. Proteins 2023:1-9.
4. Ng, J.D. (2023). Laboratory workbook using AlphaFold and biochemical considerations for protein crystallization. University of Alabama in Huntsville.

L4

INCELLCRYST - A STREAMLINED APPROACH FOR PROTEIN CRYSTALLIZATION IN LIVING CELLS.

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During the past decades, protein crystallization in living cells has been observed surprisingly often in all domains of life as a native assembly process, and emerging evidence indicates that this phenomenon is also accessible for recombinant proteins [1]. The advent of high-brilliance synchrotron sources, X-ray free-electron lasers, and improved serial data collection strategies has allowed the use of these micrometer-sized crystals for structural biology [2-7]. Thus, *in cellulo* crystallization could offer exciting new possibilities for proteins in a quasi-native environment, complementing conventional crystallization approaches.

This lecture will present an overview of the current knowledge about *in cellulo* crystallization of native and recombinant proteins, complemented with a discussion of the current method developments to successfully collect X-ray diffraction data from intracellular crystals. Efforts to systematically exploit living insect cells as protein crystallization chambers and to streamline this process for structural biology resulted in the establishment of a pipeline to elucidate the structural information of *in cellulo* crystallized target proteins in short time, denoted as '*InCellCryst*' [8]. After cloning of the target gene into baculovirus transfer

vectors, the associated recombinant baculoviruses are generated to infect insect cells, and crystal formation is detected at day 4 to 6 after infection. If intracellular crystal -lization is successful, diffraction data are collected after crystal isolation or directly within the living cells using recently developed serial crystallography approaches at XFELs [2, 3, 6, 7] or synchrotron sources [4, 8], depending on the obtained crystal size. However, low numbers of crystal containing cells within a cell culture and limitations in crystal detection techniques represent the main bottlenecks currently restricting a more broad application. Innovative approaches for direct time-efficient screening of cell cultures using X-rays [9] as well as for insect cell fusion to increase intracellular crystal size are finally presented. This will allow a more efficient use of crystal containing cells as suitable targets for structural biology in the future.

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L5

CRYSTALLIZATION OF MEMBRANE PROTEINS IN LIPIDIC SYSTEMS

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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. Emphasis will be placed on crystallization approaches which make use of the lipidic systems. In my talk I will describe these methods and our progress in understanding how they work at a molecular level. The practicalities of implementing these methods in low- and high-throughput modes will be examined. A practical demonstration of the lipid cubic phase or *in meso* method will be given at the *FEBS Lab Excercises* on Tuesday, June 11.

Useful References and Links

Caffrey, M. 2015. A comprehensive review of the lipid cubic phase or *in meso* method for crystallizing membrane and soluble proteins and complexes. *Acta Cryst. F71*, 3-18. https://doi.org/10.1107/S2053230X14026843

Caffrey, M. 2021. Membrane protein crystallization. In **Protein Crystallization**. 3rd Edition (Ebook), Bergfors T. M. (ed.) IUL Biotechnology Series, 10. Chapter 19, pp 373-410. ISBN: 978-0-9720774-7-7. https://iul-press.us/product/protein-crystallization-third-edition-ebook/

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Lab Publications: http://www.tcd.ie/Biochemistry/research/publications_mcaffrey.php

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L6

SYNTHETIC MACROCYCLES AS MEDIATORS OF PROTEIN CRYSTALLIZATION

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Of the many innovations in protein crystallization, water soluble macrocycles are proving useful. Calixarenes, crown ethers and cucurbiturils can function as "molecular glues" that bridge two or more protein surfaces, thereby enabling assembly/crystallization [1-3]. Symmetric and chemically-uniform, the macrocycle can mask the protein, simplifying the surface features and providing a homogenous scaffold for assembly. Recent work with sulfonato-calix[8]arene (**sclx$_8$**) will be illustrated, including examples of macrocycle oligomerization and macrocycle-mediated protein frameworks (Figure 1).

1. Ramberg, Engilberge, Skorek, Crowley, *J. Am. Chem. Soc.* 2021, 143, 1896-1907.
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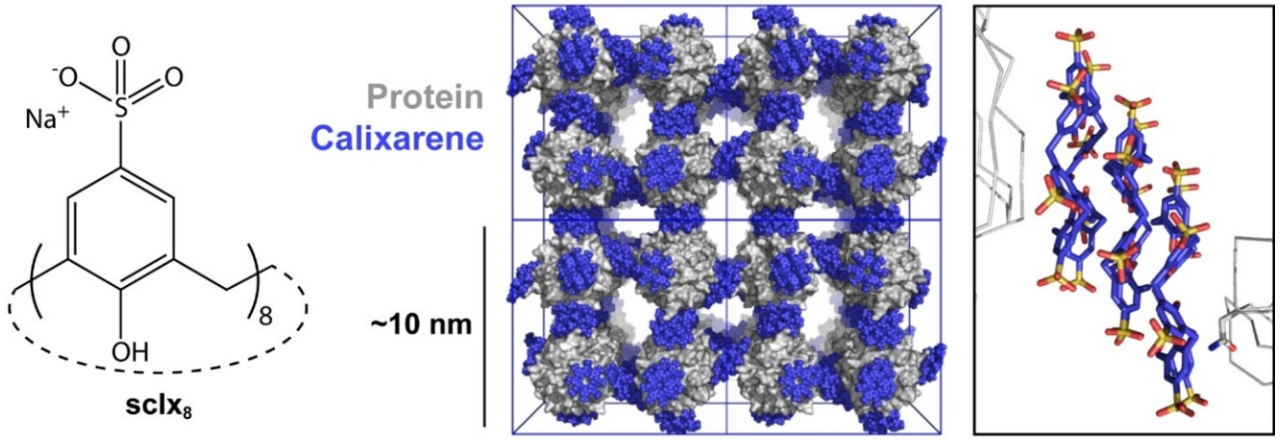

Figure 1. A cubic co-crystal structure of the 6-bladed â-propeller RSL and **sclx$_8$**. Protein *nodes* are connected by trimeric calixarene *links*.[3]

L7

FROM TARGET STRUCTURES TO DRUGS

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Drug discovery is a high-risk, high-gain enterprise. Computer-aided drug design (CADD) helps lower the risk element and has now become an integral part of the modern drug discovery process. Among CADD methodologies, structure-based drug design (SBDD) has emerged as a fundamental approach in drug design, offering unparalleled insights into molecular interactions and enhancing the efficiency of lead optimization. SBDD harnesses the three-dimensional structures of biological targets, facilitating the rational design of small molecules that are able to bind to them efficiently. This talk will delve into the principles and methodologies of SBDD, illustrating their pivotal roles in expediting drug discovery processes across a spectrum of therapeutic areas. Through case studies and advancements in computational methodologies, we will explore how these approaches are contributing to enriching the pharmaceutical landscape, driving innovation, and ultimately, bringing transformative therapies to patients in need.

L8

THE CHEMISTRY OF MUSHROOM MAGIC (AND WHY YOU SHOULD NOT LICK TOADS)

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Psychoactive substances have been around for ages and hallucinogenic experiences are described in many ancient texts. We will explore the history, chemistry, and pharmacology of various natural and designer drugs (and find that crystallization experience is not entirely useless in the process). We learn that some mushrooms kill, and that kissing of toads or chewing cacti could endanger your health. The magic mushroom hallucinogen Psilocybin, which has a long history of recreational use, has also shown some promise in the treatment of therapy resistant depressions and PTSD. The elucidation of its fungal biosynthesis provides a rich tale of crystallization adventures.

Disclaimer: Many substances discussed in this talk and in the magic kingdom of fungi are CONTROLLED (SCHEDULED) SUBSTANCES, meaning that their production, possession, and consumption are VERBOTEN. Even for SCIENTIFIC RESEARCH an exemption to possess and produce (also via biosynthesis) any of these controlled substances – including many basic precursor chemicals – is required in almost all jurisdictions.

No toads were hurt during the production of this presentation, and no mushrooms were consumed during structure determinations.

