International School on Biological Crystallization

Abstracts eBook, 8th Edition
ISBC2023

Granada, Spain May 21st -26th, 2023

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WELCOME TO ISBC GRANADA 2023

Dear Participant,

On behalf of the Organising Committee, it is with great pleasure to welcome you to the 8th International School of Biological Crystallization, ISBC2023.

After a long gap due to the pandemic, we are thrilled to finally be able to gather in person once again and continue our pursuit of knowledge in this renewed field. The School is held at Hotel Abades Nevada Palace de Granada at close distance from the Centre of the beautiful City of Granada by metro, aiming to facilitate close scientific and social interactions between attendees, offering plenty of opportunities to catch up with old friends and make many new ones. At the same time, you will be able to enjoy the lively atmosphere of the city, its tapas' bars, and historical monuments.

This year's School will provide five days of lectures and practical demonstrations related to the crystallization of biological macromolecules with special attention to the crystallization of membrane proteins, large crystals for neutron diffraction and tiny crystals for XFEL and SSX studies but also including lectures and demos on CryoEM, the current focus of many PhD students.

We have tried our best to make ISBC2023 as valuable as possible for the participants by selecting some of the best lecturers for the wide range of topics covered. We expect that the programme will trigger fruitful discussions and that this will be an enriching experience for all of us. The only piece missing in this recipe is your positive approach; so please, be open and participative, ask as many questions as necessary, be proactive and leave formalities aside.

Finally, we would like to thank all of you for your participation; to the teachers for their endeavor to leave behind their duties and to the students and colleagues from academic and industrial backgrounds for believing in this scheme of Crystallization School. We also wish to express our heartfelt thanks to the Organizing Committee and to all the people who have contributed to the School. Last but not least, we are very grateful to all the Sponsors and Exhibitors for their essential and continuous support, without which the School wouldn't be possible.

We look forward to seeing you in Granada and wish you a safe trip to enjoy the Granada crystal growth experience!

Yours faithfully,

Juan Manuel García Ruiz & Jose A. Gavira Gallardo
Directors of the School

Table of Contentes

Table of Contentes

GETTING STARTED

The school will take place on the ground floor of the 'Abades Nevada Palace' hotel. During the School, two main rooms will be used: the Lecture Room and the Exhibitors/Posters Room. On Thursday, both spaces will be connected for the Demonstrations Fair.

Once in Granada, speakers should contact the ISBC reception desk as soon as possible to set up and test their presentations in advance. Any special requirement other than a computer and a pointer must be clearly requested in advance.

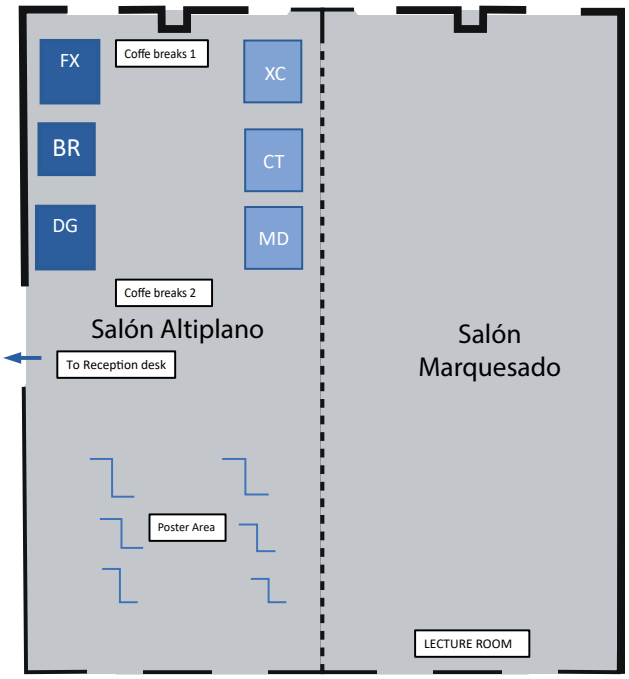
Students presenting a poster should ask at the ISBC reception desk for their allocated panel. Posters will be displayed from Monday afternoon for the whole duration of the School. The posters sessions will take place in the evenings at the end of each day and also during coffee breaks so that there are enough opportunities for the students to discuss their work with the rest of the participants and with the lecturers.

Important: Please be aware that a few posters will be selected to make a short presentation (2-3 min) on the final day of the School. We advise you to prepare a couple of slides of your poster in advance.

Free Wireless Internet connection is available at any location within the Hotel (the WiFi key can be obtained from the ISBC reception desk).

Coffee will be served in or near the Exhibitors/Posters Room. Lunch will be served at the Restaurant located on the ground floor of the hotel. Lunch tickets are distributed to each participant together with the identification badge during registration.

Please bear in mind that accommodation payments and invoicing are not handled by the organization (contact directly with the reception of your hotel).



DG: Douglas Instruments
BR: Bruker
FX: Formulatrix
XC: XtalConcepts
CT: Cytiva
MD: Molecular Dimensions

Programme

Sunday, May 21st

WELCOME

18:00 – 20:00 Registration *(Abades Nevada Palace Hotel)*

20:00 *Welcome Cocktail (Abades Nevada Palace Hotel)*

Monday, May 22nd

FROM SOLUTION TO PROTEIN CRYSTALS

08:00 – 09:00 Registration *(Abades Nevada Palace Hotel)*

09:00 – 09:15 **Overview of the School**
J.A.G. & J.M.G-R

09:15 – 10:00 **Protein purification strategies intended for crystallization**
S. Martínez-R

10:00 – 10:30 *Coffee break and Poster Session*

10:30 – 11:15 **From protein solution to crystals: Nature and formation of protein crystals**
B. Rupp

11:15 – 12:00 **Preparation of protein samples for crystallization experiments**
P. Řezáčová

12:00 – 12:45 **Nucleation of Macromolecular Crystals**
J.M. García-Ruiz

12:45 – 13:30 **Analyzing, Scoring and Optimizing Crystallization Conditions**
C. Betzel

13:30 – 15:00 *Lunch*

15:00 – 15:45 **Dialysis, crystallization and the Hofmeister series**
J. Mesters

15:45 – 16:30 **Protein Crystallization by capillary Counter-diffusion technique**
J.A. Gavira

16:30 – 17:15 **Unpacking how to grow protein crystals**
J. Newman

17:15 – 18:00 **Seeds of success: An overview of the Microseed Matrix Screening technique**
M. Marsh

18:00 – 19:00 *Coffee break and Poster Session*

Tuesday, May 23rd

TINY & LARGE CRYSTALS, MEMBRANE PROTEINS, COMPLEXES, SAXS, CryoEM...

09:00 – 09:30 **A guide to choosing your method for crystallization**
L. Govada

09:30 – 10:00 **Microfluidics in action: crystallization and crystallography in microchips**
C. Sauter

10:00 – 10:30 **Trace fluorescent Labeling and Low-Cost Imaging of Protein Crystals**
C. Tarver

10:30 – 11:00 *Coffee break and Poster Session*

11:00 – 12:00 **Femtosecond Crystallography, a New Era in Structural Biology**
P. Fromme

12:00 – 12:45 **Crystallization of Membrane Proteins in Lipid Mesophases**
M. Caffrey

12:45 – 13:30 **Helicobacter pylori Acid Acclimation: The Evil Duo of a pH-Gated Urea Channel and a Cytoplasmic Urease.**
H. Luecke

13:30 – 15:00 *Lunch*

15:00 – 15:45 **Screening Data Analysis and Crystallization Condition Prediction**
M. Pusey

15:45 – 16:30 **Best practices to prepare and deliver samples in serial femtosecond crystallography experiments at XFELs**
J.M. Martin-G.

16:30 – 17:15 **Crystallization of Protein-Nucleic Acid Complexes**
C. Biertümpfel

17:15 – 18:00 **AlphaFold and biochemical considerations for protein crystallization**
J.D. Ng

18:00 – 19:00 *Coffee break and Poster Session*

22:00 *Night guide walk of Albaicín & Sacromonte*

Wednesday, May 24th

TINY & LARGE CRYSTALS, MEMBRANE PROTEINS, COMPLEXES, SAXS, CryoEM…

09:00 – 09:30 **Differences in crystallization of various haloalkane dehalogenases**
I.K. Smatanova

09:30 – 10:00 **Optimisation of Crystal Growth for Neutron MX**
M. Budayova-Spano

10:00 – 10:30 **Protein crystallization in micro- and meso-scale devices**
F. Castro

10:30 – 11:00 *Coffee break and Poster Session*

11:00 – 11:45 **Intracellular protein crystallization**
L. Redecke

11:45 – 12:30 **Molecular movies of enzyme catalysis**
G. Calero

12:30 – 13:30 **Small Angle Solution Scattering as a complementary technique in structural biology studies.**
E. Snell

13:30 – 15:00 *Lunch*

15:00 – 15:45 **Cryo-Electron tomography – method to bridge scales in biological system**
N. Mizuno

15:45 – 16:30 **Introduction to single particle cryoEM**
E. Cunha

16:30 – 17:15 **How do we get fragments or ligands into structures?**
T. Peat

17:15 – 18:00 **Microcrystal electron diffraction: methods and applications**
B. Nannenga

18:00 – 19:00 *Coffee break and Poster Session*

Thursday, May 25th

DEMONSTRATION FAIR

09:00 – 10:30 **Practical Demonstration "a la carte"**

10:30 – 11:00 *Coffee Break*

11:00 – 13:30 **Practical Demonstration "a la carte"**

13:30 – 15:00 *Lunch*

15:00 – 16:30 **Practical Demonstration "a la carte"**

17:00 – 17:45 **Practical Demonstration "a la carte"**

20:00 *Gala Dinner (Carmen de la Victoria)*

Friday, May 26th

CLOSING LECTURES & STUDENTS PRESENTATIONS

09:30 – 10:15 **The Chemistry of Mushroom Magic** *(Why you should not lick toads.)*
B. Rupp

10:15 – 11:00 **Round Table: Teaching Crystallography**

11:00 – 11:30 *Coffee Break*

11:30 – 12:30 **Oral Presentation of finalist posters**

12:30 – 13:30 **Poster Prizes and Closing of the School**

13:30 – 15:00 *Lunch*

Lectures

L1 Overview on protein purification strategies intended for crystallization

Sergio Martínez-Rodríguez

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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 (Chayen & Saridakis, 2008; Khurshid et al., 2014; McPherson & Gavira, 2014;). Purification techniques have improved dramatically in the past decades, including advances in statistical factorial approaches allowing to avoid the usual trial-and-error method in protein purification workflow (Papaneophytou & Kontopidis, 2014); however, 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 (Chen *et al.*, 2015; Papaneophytou, 2019). 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.

References

- Chayen, N.E., & Saridakis, E. (2008). Nat Methods. 5:147-53.
- Chen ,R.Q., Lu, Q.Q., Cheng, Q.D., Ao, L.B., Zhang, C.Y., Hou, H., Liu, Y.M., Li, D.W., & Yin, D.C. (2015) Sci Rep. 5:7797.
- Khurshid, S., Saridakis, E., Govada, L., & Chayen, N.E. (2014) Porous nucleating agents for protein crystallization. Nat Protoc. 9:1621-1633.
- McPherson, A. & Gavira, J.A. (2014) Acta Crystallogr F Struct Biol Commun. 70:2-20.
- Papaneophytou, C.P. & Kontopidis G. (2014). Protein Expr Purif. 4:22-32.
- Papaneophytou C. (2019). Mol Biotechnol. 61(12):873-891.

L2 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. Protein molecules however are large, complex, and dynamic molecules, and most proteins are therefore difficult to crystallize. To effectively explore conditions that allow crystal formation, we need to examine the chemical and physical properties of proteins, in particular how to modify their solubility and local surface property distribution. Once we understand what conditions must be fulfilled for crystallization to occur, the question is how to (a) design and obtain a protein that actually can crystallize; (b) how to efficiently sample the multitude of possible reagent combinations to find the right macroscopic or thermodynamic conditions; and (c) how to 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. We will examine how to conceptualize the crystallization process with the assistance of crystallization 'phase diagrams' for different crystallization techniques. Initial screening or sampling of crystallization space informs us how to proceed towards optimization of crystal growth, and often also indicates that further examination and modification of the protein itself will be necessary to achieve successful crystallization.

L3 Preparation of protein samples for crystallization experiments

Pavlína Řezáčová

Institute of Molecular Genetics, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague

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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 introduced: (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), and (h) CD spectroscopy.

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).

References

[1] McPherson A. (1999). Crystallization of Biological Macromolecules, Chapter 3. The Purification and Characterization of Biological Macromolecules, pp. 67 – 126, Cold Spring Harbor Laboratory Press, New York, USA

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

L4 Nucleation of macromolecular crystals

Juan Manuel García-Ruiz

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This lecture provides an introduction to the physical and chemical mechanisms by which protein crystals are formed. We will start by defining the main concepts, parameters and variables required to describe properly the driving force for crystallization. The lecture will approach the nucleation problem using the classical theory considering that small unstable clusters of molecules form stochastically in the solution until some of them reach the critical size to endure as a solid phase in the solution. Then, I will introduce the two-step nucleation approach and discuss the recent results on the formation of crystalline nuclei via amorphous precursors.

The second part of the talk is devoted to apply the nucleation theory to understand how do crystallization techniques work, namely vapour diffusion, batch technique and counter-diffusion technique. The learning from nucleation theory will be used in order to understand the output of a protein crystallization experiment and to improve the yielding in terms of crystal size and crystal quality.

L5 Analyzing, Scoring and Optimizing Crystallization Conditions

Mengying Wang, Hévila Brognaro, Prince Prabhu, Susanna Georgyan and Christian Betzel

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Today at micro-beam synchrotron (SR) and X-ray Free-Electron-Laser (FEL) beamlines, beside conventional single crystal diffraction data collection micro-sized crystals are preferred and mandatory for the merging technique of serial diffraction allowing data collection at room temperature (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 required and need to be established. In this context the understanding of the initial steps of a crystallization process, including the early events of phase separation and the nucleation process is of fundamental importance to successfully grow and obtain crystals with high diffraction quality and for serial experiments to obtain crystals in sufficient quantities having homogeneous dimensions. In this context latest light scattering and imaging techniques are most suitable to obtain insights about crystallization processes, to score and optimize crystallization of biomolecules. Corresponding procedures and techniques will be presented.

Latest data and publications indicate that prior to nucleation a distinct phase separation of biomolecules can be observed [2,3]. Therefore, and more recently the nucleation process is discussed in theory and experiment differently [4,5]. In order to obtain also insights about the initial molecular pathways of crystal nucleation we investigated the early and pre-crystallization molecular events for various proteins applying particular in situ dynamic light scattering, small-angle X-ray scattering and transmission electron microscopy [6,7] The data obtained strongly support the existence of initial phase separation and formation of liquid dense clusters prior to nucleation, followed by the transition to higher order assemblies inside these clusters [8].

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 strongly raised the necessity to establish also new methods to image and monitor nano- and micro-crystal suspensions. We designed and constructed a imaging setup including hardware to detect second and third harmonic generation (SHG, THG) signals combined with a UV-fluorescence option [9]. 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 instrumentation, methods, procedures and examples will be presented.

References:

[1] K.H. Nam, Serial X-ray Crystallography, Crystals, 2022, 12(1), 99

[2] H. Brognaro, S. Falke, C.N. Mudogo, Ch. Betzel, Multi-Step Concavalin A Phase Separation and Early Stage Nucleation Monitored via Dynamic Light Scattering, Crystals, 2019, 9.

[3] P. G. Vekilov, Dense Liquid Precursor for the Nucleation of Ordered Solid Phases from Solution, Cryst. Growth Des., 2004 (4), 671–685.

[4] F. Zhang, Nonclassical Nucleation Pathways in Protein Crystallization, J. Physics Condesn. Matter, 2017, 29, 443002

[5] D. Gebauer, H. Gölfen, Prenucleation clusters and non-classical nucleation, Nano Today, 2011, 6, 564-584.

[6] S. Falke H. Brognaro, Ch. Betzel, Latest Insights and Methods in Analyzing Liquid Dense Cluster and Crystal Nucleation, Encylopedia Analytical Chemistry 2019, https://doi.org/10.1002/9780470027318.a9547

[7] R. Schubert, A. Meyer, D. Baitan, K. Dierks, M. Perbandt and C. Betzel, Real-Time Observation of Protein Dense Liquid Cluster Evolution during Nucleation in Protein Crystallization, Cryst. Growth Des., 2017 (17), 954–958.

[8] M. Wang, A.L. Barra, H. Brognaro, Ch. Betzel, Exploring Nucleation pathways in Distinct Physicochemical Environments Unveiling Novel Options to Modulate and Optimize Protein Crystalliazion, Crystals 2022, 12, 437.

[9] Q. Cheng, H.-Y. Chung, R. Schubert, S.H. Chia, S. Falke, C.N. Mudogo, F.X. Kärtner, Ch. Betzel, Protein Crystal Detection with a compact multimodal multiphoton microscope, Comm. Biol., 2020, 3, 569

L6 Dialysis, crystallization and the Hofmeister series

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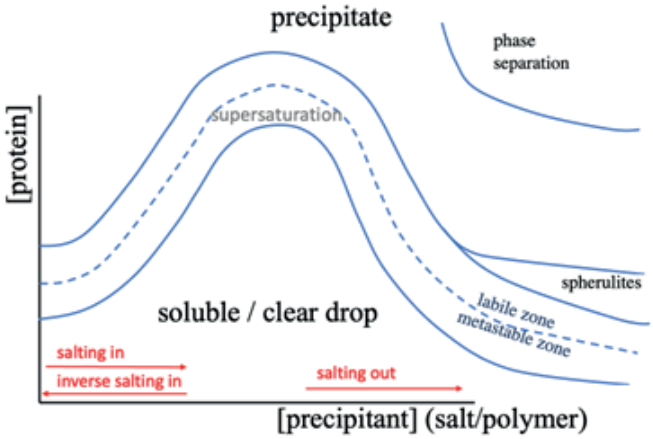
The oldest methods used for the crystallization of macromolecules are the batch (simply adding the precipitant to the solute) and the *dialysis* method (protein in dialysis tube against a buffer). The batch method is still being used in industry for the large-scale purification of pharmaceutical compounds. What some consider as an advantage, others think of as the most important limitation of the batch method; "the static sampling of the phase diagram". Also the dialysis methods suffers from prejudices and yet, it has many advantages to offer. The technique easily allows for simple adjustments in salting-in and salting-out proteins, as well as introducing pH-shifts to trigger and promote crystal growth.



The usefulness of the dialysis method will be discussed for exploiting the phase diagram and the influence of solute components, *i.e.* Hofmeister series, which are fundamental for achieving solubility, nucleation, and crystal growth.

Primers

I. Zeppezauer et al. (1968) Micro diffusion cells for the growth of single protein crystals by means of equilibrium dialysis. Arch. Biochem. Biophys. 126, 564–573.
II. Zeelen & Wierenga (1992) The growth of yeast thiolase crystals using a polyacrylamide gel as dialysis membrane. J. Cryst. Growth 122, 194-198.
III. Ducruix & Giegé. (1999) Editors. Crystallization of Nucleic Acids and Proteins. A Practical Approach. Oxford: IRL Press.
IV. McPherson, A. (1999) Crystallization of Biological Macromolecules. New York: Cold Spring Harbor Laboratory Press.
V. Giegé (2002) A historical perspective on protein crystallization from 1840 to the present day. FEBS J. 280(24):6456-6497.
VI. https://hamptonresearch.com/uploads/cg_pdf/CG101_Microdialysis_Crystallization.pdf

L7 Protein crystallization by capillary counter-diffusion technique

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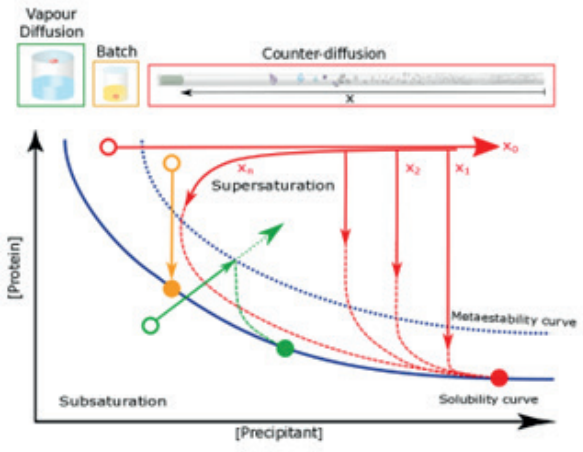
Vapor diffusion and micro-bath under-oil are the most used techniques in Structural Biology laboratories for protein crystallization. In vapor diffusion technique, the slow evaporation of a drop with a mixture of protein and precipitant brings the system towards the supersaturated region at certain rate while in batch, 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 counter-diffusion (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 capillary [2-4].
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 [5].



References
[1] Gavira, J. A. (2016) ABB, 602, 3-11.
[2] Garcia-Ruiz, J. M. (2003) Method. Enzymol. 130-154.
[3] Ng, J. D., Gavira, J. A. & García-Ruiz, J. M., (2003) JSB, 142, 218-231.
[4] Otálora, F., Gavira, J. A., Ng, J. D. & García-Ruiz, J. M., (2009) Prog. in Biophy. and Mol. Biol. , 101, 26-37.
[5] Gonzalez-Ramirez, L., et al., (2017) CG&D, 17, 6780-6.

L8 Unpacking how to grow protein crystals

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Growing protein crystals was described many decades ago by Max Perutz:

“Crystallisation is a little like hunting, requiring knowledge of your prey and a certain low cunning”

Unfortunately, too many practitioners now believe that the protein crystallisation bottleneck has been solved. Further, they believe that all that is required to grow appropriate crystals is sufficient screening with commercially available kits.
In this lecture, we will discuss why simply screening is most likely not going to give you what you want, but is only the one of the steps needed in order to produce well diffracting crystals. We will touch on sample preparation, sample modification, screening kits, screening strategies and techniques that extend basic screening.

L9 Seeds of success: An overview of the Microseed Matrix Screening technique

May Marsh Sharpe
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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.

L10 A guide to choosing your method for crystallization

Lata Govada and Naomi E. Chayen

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Anyone who has ever attempted to crystallise a protein or other biological macromolecule has encountered at least one, if not all of the following scenarios: No crystals at all, tiny low quality crystals; phase separation; amorphous precipitate and the most frustrating; large, beautiful crystals that do not diffract at all. This talk will highlight a few simple ways to overcome such problems, which have worked well in our hands and in other laboratories.

L11 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 potentially 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, 2021). The perspective of using inexpensive 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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L12 Trace Fluorescent Labeling and Low-Cost Imaging of Protein Crystals

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A crucial step in X-ray crystallography is successful protein crystallization. Once crystallization screening plates are prepared, they are carefully examined for crystals. Automated systems for imaging are very expensive, and most are reliant on the protein containing tryptophan. Sensitivity of this approach depends on the number of tryptophan residues in the protein and crystal size. The use of trace fluorescent labeling[1] can be an affordable, fast, and powerful tool when evaluating the results of crystallization screening plates. We have now coupled it to a smartphone-based imaging method[2] that can be implemented for as low as $50. This visible fluorescence approach can give unambiguous macromolecule crystal identification (salt vs protein crystal). As only the macromolecule has fluorescent probe attached to it, images can give a direct indication of what the protein is doing in response to the imposed screening conditions in each well. Additionally, one can use various color fluorescent probes visible at different wavelengths for the imaging of protein complexes, the presence of each color being verification that those molecules are present in a single crystal. The labeling method is simple and takes about 15 min to carry out, and the target labeling level of 0.1–0.5% is achieved if it is performed according to protocol. Labeling at this level has been shown to not affect the nucleation rate or the diffraction quality of the crystals[3]. Furthermore, no changes were observed between the crystal structures of TFL and unlabeled proteins in our studies.

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L13 Femtosecond Crystallography, a New Era in Structural Biology.

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The Biodesign Center for Applied Structural Discovery aims to develop new revolutionary techniques that reveal the structure and dynamics of biomolecules towards new visionary discoveries in Medicine and Energy Conversion. Biological processes are highly dynamic, while most of the structures of biomolecules determined by X-ray crystallography represent a static picture of the molecule. Serial Femtosecond crystallography (SFX) provides a novel concept for structure determination, where X-ray diffraction "snapshots" are collected from a fully hydrated stream of nanocrystals, using femtosecond pulses at the high energy X-ray free-electron laser, the Linac Coherent Light Source [1,2]. As femtosecond pulses are briefer than the time-scale of most damage processes, femtosecond crystallography overcomes the problem of X-ray damage in crystallography [3]. The concept of fs crystallography extends to atomic resolution [4],[5] and has been applied to important membrane protein drug targets crystallized in lipidic environments [6-10]. First experiments on the proof of principle for time resolved serial femtosecond crystallography [11-16] pave the way for the determination of molecular movies of the dynamics of proteins "at work".

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L14 Crystallization of membrane proteins in lipid mesophases

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

Caffrey, M. (2021) Membrane protein crystallization. Bergfors T. M. (Ed.) Protein Crystallization. 3rd Edition (Ebook), IUL Biotechnology Series, 10. Chapter 19, pp 373-410. ISBN: 978-0-9720774-7-7

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L15 *Helicobacter* pylori drug discovery using cryo EM: Targeting the evil duo of a pH-gated urea channel and a cytoplasmic urease

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Helicobacter *pylori's* proton-gated plasma membrane urea channel and cytoplasmic urease are essential for the survival of this carcinogen the human stomach. The channel is closed at neutral pH and opens at acidic pH to allow the rapid access of urea to cytoplasmic urease. Urease hydrolyzes urea into 2 NH_3 and CO_2, neutralizing protons and thus buffering the cytoplasm even in gastric juice when the pH is below 2.0. We determined the crystal structure of the channel, revealing six protomers assembled in a hexameric ring surrounding a central bilayer plug of ordered lipids. Each protomer encloses a channel formed by a twisted bundle of six transmembrane helices. The bundle defines a previously unobserved fold comprising a two-helix hairpin motif repeated three times around the central axis of the channel, without the inverted repeat of mammalian-type urea transporters. Both the channel and the protomer interface contain residues conserved in the AmiS/UreI superfamily, suggesting the preservation of channel architecture and oligomeric state in this superfamily. Predominantly aromatic or aliphatic side chains line the entire channel and define two consecutive constriction sites in the middle of the channel. Mutation of Trp153 in the cytoplasmic constriction site to Ala or Phe decreases the selectivity for urea in comparison with thiourea, suggesting that solute interaction with Trp153 contributes specificity. The structure suggests a new model for the permeation of urea and other small amide solutes in prokaryotes and archaea. Follow-up microsecond-scale unrestrained molecular dynamics studies provide a detailed mechanism of urea and water transport by the channel. More recently, we have determined the structure of the 1.1 MDa urease with various bound inhibitors to resolutions up to 1.5 Å using cryo electron microscopy. Inhibitor discovery against both targets is in progress.

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L16 Crystallization Screening Analysis and Condition Prediction

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Screening is the first stage in determining the significant factors to a process. For macromolecule crystallization, this involves subjecting the target to a random campaign of chemical warfare, to bend it to your will and induce self-association in an ordered array – a crystal. Most often the protein ignores your will and either does nothing, randomly aggregates, or denatures and dies. On occasion one hits upon conditions that lead to some form of self-associated outcome, which may range from nice faceted crystals to ugly lumps of something that is crystalline in name only. After screening comes the second stage, which may range from optimizing the hits obtained to leaving structural biology for some other field of endeavor.

A crystallization screening experiment contains a wealth of data about the proteins response to the chemical assault made upon it. In the absence of clear and faceted crystals there are a range of outcomes that may indicate whether or not the molecules have at been nudged into doing one's bidding. These can be revealed by careful observation, a process facilitated by using methods beyond simple transmission microscopy. Numerically ranking the outcomes of a crystallization plate enables analysis of those results. We have implemented several methods for carrying out this analysis, and are now working on putting them on a server so that it can be publicly accessed.

A byproduct of the development of the trace fluorescent labeling method and of screening data analysis methods has been the growth of a (currently) modest database of crystallization conditions. Initially assembled from screening experiments using a set of 4 x 96 condition screens, we are currently expanding it to 8 x 96 condition screens carried out in duplicate, which reduces the loss of conditions due to the stochastic nature of the crystal nucleation process. It is clear even from the nascent database that the distribution between PEGs, Salts, Alcohols, etc. varies widely between proteins.

Protein-protein interactions are formed through the amino acids on the surface. The approach being taken is to correlate the surface amino acids with the propensity to crystallize from a given reagent or reagent class. The analysis uses a ratio consisting of 5 amino acids each in the numerator and denominator, with every possible combination being tested. For each protein the surface population for each amino acid was estimated and these were summed for the numerator and denominator. Additionally, a null amino acid having a value of 0 was included to capture ratios consisting of 1 to 4 amino acids in either or both the numerator and denominator. Use of the initial database, 30 proteins and ~2500 conditions, showed surprisingly good correlations, with R^2 values often in the 0.65 to 0.8 range. An analysis of the frequency of occurrence for each amino acid could be extracted from the highest correlation results. These results suggest those that are most likely to be responsible for the effectiveness (or not) of that precipitant. The reverse process, where the amino acid ratios for a given protein are fed back through the correlation coefficients, is proposed as a means of estimating the propensity of that protein to be crystallized by that precipitant.

L17 Best practices to prepare and deliver samples in serial femtosecond crystallography experiments at XFELs

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Methods for serial femtosecond crystallography (SFX) at X-ray free electron lasers (XFELs) have been developed to obtain room-temperature structural information from crystals that are too small, weakly scattering, or radiation damage-sensitive to be probed at synchrotrons. In SFX, each crystal is typically exposed only once because the intense, ultrashort XFEL pulse triggers a cascade of ionization events that ends with the crystal exploding. However, diffraction patterns are formed before structural damage becomes evident since the atomic motions are slower than the XFEL-pulse duration. The success of SFX experiments strongly depends on the ability to produce large amounts of a homogeneous size distribution of well-diffracting nano/micro-crystals, so that SFX has also cultivated new methods for nano-crystallogenesis, which have been applied in numerous SFX experiments. A brief introduction to these methods as well as to the most used detection and characterization techniques of nano/micro-crystals will be discussed.

A major drawback of SFX experiments is the large amount of sample required. The choice of a sample delivery method is thus crucial for the success of SFX experiments. An ideal sample delivery method must: 1) replenish crystals in the interaction region ideally at the same rate of the XFEL pulses; 2) consider sample characteristics such as crystal size and morphology, fragility, and concentration; and 3) fulfill seemingly incompatible requisites, such as the need to work in vacuum to avoid background scatter from air, while preventing the sample from drying, freezing, or clogging. Sample delivery methods roughly fall into three categories: injection methods, fixed-target methods, and hybrid combinations of these two methods. All these methods will be briefly discussed during my talk.

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L18 Crystallization and Structural Studies on Protein-Nucleic Acid Complexes

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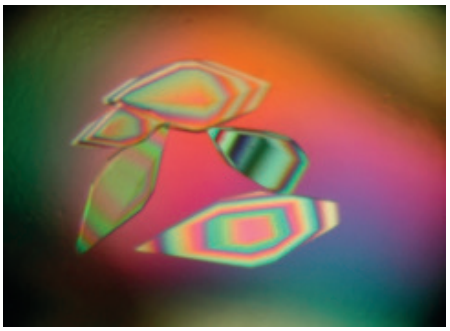
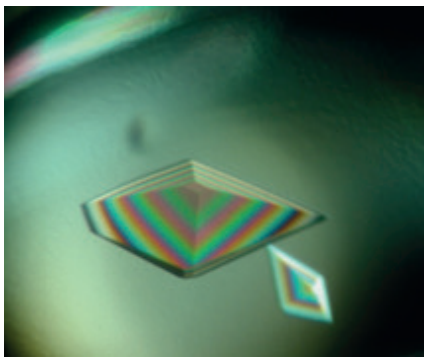
Structural studies on protein-nucleic acid complexes provide important insights into our understanding of processes that store, maintain, replicate, read, regulate, sense, transcribe, translate, and ultimately modify biological information. Protein-nucleic acid complexes are not only at the core of biology, they also impact synthetic, biotechnological and therapeutic approaches.



Obtaining stable complexes that yield diffraction quality crystals is still a bottleneck for most crystallographic studies, and it is also a prerequisite to characterize complexes biochemically and biophysically. Crystallizing protein-nucleic acid complexes depends to a large extent on the behavior of the protein part; however, nucleic acids play also a critical role for a successful experiment. Here, careful construct and experimental design using prior knowledge can have a tremendous impact on crystallization outcome and the understanding of a biological system. In particular, protein-nucleic acid complexes offer the possibility to exploit biochemical information for analyzing complexes in different functional states or take 'snapshots' of a biological process. In this context, complementary structural methods to gain additional information are highly valuable and can provide a comprehensive view on a complex of interest. Moreover, recent advances in cryo-electron microscopy (cryo-EM) open exiting new avenues to analyze protein-nucleic complexes at near atomic resolution.



The lecture and demonstration will give an overview of the work with protein-nucleic acid complexes covering topics from experimental design, sample preparation, sample characterization, stabilization of complexes and the optimization of poorly diffracting crystals to phasing techniques. An emphasis will be on strategies for designing suitable nucleic acid substrates for a thorough biochemical and biophysical characterization as well as for promoting complex crystallization. It is possible to engineer crystal contacts and to fix complexes in different functional states. Moreover, cryo-EM experiments greatly benefit from approaches for efficient complex formation and stabilization. Therefore, we will also discuss practical considerations for observing protein-nucleic acid complexes by cryo EM.

L19 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, CryoEM 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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L20 Differences in crystallization of various haloalkane dehalogenases

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Knowledge of the structure of proteins is a key in identifying and describing the detailed mechanism of biological processes, the development of therapeutics, the degradation of pollutants from the environment, etc. One of the methods used to determine the structure of proteins on atomic resolution is the X-ray crystallography. For many years, our laboratory has been researching structures of different types and mutant variants of haloalkane dehalogenases (HLDs). HLDs are microbial enzymes exhibiting catalytic activity for the hydrolytic conversion of xenobiotics and toxic halogenated aliphatic compounds to the corresponding alcohols. To date, several tertiary structures of these enzymes have been solving by X-ray diffraction analysis. Although we have several types of newly cloned enzymes and their mutants, to crystallize and structurally characterize these enzymes is not trivial. In the lecture will be discussed the complications in the crystal's preparation and combination of crystallization conditions to improve quality of crystals, also enzyme structures and reaction mechanism of the dehalogenation reaction will be outlined.

L21 Optimisation of Crystal Growth for Neutron Macromolecular Crystallography

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The use of neutron macromolecular crystallography (NMX) is expanding rapidly with most structures determined in the last decade thanks to new NMX beamlines having been built and increased availability of structure refinement software. However, the neutron sources currently available for NMX are significantly weaker than equivalent sources for X-ray crystallography. Despite advances in this field, significantly larger crystals will always be required for neutron diffraction studies, particularly with the tendency to study ever-larger macromolecules and complexes. Further improvements in methods and instrumentation suited to growing larger crystals are therefore necessary for the use of NMX to expand.