Lectures - Tuesday, June 11

L9

FROM PROTEIN SOLUTION TO CRYSTALS: NATURE AND FORMATION OF PROTEIN CRYSTALS

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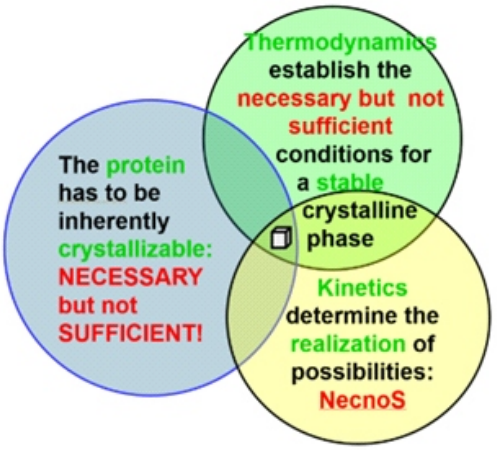
Protein crystallization is the self-assembly of protein molecules into an ordered, periodic 3-dimensional structure, the protein crystal. Unfortunately, protein molecules are large, complex, and dynamic molecules, and most proteins are therefore difficult to crystallize. Three fundamental conditions need to be met for a protein to self-assemble into an ordered crystal: (a) the protein must be soluble, stable, and inherently crystallizable; (b) we need to find the macroscopic thermodynamic conditions that allow formation of a protein rich phase in form of a protein crystal; and (c) control, at least within limits, the microscopic kinetic parameters governing the realization of the most desirable outcome of a crystallization trial: a well-diffracting protein crystal.

In most cases, modification of the protein itself will be necessary to achieve successful crystallization. We will examine how fundamental physico-chemical properties of a protein influence crystal formation and appreciate the role of specific properties affecting key determinants such as solubility and intermolecular contacts. To efficiently sample the almost infinite combinations of reagents and environment providing the right macroscopic conditions, we will conceptualize the crystallization process with the assistance of crystallization 'phase diagrams' for various crystallization techniques and examine how to proceed towards optimization and control of crystal growth.



Crystallization is ruled by three fundamentals, each necessary but not sufficient: logical AND operation

L10

FROM PROTEIN EXPRESSION AND PURIFICATION TO CRYSTALLIZATION

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Protein crystallization continues being a major bottleneck in the X-Ray Structural Biology field, highly dependent on the production of abundant, pure and homogeneous protein samples [1]. Whereas purification methodologies have improved dramatically in the past decades, purification optimization continues being a time-consuming multifactorial process, where factors such as (but not limited to) protein production hosts, recombinant construction design, lysis methods, protein stability (degradation, aggregation propensity, thermostability,…) and/or sample composition (pH, ionic strength, temperature, additives,…) play important roles [2, 3]. Thus, protein sample preparation continues being of critical consideration for the Structural Biology field. Classical and new methodologies in protein purification workflow will be discussed, emphasizing crucial considerations in the purification of protein complexes.

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L11

CRYSTALLIZATION FOR THE DESPERATE

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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?
- So many crystallization kits! Which one to choose?
- Chemical space or the kinetic pathway: which one matters most?
- 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?

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.*

Bergfors, T. M., ed. **Protein Crystallization, 3rd Edition,** 2022, International University Press, La Jolla California.

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L12

HOFMEISTER ION SERIES AND THE PROTEIN PHASE DIAGRAM: CONSEQUENCES FOR SOLUBILIZATION AND CRYSTALLIZATION

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The higher the supersaturation concentration of the macromolecule, the higher the likelihood smaller nuclei will advance and sustain crystal growth. Initial poor solubility typically culminates in precipitates or numerous small crystals of reduced quality. Hence, as a first step on the road to obtaining good crystals, the protein solubility should be rigorously maximized before embarking to evoke supersaturation of the solution by means of temperature change, pH shift, polymer addition or (inverse) salting-out.

The Hofmeister series (1888) provides a basic but fundamental tool to manipulate on the one hand solubility and on the other supersaturation by means of salt ions. Maximally optimized states of the pair – solubility and supersaturation – are rarely attainable by applying one type of salt only. Depending on the pI of the protein and the pH of the solute, a more strategic choice of salt ions instead of the widely used sodium chloride is critical for maximum solubility, namely salts that according to the outcomes of the Hofmeister series de-stabilize the protein. Note, de-stabilization increases flexibility and hence, endorses enzymatic activity. Finally, a salt that stabilizes the protein has to be identified and added in order to reach supersaturation and thus suitable nucleation and crystal growth.

Besides a theoretical overview, the practical use of the Hofmeister ion series will be addressed and examined in more depth.

Primers and Links:

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

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

III. A. McPherson, *crystallization of biological macromolecules*, Cold Spring Harbor Laboratory Press. http://www1.lsbu.ac.uk/water/hofmeister_series.html by Martin Chaplin.

L13

PROTEIN CRYSTALLIZATION BY CAPILLARY COUNTER-DIFFUSION METHODS

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Vapor diffusion and micro-batch 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, protein and precipitating 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 [1]. Chaotic mixing and convection can be reduced when crystallization proceed in any media with mass transport controlled by diffusion: gels, capillaries, microfluidic devices or microgravity. This effect can be achieved with liquid-liquid diffusion (free-interface diffusion) techniques in which protein and precipitant are allowed to diffuse one against each other [2].

Among different ways to implement this technique, the most effective configuration proven to be useful for growing macromolecules crystals is the capillary counterdiffusion (CCD) technique. Unlike other techniques aimed at finding initial conditions close to equilibrium, counter-diffusion 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 events of diffusion and crystallization giving rise to a supersaturation gradient along the length of the protein chamber (capillary, microfluidic channel, etc.) [2-5].

In this talk we will discuss the effectiveness of counter-diffusion technique not only for improving crystal quality but also for the search of initial crystallization conditions when compared with traditional crystallization technique [6].

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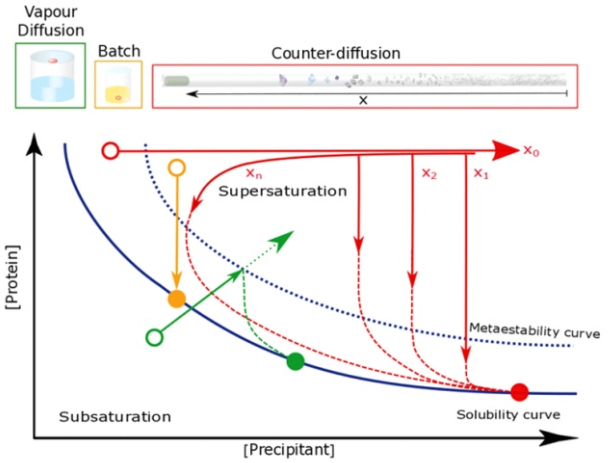

L14

CONSTRUCT DESIGN AND LIMITED PROTEOLYSIS STRATEGIES

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The crystallisation of proteins or protein complexes is the process of forming a regular network of individual macromolecules stabilised by crystal contacts. It is reasonable to assume that the more organised and compact the molecules that make up this network, the higher the probability of forming macromolecular crystals. Similarly, the chances of performing usable diffraction experiments are increased with compact macromolecules. In this talk, we will try to illustrate different approaches to design constructs on target proteins/protein complexes in order to turn them into favourable candidates for macromolecular crystallisation. We will review online bioinformatics resources that can assist and guide the design of constructs with highlighting the use of AF2 to design protein protein complexes constructs . In addition, limited proteolysis is a very powerful approach to obtain more compact constructs. It is a particularly powerful technique for studying protein complexes. Several examples will be presented.

L15

A CRYSTALLOGRAPHER'S GUIDE TO THE GALAXY: STRATEGIES FOR PROTEIN CRYSTALLISATION USING MOLECULAR DIMENSIONS CRYSTALLOGRAPHY SCREENS

Paul Driver

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X-ray crystallography is a cornerstone technique in structural biology, requiring high-quality crystals to determine molecular structures with atomic precision. This talk will guide attendees through the strategic selection of crystallisation conditions using Molecular Dimensions' commerical screens. Emphasizing practical approaches, we will explore how to optimize crystallisation conditions to increase the likelihood of producing suitable crystals for diffraction studies. By understanding the principles behind crystallography screens and leveraging their potential, researchers can significantly enhance their success rates in protein crystallisation, paving the way for more accurate and detailed structural analyses. This session aims to provide an insights to the wide choice of commerically available screens offering a selection strategy to achieve excellence in crystallisation outcomes.

Lectures - Wednesday, June 12

L16

ANALYZING, SCORING AND OPTIMIZING CRYSTALLIZATION CONDITIONS APPLYING ADVANCED DYNAMIC LIGHT SCATTERING (DLS) TECHNIQUES

Karsten Dierks[1] Hévila Brognaro[2], Susanna Georgyan[2] and Christian Betzel[2],

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Beside conventional single crystal diffraction data collection, which appreciates larger crystals, today at micro-beam synchrotron (SR) and X-ray Free-Electron-Laser (XFEL) beamlines micro-sized crystals are preferred and mandatory. Micro-sized crystal suspensions are required for the emerging techniques of serial diffraction data collection (SX, SSX) and particular for time resolved data collection (TRX) [1]. Therefore, reliable methods to prepare, detect and score routinely micro- and nano-sized crystals and crystal suspensions are a prerequisite. In this context the understanding of the initial steps of a crystallization process, including the early events of phase separation and nucleation is of fundamental importance to successfully grow and obtain crystals and/or crystal suspensions with homogeneous crystal dimensions. Advanced light scattering (DLS) techniques are most suitable to obtain insights about crystallization processes to score and optimize crystallization of biomolecules [2]. Corresponding DLS procedures and techniques will be presented and discussed.

Latest data and publications indicate that prior to nucleation a distinct phase separation of biomolecules can be observed, followed by the transition to higher order assemblies inside these condensates [3,4,5]. Hence, and more recently the nucleation process is discussed intensively in theory and via experimental results [6,7]. DLS methods to obtain insights about the initial molecular pathways of crystal nucleation will be presented for selected proteins, applying particular *in situ* dynamic light scattering, small-angle X-ray scattering and transmission electron microscopy [8,9].

As mentioned before, as the desired crystal dimensions for serial experiments, applying on chip data collection, tape drive, or jet sample/crystal delivery, are today preferably in the upper nanometer or lower micrometer regime it is necessary to apply and to establish also appropriate imaging procedures to monitor nano- and micro-crystal suspensions. We designed and constructed an imaging setup including hardware to detect second and third harmonic generation (SHG, THG) signals combined with a UV-fluorescence option [10]. This imaging system allows the reliable detection of tiny and small crystals via SHG, even of nano-sized crystals with high symmetry and allows to distinguish between amorphous material and crystalline particles.

The combination of complementary DLS and imaging instrumentation, methods, procedures, and examples will be presented.

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L17

PROTEIN AS THE MAIN VARIABLE IN CRYSTALLIZATION

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Before X-ray protein crystallography was born, crystallization was used to purify and /or confirm the purity of a protein sample. Nowadays, protein crystals are mainly used for determining the structure by diffraction methods and, more recently, also for therapeutic purposes for delivering biopharmaceuticals.

Crystallization is influenced by many physico-chemical and chemical parameters, the most important of which is the protein itself, its purity, homogeneity and specific properties, namely its propensity to form crystals [1]. In the past, crystallization of the same type of protein, but isolated from several different sources, was recommended in case of unsuccessful attempts. Currently, in the era of molecular biology, experiments are not limited by the presence of natural variants, but it is possible to modify the proteins and prepare mutants with an increased ability to form high-quality crystals. This may include preparing proteins with different types of fusion partners or tags, removing their most flexible parts (N- and C- termini or flexible loops), increasing homogeneity by modifying free cysteines or potential glycosylation sites, replacing unfavorable amino-acid residues on the surface of the molecule, etc. One of the most successful strategy of enhancing the crystallizability of proteins is mutational surface engineering based on the reduction of surface entropy [2,3].

Statistics from structural genomics projects show that the success rate of high-throughput crystallization is only 10-30%. Experimentally and more or less accidentally, interesting proteins showing a high tendency to crystal formation were also discovered. The best known is lysozyme, which is used as a model protein for the study of crystallization. A number of other proteins were found that were present as protein impurities in concentrated solutions of the investigated proteins, but unexpectedly crystallized, thereby complicating the solution of the structure [4]. For early identification of unwanted crystallized protein contaminants, the *ContaMiner* web server and the *ContaBase* contaminant database were created [5].

The requirement of protein purity and homogeneity will be discussed and emphasized. The effect of protein modifications on its crystallization and/or crystal packing and quality will be documented based on results obtained in our laboratory and examples from the literature. Some rational approaches and strategies aimed at increasing protein crystallizability will be presented.

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L18

UNCONVENTIONAL CRYSTALLIZATION STRATEGIES AND TECHNIQUES FOR SCREENING AND OPTIMIZATION

Lata Govada and Naomi E. Chayen

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All prescription drugs on the market today, which account for billions of pounds in annual sales worldwide, directly or indirectly target proteins. Protein functions are determined by their three-dimensional structures, hence detailed understanding of protein structure is essential for rational design of therapeutic treatments. Examples include cancer, obesity, cardiovascular disorders, autoimmune diseases and a multitude of other ailments.

The most powerful method for determining the structure of proteins is X-ray crystallography which is totally reliant on the availability of high quality crystals, but producing useful crystals has always been, and still remains, the bottleneck to structure determination.

There is no 'magic bullet' that will guarantee the yield of good crystals, hence rational approaches leading to the development of new and improved technologies for obtain-

ing high quality crystals is of crucial importance to progress.

This talk will present strategies for increasing the chances of success and highlight a variety of practical methods that resulted in successful crystallization in cases where standard procedures have failed. The methods involve active influence and control of the crystallization environment, in order to lead crystal growth to the desired result. Many of the techniques can be automated and adapted to high throughput mode and several have been patented and commercialised.

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L19

MICROFLUIDICS IN ACTION: CRYSTALLIZATION AND CRYSTALLOGRAPHY IN MICROCHIPS

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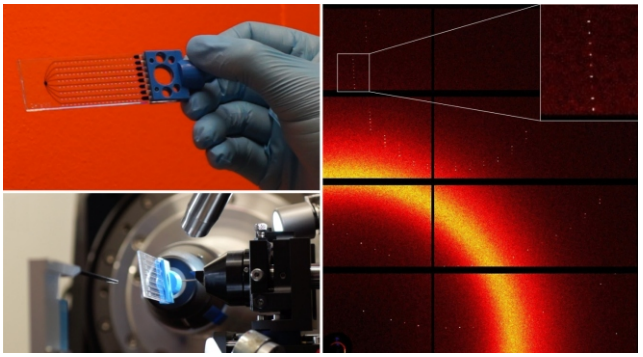
Two decades ago microfluidic technologies opened new possibilities for the crystallization of biological macromolecules. Indeed, microfluidic systems offer a lot of advantages for crystal growth: they enable an easy handling of nano-volumes of solutions as well as an extreme miniaturization and parallelization 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 and nano-batch (Hansen et al. 2002; Zheng et al. 2004) crystallization in microfluidic chips have already demonstrated the value of this technology, especially for high throughput screening applications in structural genomics.

Examples of microfluidic devices available on the market or in development will be described to illustrate how different steps of a structural study can be carried out 'on chip' from the crystallization to the observation of crystals and their characterization using synchrotron radiation (Sauter et al. 2007; de Wijn et al. 2019). The perspective of using affordable microfluidic chips for screening best crystallization agents and for automated X-ray diffraction analysis and their complementarity with conventional crystallization setups will be discussed.



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L20

WHAT'S THIS IN MY DROP? INTERPRETATION OF CRYSTALLIZATION PHENOMENA

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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 crystallization drop phenomena and videos can be accessed at: xray.teresebergfors.com.

L21

MICROSEED MATRIX SCREENING AND ITS USE IN STRUCTURE BASED DRUG DISCOVERY

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Protein crystals obtained in initial screens typically require optimization before they are of X-ray diffraction quality. Seeding is one such optimization method. In classical seeding experiments, the seed crystals are put into new, albeit similar, conditions. The past decade has seen the emergence of an alternative seeding strategy: microseed matrix screening (MMS). In this strategy, the seed crystals are transferred into conditions unrelated to the seed source.