In my lecture, I will introduce rational strategies and devices for crystallization [1-4], developed especially with the focus on NMX, that combine precise temperature control with real-time observation through a microscope-mounted video camera. Latest instrument consists in the crystal growth bench that, in addition to accurate temperature control, also allows composition of the crystallisation solution (e.g. precipitant concentration, pH, additive) to be controlled and changed in an automated manner [2, 4] by dialysis. I will demonstrate how this control of temperature and chemical composition facilitates the search for optimal crystallization conditions using model soluble proteins [3]. Thorough knowledge of the crystallization phase diagram is crucial for selecting the starting position and the kinetic path for any crystallization experiment. I will show how these established rational approaches are used to guide our crystallisation experiments in order to control the size/volume and number of crystals generated based on knowledge of multidimensional phase diagrams. [3, 4].

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L22 Protein crystallization in micro- and meso-scale devices

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Protein crystallization is mainly known for two purposes: protein three dimensional (3D) structure determination by X-ray crystallography and bioseparation [1]. While for structural biology diffraction-quality crystals are often obtained by slow-growing processes under quiescent conditions [2], high yields and uniform-sized crystals are sought in bioseparation in a short operation time and under efficient mixing [3]. Although the demands on crystals and processes are quite different, the initial step for both purposes is to establish the conditions for protein crystallization. Currently, protein crystallization experiments have a rather empirical design, mostly attributed to the complexity of the underlying phenomena [4]. Due to the highly variable nature of crystallization, each process case must be evaluated individually.

In this context, micro- (microliters) and meso- (milliliters) scale systems offer unequalled conditions to explore protein crystallization, independently of the ultimate purpose. One of the most remarkable advantage of scaling down is enhanced transport phenomena, which is a key factor for the success of crystallization processes because it significantly affects process reproducibility and product quality [3], [5]. On the one hand, micro-scale devices provide a unique platform for investigating protein crystallization mechanisms, since they enable high-throughput experimentation and optical access for in situ characterization. On the other hand, protein crystallization assays on the liter scale are often ruled out, whereas representative experiments in meso-scale devices can provide important information for industrial-scale crystallization [3].

Our research group has been developing devices for protein crystallization experimentation at both micro- and meso-scale. A droplet-based microfluidic platform was developed for the screening and optimization of crystallization conditions [6]–[8] and the establishment of phase diagrams [9]. The results also provided key scientific insights on the influence of hydrodynamics [9] and physical parameters (e.g., droplet size [7] and ultrasounds [6]) on protein crystallization. In addition, lysozyme [10]–[12] and insulin [13] crystals with consistent characteristics (e.g., crystal size distribution, shape) have been successfully produced in an oscillatory flow-based platform, where control strategies such as in-line turbidity monitoring [10-13], seeding [12] and supersaturation control have been implemented [13].

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L23 Intracellular protein crystallization

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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 in a quasi-native environment in combination with serial diffraction data collection could offer exciting new possibilities for the structure elucidation of proteins, complementing conventional crystallization approaches.
This lecture will present an overview of the cellular functions, the physical properties, and, if known, the mode of regulation of native intracellular crystal formation, complemented with a discussion of the reported crystallization events of recombinant proteins and the current method developments to successfully collect X-ray diffraction data from in cellulo 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 the InCellCryst pipeline to elucidate the structural information of in cellulo crystallized target proteins in short time. 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 crystallization is successful, diffraction data are collected after crystal isolation or, nowadays, directly within the living cells using recently developed serial crystallography approaches at XFELs [2,3,5] or synchrotron sources [6,7], depending on the obtained crystal size. Particularly fixed-targets have been qualified for efficient sample delivery [5,7]. However, low numbers of crystal containing cells within a cell culture and limitations in crystal detection techniques represent the main bottlenecks currently restricting a broader application. An innovative approach for direct time-efficient screening of cell cultures using X-rays, denoted as XRPD-SAXS [8], is finally presented, as well as fluorescence-based sorting of crystal-containing cells. This will allow a more efficient use of crystal containing cells as suitable targets for structural biology in the future.

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L24 Towards Molecular Movies of Enzyme Catalysis

Guowu Lin[1], Sandra Vergara[1], Paola Zinser[1], Brisa Chagas[1], Xiaohong Zhu[1], Silvia Russi[3], Ulises Santiago[1], Nicolas Sluis-Cremer[2], Aina Cohen[3] and Guillermo Calero[1]

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Amino acid residues in their binding pockets *undergo transient ultra-fast (pico- to milli-seconds) vibrational, rotational and translational motions to allow substrate binding in active sites.* These transient-state intermediates are critical for enzymatic activity, drug action and drug resistance. Moreover, contemporary structural biology approaches, such as X-ray crystallography and single particle cryo-electron microscopy (cryoEM) can only resolve the structures of thermodynamically stable species, and cannot inform of kinetic intermediates. One of the next challenges in structural biology is advancing from a static picture to the observation of enzymes in action. Time-resolved (TR) structural studies can uncover conformational changes occurring in the nano-to milli-second timescale (real time) revealing hidden transition-state intermediates and hence increasing the "druggable landscape" available for molecular modelling and inhibitor testing and development. Albeit its significance, TR structural studies have been scarce as they present technical challenges. Recently my laboratory has embarked in performing TR studies of RNA polymerase II and the oncogenes N- and K-RAS, using X-ray crystallography; and HIV-1 reverse transcriptase (RT), using single particle cryoEM. We employed UV photolysis of caged substrates and data collection at 140 degrees Kelvin to create the first structural movies of nucleotide addition by Pol II and RT, and GTP hydrolysis by N-RAS.

L25 Small Angle Solution Scattering as a Complementary Technique in Structural Biology Studies

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Small Angle Solution Scattering provides low-resolution information about particles in solution. Small Angle X-ray Scattering (SAXS) is commonly used to characterize macromolecular samples, test structural hypotheses, and provide molecular envelopes, etc. More recently, methods have been developed to extract electron density from the scattering profile. When combined with high-resolution structural information it can be used to place components of complexes, visualize structural elements that may be constrained by crystal packing artifacts, and yield information on dynamical regions not observed crystallographically. SAXS is sensitive to molecular interactions and can be used to probe systems under physiological conditions and potential crystallization space. Small Angle Neutron Scattering (SANS) can be used in the same manner but is most often used with contrast matching. By varying the ratio of H_2O and D_2O it is possible to mask structural components and determine the makeup of those structural components within the whole system. Small Angle Solution Scattering, X-ray and neutron, is a complementary technique that can provide information guiding structural studies and illuminating mechanisms. The sample requirements are very similar to crystallization with data collection and analysis amenable to those used to single crystal techniques. This lecture will discuss the basics of the technique, practical guidance, and potential uses complementary to crystallography, but also other structural approaches.

L26 Introduction to single particle cryoEM

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We will cover the recently revolutionized technique of Cryo-EM whose leading developers were awarded the Nobel prize in Chemistry in 2017. The “resolution revolution” in cryo¬EM, driven by developments in instrumentation such as direct detectors, coupled with major improvements in data analysis, has put Cryo-EM at the forefront of structural biology, a novel method for attaining high-resolution models of difficult targets (low yield, moderately flexible). Since 2015, several records have been achieved including a structure of a membrane protein, the β3 GABAA receptor homopentamer, reconstructed to a resolution of 1.7 Å. Another advantage is the possibility of studying glycosylated proteins as well as the option of reconstituting membrane proteins into nanodiscs, providing a protein-enclosed lipid bilayer and thus a more native-like environment as opposed to detergents.

We will cover sample preparation and the theoretical aspects of data processing for single particle cryo-EM such as CTF estimation; manual and automated particle picking, particle extraction, 2D classification, initial model generation and 3D classification.

L27 Cryo-Electron tomography – method to bridge scales in biological system

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Cryo electron microscopy (cryo-EM), is an advanced structural biology technique. This technique is advantageous as it can bridge between light-microscopy and methods for gaining information on a molecular level (X-ray crystallography/NMR). Importantly, the recent development of direct detectors for EM pushed the targeted resolution limit to near atomic level, opening tremendous opportunities for the visualization and analysis of biological assemblies.

My laboratory employs a wide range of the spectrum of the imaging techniques to understand molecular actions in various contexts.

In this talk, introducing our new pipeline of structural cell biology, I will discuss about our recent molecular findings of neuronal regeneration. Using in situ cellular cryo-electron tomography on primary neurons, we aim to understand the molecular actions of the remodelling of organelles and cytoskeleton structures during the critical events of neuronal morphogenesis and regeneration.

L28 How do we get fragments or ligands into structures?

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The first part of my talk will delve into the most common techniques to obtain fragment/ligand bound structures. The second part of the talk will look at how we determine whether a ligand is present in a structure? Is that blob of density the ligand you expect or something else? What techniques do we use to determine whether a ligand is present and what we as structural biologists do to validate a given assertion.

L29 Microcrystal electron diffraction: methods and applications

Brent L. Nannenga

Electron diffraction is able produce diffraction data from crystals that are orders of magnitude smaller than those needed for conventional X-ray crystallographic experiments, which can alleviate structure determination bottlenecks associated with the growth of large crystals. Microcrystal electron diffraction (MicroED) is a cryo-electron microscopy technique that is used for high-resolution structure determination from very small microcrystals. In this presentation, the MicroED workflow and how to use the method will be covered. Additionally, applications of MicroED to samples from diverse fields including structural biology, organic chemistry, and materials science will be presented

L30 The Chemistry of Mushroom Magic (and why you should not lick toads)

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Psychoactive hallucinogens produced by 'Magic Mushrooms' have been used as recreational drugs since ages in many parts of the world. Psilocybin, the active ingredient of the psilocybe mushrooms and related species, has recently gained traction both for recreational use and as a therapeutic drug in controlled trial settings for the treatment of resistant severe depression and PTSD. To appreciate the risks and possible benefits of these hallucinogenic drugs, we first explore how different types of psychoactive drugs work and illustrate their history and the development and (crystal) chemistry of related designer drugs. We learn more about magic mushrooms, cacti, the art of toad kissing, and anecdotal lore and tales of mushroom consumption. The magic is made by key biosynthetic enzymes of the mushrooms in a stepwise reaction pathway, and the structures of some key enzymes have been recently determined. A combination of many of the techniques introduced in previous lectures had to be applied to yield the high-resolution crystal structures necessary to decipher the unusual final methylation steps to yield the hallucinogen psilocybin.

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 talk, and no mushrooms were consumed during the structure determinations.

Demonstrations Fair

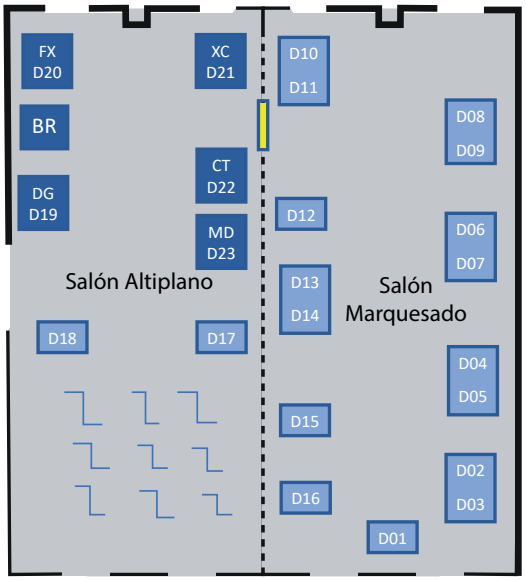
Demonstrations Fair

The practical training during ISBC2023 has been organized in our innovative and lively design called Demonstrations Fair. A number of stands will simultaneously offer the opportunity to attend 23 short practical sessions carried by specialists (see tables in next two pages). D1-D16 will take place in the Salón Marquesado; and demonstrations D17-D23 will be located in the Salón Antiplano (see scheme below).

The Demonstrations Fair will run from 09.20h until 17.40h on Thursday 25th. Each demo takes about 40 minutes and will be repeated a number of times during the day according to the schedule in the next page. Active participation of all participants is desirable to get the most out of the Fair.

Arrange your own Practical Training Session and enjoy it!

Nº	TITLE	TUTORS	9:20 10:00	10:00 10:40	Coffee Break	11:00 11:40	11:40 12:20	12:20 13:00	Lunch	15:00 15:40	15:40 16:30	16:30 17:10
D01	The secret life of your crystallization drop: Do you know what really happens in your drops?	Bernhard Rupp	■				■				■	
D02	Characterization, analysis and use of crystallization screening outcome	Edward H. Snell		■		■		■				
D03	AlphaFold and biochemical considerations for protein crystallization	Joe Ng	■				■			■		
D04	Microfluidic tools and crystallization: from fabrication to application	Isaac Rodríguez		■				■				■
D05	Crystallization and crystallography in microchips	Claude Sauter	■				■				■	
D06	Gels for Crystallization	Ángeles Hernández		■		■		■				
D07	Protein crystallization by capillary counterdiffusion	Luis González	■				■			■		
D08	Hanging drop setup – you probably think you know how to do this already	Janet Newman		■		■		■				■
D09	How to perform vapour diffusion experiments: Hanging and Sitting drops	Ivana K.S. / Paulina R	■				■				■	
D10	The Temperature-controlled Optimization of Crystallization	M. Budayova-Spano		■				■		■		■
D11	Microbatch crystallization under oil	Lata Govada	■				■				■	
D12	Dialysis, crystallization and the Hofmeister series	Jeroen Mesters		■		■				■		
D13	Crystallization and Structural Studies on Protein-Nucleic Acid Complexes	C. Biertümpfel	■				■				■	
D14	Cryo-Electron tomography – method to bridge scales in biological system	Naoko Mizuno		■				■		■		■
D15	Growth of microcrystals of Photosystem I for serial femtosecond crystallography	P. Fromme / J.M. García / J-H. Yang	■			■					■	
D16	Labelling and Low-Cost Imaging of Macromolecule Crystals	M. Pusey / C. Tarver		■			■			■		
D17	Crystallization of membrane proteins in lipid mesophases	Martin Caffrey	■			■					■	
D18	The European X-Ray Free-Electron Laser Facility (EuXFEL)	Diogo Melo / Faisal H. Mekky Koua		■				■		■		■
D19	Microseed Matrix Screening experiments using a robot	S. Kolek / M. Sharpe	■				■				■	
D20	Formulatrix µPulse TFF - for Sample Concentration - Diafiltration (Buffer Exchange, Desalting)	Kate Lewis		■				■		■		
D21	The most sample efficient sample qualification method. Automated DLS	Karsten Dierks	■			■					■	
D22	Chromatography Principles and ÄKTA™ chromatography systems	P. Braga / E. García		■			■			■		
D23	Crystal fishing and mounting	Simon Tanley	■					■			■	



D01 The secret life of your crystallization drop: Do you know what really happens in your drops?

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The chemical composition of a crystallization cocktail determines whether a protein solution can reach a thermodynamically metastable state of supersaturation from which it can return to an equilibrium in which the separating protein-rich phase may be a ordered protein crystal. Whether this state can be actually reached, depends on the kinetics which are much harder to control. Many method-dependent parameters such as drop size, protein-precipitant ratio, or mixing rates, are not known, and affect kinetic phenomena such as nucleation and convection. With a digital microscope camera we will observe instant nucleation and crystallization in a protein drop showing convection and diffusion patterns with microcrystals floating and moving through the crystal while they grow. During the growth phase of the crystals we will review again selected problems in crystallization space using the phase diagrams.

D02 Characterization, analysis and use of crystallization screening outcome

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The National Crystallization Center is based at the Hauptman-Woodward Medical Research Institute in Buffalo New York and has worked with approximately 2,000 laboratories worldwide on the crystallization screening of almost 20,000 different biological macromolecules. Each is screened against 1,536 different conditions using the microbatch-under-oil technique with outcomes imaged visually, and in the later decade of operation with second order non-linear imaging of chiral crystals (SONICC) and ultraviolet two-photon excited fluorescence (UV-TPEF). A comprehensive laboratory information management system, coupled with the extensive imaging available, and a large-scale human analysis of outcome, enabled machine learning techniques to classify outcome with accuracy and precision equal or greater to a human. In this demonstration, the strategies of different crystallization screens and what they tell you will be discussed. Using imaging software developed by the Crystallization Center, we will look at outcomes and determine what lessons can be derived, not from a single result, but from the overall screening process and how that can provide information that can be used to optimize crystallization conditions and yield information on important biology. We will also discuss different crystallization methods, i.e. vapor diffusion and micro batch approaches, and how outcomes can define location on the macromolecular solubility phase diagram. Examples will be illustrated from macromolecules that have gone through the crystallization center and become structures deposited in the PDB.

D03 *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].

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 demonstration, 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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D04 Microfluidic tools and crystallization: from fabrication to application

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Novel technologies are allowing the democratization and the routine use of microfluidic tools, formerly exclusive of laboratories with access to high-end facilities and/or expensive equipment and materials. As a result, microfluidics are becoming more and more accessible to regular laboratories, and are gaining relevance in crystallization studies due to clear advantages and a smaller environmental footprint with respect to macroscopic experiments. Indeed, the low reagent consumption, the possibility of simultaneously running a large number of independent experiments in nanoliter volumes, and the precise control on heat and mass transfer provided by the microfluidic scale, offer an unparalleled environment for the research on crystallization (e.g. nucleation and crystal growth studies, or crystallization conditions and polymorph screenings). In this regard, the application of high throughput techniques, like the micro-batch droplet methods [1, 2], or on-chip counter diffusion methods [3, 4], coupled to different photonic techniques for in-situ characterization (UV-vis spectroscopy, X-Ray diffraction, or Small angle X-Ray scattering [4, 5]) is giving new insights in the fields of protein and small molecule crystallization.