MMS can optimise crystallisation starting from many kinds of crystalline aggregate, can expedite the search for new crystal forms, and can allow the crystallisation of previously uncrystallisable mutants or complexes.

In this presentation I will give an introduction to MMS, and show examples of its successful application to a wide variety of real-life crystallisation projects in industry and academia. I will also show how the method is particularly valuable in optimising crystals systems for the demands of crystal-based fragment screening.

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L22

SAMPLE PREPARATION FOR ROUTINE AND ADVANCED STRUCTURAL BIOLOGY, INCLUDING SERIAL DATA COLLECTION, MICROED, AND CRYOEM

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Serial data collection and microED techniques usually require "slurries" of tiny, well-ordered crystals. Neutron diffraction requires very large single crystals. Microseeding effectively generates such samples since the seed stock can be concentrated or diluted as necessary. During the 16 years since the random microseed matrix-screening (rMMS) method was published, understanding of the theoretical advantages of the method has increased, and several practical variations of the technique have emerged. Moreover, seeding can be carried out in a microbatch-under-oil setup, which has two important advantages: (1) easily interpreted phase diagrams can be constructed in a few minutes; (2) batch experiments are easy to scale up. We present case studies using these approaches to increase control and crystal quality for routine and advanced data collection.

Protein structure determination by cryo-EM requires expensive equipment that has low throughput. It is, therefore, wasteful to examine samples that can be shown in advance to be aggregated since such samples are unlikely to be suitable. We used a high-throughput screening approach with dynamic light scattering to explore 96 chemical conditions, with as little as 10 µL of protein solution in total, to identify conditions with reduced aggregation.

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Lectures - Thursday, June 13

L23

USING FLUORESCENCE TO FIND YOUR CRYSTALS

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A wide variety of crystallization solutions are screened to establish conditions that promote the growth of a diffraction-quality crystal. Screening these conditions require the assessment of many crystallization plates for the presence of crystals. A range of optical techniques and automated systems for screening are available. One disadvantage of some automated imaging systems is the need for certain characteristics, such as the presence of tryptophan, for crystal detection. Another disadvantage is the cost of the instrumentation, which is typically greater than $50,000.

We have developed a visible fluorescence approach that can give unambiguous macromolecule crystal detection and have now coupled it to a smartphone-based imaging method [1] that can be implemented for as low as $25-$35. Since the method uses trace fluorescent labeling (TFL) [2] with visible wavelength fluorescent probes, one can use different colors for the imaging of complexes as the presence of each color in the crystal being verification that those molecules are present. The TFL method involves the covalent attachment of a fluorescent dye to ~0.1 to 0.5 % of the protein molecules and has been shown to not affect crystal nucleation or diffraction quality at these levels.[3] The images give a direct indication of what the protein is doing in response to each screening condition as only the macromolecule has fluorescent probe attached to it.

There are several advantages of the TFL technique for detecting crystals. The ability to distinguish salt crystals from macromolecule crystals, facilitating the analysis of screening results, and the low-cost of imaging. This approach can be used for the imaging of protein complexes, which can save beam time.

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L24

ANALYSIS OF PROTEIN CRYSTALLIZATION SCREENING RESULTS

Marc L. Pusey

Dept. of Chemistry, University of Alabama in Huntsville

Having a crystal is a defining requirement for structure determination by crystallography. Beyond events such as this workshop, proteins do not come with instruction manuals attached that clearly delineate how to go about obtaining such an outcome. As a result one must carry out an experimental program with the goals of producing the required crystal(s). The crystallization process can be broken down into several stages. First is the screening process, which by definition is where the primary parameters for a given system are identified. Although it often is, this is not meant to be the primary process by which crystals are obtained. For proteins this is where we (hopefully) reduce hundreds of parameters down to a more tractable number. Analysis of the screening results is where this reduction occurs. The screening process as likely as not does not give any clear crystalline outcomes, and as a result we proceed to an analysis stage to try and extract crystallization conditions from these apparently dismal results. The analysis process is used to generate test conditions for the production of crystals, the second stage of the crystallization process. This presentation will cover several of these analysis method which were developed in collaboration with computer scientists. We are working on implementing these on a server for broad community access. Additionally, we are investigating correlating the protein surface amino acid composition with successful crystallization cocktail components. The starting purpose of this is to add another predictive parameter to the above analysis processes. The secondary, longer term, purpose is to begin exploring methods for predicting likely protein crystallization conditions.

L25

FRAGMENT-SCREENING BY CRYSTALLOGRAPHY AT THE HZB: WORKFLOW, TOOLS AND PROCEDURES

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Fragment screening is a technique that helps to identify promising starting points for ligand design. Given that suitable crystals of the protein of interest are available and exhibit reproducibly good X-ray diffraction properties, X-ray crystallography is nowadays the preferred method for fragment screening, because - in addition to a simple yes/no answer with respect to binding - it provides detailed 3D information of the binding mode. In the presentation, the complete practical workflow and the included tools on how to conduct a crystallographic fragment screening campaign at the BESSY II synchrotron radiation facility at the Helmholtz-Zentrum Berlin (HZB) are presented. This includes the use of a suitable library, the use of special tools for the handling of many crystals with ease and reliably, automated facilities for diffraction data collection and processing as well as automated structure refinement and ligand identification. Also, glimpses will be provided in how to evolve the initial fragment hits into more tightly binding compounds.

L26

INTRODUCTION TO SINGLE PARTICLE ANALYSIS BY CRYO-EM

Oksana Degtjarik

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Cryo-electron microscopy (cryo-EM) has emerged as a powerful tool for visualizing biological molecules at near-atomic resolution. Nowadays, Cryo-EM has three main directions: single particle cryo-EM, cryo-electron tomography, and microcrystal electron diffraction. Single particle cryo-EM analysis (SPA) enables study of individual particles, such as proteins and macromolecular complexes, in their native state. This talk will cover the principles and methodologies of SPA by cryo-EM, explore the SPA workflow, starting from sample vitrification and image acquisition to data processing. Additionally, we'll discuss negative stain TEM as a complementary technique, and highlight the advantages, challenges, and recent advancements in cryo-EM.

L27

SAMPLE PREPARATION FOR SINGLE-PARTICLE CRYO-ELECTRON MICROSCOPY

Iuliia Iermak

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High-resolution structure determination by single-particle analysis (SPA) in cryo-EM is highly dependent on successful sample preparation, just as in X-ray crystallography, although the sample requirements are different. Ideally, the macromolecules or their complexes to be analyzed should be uniform in size, evenly distributed in the vitreous ice on the cryo-EM grid, and be represented a variety of orientations.

The first step to successfully prepare a sample for cryo-EM is to ensure the homogeneity and stability of the macromolecular complex in the bulk solution by optimizing the buffer composition and verifying the size of the complex using size exclusion chromatography or light scattering techniques. Further steps may include the search for ligands, additives, and other components of the complex, as well as chemical crosslinking to stabilize the complex.

The process of preparing cryo-EM grids, however, can be quite harmful to macromolecules and their complexes (1). For example, macromolecules can be damaged by the shear forces that occur during the removal of excess sample from the grid. As a result, fewer intact particles and more heterogeneous particles will remain. Another common problem is the denaturation of proteins at the air-water interface, which leads to more artifacts such as aggregation, preferential orientation of protein molecules, and even the absence of intact particles (3).

In this lecture, we will review general sample preparation considerations, the most common problems encountered in cryo-EM grid preparation (1-3), and the optimization techniques used to overcome them. These include cross-linking complexes for stabilization, adding surfactants to block the air-water interface, and using grids with support films.

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L28

ADVANCEMENTS IN IMAGING TECHNOLOGIES AND MICROSCOPY: EXPLORING THE INVISIBLE WORLD WITH NIKON AND NANOLIVE

Barbora Kobidová, Zbyněk Halbhuber

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The rapid advancements in imaging technologies have revolutionized the field of life sciences, enabling unprecedented insights into biological processes at the cellular and molecular levels. Nikon's broad scale of technologies allows researchers to target many applications. The microscopes known for their precision, versatility and high-resolution capabilities facilitate detailed examination of biological specimen. With cutting-edge optical technologies and advanced fluorescence features they represent a significant advancement in the field of biological and medical research, offering high-resolution, real-time visualization of dynamic processes within living cells and tissues.