Considering that microfluidics could soon represent a useful element available in the toolbox of every laboratory/researcher, in this workshop, we will take the first steps along the technical aspects concerning microfluidics conception and operation. The most widespread techniques and technologies for microfluidics manufacturing will be reviewed, from the initial design to the final product. The pros and cons involved in its manufacturability and operability will be compared, from standard clean room processes to novel fabrication approaches, affordable to any laboratory. In addition, the choice of microfluidics fabrication materials will also be discussed, as a function of their compatibility with different applications and in-situ characterization techniques. During the discussion, different equipment, materials and microfluidic tools and platforms will be displayed, together with alternatives and solutions to go through the most frequent issues appearing when operating microfluidic tools.

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D05 Crystallization and crystallography in microchips

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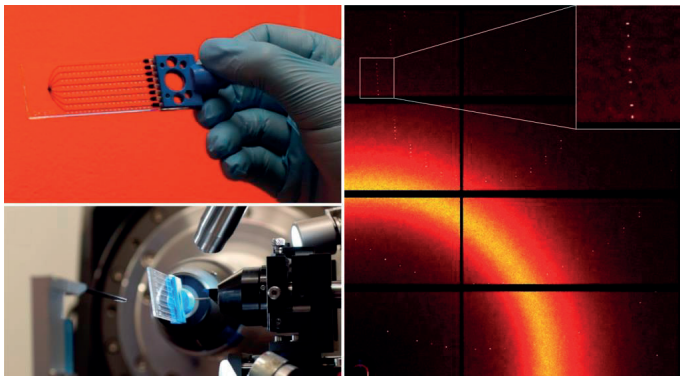
Microfluidic devices offer many advantages for crystal growth: they can easily handle nano-volumes of solutions and have been used to miniaturize crystallization methods such as batch, free interface diffusion, counter-diffusion or dialysis. Thus, they provide a very efficient parallelization of crystallization assays for high throughput screening applications. In addition, due to their small cross-section, microfluidic channels and chambers constitute convection-less environments that are a priori favorable to the growth of high quality crystals. Finally, some of these microfluidic chips have been designed to be compatible with the in situ crystal analysis by X-ray diffraction [1,2].



Different microsystems, their manufacturing processes and function will be presented during practical work.

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D06 Gels for crystallization

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Silica gels have been used for over a century to grow inorganic crystals [1]. A decade ago silica and, mainly, agarose gels were introduced in the field of protein crystallization [2]. Generally, gels behave as a neutral network in which convection is reduced or eliminated depending on the gel concentration [3, 4], and sedimentation of crystals is avoided which favors the three-dimensional growth of the crystals. The combination of the two former properties, together with the reduced incorporation of impurities [5] leads to better diffracting crystals [6].

This demonstration will show how to prepare agarose and silica gel correctly. These gels can be applied in various experimental crystallization setups such as batch, counter diffusion, and hanging and sitting drop experiments.

Agarose gels are obtained when an aqueous sol of the poly galactoside (purified from seaweed) is cooled below their gelling temperature and produces a hydrophilic and thermoreversible hydrogel [7]. A common example of how to prepare quickly and easily an agarose gel for crystallization experiments will be shown in this demonstration. The influence of the salt concentration (i.e. ionic strength) and pH of the buffer on the process of silica and agarose gel [8] will be discussed.

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D07 Protein crystallization by capillary counterdiffusion

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Counterdiffusion crystallization (CDC) in the capillary is a very simple, cost-effective, and practical procedure for obtaining protein crystals suitable for X-ray data analysis. The counterdiffusion process has been used to simultaneously screen for optimal conditions for protein crystal growth, incorporate strong anomalous scattering atoms, and mix in cryogenic solutions in a single capillary tube. The crystals obtained in the capillary have been used in situ for X-ray analysis.

Counterdiffusion techniques have been very effective in crystallizing a wide number of macromolecules including proteins, viruses, and nucleic acid–protein complexes with different molecular weights and isoelectric points under a wide range of chemical and physical conditions. Counter diffusion is a non-equilibrium crystallization technique that allows the precipitating agent and the protein solution to counter-diffuse one against the other. This process provokes subsequent nucleation events along the protein chamber at progressively lower supersaturation, producing fewer crystals of larger size and higher quality [2-4].

The advantages of this technique can be summarized as follows:
a. It works under diffusion-controlled mass transport, which is known to produce better-ordered crystal lattices provided the growth proceeds in the diffusion-controlled or mixed regime.
b. It automatically searches for optimal crystallization conditions.
c. It reduces the consumption of macromolecules as thin glass capillaries are used

This technique has proved its ability to increase protein crystal quality and it is regularly used for optimization after initial crystallization conditions have been found with other techniques.

In this demonstration, it will be shown the preparation of experiments of the crystallization of proteins by means of the CD technique in capillaries. Likewise, different experimental variants for the implementation of this method will be described.

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D08 Hanging drop setup – you probably think you know how to do this already

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Setting up vapour diffusion (hanging or sitting drop) experiments by hand continues to underpin crystal growth in most laboratories. Setting up these experiments well is non-trivial, and in this demonstration some tips and tricks for streamlining the process will be shown. These experiments can be set up in many different ways, and we will discuss how you might use different consumables (i.e. what you have lying around in the lab) and what the limitations and or advantages might be of the different consumables might be.

We will discuss how to set up the crystallization experiments, how to modify them, and how to get crystals out of the experiment. We will also talk about how to best organize your workflow, and simple tricks and tips for speeding up the experiment.

D09 How to perform vapour diffusion experiments: Hanging and Sitting drops

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Obtaining crystals suitable for X-ray diffraction analysis is still the least understood step in the determination of protein structures by biocrystallography methods because the technique relies a lot on a trial-and-error approach. However, there are several principles that form a basis of all crystallization techniques.

Firstly, properties of the protein itself are critical for the success of crystallization. Surface amino acid residues that can participate in crystal contacts; disordered regions in protein molecule (flexible loops) can perturb or prevent the formation of stable crystal contacts; cofactors and post-translational modifications either help or hinder crystal formation. Supersaturation of protein solution is achieved by increasing the effective protein concentration. For this purpose, precipitant is added to the protein solution, which affects the packing of water molecules in the protein hydration shell.

External parameters that affect crystallization are: (1) concentration of protein and precipitant; (2) type of precipitant; (3) pH, which affects charges of surface amino-acid residues and therefore electrostatic forces between protein molecules; (4) temperature, that influences kinetics of crystal growth and strength of hydrophobic interactions.

If the protein and/or precipitant concentration is too high, protein molecules will aggregate and form amorphous precipitate. At even higher concentrations phase separation of protein and precipitant solutions occurs. If a protein solution is not saturated, no precipitation or crystallization will occur and the crystallization drop will remain clear. The phase diagram (Figure 1) illustrates solubility of a protein in a precipitant solution.

There are many methods to crystallize biological macromolecules, all of which aim at bringing the solution of macromolecules to a supersaturation state. The most common crystallization techniques successfully used for crystallization screening and optimization experiments are standard crystallization techniques such as sitting, hanging and sandwich drop based on vapor diffusion (Figure 1A, B, C). Sitting and hanging drop methods are popular because they are easy to perform manually as well as automatically, they require small amounts of sample and allow for various modifications during screening and optimization.

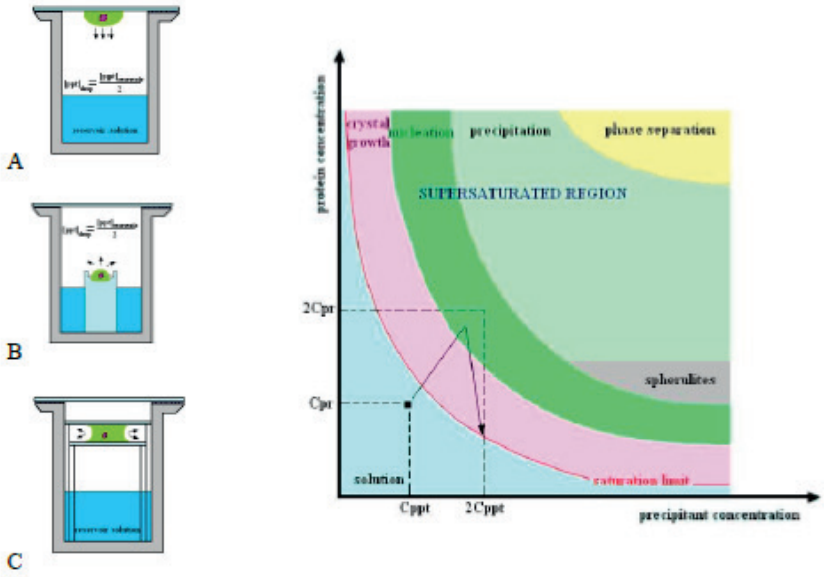

Figure 1. Phase diagram and representation of standard vapor diffusion crystallization methods (hanging, sitting and sandwich drop). Through loss of water vapor to the larger volume reservoir, the droplet comes into equilibrium with the precipitant concentration in the reservoir, thus inducing crystallization.

During optimization, a variable set of parameters is screened to determine appropriate conditions for nucleation and growth of single crystals suitable for X-ray diffraction analysis. A newly applied alternative cross-crystallization method (Figure 2) also known as cross-influence procedure (CIP) is based on addition of metal ion salts simultaneously to the protein drop and even to neighboring drops, which allows a cross-influence effect of additives during crystallization experiment. The presence of metal ions significantly influences the crystal growth, as the modification of crystal morphology and internal packing were observed.

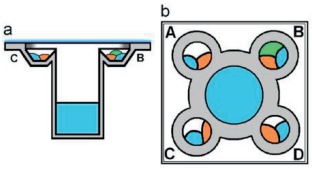

Figure 2. Schematic side and top view of Emerald BioStructures Combi Clover Crystallization Plate (EBS plate) for sitting drop experiments. Blue color presents reservoir solution, red areas indicate each additives and green color represents protein-containing solution.

D10 The Temperature-controlled Optimization of Crystallization

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The success of diffraction experiments in protein crystallography is directly related to the quantity and quality of the data recorded from the crystal samples. New and emerging uses result in specific challenges for crystallization of proteins, in which precise control of crystal size is essential. New approaches to serial synchrotron crystallography and to solving structures including time-resolved studies of short-lived intermediates require small crystals, typically in the 0.2–10 μm size range. At the other extreme are the requirements of the next-generation flagship neutron sources, such as the European Spallation Source (ESS, Lund). Because neutrons interact very weakly with matter, much larger, and ideally bulky, crystals are needed with volumes of about 0.1 to 1.0 mm^3 for neutron crystallography. A rational way to find the appropriate conditions to grow optimal crystal samples for bio-crystallography is to determine the crystallization phase diagram, which allows precise control of the parameters affecting the crystal growth process. There are various approaches for obtaining protein crystals, each crystallization technique uses different kinetic trajectory to reach nucleation and metastable zones of the crystallization phase diagram.

The practical demonstration will expand on the principles of temperature-controlled protein crystallization [1, 2] and established relative strategies [3, 4] that have been successfully used to provide sufficient scattering volumes for neutron diffraction studies that require large-volume well ordered single crystals as well as to generate homogeneous populations of uniformly sized small protein crystals required for use by other advanced serial diffraction techniques.
Due to time constrains, the practical session will focus on the use of Centeo's electronic temperature controlled microplate (TG40 System) to carry out a simple temperature screening using a model protein.

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D11 Microbatch crystallization under oil

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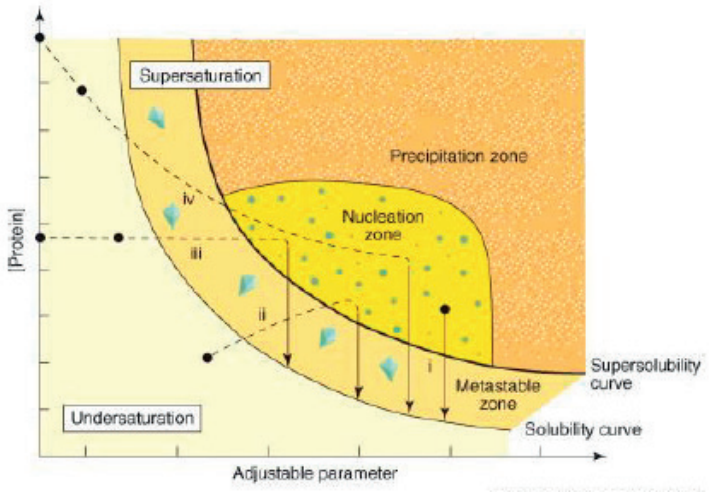
The oldest method used for the crystallization of macromolecules was the batch method (simply adding the precipitant to the protein). Over time the batch method was miniaturized to fit to 72-well Terasaki plates (microbatch method under oil) to reduce protein consumption. It was the first method to lend itself to automation for both screening and optimisation trials. A layer of oil is covered over the drops to prevent the drops from evaporation and drying out. To enable some evaporation, a 1:1 mixture of Silicon and paraffin oil is used in screening experiments (modified microbatch) and paraffin oil for optimisation trials.



During the demonstration fair, all practical aspects and benefits of the microbatch method will be discussed and demonstrated.

Primers
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II. D'Arcy et al. (2004) Modified microbatch and seeding in protein crystallization experiments, J. Synchrotron Radiat. 1;11(Pt 1), 24-26.
III. D'Arcy et al. (1996) A novel approach to crystallizing proteins under oil, J. Cryst. Growth 168, 175-180.
IV. JR Luft & GT DeTitta (1999) A method to produce microseed stock for use in the crystallization of biological macromolecules. Acta Cryst. D55, 988-993.

D12 Dialysis, crystallization and the Hofmeister series

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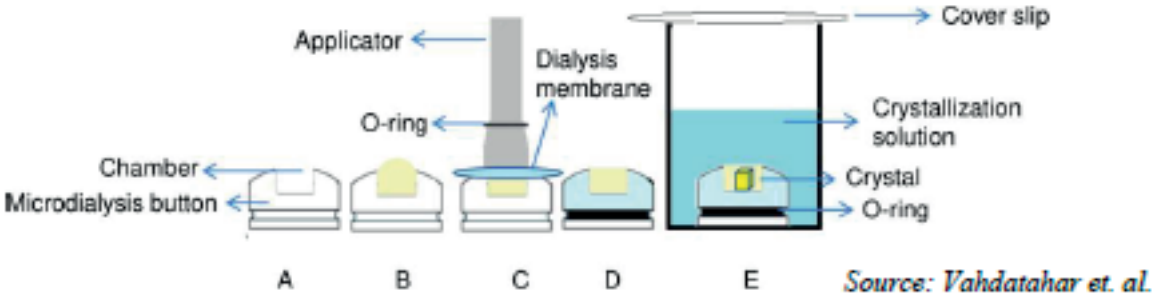
One of the oldest methods used for the crystallization of macromolecules is the *dialysis* method (protein in dialysis tube against a buffer). Nowadays, dialysis methods suffer from prejudices and yet, it has many advantages to offer: The technique easily allows for simple adjustments in salting-in and salting-out, as well as pH-shift crystallization experiment.



Especially useful equipment for exploiting the phase diagram in combination with solute components, *i.e.* Hofmeister series, are microdialysis buttons. These help to establish the fundamentals for deepening our understanding of protein solubility, nucleation, and crystal growth. During the demonstration fair, all practical aspects and benefits of the dialysis method will be examined and demonstrated.

Primers

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D13 Crystallization and Structural Studies on Protein-Nucleic Acid Complexes

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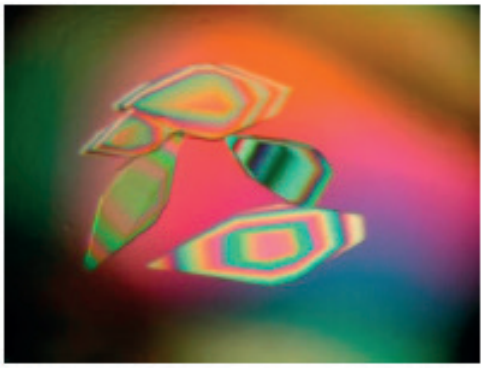
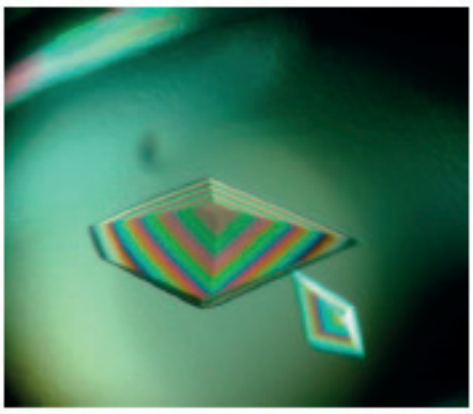
Structural studies on protein-nucleic acid complexes provide important insights into our understanding of processes that read, store, maintain, replicate, regulate, sense, transcribe, translate, and ultimately, modify biological information. Protein-nucleic acid complexes are not only at the core of biology; they also impact synthetic, biotechnological, and therapeutic approaches.



Obtaining stable complexes that yield diffraction quality crystals is still a bottleneck for most crystallographic studies, and it is also a prerequisite to characterize complexes biochemically and biophysically. Crystallizing protein-nucleic acid complexes depends to a large extent on the behavior of the protein part; however, nucleic acids also play a critical role for a successful experiment. Here, careful construct and experimental design using prior knowledge can have a tremendous impact on the crystallization outcome and the understanding of a biological system. In particular, protein-nucleic acid complexes offer the possibility to exploit biochemical information for analyzing complexes in different functional states or to take 'snapshots' of a biological process. In this context, complementary structural methods to gain additional information are highly valuable and can provide a comprehensive view on a complex of interest. Moreover, recent advances in cryo-electron microscopy (cryo-EM) open exiting new avenues to analyze protein-nucleic complexes at near atomic resolution.

Designing suitable nucleic acid substrates and optimizing conditions for a successful biochemical and structural analysis is not trivial. The demonstration will focus on strategies for designing nucleic acid substrates for efficient complex formation, how to characterize and optimize crystallization- as well as cryo-EM experiments, how to avoid pitfalls, and we will discuss practical considerations for test cases. In addition, it will be possible to discuss individual cases or projects of the workshop participants.



D14 Cryo-Electron tomography – method to bridge scales in biological system

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Cryo electron microscopy (cryo-EM), is an advanced structural biology technique. This technique is advantageous as it can bridge between light-microscopy and methods for gaining information on a molecular level (X-ray crystallography/NMR). Importantly, the recent development of direct detectors for EM pushed the targeted resolution limit to near atomic level, opening tremendous opportunities for the visualization and analysis of biological assemblies.
My laboratory employs a wide range of the spectrum of the imaging techniques to understand molecular actions in various contexts.
In this talk, introducing our new pipeline of structural cell biology, I will discuss about our recent molecular findings of neuronal regeneration. Using in situ cellular cryo-electron tomography on primary neurons, we aim to understand the molecular actions of the remodelling of organelles and cytoskeleton structures during the critical events of neuronal morphogenesis and regeneration.

D15 Growth of microcrystals of Photosystem I for serial femtosecond crystallography

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The success of serial crystallography experiments strongly depends on the ability to produce large amounts of crystals of well diffracting nano- or micro-crystals with a homogeneous size distribution. Traditional crystallization methods such as vapor diffusion, dialysis or counter-diffusion, have been focused on the production of a few large crystals (>100µm). However, methods for growth nano- and micro-crystals of high quality and homogeneity are still underexplored. Over the past few years, our group has developed several micro- crystallization methods including the batch method, the free interface diffusion and the free interface diffusion centrifugation[1-3]. These methods have been applied in numerous serial crystallography experiments both at XFELs[2] and synchrotron sources[4-6]. During our demonstration, the students will have the opportunity to grow and manipulate very small crystals of photosystem I, which is a giant integral membrane protein complex of 1 MDa (36 proteins and 381 cofactors), and one of the most important membrane protein complexes in the process of photosynthesis along with photosystem II. PSI protein will be isolated from the natural thermophilic cyanobacterium *Thermosynechococcus elongatus* in our labs at Arizona State University by a complex multi-step isolation procedure, which includes crystallization as a final purification step. Due to fact that PSI cannot be frozen at any step during the isolation and crystallization process, the protein will be freshly isolated and shipped to ISBC as a crystal suspension at 4°C. During the demonstration, the crystals will be dissolved and re- crystallized by reduction of the ionic strength. Live crystal growth of the gigantic PSI will be monitored by conventional microscopy techniques.

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D16 Labeling and Low-Cost Imaging of Macromolecule Crystals

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University of Alabama in Huntsville – Trace Fluorescent Labeling
Crissy Tarver
Stanford University – Low Cost Imaging

Transmission microscopy using white light is the "standard" method for reviewing the results of crystallization screening plates. Careful observation is always required to maximize the results of a crystallization screening experiment. However, this observation is complicated using white light imaging only as one can often miss clues as to the true nature of the results obtained. Methods that emphasize the nature of the observed features, protein or not and crystalline or not, facilitate and speed up the data analysis process. We have put forth the trace fluorescent labelling method, where ~0.1 – 0.2% of the protein molecules are derivatized with a fluorescent probe the excites and emits in the visible spectrum. This method has the added advantage in that different species of a complex can be labeled with different colors and as a result one can quickly determine their presence in a crystal. This practical will focus on how to carry out trace fluorescent labeling. Fluorescent imaging is not beyond the financial reach of a crystal grower. The practical will show how a labeled sample can be imaged using a low cost (typically < $50) method. A smart phone based approach to implementing this method will be demonstrated.

D17 Crystallization of membrane proteins in lipid mesophases

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

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• Lab Publications: https://www.tcd.ie/Biochemistry/research/caffrey/publications/
Supported in part by Science Found
ation Ireland (16/IA/4435

D18 The European X-Ray Free-Electron Laser Facility (EuXFEL)

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The European XFEL in the Hamburg area is a new international research facility of superlatives: 27,000 X-ray flashes per second and a brilliance that is a billion times higher than that of the best conventional X-ray sources open up completely new opportunities for science. Research groups from around the world are able to map the atomic details of viruses, decipher the molecular composition of cells, take three-dimensional "photos" of the nanoworld, "film" chemical reactions, and study processes such as those occurring deep inside planets. The operation of the facility is entrusted to European XFEL, a non-profit company that cooperates closely with its main shareholder, the research centre DESY, and other organisations worldwide. European XFEL has a workforce of more than 450 employees and started user operation September 2017. With construction and commissioning costs of 1.25 billion euro (at 2005 price levels) and a total length of 3.4 kilometres, the European XFEL is one of the largest and most ambitious European new research facilities to date. At present, 12 countries have signed the European XFEL convention: Denmark, France, Germany, Hungary, Italy, Poland, Russia, Slovakia, Spain, Sweden, Switzerland, and the United Kingdom. For more information on European XFEL go to www.xfel.eu or visit the European XFEL booth where you get the chance to virtually walk through the X-ray laser facility with VR goggles.and to ask your questions.

D19 Microseed Matrix Screening experiments using a robot

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The aim of this practical is (1) to teach students how to make crystal seed stocks suitable for Microseed Matrix Screening experiments.

(2) Setting up the MMS experiments using a robotic liquid handling device to seed into sparse matrix screens and establish optimal seed dilutions.

Making the seed stock

- We will demonstrate the different methods of making seed stocks by manual crushing and using various bead materials to make seed suspensions
- Students can make the seed stocks and check the preparations under a microscope
- A dilution series will be made from these stocks
- A pre-prepared plate will illustrate the effect of introducing different seed concentrations.

MMS using a Douglas Instruments liquid handling system

- We will demonstrate how to set up and perform automated Matrix Microseed Screening and subsequently demonstrate the differences compared to a control plate with no seeds added.
- In addition, we will show students how the correct dilution of seeds can be experimentally determined using the same instrument.

References

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D20 Formulatrix µPulse TFF - for Sample Concentration - Diafiltration (Buffer Exchange, Desalting)

Kate Lewis
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Visit the Formulatrix stand to see the µPULSE® - TFF System in action.

The µPulse is a fully automated, walk away system that uses tangential flow filtration (TFF) for sample concentration and diafiltration (buffer exchange, desalting).

Simply enter your starting and final volume with optional diafiltration - The µPULSE® - TFF System takes care of the rest.

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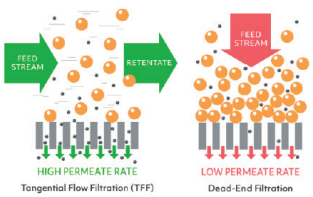
Walk-Away System
Set your desired parameters and let the system do 95% of the process automatically until it is time to collect your sample; Remotely monitor via a web-based application.
Fast
Permeate flow rate up to 2.5x faster than centrifuges with dead-end filters.
Scalable Process
Whether you are trying to scale up or down, µPULSE allows you to control all the parameters of an industrial TFF, at the research scale.
Wide Range of Operating Volumes
Concentrate from 100mL to 1mL with up to 250 mL of buffer exchange.
Lowest Hold-up Volume in the Industry
Our patent-pending pump design offers just 0.65 mL of hold-up volume. Up to 100% hold-up recovery with automated recovery options.
Easy Maintenance
All components in the fluid path are disposable consumables.
Cost-Effective
The µPULSE consumable chips can be cleaned in place for re-use up to 300 mL of permeate (sample dependent).



Unlike centrifuges with traditional dead-end filtration systems, the µPULSE prevents a high concentration gradient from forming at the filtration membrane. This means no reduction in the permeate flow rate and increased sample recovery.

Sample Concentration, Diafiltration - µPULSE TFF System (formulatrix.com)

D21 The Most Sample Efficient Sample Qualification Method. Automated Scanning DLS

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In situ Dynamic Light Scattering (*in situ* DLS) [1, 2] is a convenient tool for protein size determination and the basis for automated scanning DLS. DLS itself is a standard non-invasive method that provides information about the particle size and, in particular, the aggregation state of biological macromolecules. The monodispersity of such samples is a prerequisite for almost all subsequent structure determination methods, e.g. crystallisation, cryo-electron microscopy (cryo-EM), small-angle X-ray scattering (SAXS) and nuclear magnetic resonance (NMR). After purification, biological macromolecules, i.e. proteins, often exhibit a degree of aggregation. This can be due to an unsuitable buffer, partially denatured proteins or incomplete folding. With automated scanning DLS, the response of a protein to a large number of different buffers or other additives can be rapidly investigated, even with very small sample volumes.

Standard microbatch plates are used for automated scanning DLS. The sample droplets are kept under paraffin oil to prevent evaporation. In this setup it is possible to perform routine DLS measurements with droplet volumes down to 100 nL. Such small sample volumes can cope with the variety of conditions while ensuring maximum sample efficiency in a formulation process. More recently, routine DLS measurements can be performed by placing a sample on a cryo-EM grid using the same hardware as for automated scanning DLS. This on-grid DLS is the very last step in the single-particle 3D cryo-EM process where information can be obtained from a sample prior to freezing. These investigations are made possible by the unique optical arrangement of the DLS optics in the SpectroLight 600, which underlines the versatility of the hardware. This method will help to increase the success rate of future cryo-EM approaches.

References

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D22 Chromatography Principles and ÄKTA™ chromatography systems

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Chromatography is a powerful method that allows users to separate biomolecules according to their differences in specific properties.

In this practical course you will learn more about the different techniques that are commonly used today for protein purification, as well as participate in a live demonstration of an affinity chromatography (AC) purification method using a modern FPLC ÄKTA™ system.

The main objective of this course will be to learn about the different components of ÄKTA™ systems, how they work, and what techniques can be implemented by the user depending on the biomolecules' characteristics and the equipment at hand. Sample preparation will be also reviewed during this course and its importance in prolonging column lifetime and obtaining better results.

Cytiva is a global provider of technologies and services that advance and accelerate the development and manufacture of therapeutics. We have a rich heritage tracing back hundreds of years. Our customers undertake life-saving activities ranging from fundamental biological research to developing innovative vaccines, biologic drugs, and novel cell and gene therapies. Our job is to supply the tools and services they need to work better, faster and safer, leading to better patient outcomes.

Join our team of curious experts to discuss and learn together at every step of the process, seeing the whole picture, understanding both the science and the application.

D23 Crystal fishing and mounting

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Proteomics Lead. BioServUK / Molecular Dimensions / Protein Ark
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This demo will allow students to learn the essential technique of fishing crystals from drops and transferring them to a cryo-solution.

Posters

P01 (Further) Development of lead structures for the inhibition of the class-II-chaperone IpgC in Shigella

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Shigellosis, an infection of the large intestine, is caused by bacteria of the genus Shigella. These bacteria possess the class-II chaperone IpgC, whose interactions with a number of pathogenicity factors are prerequisite for virulence. Therefore, we have chosen IpgC as a target for drug design. So far, we determined a high resolution crystal structure of IpgC, which provided the basis for crystallographic fragment screening. Subsequently, fragments identified as binders were enlarged via in-silico docking. Three of the resulting molecules could be crystallographically validated as IpgC ligands. In the current work, these compounds will serve as lead structures for further design. To analyse the interactions of IpgC and its substrates the recombinant production and purification of those proteins is required.

P02 Application of advanced crystallization techniques on naughty proteins and their complexes

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When the conventional vapor-diffusion techniques of protein crystallization do not yield crystals of sufficient quality, it does not need to lead to hopelessness as well. Instead, we can apply advanced crystallization techniques such as capillary counter-diffusion and micro-seed matrix screening. We applied these methods to problematic proteins whose crystallization by basic methods was not successful. We selected proteins of our interest, whose revealed structure would be beneficial in addition to their use in validation of the advanced crystallization methods as tools for proteins resisting crystallization by more conventional crystallization methods. The protein targets specifically are:

1. Medicinally relevant human enzymes: purine nucleoside phosphorylases (PNP) in complex with inhibitors and cyclic GMP-AMP synthase (cGAS)
2. A representative of generally difficult-to-crystallize protein-DNA complexes: lactate-utilization repressor (LutR) from *Bacillus subtilis* in the complex with its DNA operator.

Poster will present the results we have achieved in this still-going project.

The project was supported by Mobility Plus project number of Czech Academy of Sciences (CSIC-22-02) and the i-LINK 2021 program of the Spanish National Research Council (CSIC) LINKC20027.

P03 *Arabidopsis thaliana* Aldo-keto-reductases 4C (AKR4C): a mostly uncharacterized family of proteins expressed, purified and successfully crystallized thanks to the SUMO tag system

Giuseppe Gabellini[1] Ginevra Marie Eloise Peppi[2] Lauren Velie[3] Patrick Treffon[3] Elizabeth Vierling[3] Mirko Zaffagnini[2] and Simona Fermani[1]

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Protein characterization has always been a challenge in the world of physiology given the sheer number of proteins involved in metabolism, especially the main key issues have always been the yield and solubility/stability required to perform adequate structural analysis. For this reason there are optimized expression and purification systems that involve the use of large TAGs covalently bound to the protein of interest (1). One such system is the SUMO system, which involves binding of a modified SUMO1 (small ubiquitin like modifier 1), a small eukaryotic protein with a cell signaling function, to the end of the protein of interest to increase its expression yield as well as its solubility in vitro. Although the advantages of this system have been extensively proven for the yield, it has also been surmised that, given the special chemical/physical properties of SUMO, TAG SUMO system can largely promote crystallization of the protein of interest, although this has never been proven (2).

In this work, we were able to express, purify and obtain crystals of an entire subfamily of Aldo keto reductases (AKRs) from *Arabidopsis thaliana*, proteins present in a large number of isoforms involved in the metabolism of toxic substances and with potential biotechnological applications, but almost uncharacterized in plants due to structural instability in vitro and the lack of obvious phenotypes in vivo (3). The subfamily in question is subfamily 4C comprising members AKR4C8, AKR4C9, AKR4C10 and AKR4C11, which were expressed as fusion proteins with SUMO. All 4 proteins were successfully crystallized using a single condition giving in return very similar crystals in shape and size despite primary sequence homology being around 70 percent. For AKR4C8 and AKR4C9 there are already SUMO-free structures obtained by another research group in which the crystallization conditions are different, in accordance with the different amino acid composition and different chemical and physical properties. Screening on two other AKRs fused to SUMO, hsAKR1A1 and AtAKR4B9, whose overall homology is around 40 percent for both did not yield crystals under the same conditions as AKR4C. Our hypothesis is that SUMO is able to compensate for differences between proteins by acting on fundamental properties such as isoelectric point by tending to equalize crystallization conditions, limiting the influence of variability due to differences in primary structure but not deleting them out. These preliminary data suggest that the SUMO purification system can be considered to optimize not only the purification conditions but also the subsequent obtaining of crystals, facilitating the study of large and poorly characterized protein families.

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P04 Arsenite oxidase interaction with substrates and partners: a structural and biophysical characterization

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Water contamination with arsenic (As) presents a global threat both to the environment and to public health. A study published in Science (1) identified that up to 220 million people are at risk thanks to groundwater consumption that exceeds the recommended WHO maximum value of 10 µg/L for As (2). The arsenite oxidase (Aio) (3-5), a complex metalloenzyme, is a good candidate to be used as a biosensor and in remediation since it oxidizes arsenite (As^{III}) and antimonite (Sb^{III}) (6), to less toxic and easier to remove species (As^{V}/Sb^{V}).

Our goal is to unravel Aio's catalytic mechanism and characterize its interaction with the electron acceptor partners – either cytochrome c552 or azurin – paving the way to develop new bioengineered solutions simultaneously effective, clean, and economically sustainable (7).

Only Aio's from P*seudorhizobium banfieldiae* NT-26 (NT-26 Aio) and *Alcaligenes faecalis* (*Af* Aio) have had their X-ray structures determined (PDB IDs: 4AAY, 5NQD and 1G8K/1G8J (3-5)); these correspond to ligand-free forms, revealing very little regarding the catalytic reaction. We obtained 4 high-resolution structures (up to 1.44 Å) for complexes of NT-26 and *Af* Aio with As/Sb oxyanions bound to the active site that allowed us to revisit the catalytic mechanism of AsIII oxidation. Additionally, we determined the dissociation constants of Aio with both physiological binding partners, using microscale thermophoresis and NMR spectroscopy assays are currently being conducted for the identification of the amino acids involved in electron transfer and interaction between *Af* Aio and azurin.

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P05 Assay development for JAK2 inhibitor screening and identification of allosteric inhibitors

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Janus kinase 2 (JAK2) mediates cytokine signaling via the JAK-STAT pathway regulating many cellular functions, including immunity. Several mutations have been identified in JAK2, leading to serious diseases such as cancer, highlighting the need for specific JAK2 inhibitors. Currently, there are few homogenous activity-based inhibitor screening assays for JAK2, emphasizing the need for a straightforward and affordable assay for JAK2 inhibitor discovery.

We optimized a general kinase activity assay for JAK2 inhibitor screening. The assay measures ADP, the universal product of kinase reactions, via enzyme-coupled reactions leading to the production of fluorescent resorufin. Measuring the fluorescence intensity of resorufin facilitates the direct detection of the amount of ADP produced, and therefore, the quantification of kinase activity. The assay performs well with a Z' value of 0.87 and a signal-to-background of 7.4.

The optimized assay was used for compound screening of JAK2 inhibitors. The hits included both allosteric and ATP-competitive compounds, based on in vitro experiments. Strikingly, the majority of the hits were allosteric, which is exciting considering that there have not been any reliably characterized allosteric JAK2 inhibitors to date. Identifying this type of inhibitor would be of great benefit for developing better treatments for JAK2 mediated blood cancers.

X-ray crystallography is used for determining the binding modes of these hit compounds. The structures obtained will provide information on the presumed allosteric pockets, allowing for their structure-based characterization and utilization in inhibitor development.