In parallel, the Nanolive holographic microscope represents a groundbreaking approach to live-cell imaging. Utilizing a non-invasive, label-free technology, it captures high-contrast, three-dimensional images of living cells, providing insight into cell morphology, growth and interactions without the need for dyes or markers.

Lectures - Friday, June 14

L29

PREPARATION OF PROTEIN SAMPLES FOR CRYSTALLIZATION EXPERIMENTS

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Protein crystallization is a process influenced by large number of various factors. Property of the protein sample is among the important and belongs to factors the crystallizer (mostly) can control. Preparation and characterization of the protein sample before experiment thus play a crucial role in protein crystallization.

In the lecture, the most widely used techniques to 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 fine-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, however most protein can be stored at -70 C or 4 C. Pooling of different purification bathes is not recommended. 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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L30

PREPARATION AND CRYSTALLIZATION OF PROTEIN COMPLEXES: TRICKS AND EXAMPLES FROM OUR HOST-VIRUS STUDIES

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The action of one macromolecule on another is predominantly involved in all biological events, thus triggering a series of recognition, signaling and modification events. While compared to the relatively well-conserved processes found in cellular organisms, viruses demonstrate huge variations in terms of genomic composition, patterns of evolution, and protein function. While studying protein–protein interactions in virus–host systems, these variations on the pathogen side must be considered. The details of such macromolecular interactions are critical to our understanding of biological function and bestow greater knowledge than the three-dimensional structures of single macromolecules. Although substantial progress has been made in macromolecular docking, it still remains difficult to predict the mode of interaction between macromolecules even when the structures of the interacting partners are known [1-2, 4]. Given the large size of such complexes, crystallography remains the method of choice to determine their structure, and therefore crystals for such complexes need to be grown.

In an era that has been dominated by structural biology for the last 30-40 years, a dramatic change of focus towards sequence analysis has spurred the advent of the genome projects and the resultant diverging sequence/structure deficit [3-4]. The central challenge of computational structural biology is therefore to rationalize the mass of sequence information into biochemical and biophysical knowledge and to decipher the structural, functional and evolutionary

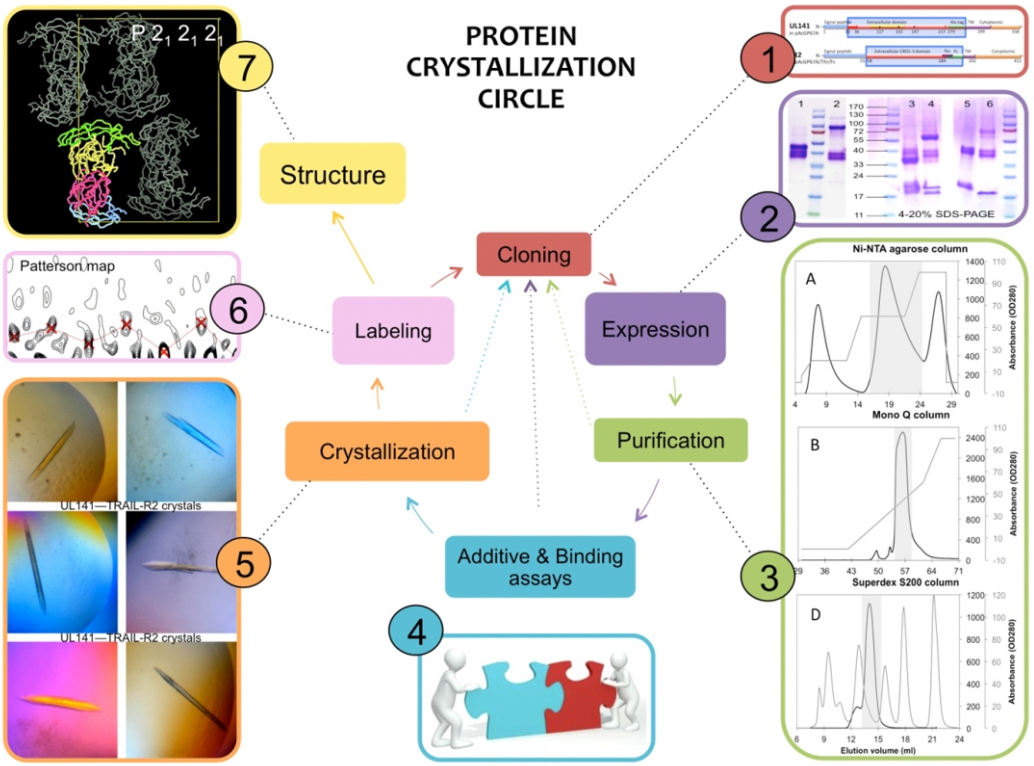

Figure 1. Protein crystallization circle: A number of ways to influence protein crystallization.

clues encoded in the language of biological sequences. However, many other efforts have been performed to understand the relationship between the structure of proteins and their biological function. In addition, a number of protein candidates generated by genomics programs, has increased the interest in all the aspects of gene design, protein expression, purification and crystallization [Figure 1].

In this lecture, we attempt to provide a critical assessment of what one may experience during protein crystallography and to identify major issues yet to be resolved in attempt to crystallize multiprotein complexes. The presentation is organized under several subtitles like definition of ligand, receptor and their affinity; methods to determine binding properties; strategy for expression, purification and crystallization of macromolecular complexes and structure determination, choice of expression vector and/or system; expression and solubility analysis; protein characterization; binding properties and assays; complex formation.. all included in the 'crystallization circle' shown in Figure 1.

We will also discuss a number of ways to stabilize proteins for crystallography that we have been experiencing, including genetic engineering, co-complexing with natural ligands and binding of antibody fragments or alternative scaffolds [2–5]. It is important to remember that in a three-dimensional crystal four or more (in a two-dimensional case we need at least three) different molecular contacts are needed to be able to form a lattice with a non-symmetrical object, and that the lattice interactions will always outnumber the specific contacts that give rise to the physiological complex. However, obtaining diffraction-quality crystals has long been a bottleneck in solving the three-dimensional structures of proteins. Often proteins may be stabilized when they are complexed with a substrate, nucleic acid, cofactor or small molecule. These ligands, on the other hand, have the potential to induce significant conformational changes to the protein and *ab initio* screening may be required to find a new crystal form. This lecture presents an overview of strategies in the following areas for obtaining crystals of protein–ligand complexes: (1) co-expression of the protein with the ligands of interest, (2) the use of the ligands during protein purification, (3) co-crystallization and (4) soaks.

Literature (Suggested readings)

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L31

NEUTRON MACROMOLECULAR CRYSTALLOGRAPHY FOR BIOLOGICAL SAMPLES

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Knowledge of hydrogen locations and protonation states is critical for a fundamental understanding of biological macromolecular function/interactions, and neutron macromolecular crystallography (NMX) is uniquely suited among the experimental structural determination methods to provide this information. However, despite its potential, NMX remains a relatively niche technique due to substantial limitations. The lecture will explore NMX's role amongst the evolving landscape of structural biology, comparing and contrasting it to the historical gold standard of X-ray macromolecular crystallography and the increasing prevalent electron-based methods – i.e., electron microscopy and electron diffraction. Forthcoming developments (e.g., the European Spallation Source in Lund, Sweden coming online) are expected to substantially address current limitations and ensure NMX will remain relevant in the coming decades. The lecture will especially detail the practicalities of implementing NMX for biological macromolecular targets, such as sample crystallization, sample deuteration, and data collection at room and cryogenic temperature.

L32

EXTREMELY BRILLIANT X-RAY SOURCES AND NEW OPPORTUNITIES IN MACROMOLECULAR CRYSTALLOGRAPHY

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Over the past decade, the advent of X-ray free electron lasers delivering ultra intense X-ray beams has revolutionized biocrystallography. With a brilliance a billion times higher than at synchrotrons, the XFEL beam destroys the sample just after the emission of its diffraction signal in a process called "diffraction before destruction". While this firepower allows the characterization of smaller crystals than ever (micro or even nanocrystals), the sample needs to be refreshed after each shot and the collection of a full dataset requires series of thousands of crystals. Also, crystal cryocooling is no longer necessary and this type of analysis is mostly performed at room temperature. In these near-to-physiological conditions and thanks to the temporal resolution of XFEL pulses (<100 fs), the dynamics of biological systems (conformational changes, catalytic events) can be probed in crystallo. Similar protocols have been implemented at synchrotron facilities and are widely accessible.