P06 Biophysical and Structural Characterization of HR1-HR2 Interaction of the SARS-CoV-2 Spike Protein Using Mimetic Recombinant Constructs

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The COVID-19 pandemic has been recognized as the most significant global outbreak in recent decades. While several vaccines have been developed and administered worldwide, they face persistent challenges such as declining immunity and emergence of new viral strains that can evade them. To combat these obstacles, it is crucial to develop new antiviral treatments and vaccines that target highly conserved regions of the virus. One promising therapeutic approach against SARS-CoV-2 is to disrupt the membrane fusion mechanism that enables the virus to enter the cell. This process is promoted by the Spike (S) protein, which is a trimer of heterodimers (with S1 and S2 subunits), and involves the interaction between two highly conserved heptad-repeat regions (HR1 and HR2) of S2 to form a 6-helix bundle (6HB) structure. Our previous research with HIV-1, a virus that shares a similar fusion mechanism with SARS-CoV-2, has demonstrated that single-chain chimeric proteins that mimic the HR1 region of the gp41 subunit, functionally equivalent to SARS-CoV-2 S2 subunit, can bind strongly HR2 and constitute potent fusion inhibitors [1].

In this work, we describe the design, engineering, and biophysical characterization of a series of single-chain proteins (named CoVS-HR1) that imitate the HR1 region in its trimeric coiled-coil conformation. While a first generation of proteins folded in-helical structures as expected, they tended to oligomerize and some of them showed low stability [2], a second generation of re-engineered proteins showed much better biophysical characteristics in terms of structure, stability and oligomerization state and allowed a detailed study of their binding to their complementary HR2 peptides [3].

Finally, we present the high-resolution crystallographic structure of the complex between a CoVS-HR1 protein and an HR2 peptide, which allowed a deep analysis of the interaction and highlighted the most implicated residues. It also allowed us to identify several interfacial water molecules and three mayor water-filled cavities within the HR1 structure, which could have an interesting role in the conformational changes that this region undergoes during the virus fusion. All these results aid in understanding the HR1-HR2 interaction and show the feasibility of our approach to design protein mimics of the SARS-CoV-2 Spike HR1 region, with great potential as fusion inhibitors.

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P07 Catalytic process of Anhydro-N-Acetylmuramic Acid kinase (AnmK) from Pseudomonas aeruginosa

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The cell envelope of bacteria is the structure with which the bacterium both engages with, and is protected from, its environment. Within this envelope for many bacteria and notably, for many of the most virulent bacterial pathogens such as *Pseudomonas aeruginosa*—is a peptidoglycan polymer conferring both shape and strength to the cell envelope. The enzymatic processes that build, remodel, and recycle the chemical components of this cross-linked polymer are preeminent targets of antibiotics, and exploratory targets for emerging antibiotic structures. We report comprehensive kinetic and structural analyses for one such enzyme, the P. aeruginosa anhydro-Nacetylmuramic acid (anhNAM) kinase (AnmK). AnmK is an important enzyme in the peptidoglycan-recycling pathway of this pathogen. It catalyzes the remarkable pairing of hydrolytic ring-opening of the anhNAM with concomitant ATP-dependent phosphoryl transfer. Crystallographic analyses demonstrate that both substrates enter the active site independently in an open conformation of the substrate subsites, with protein loops acting as gates for anhNAM binding. A remarkable X-ray structure for the dimeric AnmK sheds light on the pre-catalytic and post-catalytic ternary complexes, one in each subunit. Computational simulations in conjunction with the four highresolution X-ray structures reveal the full catalytic cycle.

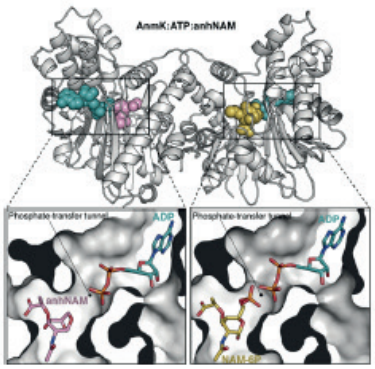

Figure 1. Crystal structure of the AnmK:ATP:anhNAM homodimer complex. The left panel shows the active site in monomer A with bound substrate anhNAM and bound product ADP. The right panel shows the active site in the monomer B with both products of catalysis (ADP and NAM-6P). All ligands are shown as capped sticks.

P08 Cathepsin proteases from *Schistosoma mansoni parasite* as drug target

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Schistosomiasis, a parasitic disease caused by blood flukes of the genus Schistosoma, is a global health problem with over 240 million people infected. Treatment relies on just one drug, and new therapies are needed. In the schistosome gut, a cascade of cathepsin-type proteases is responsible digesting host blood proteins. Our research aims at the structural analysis of these proteases and the rational design of their inhibitors as new drug leads. The project is focused on cathepsins L and B, which are involved in the early and late stages of the digestive cascade in Schistosoma mansoni, respectively. As a proof of principle, we identified several peptidomimetic reactive inhibitors of cathepsin B (SmCB1) with low nanomolar potency and solved the high-resolution crystal structures of their complexes with SmCB1. These inhibitors were successfully tested as antischistosomal compounds, thus validating SmCB1 as a drug target. We continue to investigate three isoenzymes of cathepsin L (SmCL1, SmCL2, and SmCL3) and prepared their fully active recombinant forms, which provide a starting point for ongoing comparative studies of active site structure and inhibitory specificity

P09 Challenges in the crystallization process of two acidic fungal unspecific peroxygenases

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Fungal unspecific peroxygenases, UPOs, (EC 1.11.2.1) have been studied recently as potential biocatalysts due to their dual peroxidative and peroxygenative activity and their high stereoselectivity, essential features in chemical and pharmaceutical industries. Following our previous work on the crystallization of laboratory-evolved peroxygenases, we have determined the crystallographic structure of two unusual acidic long unspecific peroxygenases. After several unsuccessful attempts to crystallize the deglycosylated form of the enzymes, both peroxygenases have been crystallized with a high glycosylation level that has caused an important disorder in the crystal structure.

In order to improve the quality of crystals, we have made use of a wide variety of additives that have improved the aspect and internal order of the crystals in one of the peroxygenases. However, the improvement of the crystal quality in the other UPO continues being a challenge to be solved since elucidating the molecular basis and the mechanisms of these enzymes is our main goal. For that purpose, high-quality crystals are required to perform soaking experiments and obtain complexes with ligands of interest.

P10 Characterization of Fragile X Messenger Ribonucleoprotein 1, a protein involved in Fragile X Syndrome, and its pathological mutants

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Fragile X syndrome (FXS) is an inherited genetic condition and is a major form of intellectual disability. It is generally related to the expansion of the CGG trinucleotide repeat in the 5′ untranslated noncoding region of the FMR1 gene, which encodes for Fragile X messenger ribonucleoprotein 1 (FMRP), leading to hypermethylation of the gene, its transcriptional silencing, and thus loss of FMRP [1].
FMRP is an RNA-binding protein involved in cognitive development and plays a key role in RNA metabolism, and synaptic plasticity; however, the molecular mechanisms by which it operates remain largely elusive.
FMRP is organized into three different regions: the N-Terminus contains two Tudor Domains, one nuclear localization signal (NSL), and a K-homology domain (KH0); the central region consists of two KH domains (KH1 and KH2); the C-Terminus domain presents an intrinsically disordered region containing arginine- and glycine-rich (RGG) motifs [3]. However, the 3D structure of the entire protein is still lacking.
The goal of my project is to crystallize the full-length protein, solve its structure and characterize the KH single domains, which are essential for the function of FMRP. Interestingly, the literature has recently reported FXS-like phenotype attributable to X-Fragile due to single point mutations in KH domains [4].
Among the missense mutations, the G266E substitution is found in KH1 of FMRP.
Therefore, it was chosen to study the KH1 domain and its pathogenic mutation G266E to understand their relevance in pathology by performing Circular Dichroism experiments, SAXS analysis, and Molecular Dynamics simulations. Our preliminary data show a marked difference between the WT and the mutant suggesting that the mutation may have significantly altered the secondary structure.
Therefore, given its many functions and its implication in FXS, FMRP represents an interesting object of study.

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P11 Combining X-ray cryo-crystallography and in crystallo microspectrophotometry to study light-activation in a B12-dependent photoreceptor

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The newly discovered family of B_{12}-dependent photoreceptors repurpose vitamin B12 derivatives from enzymatic cofactors as light sensors [1]. CarH is a member of this family [2], acting as a bacterial transcription regulator that controls the light-dependent biosynthesis of carotenoids. The conformational changes upon photoexcitation start with the cleavage of the cofactor's upper axial ligand (5'-deoxyadenosyl group). This leads to the modification of the protein's oligomeric state, from a DNA-bound tetramer to free monomers, thus allowing gene transcription. We investigated CarH dark and light states by collecting cryo-crystallographic X-ray and online in *crystallo* absorption spectroscopy data at the FIP2 beamline of the ESRF synchrotron. Structural and spectroscopic changes are shown to occur upon light-illumination at 100 K.

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P12 Cross-linked protein crystals doped with metal nanoparticles as sustained antibacterial

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The accelerating prevalence of antimicrobial resistance is challenge the global healthcare system (1). The overuse and misuse of antibiotics have resulted in multi-drug resistance pathogens leading the world to a pre-antibiotic era (2,3).

To tackle severe infections, novel therapeutics as well as novel delivery systems are needed. Hence, the objective of this work is to develop an innovative drug delivery vehicle able to tackle multidrug-resistant bacterial infections. We have created novel cross-linked lysozyme composite crystals containing metal nanoparticles (MNPs), i.e. silver or gold. These protein crystals will behave as delivery vehicles able to tackle infections by their chemical composition (lysozyme and nanoparticles) and by the rate of release at which these two components are liberated in the affected area. The rate of release would be controlled by the crosslinking degree of the crystals. We anticipate that these synergistic antimicrobial particles can be an excellent strategy to combat biofilms formation.

To obtain this material we followed a bottom-up protocol in which MNPs were firstly obtained in a gelled that was subsequently used as crystallization media to obtaining lysozyme (Lzm) crystals. Composite Lzm@MNP crystals were later crosslinked at different degrees to control their dissolution rate.

Herein, we present a proof of concept of a novel active compounds delivery vehicle in which the vehicle and their cargo has an active and remedial role. This strategy can be easily extended to other proteins and cargos to treat other diseases, to protect goods, etc.

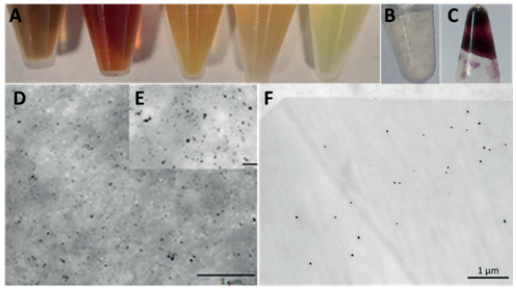

Fig 1. A) AgNPs grown in situ in agarose gel. Concentration of NaBH4 decreases from left to right. B) Lzm crystals grown in agarose with AgNPs. C) AuNPs grown in situ in Fmoc-FF hydrogel. D) TEM image of AgNPs inside gel. E) The insert shows a closer view of AgNPs dispersion within the lysozyme crystal. F) TEM image of AuNPs inside lzm crystals.

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[2] Kutta, R. J., S. J. O. Hardman, L. O. Johannissen, B. Bellina, H. L. Messiha, J. M. Ortiz-Guerrero, M. Elias-Arnanz, S. Padmanabhan, P. Barran, N. S. Scrutton and A. R. Jones (2015). "The photochemical mechanism of a B12-dependent photoreceptor protein." Nat Commun 6: 7907.

P13 Crystal based lead identification by fast fragment and compound screening at the Swiss Light Source

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Over the last two decades, fragment-based drug discovery has emerged as an effective and efficient method to identify chemical scaffolds for the development of novel lead compounds. The inherent "start small – elaborate efficiently" approach allows to address issues like compound-selectivity, -toxicity and -efficiency from a very early development state on, while saving time and resources compared to classical high throughput screening of larger compounds. X-ray crystallography has been used for a long time as an important orthogonal method for validating binders discovered by higher throughput screening methods and to elucidate corresponding ligand-target interactions. Advances in beamline- and crystal-harvesting-instrumentation have tremendously increased the throughput of X-ray crystallography in the last few years. This has facilitated the establishment of macromolecular crystallography as a powerful primary screening method for the identification of ligand binding. Combining fragment screening with the high information content derived from X-ray crystallography results in a powerful platform for structure-based drug discovery. The involved crystals system is the heart of each CBFS campaign and the handling required to achieve reliable nucleation and stable, comparable soaking conditions in a high throughput setup often demands preceding optimization of crystals and soaking conditions alike. Here, we proudly present the Fast Fragment and Compound Screening pipeline (FFCS)1 at the Swiss Light Source (PSI, Switzerland) and discuss the difficulties of crystal handling in a crystal based fragment screening campaign.

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P14 Crystal structure of smolstatin – protease inhibitor from the myxozoan parasite sphaerospora molnari

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The cystatin superfamily is a large group of cysteine protease inhibitors (including type 1 cystatins – stefins, type 2 cystatins – true cystatins and type 3 cystatins – kininogens), present in various organisms. Parasite cystatins are involved in the active parasitism in the host by supressing host immune responses. Thus, cystatins are critical for the interactions between host and parasite during the infection [1, 2].Here, we structurally characterized stefin Smolstatin from Sphaerospora molnari, which is a myxozoan parasite of common carp Cyprinus carpio. From evolutionary point of view, myxozoans are parasites that stand at the base of the metazoan evolution, therefore structural information can bring insights into the evolution of the cystatin superfamily as well as it can contribute to the aquaculture field for studying host- parasite interactions [3]. Smolstatin is a 13.5 kDa large single domain protein, which consists of typical cystatin-like domain, but unlikely for stefins, it also carries a signal peptide.Smolstatin was recombinantly produced, purified and crystallized using sitting drop vapour diffusion technique. Diffraction data were collected on BL14.1 at the BESSY II electron storage ring operated by the Helmholtz-Zentrum Berlin [4]. Smolstatin crystallized as a domain swapped dimer. The crystal structure was determined by molecular replacement, refined and deposited to the PDB database under the accession code 8and.

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P15 Crystallization of Cancer-related Drug Target Carbonic Anhydrase IX with Sulfonamide-based Inhibitor

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During tumor development cancer cells must cope with several forms of stress, such as hypoxia-related glycolytic metabolism resulting in high concentration of protons and lactate, and consequent low pH. Overcoming this difficulty is provided by overexpression of carbonic anhydrase IX (CA IX) in various types of solid tumors.

CA IX is a member of α-CA metalloenzymes family with activity of reversible hydration of CO2 to HCO3- and H+. These sequentially highly homologous enzymes share typical antiparallel β-sheet fold of catalytic domain. The activity of CAs is efficiently inhibited by the molecules containing a sulphonamide group, that is based on the electrostatic coordination to Zn2+ ion in the active-site cavity. Comparably to some other isoenzymes CA IX forms dimer on cytoplasmic membrane, on the contrary CA IX possess unique proteoglycan-like (PG) domain. Until now, only structure of CA IX catalytic domain was determined, no structural information on PG domain is available. Structural characterization of whole CA IX, however, is crucial for understanding CA IX function and for finding inhibitors that would be isoformspecific.

We expressed and purified recombinant CA IX consisting of both catalytic and PG domain with various mutations that enable to obtain protein in sufficient amount and purity for crystallography experiments. Initial crystallization screening was performed via robotic system and commercial conditions sets (JCSG-plus, PEGs Suite and Morpheus) with the addition of the sulfonamide-based inhibitor. Initial crystals were plates or sea urchins at best. Thus, various types of optimization experiments were carried out, namely, change of protein buffer solution, lower concentration of protein or precipitant, additive screen, seeding, as well as different vapor diffusion technique set-ups with various protein-precipitant ratios. Further optimization of crystal quality is now in progress. In parallel we aim to employ other biophysical experimental methods, including NMR to gain structural information on CA IX PG domain.

P16 Crystallization of metal-dna-based nanomaterials

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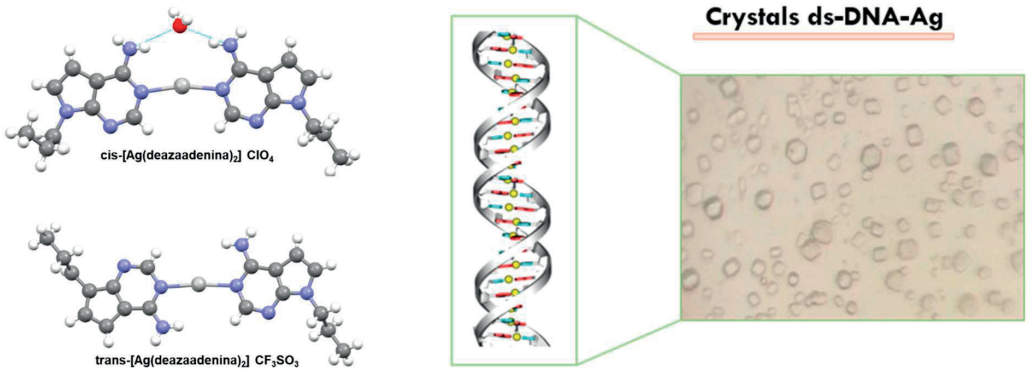
Keywords: DNA, metal ions, crystals, nanostructures, nucleobases.

DNA has become an important tool in nanoscience and nanotechnology. The possibility of programming specific sequences to self-assemble in customized patterns makes it an ideal candidate for building new nanomaterials. Many efforts have recently focused on the structural modifications of nucleic acids to provide unique properties. In this area, the metallization of DNA has been demonstrated to offer many possibilities due to the various properties metal ions can provide. However, understanding the structure of the metal-DNA system is crucial to determining the relationship between structure and properties. In this regard, the crystallization of metallized DNA molecules is an important area of research to understand the design of this new material. Although obtaining crystals of metal-DNA molecules can be an ambitious challenge, some silver-DNA systems have been recently crystallized and fully characterized. [1]

Our group works on developing unique metal-DNA systems with silver-modified Watson-Crick base pairs, where a coordination bond replaces canonical hydrogen bonds.[2] We also aim to prepare metal-DNA systems using metal complexes complementary to the nucleobases to prepare highly customizable assembly.[3] This communication presents our results on crystallizing isolated metalmodified base pairs (Figure, left), as well as crystallizing double-stranded Ag-DNA molecules (Figure, right) and single-stranded DNA-Pd-complex systems. We have used conventional crystallization methodologies, including slow evaporation, solvent diffusion, and more subtle hanging drop and sitting drop procedures.



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P17 Detection of Semiquinones in Ferulic Acid Decarboxylase

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With the effects of climate change becoming more apparent, the need to mitigate increasing quantities of greenhouse gas emissions is crucial. Biofuels are a viable option in achieving net-zero emissions.[1] To obtain biofuels, fatty acids may be converted to alkanes or alkenes through decarboxylation. When co-expressed for UbiX, UbiD/ferulic acid decarboxylases (Fdc) bind prenylated flavin mononucleotide (prFMN) which is used to decarboxylate sorbic acid, a common food preservative.[2] However, the enzyme becomes catalytically inactive when illuminated with UV light.[3] UbiD/Fdc enzymes bind flavin mononucleotide (FMN) similarly to prFMN when singly expressed. could perform light-driven decarboxylation using flavin mononucleotide (FMN). Therefore, the redox properties of UbiD-FMN complexes were studied. The bound FMN was reduced with solutions of sodium dithionite and monitored using UV-Vis spectroscopy. All complexes were successfully reduced; however, reduction of FMN bound to variants of Fdc1 found long-lived anionic and neutral semiquinones.

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P18 Discovery of allosteric JAK2 inhibitors

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Protein-protein interactions are important in cellular signal transduction, and structural information about these interactions is also crucial for understanding how cellular machinery function. Oncogenic gain-of-function mutation V617F is found within the pseudokinase domain (JH2) of JAK2 kinase, which causes hyperactivity of the adjacent catalytic kinase domain (JH1). This mutation causes the majority of all myeloproliferative neoplasms (MPNs) and is found also in several leukemias. We combine both computational and experimental methods to screen small molecules inhibitors against JAK2 pseudokinase domain to identify novel compounds that specifically inhibit the hyperactivation of V617F-induced JAK2. We have developed a biolayer interferometry assay for inhibitor screening against JAK2 pseudokinase domain and utilized it to screen small molecule compounds. We have also evaluated the compounds in human myeloproliferative cell lines carriers of JAK2-V617F mutation for their inhibition of JAK-STAT pathway and cell viability. Crystallographic analysis is on-going to determine the exact inhibitory mechanism of the compounds. These results will contribute to the understanding on how JAK2 pathogenic mutation driven hyperactivation occur, contributing to the optimal design of highly selective compounds with novel binding modes (targeting alternative sites to the ATP-pocket of the kinase domain) and mechanisms-of-action, with disease-course modifying properties. Thus, with relevant pharmacological and direct translational impact.

P20 Eu3+ and Tb3+doped apatite nanoparticles prepared by hydrothermal transformation of oyster shells calcium carbonate. Solid-state, luminescence, and in vitro biological characterization

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Oyster shell wastes from the fishing industry represent a major environmental problem, as 7 million Tm/year are produced with no efficient recycling [1]. They are composed of Mg-calcite embedded in an organic matrix formed of proteins and polysaccharides. On the other hand, nanocrystalline apatites (NAp), which are structural and compositional analogues to the major inorganic component of human bones, exhibit excellent bioactivity and biocompatibility, and present a broad range of applications in hard tissue engineering, biomedicine, drug-delivery system and even in analytical sciences [2].

In this work, biogenic calcite from oyster shells was used to obtain apatite NPs by a "one-pot" hydrothermal process. Eu^{3+} and Tb^{3+} ions were added to the formulation to obtain Eu^{3+}- and Tb^{3+}-doped apatite NPs with luminescent properties. Solid-state characterization of the nanoparticles was performed by powder X-ray diffraction (PXRD), FT-IR and Raman spectroscopy, SEM and TEM microscopy, dynamic light scattering (DLS) and inductively coupled plasma (ICP) spectrometry, while luminescence properties were evaluated by luminescent spectroscopy. Finally, the biological behaviour of the NPs was evaluated by cytotoxicity and osteogenic differentiation assays, in murine mesenchymal (M17.1) and murine endothelial (MS-1) cells. Results demonstrated the efficiency of this novel "one pot" hydrothermal process to obtain nanoparticles of apatite with improved luminescent properties, while, in vitro assays demonstrated the excellent cytocompatibility and the impact of the metal-doping over the osteogenic differentiation capacity of the nanoparticles.

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P21 Hydrothermally Synthesized Calcium Phosphate Micro-Nanoparticles from Eggshell Calcite for Biomedical Applications

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Eggshell (ES) is a biomaterial mainly composed of an inorganic component (CaCO3, calcite phase) and an organic matrix. This material is mostly discarded during food processing cycles. Hydrothermal processing can transform them into calcium phosphate (CaP) salts for industrial and technological applications. This research aims to study the effect of the ES organic matrix during the mineral replacement of CaCO3 into CaP. Additionally, this research explores the biological properties of the resulting CaP particles to determine their potential use for biomedical applications. ES pieces were collected and divided into two groups: untreated ES and ES treated with NaClO for selective removal of the organic component of the material. Subsequently, the ES were milled and sieved into different sizes (50-200 μm). The particles were then subjected to hydrothermal treatment using phosphate-bearing solutions at varying temperatures (100°C-200°C) for 7 days. The obtained precipitates were analyzed using scanning electron microscopy (SEM), spectroscopic (ATR-FTIR and Raman), and X-ray diffraction (XRD) techniques. Biological tests were also conducted to evaluate their cytocompatibility on MG-63 human osteosarcoma tumor cells and m17.ASC murine mesenchymal stem cells, as well as the osteogenic differentiation of m17.ASC cells. The CaP particles presented crystallographic and morphological characteristics compatible mainly with the apatite. This conversion occurred differently for the CaCO3 particles in which the organic matrix was preserved, presenting a mineral deposit with hexagonal and nanostructured crystals. Spectroscopic and X-ray diffraction analyses evidenced the control of the organic matrix in the conversion of the ES particles to CaP. The pristine and treated particles were cytocompatible on MG-63 and m17.ASC cells and promoted osteogenic differentiation of m17.ASC cells. In conclusion, the hydrothermal technique allows the conversion of ES particles into CaP phases. The retained organic matrix of the CaCO3 particles mainly affects the morphological properties of the resulting CaP particles and could be useful in creating microstructured biomaterials.

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P22 Investigating the Conformational Flexibility of the gp41 Hydrophobic Pocket through Crystallography and Molecular Dynamics Simulations

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Gp41 protein from the HIV-1 virus is ultimately responsible for viral infection and contains a conserved hydrophobic pocket that is a promising target for small-molecule inhibitors to prevent viral fusion and host cell entry. The conformational flexibility of this pocket and its potential as a drug target were investigated using a crystallographic structure of a protein derived from gp41, called covNHR, that was rationally designed and recombinantly produced in E. coli, with specific mutations to enhance its stability and solubility for structural studies. The protein was successfully expressed, purified and crystallized for X-ray diffraction analysis, revealing a detailed three-dimensional structure of the hydrophobic pocket. Molecular dynamics experiments were also conducted using the crystal structure to explore the dynamics and flexibility of the pocket over time as well as in complex with its target peptide. The results demonstrate significant flexibility of the pocket and its ability to adopt different conformations, indicating that it may be amenable to modulation by small molecules. Key residues were identified within the pocket that stabilize its conformation and interactions with ligands. These findings provide valuable insights into the molecular basis of viral fusion and may pave the way for the development of new strategies to design small-molecule inhibitors targeting the gp41 hydrophobic pocket. The study highlights the potential of rationally-designed proteins, crystallography, and molecular dynamics simulations to advance our understanding of the mechanism underlying gp41 function and drug discovery of inhibitor directed against HIV-1 targeting gp41.

P23 Investigation of proteinaceous inhibition of M. tuberculosis dUTPase

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The dUTPase enzyme plays a key role in the maintenance of genomic integrity in various species by preventing the uracil incorporation into DNA. The decreased activity of the enzyme can indirectly lead to DNA double-strand brakes and to cell death (1). The design of species-specific dUTPase inhibitor could help in defence against human pathogens, like Mycobacterium tuberculosis, as the emergence of multidrug-resistant strains causes an increasing problem in the treatment of tuberculosis (2). A known dUTPase inhibitor, the staphylococcal protein Stl strongly inhibits the Mycobacterium tuberculosis dUTPase (MtDUT) (3,4). The understanding of their interaction may help in the design of efficient MtDUT inhibitors.

For this reason, we have crystallized the complex of MtDUT and a truncated Stl protein variant, Stl1-159 and obtained an X-ray dataset at Elettra Trieste with 3.4 Å resolution. Bio-layer interferometry measurements were performed in favour of the analysis of the protein-protein binding kinetics. In order to examine whether shorter, truncated versions of Stl possess a more effective inhibitory effect on MtDUT activity than the full-length protein, we tested the effect of three truncated versions of Stl by steady-state activity measurements.

According to the result of the Bio-layer interferometry and steady-state activity measurements, the full-length Stl protein possesses the highest affinity to MtDUT and has the most effective inhibitory effect on dUTPase activity. The structural model of the Stl1-159-MtDUT complex reveals the main amino acid residues involved in the interaction. However, there may still be unknown interacting residues on the C-terminal region of Stl, which contribute to the formation of the strong complex explaining the most effective inhibition of MtDUT.

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P24 Lipolytic system of the hard tick Ixodes ricinus

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Ticks are obligatory blood-feeding ectoparasites capable of transmitting a wide variety of pathogens comprising bacteria, viruses, and protozoa to humans and animals. After mosquitoes, ticks are the second most dangerous vectors of arthropod-borne diseases. Hard tick *Ixodes ricinus* is a typical representative of the 3-host tick and its life cycle comprises three life stages. Adult ticks are capable to imbibe and digest huge amounts of host blood exceeding hundred times their unfed weight.

Physiology, development, reproduction, and vectorial capacity of ticks depend entirely on the ingestion and processing of host blood as the sole source of nutrients. In contrast to the relatively well-studied digestion of proteins, almost nothing is known about the uptake and utilization of host lipids. Several genes encoding lipolytic enzymes (lipases and hydrolases) have been identified in the transcriptomes of the midgut of our model tick, I. *ricinus*. However, identification of the corresponding proteins by classical proteomic approaches is hindered by the presence of other highly abundant proteins.

To deeply investigate lipolytic system in the I. *ricinus* ticks, label-free quantitative (LFQ) proteomics extended with activity-based proteomics and lipase activity assays were carried out. Midgut homogenates from different feeding time points (unfed, fed for 3 and 5 days, and fully fed) were labeled with C6 activity based probe and active hydrolases were isolated using streptavidin-agarose beads. On-bead digestion using trypsin was followed by the peptide analysis carried out on the timsTOF Pro (Bruker). LFQ proteomics was used to investigate the dynamic changes during the tick blood meal. Data were analyzed using Perseus and MaxQuant programs. For the measurement of total lipase activity (TLA), fluorescent substrate (4-Methylumbelliferyl oleate) was used. To the 100 µl of substrate, 100µl of midgut homogenates were added and consequently measured using Tecan Plate Reader at the fluorescence (excitation/emission wavelengths of 355/460) up to 30min, 37°C. For data evaluation Magellan software (Tecan) was used. Data (RFU/min) were related to the protein concentration and number of midguts used per each sample.

Our data revealed that TLA activity reaches its maxima in ticks that were feeding for five days on the host. More importantly, several hydrolases from different classes were identified during the tick blood meal. LFQ values showed different abundance of some hydrolases at different feeding timepoints. Our first-ever activity-based proteomics study of lipid hydrolases and study of TLA contribute to the limited understanding of lipid metabolism in ticks and holds promise for finding susceptible targets for effective interventions against ticks. Identified hydrolases will be further functionally investigated including the elucidating of tick lipases crystal structures.

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P25 Microorganisms transported in saharan dust plumes and deposited by red rain in Granada (Southern Spain)

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Dust storms are natural phenomena characteristic from arid and desert regions which act as sources of atmospheric dust. Suspended dust can be transported and distributed across the world provided the weather conditions are appropriate, being subsequently deposited in other continents by red rain or dry deposition [1].

Aerobiology is a branch of Biology which focuses on the study of airborne bioaerosols and namely, bacteria, fungal spores, small insects or pollen. The transport and spread of microorganisms bound to dust storms is remarkable, given that they are able to survive under extreme conditions such as UV radiation, desiccation or nutrients scarcity [2].

Nowadays, numerous works study the microbiota present in atmospheric dust using different methodologies and culture media [3, 4]. In recent years, we have carried out important studies on atmospheric phenomena related to Saharan dust intrusions in Granada basin (Southern Iberian Peninsula) and we have described "iberulites" as potential carriers of microorganisms and other living beings [5, 6].

In the present work, rainwater samples with significant amounts of Saharan dust have been collected in Granada after red rain episodes registered in the last years (2021-2022). Airborne microbes attached to dust particles through EPS have been observed using FESEM imaging. By means of 16S rRNA sequencing, 18 culturable microorganisms have been identified. In general, members of *Pseudomonadota and Bacillota* phyla have been mainly found, with a good representation of the genus *Bacillu*s. Species such as *Peribacillus frigoritolerans and Bacillus halotolerans*, both capable of surviving under extreme conditions [7], have been isolated in different events.

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P26 Molecular basis of pathogenic JAK2 activation in myeloproliferative neoplasms

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Janus kinase 2 (JAK2) mediates cellular signaling via type I and type II cytokine receptors but JAK2 is also activated by somatic mutations resulting in myeloproliferative neoplasms by still incompletely elucidated mechanisms. Quantitative super-resolution microscopy and FRET assays showed that erythropoietin receptors (EpoR) exist as monomers on the cell surface and they dimerize upon Epo stimulation or through predominant JAK2 mutations (V617F, K539L, R683S). JAK2 V617F showed stronger propensity for EpoR dimerization than the other mutants, and interestingly K539L and R683S, but not V617F showed increased in vitro kinase activity suggesting distinct activation mechanisms. Crystallographic analysis of the hyperactivating JAK2 pseudokinase mutants complemented by extensive molecular dynamics (MD) simulations revealed molecular basis of the different dimerization interfaces for JAK2 V617F and K539L. Furthermore, artificial intelligence-guided modeling and MD simulations revealed that the pseudokinase mutations impose differences in the pathogenically activated full-length JAK2 dimers. Structural insights of the molecular changes in mutation-driven JAK2 hyperactivation may open new possibilities in therapeutic targeting of pathogenic JAK2 signaling.

P27 Molecular Rotors to Probe Intermolecular Protein Interactions

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In protein solutions, the interplay between short-range attraction and long-range molecular repulsion leads to a complex phase behavior with the formation of transient dense liquid phases, equilibrium clusters, and kinetically arrested states. The role of liquid-liquid phase separation (LLPS) in crystal nucleation is still debated, and the high viscosity of dense phases is known to be a parameter limiting nucleation within the dense phase.

We propose a methodology based on fluorescence lifetime imaging microscopy (FLIM) to characterize protein interactions in solution and study the role of LLPS in crystal nucleation mechanisms. As phase transition implies local changes in solution structure, we propose to use environment-sensitive fluorophores, molecular rotors, which fluorescence lifetime correlates with local viscosity or free volume available in its immediate vicinity. We have studied the relation between protein-protein interactions and fluorescence lifetime in lysozyme solutions using Sulforhodamine-B (SRh-B). Fluorescence lifetime evolution showed a nonmonotonic trend at constant protein concentration (Fig.1A), with the increase of sodium chloride concentration. SAXS analyses revealed changes in solutions structure factors near the minima of lifetime curves, confirming that the observed tendency is associated with the transition from a repulsive to an attractive interaction regime.
We have analogously characterized the dense liquid phase formed upon lysozyme LLPS. Preliminary results have shown higher fluorescence lifetime and intensity inside the protein-rich droplets (Fig.1B), indicating a crowded environment for the rotor.

Rotor-protein interactions have additionally been studied using X-ray Crystallography and found to be non-specific. SAXS data has shown no changes in the structure factors of the protein solutions in presence or absence of SRh-B. Therefore, we propose fluorescence lifetime of molecular rotors as an indicative of protein interactions, and FLIM as a promising tool for characterizing transient phases in nucleation studies.