To take advantage of these new approaches, crystal growers need to adapt current protocols mainly devoted to the production of large single crystals, to the preparation of showers of microcrystals with homogeneous size and diffraction quality. Based on crystal growth principles and examples of alternative crystallization approaches including advanced crystallization control or microfluidics technologies [1, 2, 3].

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L33

HOW TO TRAP SMALL OBJECTS IN A BEAM OF LIGHT

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The use of optical tweezers for manipulating microscopic objects, such as microcrystals, is becoming an increasingly common method in various scientific fields. Optical tweezers allow precise manipulation of micrometric and sub-micrometric objects using a highly focused laser beam that creates a gradient trap. This technology has proven effective for selecting and mounting microcrystals for X-ray crystallography, where traditional manual techniques often fail.

Optical tweezers are not limited to manipulating microcrystals alone. In biological and chemical sciences, they are also used for measuring small forces in the range of piconewtons, enabling the study of mechanical properties of molecular bonds and cellular structures. Additionally, optical tweezers can be employed for cutting and shaping microscopic objects, opening new possibilities in microengineering and nanotechnology.

Other applications include cell sorting, manipulation of individual molecules, and studying the dynamics of biological processes in real-time. Due to their precision and versatility, optical tweezers are becoming an essential tool for advanced research and development in various scientific disciplines.

Posters

P1

NEW MOLECULAR MECHANISMS FOR REGULATION OF BACE1 IN ALZHEIMER'S DISEASE

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The project is focused on BACE1, the major beta-secretase that generates toxic amyloid-beta in neurons. BACE1 is a target for the treatment of Alzheimer's disease; however, relevant molecular information about BACE1 regarding the natural regulation of its proteolytic activity is urgently needed. The research identifies novel structural mechanisms that control the activation and inhibition of BACE1. These are characterized using the 3D structures determined by protein crystallography. The discovered structural inhibitory motifs are exploited for the design of selective biomimetic inhibitors of BACE1. The project provides fundamental insights into the structural biology of BACE1 and potent lead compounds for the development of new strategies for the treatment of Alzheimer's disease.

P2

REGULATION OF THE (ADP-RIBOSYL)TRANSFERASE, AF-PARP, FROM ASPERGILLUS FUMIGATUS

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Aspergillus fumigatus (*Af*), an ubiquitous, airborne fungal pathogen, designated in 2022 as a critical priority pathogen by the WHO, due to the high morbidity and mortality associated with invasive infections as well as the rapid emergence of antifungal resistance. This underscores the urgent need to identify novel therapeutic targets and treatment strategies. A key process for Af survival in the host niche is the DNA damage response (DDR), which supports adaptation and immune evasion. A master regulator of the DDR is the essential *Af*-Parp enzyme, which can recognise DNA damage and create a localised ADP-ribosylation signal, which in turn facilitates the DNA repair process. We have showed *Af*-Parp acts as a (poly-ADP-ribosyl)transferase, which is preferentially activated by 5'-phosphorylated DNA nicks. This is in contrast to human homologues that have a broader target spectrum. Therefore, the highly specific DNA damage recognition and activation of *Af*-Parp coupled with its sole presence as a PARP homologue in *Af*, prompt inquiries into potential cooperative binding modes with other proteins to broaden its spectrum of DNA damage recognition. Future endeavours, including elucidation of atomic structures of DNA bound *Af*-Parp and identification of interacting partners hope to shed light on these questions, advancing our understanding of *Af*-Parp function and aiding in the development of targeted therapies against Invasive Aspergillosis.

P3

INSIGHT INTO INHERITED ANEMIA CDA-I: DISEASE-ASSOCIATED MUTATIONS DISRUPT CODANIN1-CDIN1 COMPLEX

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Congenital dyserythropoietic anemias (CDAs) are characterized by ineffective erythropoiesis and morphological abnormalities in erythrocytes and erythroblasts. One of them, CDA type I (CDA-I), is rare hereditary anemia described by congenital abnormalities like interchromatin bridges and Swiss-cheese-like heterochromatin.

CDA-I is associated with mutations in two different loci, CDAN1 and CDIN1. CDAN1, encoding Codanin1, is involved in nucleosome assembly and disassembly. CDIN1 is a recently discovered protein predicted to be a divalent metal ion-dependent restriction endonuclease. Despite their undeniable importance for CDA-I progression, both proteins and their mutual interaction are poorly described.

Here, we present a pioneer study of the essential interaction between CDIN1 and Codanin1. Firstly, We employed biophysical techniques to characterize the homoand heterodimerization of these proteins, along with their structural features. Additionally, we quantified CDIN1-Codanin1 binding affinity in the low nanomolar range. Finally, we mapped the specific interaction regions on both proteins and demonstrated that disease-associated mutations within these regions disrupt the CDIN1- Codanin1 complex formation.

These findings represent a significant step forward in elucidating the molecular mechanisms underlying CDA-I activation and progression. This knowledge holds promise for the future development of targeted therapies for this rare disease.

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P4

NEW MECHANISMS OF REGULATION OF THE ADAM17 METALLOPROTEASE AND THEIR APPLICATION IN THE TREATMENT STRATEGIES FOR INFLAMMATION AND OBESITY

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Metalloprotease ADAM17 plays a crucial role in the proteolytic release of over 80 cell-surface proteins, including cytokines and growth factors, essential for numerous physiological processes. In macrophages, ADAM17-mediated shedding of TNF initiates inflammatory responses. The maturation and trafficking of ADAM17 to the plasma membrane in this context rely on the transmembrane pseudoprotease iRhom2. Additionally, cytosolic FERM-domain containing protein 8 (FRMD8) has been identified as a stabilizer of the ADAM17-iRhom2 complex on the membrane. FRMD8 binds to the disordered cytosolic N-terminus of iRhom2, yet the specific amino acids involved in this interaction and the mechanism of stabilization remain elusive.

Utilizing multimer prediction in Alphafold2, we have identified two highly conserved cavities in the structure of FRMD8 likely responsible for binding of iRhom2. Currently, we are validating this model through microscale thermophoresis with synthetic peptides. Subsequently, we plan to elucidate the structure of FRMD8 bound to the iRhom2 N-terminus using X-ray crystallography, aiming to design potent inhibitors capable of competing for binding. Such inhibitors could mitigate the production of the pro-inflammatory cytokine TNF, implicated in various human diseases, including inflammatory conditions and obesity.

To further understand the mechanism by which FRMD8 stabilizes the ADAM17-iRhom2 complex, preliminary data from proximity proteomics and Alphafold2 multimer analysis have suggested the involvement of the actin-binding protein cofilin1. Given cofilin1's destabilizing effect on the actin cytoskeleton, we hypothesize that the interaction between FRMD8 and cofilin1 is inhibitory, leading to actin cortex stabilization and reduced lysosomal degradation of ADAM17. We plan to verify this hypothesis both mechanistically and structurally.

P5

CHARACTERIZING THE INTERACTION BETWEEN GALECTIN-3 AND THE HUMAN NATURAL KILLER CELL ACTIVATION RECEPTOR NKP30

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Natural killer (NK) cells are large granular lymphocytes that play a crucial role in innate immunity by recognizing and eliminating malignantly transformed or infected cells without prior activation. To achieve this, NK cells express a set of activation and inhibition receptors on their surface, including the glycoprotein NKp30, an activation natural cytotoxicity receptor. Among the known ligands of NKp30, there is significant interest in tumor-related ligands such as B7-H6, BAG-6, and the recently discovered Galectin-3. Gal-3 can inhibit NK cell functions by binding to the NKp30 receptor, making it a promising target in antitumor therapy. Studying the interaction between NKp30 and Gal-3 is challenging due to a few factors. Firstly, wild-type Gal-3 can form dimers through an odd cysteine, which can interfere with measurements. Secondly, NKp30 carries three N-glycosylation sites (Asn-42, Asn-68, Asn-121) within its ligand-binding domain (LBD), each contributing differently to this interaction. To obtain clearer insight into this interaction, NKp30 LBD *N*-glycosylation mutants were produced in the HEK293T cell line, and a cysteine-less form of the carbohydrate recognition domain of Gal-3 (C173S) was expressed in *E. coli*. The interaction was studied using various biophysical methods. The binding of Gal-3 to NKp30 mutants was confirmed using size-exclusion chromatography and analytical ultracentrifugation, while the affinity of this interaction was determined using microscale thermophoresis. Additionally, differential scanning fluorimetry was used to study the stability of the NKp30 LBD N-glycosylation mutants. The most promising N-glycosylation mutant (NKp30_LBD_G121) was selected for crystallization trials of the Gal-3:NKp30 complex to solve its structure using X-ray crystallography.

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P6

CHARACTERIZATION OF THE ZEARALENONE DEGRADING ENZYMES

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Fungal mycotoxins can significantly reduce the quality of grains used for feed and food, and in large quantities, they can be harmful to animals or humans if consumed.

Zearalenone is a mycotoxin produced by various species of Fusarium fungi, specifically Fusarium graminearum. It poses a serious threat to crops, particularly maize, wheat, and barley. Upon consumption by livestock, zearalenone disrupts the hormonal balance, primarily affecting the reproductive system. The mycotoxin mimics the effects of estrogen, leading to reproductive disorders and negative effects on fertility in animals, mainly in swine.

Currently, various methods are known for the detoxification of zearalenone. Physical methods (adsorption or heating) or chemical degradation (use of strong oxidants or specific chemicals) are widely used. However, they require harsh conditions, they could damage the nutritional value of the feed and the full decontamination is often incomplete.

A favorable solution for the removal of zearalenone may be the use of enzymes. Lactone hydrolases attracted significant attention because they can degrade the mycotoxin to a non-toxic form by hydrolyzing the lactone bond in the molecule.

Our research focuses on the improvement of bacterial lactonases isolated from Rhodococcus erythropolis and Streptomyces coelicoflavus. These enzymes show better substrate affinity and turnover number, than the widely known fungal hydrolases, like ZDH101 from Clonostachys rosea. We aim to further improve the stability and activity of these proteins with protein engineering to make them applicable as a feed additive for livestock. Crystallizing these proteins with substrate analogs can reveal their mechanism of action. Additionally, it makes possible the rational design of active site mutations to enhance the enzyme activity towards various mycotoxins. By using macromolecular crystallization techniques, we can also investigate the protein-substrate interactions of the various mutants.

P7

STRUCTURAL STUDIES OF BIOLOGICALLY RELEVANT PROTEIN VARIANTS OF CANCERRELATED CARBONIC ANHYDRASE IX

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Carbonic anhydrase IX (CA IX) represents an attractive target for the development of anticancer drugs as it is overexpressed in various types of solid tumors. By its catalytic activity, CA IX assists the cancer cells to maintain the optimal intracellular pH and to acidify the extracellular milieu promoting tumor development. There are twelve enzymatically active carbonic anhydrases present in human body, all sharing a high sequence identity and a typical ß-sheet structural fold of the well-studied catalytic domain. Although, the activity of CAs can be efficiently inhibited, the design of inhibitor selective to the cancer-related CA IX has been hampered by the high sequence conservation. CA IX has several unique features compared to other members of the family, which investigation may help in the development of selective drug compounds. Namely, it is a type I transmembrane dimeric protein with unique N-terminal PG domain, extracellular catalytic domain, and short cytoplasmic C-terminal segment.

The above denoted traits make CA IX subject of structure-based drug design efforts. However, the expression and purification in high yield as well as crystallization experiments has been challenging. Therefore, protein variant bearing six amino acid substitutions for recombinant expression in E. coli has been published before. Nevertheless, some of the amino acid substitutions hinders the formation of the biological dimeric arrangement of CA IX. For that reason, three novel protein variants were designed and structurally characterized. The CA IX variants were expressed, purified, and characterized for their oligomeric state in solution, and confirmed to form dimers. These variants were crystallized and subjected for X-ray diffraction experiment which ultimately led to determination of three protein structures. Crystal structures revealed that when the wild-type amino acids are reintroduced to specific residues, the biological dimeric arrangement is restored.

P8

EXPLORATION OF FUMONISIN ESTERASE STRUCTURE AND ACTIVITY

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The study examined two recombinant fumonisin esterases (FE1 and FE2), expressed in Pichia pastoris. Contrary to previous hypotheses, both FEs first selectively hydrolyzed the ester bond at the C-6 position of Fumonisin B1 (FB1), resulting in partially hydrolyzed FB1_7 (pHFB1_7) intermediate, which was transformed into hydrolyzed FB1 (HFB1). The two FEs had similar and excellent turnover numbers in the first hydrolytic reaction. However, in the second step, FE2 had a 10-fold lower Michelis-Menten constant than FE1, making FE2 the more promising candidate as a decontamination agent of FB1. Thermal stability of the two FEs investigated with Thermofluor assay revealed a 4.5°C higher melting temperature of FE2 (55.5°C) than of FE1 (51.0 °C). Finally, the 3D structure of one of the FE is used to explain the substrate specificity of these enzymes and to attempt to understand their selectivity and kinetics. Understanding the structural basis of substrate specificity can accelerate the discovery and engineering of fumonisin esterases leading to a widespread application of this biocatalyst to enhance food safety.

P9

STRUCTURAL BIOLOGY OF RNA HELICASES

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Helicases play a vital role in different cellular processes and possess the ability to unwind DNA or RNA duplexes, heteroduplexes, and more complex polynucleotide structures. RNA helicases use the energy from ATP hydrolysis to facilitate the unwinding or annealing of RNA molecules, and participate in multiple aspects of RNA metabolism, including premRNA, rRNA, and miRNA processing or remodelling of ribonucleoprotein complexes. RNA helicases have also been implicated in the cellular response to viral infections.

Dysregulation and overexpression of RNA helicases have been linked to various diseases including genetic and neurological disorders, viral infections, and cancer. However, despite their important functions and implications in many diseases, the exact mechanism of action of RNA helicases is still poorly understood, primarily because of a lack of high-resolution structural data. The crystal structures of RNA helicases in their active state and bound to their interaction partners can provide important information to better understand their mechanisms. In addition, these structures may provide a good starting point for therapeutic interventions.

This project focuses on the structural biology of a cancer-related RNA helicase. The aim is to establish robust crystal systems diffracting at high resolution for this protein in different functional states, which could provide structural insights into the unwinding activity of this helicase. The protein was successfully expressed in Sf9 cells, followed by purification and biophysical characterisation by DLS and nanoDSF. A broad crystallisation screen was conducted using the sitting-drop vapordiffusion technique. Though several initial crystal hits were obtained, they only diffracted at modest resolution and their optimisation proved challenging.

P10

STUDY OF THE FUNCTION AND ORGANISATION OF ENOYL-ACP REDUCTASE DOMAINS IN SCHIZOCHYTRIUM

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Omega 3 polyunsaturated fatty acids (PUFAs) are key nutrients and essential for humans. These molecules are produced by vast enzymes called PUFA synthases (PFAS) found in marine gammaproteobacteria, and thraustochytrids among other microorganisms. Enoyl-ACP reductase domain (ER) catalyses the last modification step in the PUFA biosynthesis. In gammaproteobacteria one ER domain is present, while in thraustochytrids, such as Schizochytrium sp., this domain is duplicated in PfaB (ERb) and PfaC (ERc) subunits, but we lack information about their function. In this study we report that ERb and ERc can interact between them forming a heterodimer. Biochemical assays revealed that this heterodimer has enzymatic activity, in contrast with ERb or ERc without their homologue. ER domains could contribute to PfaB and PfaC dimerisation. These findings provide insight into the thraustochytrids PFAs mechanism of action.

P11

SELECTIVE INHIBITION OF CARBONIC ANHYDRASE IX FOR CANCER DIAGNOSIS AND THERAPY

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Carbonic anhydrase IX (CA IX) belongs to a group of 15 isoforms of the human carbonic anhydrase enzymes. Typically localized on the cell surface, CA IX is primarily found in specific tissues within the gastrointestinal tract. Its expression is induced in response to local hypoxia, aiding in the regulation of pH levels to accommodate the metabolic production of acidic by-products, thereby promoting cancer cell survival and proliferation. The overexpression of CA IX in solid tumors, coupled with its extracellular presence, suggests its potential utility in cancer diagnosis and therapy.

Within this project we plan to develop potent and selective CAIX inhibitors featuring a sulfonamide group capable of coordinating with the zinc ion within the enzyme's active site. Their active site is located within the central ß-sheet, where the zinc-binding core resides. The proposed inhibitors are designed with a scaffold capable of attaching to enzyme moieties. This scaffold comprises a binding group for metal ion interaction, an anchoring group for functional interaction with the enzyme's hydrophobic regions, and a sticky group for affinity to the hydrophilic group of the active site.

Recombinant CA IX is produced and expressed in E. coli BL21, followed by purification via chromatographic to ensure high protein purity. The purified CA and a series of inhibitors are assessed for affinity using the stopped-flow method to screen a library of inhibitors. To better understand the binding modes between selective inhibitors and the enzyme, X-ray crystallography is employed to achieve high-resolution structures of the compounds. Obtained structural information will not only guide the modification and tailoring of the anchoring and sticky groups in designing the selective model inhibitors, but also maximize affinity for tumor-specific CA IX while minimizing interactions with other carbonic anhydrase isoforms.

P12

ISOLATION, PURIFICATION AND CRYSTALLIZATION OF NAPIN FROM SEEDS OF IBERIS UMBELLATA L.

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Proteins and peptides belonging to the plant immune system can possess natural antibacterial, antifungal and antiviral properties. Due to their broad range of activity and stability, they represent promising novel alternatives to commonly used antifungal agents to fight the emergence of resistant strains. Napin from seeds of *Iberis umbellata* was identified by feeding the LC-MS/MS generated residual data to the UniProtKB online server, which showed 70% sequence identity to Napin from *Brassica napus*. *I. umbellata* Napin was further purified by using the ammonium sulfate precipitation (80%) and supernatant was loaded on Mono S column followed by gel filtration chromatography (Superdex 75 prep grade). Purified protein showed an approximately 16 kDa protein band under non-reduced condition which separated in two daughter bands of 8 and 6 kDa under reduced conditions of SDS-PAGE. Secondary structural elements were determined by Circular Dichroism (CD) spectroscopy which showed that it consists of 23.8% -helix, 44.8% ß-sheet, 31.5% random structure. Napin exhibited highly stable globular structure above 90 C. The pure protein was concentrated up to 10 mg/ml and Dynamic light scattering (DLS) showed the highly monodisperse status of the protein and hydrodynamic radius (RH)of 2.54 ± 0.25 nm confirming the monomeric form of the protein. SAXS showed that the radius of gyration is 1.48 ± 0.09 nm and confirmed the nearly globular shape. SEC-SAXS based ab initio dummy model of Iberis umbellata napin was compared with napin of Brassica napus (PDB ID: 1SM7). The pure protein (20mg/ml) was showed phase separation followed by crystallization under high salt concentration in sitting drop method. The napin crystals (0.625 × 0.324 mm) were appeared after two weeks at room temperature.

P13

STRUCTURAL STUDIES OF DISULFIDE CONTAINING PROTEINS PRODUCED IN *E. COLI*

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Prokaryotic expression systems like *E. coli* are the first choice for protein production but they lack cellular machinery for the formation of post translational modification such as disulfide bond formation. The development of CyDisCo™ (cytoplasmic disulfide bond formation in *E. coli*) technology facilitates the efficient production of disulfide containing eukaryotic proteins using *E. coli*. We attempted to test the limitations of *E. coli* using CyDisCo™ in the production of basement membrane proteins, including perlecan, nidogen-2 and fibulin-2. These proteins have been comprehensively studied at a cellular level, but unfortunately lack structural information at the atomic level.

Protein fragments were constructed, and expression tests were conducted in E. coli host using CyDisCo™. The successful production of soluble protein constructs covered approximately 84 %, 32 % and 61 % for perlecan, nidogen-2 and fibulin-2, respectively (having 83%, 40% and 79% of the total disulfide bonds). Additionally, biochemical, biophysical, and structural characterization was done. We successfully produced a large soluble construct of mouse perlecan region 3 residues G503- T1672, ~127 kDa in size having 44 disulfide bonds, which was an exceptional and novel achievement of *E. coli* using CyDisCo™. Furthermore, the first crystal structure of a ~40 kDa protein construct from perlecan region 3 having 15 disulfide bonds was solved which would aid in the better understanding of Schwartz-Jampel syndrome disease caused by mutations in perlecan. The mouse fibulin-2 construct S427-G545, a disulfide linked homodimer with a molecular weight of ~27.6 kDa and a total of 34 cysteines forming 17 disulfide bonds, was fully solubly produced and was purified. This construct was crystallized, and the structure reveals the formation of a single-domain structure which was previously suggested to have three motifs related to anaphylatoxin-like repeats.

P14

APOE, AN ENIGMATIC CELLULAR PLAYER IN NEURODEGENERATION

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Worldwide over 55 million people are affected by dementia, in which Alzheimer's disease (AD) affecting the elderly (> 65 years) comprises about 60-70% of cases. Due to the increasing elderly population, this number is expected to double every 20 years and reach 139 million in 2050. In 2019, the estimated societal cost for dementia care was around 1.3 trillion US dollars globally, 50% of this societal cost is attributed to informal care (family, friends, etc).

Over decades, research has focused on understanding the role of apolipoprotein E (ApoE), a key lipoprotein and cholesterol transporter in the central nervous system, in AD, which is generally characterised by ß-amyloid (Aß) plaque deposition leading to synaptogenesis and eventual cognitive decline. ApoE is involved in Aß clearance and a variant of ApoE, ApoE4, which differs from the common variant ApoE3 by a single amino acid substitution (C112R) was found to be incapable of Aß clearance and was found to be a high genetic risk factor in AD pathology.

To probe the influence of this single amino acid substitution on protein function and eventual proteinopathy in AD, we intend to use X-ray crystallography along with biochemical and biophysical techniques. However, ApoE's tendency to fragment poses a challenge to obtain the full length structure. As ApoE is stable in its lipid bound form, employing detergents to mimic similar environment would help in obtaining the full length structure of ApoE. This approach has helped in stabilizing the ApoE effectively and also has mitigated fragmentation.

P15

TARGETING FIBROBLAST ACTIVATION PROTEIN IN CANCER: STRUCTURAL CHARACTERIZATION OF A NOVEL PEPTIDOMIMETIC

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Fibroblast activation protein (FAP) is a membrane-bound serine protease that has emerged as a promising tumor marker. FAP is overexpressed in the tumor stroma of many cancers, including most carcinomas, and has been linked to promoting angiogenesis, tumor cell invasion, and immunosuppression. Moreover, its expression is highly increased relative to healthy tissue, making it a robust target for cancer imaging and therapy. Small-molecule FAP inhibitors have been widely employed as a targeting moiety of radiotracers, which have been successfully tested as theranostics in humans. However, limited structural information on FAP–inhibitor complexes has hampered further elaboration and refinement of inhibitor structures through rational design. In our recent work, we conducted a structure–activity relationship study to explore the chemical space in the P1' and P2' positions and developed a new class of peptidomimetic inhibitors bearing an ketoamide warhead. Besides other lead- like properties, the compound I22AP446 (IC_{50} = 89 pM) outperformed the most potent inhibitor published to that date. To gain insight into the binding mode of the -ketoamide derivative, we determined a crystal structure of the FAP–I22AP446 complex at 1.75Å resolution revealing key interaction features between the inhibitor and the enzyme. We thus present the first reported crystal structure of FAP bound to a peptidomimetic. Our findings provide a basis for structure-guided modifications of our lead compound and will fuel the development of selective peptidomimetics targeting FAP.