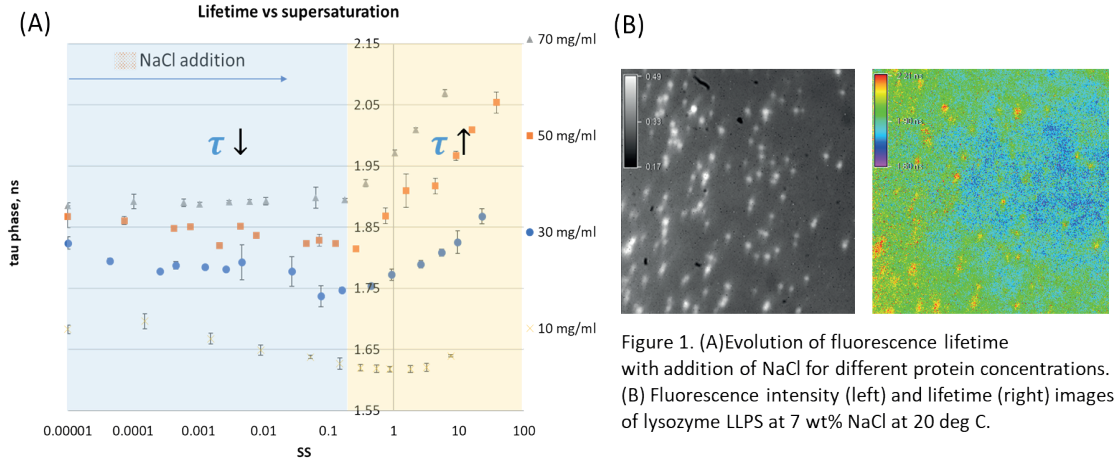

Figure 1. (A)Evolution of fluorescence lifetime with addition of NaCl for different protein concentrations. (B) Fluorescence intensity (left) and lifetime (right) images of lysozyme LLPS at 7 wt% NaCl at 20 deg C.

P28 Molecular, biochemical and structural characterization of secreted ferritin II from Ixodes ricinus

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Ferritin is a ubiquitous protein with a crucial role in tick biology. Ticks digest large amounts of host blood and are exposed to an enormous amount of free iron, which has to be treated properly to avoid its toxicity. Two types of ferritin were discovered in the tick Ixodes ricinus – tagged as ferritin I and ferritin II [1]. Ferritin I is a globular protein composing 24 subunits (25kDa each) and forming a hollow-sphere complex. [2; 3]. Ferritin I functions as an intracellular scavenger of potentially toxic free iron and is capable to sequester up to 4 500 iron atoms [2]. The function of ferritin II is not entirely clear, but it probably plays a role in the transport of non-heme iron between the tick gut and the peripheral tissues. Silencing of ferritin II using RNA interference had a detrimental effect on tick development and reproduction [1]. The vaccination of mammalian hosts with recombinant ferritin II revealed its promising potential as an efficient anti-tick vaccine [4].

This study focuses on the molecular, biochemical and structural characterization of ferritin II from Ixodes ricinus. We have cloned ferritin II into three E. coli expression vectors (pET100, pET-SUMO and pASK-37+), and optimized its production in various expression cells and conditions (e.g., temperature, times and concentrations of inducer). To obtain enough amount of pure recombinant ferritin II for following structural studies, we will concentrate on improving protein isolation and purification.

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P29 Nanocrystalline apatites doped with transition metals obtained by hydrothermal transformation of oyster shell waste

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Keywords: hydroxyapatite; calcium carbonate; recycling; magnesium; manganese; cobalt.

Waste seashells from the fishery industry represent an important environmental issue and, at the same time, a loss of potentially useful biomaterials [1]. They are biominerals made of $CaCO_3$ and an organic matrix (1-5 wt.%) composed mainly of proteins and polysaccharides. The fabrication of functional calcium phosphates such as doped nanoapatites, using seashells as a calcium source, could partially alleviate the ecological problem associated with this type of waste [2]. In this work, the one-pot hydrothermal method [2] has been used to obtain biocompatible Mg^{2+}-, $Mn2^{+}$-, and Co^{2+}-doped apatites since these metals have been shown to stimulate bone tissue regeneration [3-5]. The experiments were performed by heating aqueous suspensions composed of oyster shell CaCO3 particles (490 mM) plus 300 mM KH2PO4, in presence of 10 mM of metal (M= Mg^{2+}, Mn^{2+}, or Co^{2+}), at temperatures ranging from 25 ^{o}C to 200 ^{o}C. Full transformation of $CaCO_3$ particles was achieved at 160 ^{o}C, resulting in M-doped, platy-shaped apatite nanoparticles, and sizes within the range of 75-90 nm. When incubated with human mesenchymal stem cells for 1 and 3 days, all samples showed high cytocompatibility/biocompatibility, with only a small decrease in cell viability after 7 days of incubation at dose-dependent concentrations. Overall, this method shows promise in the preparation of biocompatible doped-apatite nanocrystals with osteogenic features, utilizing biogenic $CaCO_3$ as an unexplored calcium source for the preparation of apatite-based biomaterials.

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P30 Novel α-ketoamide inhibitors targeting fibroblast activation protein

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Fibroblast activation protein (FAP) is a membrane-bound serine protease that is highly upregulated at sites of tissue remodeling such as fibrosis and cancer. FAP overexpression in cancer-associated fibroblasts and other tumor-associated stromal cells promotes tumor progression, immunosuppression, and metastasis and is closely correlated with poor prognosis. While FAP is scarcely expressed in mammalian healthy adult tissue, it is overexpressed in a multitude of cancers, making it a robust target for cancer therapy. Thus, a great effort has been made to identify novel tools targeting FAP *in vivo*.

In our recent work [1], we conducted a structure-activity relationship (SAR) study to explore the chemical space beyond the scissile bond in the P1′ and P2′ positions and presented a new class of peptidomimetic inhibitors bearing an α-ketoamide warhead. Besides promising biological properties, the most potent FAP inhibitor IOCB22-AP446 (IC_{50} = 89 pM) showed an inhibition potency superior to that of the most potent inhibitor published to that date.

Nevertheless, the absence of a crystal structure of the FAP–inhibitor complex constitutes a major limitation to further rational design of optimized inhibitor structures. Therefore, we aim to establish a suitable protein expression platform and crystallize FAP in complex with selected α-ketoamide inhibitors from our SAR study. The resulting crystal structures will set the stage for future advances in the field of FAP-targeting strategies.

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P31 Optimizing and characterizing PBP2a and NQO1 microcrystals for protein dynamics studies by time-resolved serial crystallography experiments

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Serial crystallography at X-ray free electron lasers (XFELs) and more recently at synchrotron radiation sources, is still an emerging field for structural biology. This new field has opened the door to studying proteins that do not readily crystallize into large crystals but can crystallize as micro- and nanocrystals. One of its major impacts lies in the ability to reveal the structure of complex proteins previously inaccessible with classical crystallography at cryogenic temperatures and allowing time-resolved studies from femtoseconds to seconds. The nature of this serial technique requires new approaches for crystallization, sample delivery, and data analysis. Approaches to obtain macroscopic crystals have been perfected over decades and have become highly automated, but reliable methods for the preparation of (relatively) large quantities of nano- or microcrystals are still scarce. Also, by the nature of the serial approach, many protein crystals are delivered to the path of the XFEL, thus, there is a significant effort to optimize sample delivery methods to reduce sample waste and reduce the time required to collect sufficient data to fully characterize a protein structure. Therefore, the success of (time-resolved) serial crystallography experiments basically depend on two main factors: the ability to grow large amounts of well-ordered nano/microcrystals of homogeneous size distribution, and then the possibility to optimize the injection for saving sample.

Here, we will show how to get crystalline samples ready for a (time-resolved) serial crystallography experiment using the batch method. For that, we will use two therapeutically relevant proteins we are working with in our group: 1) the penicillin binding protein 2a (PBP2a) of methicillin-resistant *Staphilococcus aureus* (MRSA), a transpeptidase that catalyzes cell-wall crosslinking in the face of the challenge by β-lactam antibiotics; and 2) the human NAD(P)H quinone dehydrogenase 1 (NQO1), a flavoenzyme associated with several cardiovascular and neurodegenerative diseases and cancer. In addition, we will briefly show two of the sample delivery methods we have used successfully to deliver microcrystal samples of PBP2a and NQO1 in recent serial femtosecond crystallography (SFX) experiments at XFELs: 1) the 3D-printed modular droplet injection device developed by Alexandra Ros' team at Arizona State University, in which crystal slurries are encapsulated into oil droplets for an efficient and extremely low sample consumption; and 2) the double flow focusing nozzle developed by Dominik Oberthuer and co-workers.

We will also present the recent results obtained successfully for both proteins PBP2a and NQO1 at XFELs. In both cases, SFX experiments have been conducted for checking the quality of diffraction of the microcrystals and determined the room temperature structures of PBP2a at the EuXFEL (Hamburg, Germany) and the structures of apo-NQO1 at LCLS (CA, USA). We have also performed TR-SFX experiments from which we have solved, for the first time, the 3D structure of NQO1 in complex with NADH in EuXFEL. Our approach can be used to investigate crystalline samples for other proteins systems to be adapted to exploit novel scientific opportunities created by serial crystallography.

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P32 Room-temperature data collection at synchrotron: how to use protein crystals to unlock hidden conformational landscape

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Getting structural information from protein/ligand complex is one of the most efficient ways to get insights on the binding sites and modes and then further engineer optimized binders to produce therapeutic molecules. However, a single structure conformation identified at cryogenic temperatures may introduce a misleading structure as a result of cryogenic cooling artifacts. This can limit the overview of inherent protein physiological dynamics, which can play a critical role in protein biological functions, but also lead to non-biologically relevant ligand binding modes and hamper further drug development.

In order to avoid "true" structures, we facilitated room-temperature crystallography at SLS by implementing automated sample changing for efficient and reproducible data collection. We developed an ambient temperature X-ray crystallography method using temperature as a trigger to record movie-like structure snapshots on our model protein endothiapepsin (EP) [1]. Thanks to optimized EP crystals, we explored the discrepancies in ligand binding between the cryocooled and the physiological temperature structures, and captured multiple binding poses and their interplay with solvent molecules. The observations here open up new promising prospects for structure determination and interpretation at physiological temperatures, and also their implication in structure-based drug discovery and providing more accurate starting models for molecular dynamics simulations.

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P33 Structural and dynamic insights into Fascin in the development of novel anti-metastatic drugs: X-ray crystallography and XFEL studies

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Fascin is an Actin-bundling protein that is overexpressed in a variety of cancer cells and participates in cell motility, cell invasion and metastasis. To better understand the molecular mechanisms underlying Fascin's activity, we have undertaken a structural study of Fascin alone, and in complex with ligands, using X-ray crystallography.

The crystal structure of Fascin reveals a compact molecule, consisting of four beta-trefoil domains linked by flexible regions. The beta-trefoil domains facilitate binding and bundling of Actin filaments through three known binding sites, while the linker regions contribute to protein flexibility and inter-domain cooperativity. Fascin's conformational flexibility is crucial for its action and determining cooperativity in its interactions with Actin filaments.
We have identified several compounds that interact with Fascin with moderate to high affinity, using a variety of high-throughput methods and a drug repurposing strategy. Currently, we are optimizing crystallization conditions for Fascin-ligand complexes to obtain high-quality crystals suitable for X-ray crystallography experiments.

In addition, we are employing XFEL microcrystals to investigate conformational changes in Fascin upon ligand binding, to identify novel allosteric sites to be targeted in the search for novel antimetastatic agents.

Overall, our structural and dynamic studies of Fascin provide valuable insights into the molecular mechanisms underlying its activity and potential targets for the development of novel anti-cancer therapeutics.

P34 Structural and functional characterization of the Gag polyprotein of Feline Immunodeficiency Virus

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The Feline Immunodeficiency Virus (FIV) induces an immunodeficiency syndrome among feline species similar to AIDS induced by HIV in infected human beings. As for all retroviruses, this virus contains a matrix, a capsid and nucleocapsid, respectively formed by the assembly of the subunits p15, p24 and p13 of the Gag polyprotein. Immature virion assembly is triggered by the assembly of the full-length Gag proteins around a dimer of genomic RNA (gRNA). Maturation occurs through proteolytic cleavage of the Gag polyprotein which releases the subunits, which assemble into the three structures. The capsid is made of pentamers and hexamers assembling into a conical, pseudo-icosahedral form called fullerene. No efficient treatment is yet available to address FIV infections and the development of drugs inhibiting the assembly of the Gag polyprotein or the capsid after maturation is highly interesting for veterinarian medicine. Chemical library compounds were tested in vitro for their effects on the capsid assembly. We thus highlighted a compound interfering with the assembly, and whose binding site on the p24 subunit was identified using NMR. Recently, we focused on the full-length Gag protein and molecular mechanisms promoting its multimerization around the gRNA dimer during immature virion assembly. Structural information on Gag and Gag-gRNA through X-ray crystallography and cryo-electronic microscopy analysis will contribute to model the assembly of the retroviral particle, and to highlight amino acids crucial for the stabilization of the capsid pentamers-hexamers around the viral genome.

P35 Structural and Functional Characterization Proteins Involved in Siderophore Mediated Iron Uptake in *Erwinia amylovora*

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Keywords: *Erwinia amylovora, FhuD, Iron uptake, Siderophore, ViuB*

Despite being the fourth most abundant element in the earth's crust, Iron is a limiting factor for the survival of microorganisms due to its low bioavailability in host. In iron-deficient conditions, many microbes secrete low molecular weight metal-chelating compounds like siderophores. Erwinia amylovora, a causal agent of fire blight disease of rosaceous plants secretes hydroxamate-type siderophore (Desferrioxamines), which facilitates indirect iron uptake and plays a significant role in pathogenesis. Siderophore-assisted iron uptake requires outer membrane receptors (FhuA and FoxR), periplasmic binding protein (FhuD), ABC cassette type receptor components (FhuB and FhuC), and siderophore utilization protein (ViuB). Among these, ViuB and FhuD are exclusively present in rosaceous infecting Erwinia spp and are the primary targets for the current project. These proteins have been mainly studied at the genetic level by gene mutations followed by an analysis of the resulting phenotypes. However, we are the first research group using structural biology tools to study these proteins. ViuB was found soluble and purified successfully. The preliminary conditions for crystal growth were found. However, getting high-quality crystals remain a challenging step for this target. To ensure the sample quality and thermal stability, nano DSF and nano DLS were performed and the results suggested that ViuB along with added cofactor (FAD) showed better stability and was suitable for crystallization. Furthermore, Circular Dichroism and SAXS were performed which suggested that the protein is folded and has a high probability to crystallize under appropriate conditions. Some preliminary conditions in commercially available kits (XP) were found. The plate contains unique additives called TEW and it seems this additive has an important role in the crystallization of ViuB. Further optimization is ongoing to obtain high-quality crystals. Conclusively, solving 3D structures of these proteins could contribute to the discovery of novel chemical inhibitors against fire blight disease.

P36 Structural basis for clinical stage inhibitor binding to JAK2

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Janus kinases (JAKs) mediate cytokine receptor signaling through the JAK-STAT pathway, where signals from cytokine binding to their cognate receptor are transduced from extracellular space to the nucleus. There are four members of JAK family, JAK1, JAK2, JAK3 and TYK2, which play crucial roles in immunity, inflammation, cell proliferation, differentiation, survival, and apoptosis. Deregulation of JAK2 occurs in many blood disorders, most importantly myeloproliferative neoplasms (MPNs). Several drugs have been developed against JAKs, and three of them, namely ruxolitinib, fedratinib, and pacritinib have been approved by FDA for MPN treatment. Despite the impetus for developing new JAK inhibitors, structural data of JAK-inhibitor complexes has been lacking, hampering the development of more potent and selective, and thus safer and more effective JAK inhibitors.

All clinical stage JAK2 inhibitors are Type I inhibitors, which abolish phosphoryl transfer from ATP to the substrate by binding to the ATP site of kinases. We still lack detailed understanding how these compounds bind to JAK2. Here we provide a thorough, highresolution structural analysis of seven clinical stage JAK2 inhibitors and connect the structural data with potency and selectivity analysis. The results explain the differences observed with compounds currently in clinical trials and provide guidelines for the design of more potent and selective JAK2 inhibitors.

Key words: Janus Kinase, JAK2, Type-I inhibitors, co-crystallization, ITC

P37 Structural biochemistry of a bacterial collagenase from Cytophaga

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Joint Senior Authors.Collagen is the most abundant protein in mammals, and bacterial collagenases, which cleave collagen, are thus both significant soprophytic enzymes and potential virulence and pathogenicity factors. Moreover, these enzymes are widely utilized in biotechnology and biomedicine [1,2]. Our goal is to utilize structural biochemistry to explore the enzymatic mechanism and cleavage specificity of a large zinc-dependent collagenase from Cytophaga. This protease has multiple domains, which may be critical for substrate recognition, collagen unwinding, and substrate specificity. We are currently optimizing protein purification and domain boundaries for optimal proteolytic activity in gelatin zymograms. Although we have already successfully established a robust E. coli-based expression system, we are still encountering difficulties with partially incorrect disulfide network formation. We are thus attempting to develop a redox-shuffling system to obtain monodisperse protein preparations, as our first attempts at crystallization yielded mostly precipitate. Our next step is to compare different proteoforms (protein isoforms with varying numbers of accessory domains) to determine the importance of these domains for gelatinolytic vs. collagenolytic activity. Successful structure determination could guide drug design and structure-function studies to further enhance our collagenase, with the ultimate goal to develop application-specific collagenase proteoforms with excellent qualities for both biomedicine and biotechnology.

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P38 Structural characterization of Carbonic Anhydrase complexes with dual-target inhibitors

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The microenvironment of many types of tumors is often characterized by low oxygen concentration (hypoxia), which causes a shift from glucose metabolism to glycolysis leading to the acidification of the extracellular space. In these conditions, the overexpression of Carbonic Anhydrase (CA) isoforms IX and XII is a component of the complex response of cancer cells to preserve their survival and growth (1). CA IX and XII are hypoxia-regulated and tumor-specific proteins that maintain the physiological pH essential for cell function. Consequentially they are recognized as therapeutic antitumor targets and nowadays many studies aim at the development of new and more efficient CA inhibitors (CAIs). In this project, a series of dual-target compounds were designed and synthesized incorporating the zinc-binding sulfonamide moiety (specific CAIs) combined with an emerging anticancer drug: the biguanide metformin. Metformin is widely known as an antidiabetic drug, but numerous studies over the past few years have identified novel activities, including anticancer one (2). The metformin derivatives were found to be potent and selective (versus the cytosolic CA I and II isoforms) inhibitors of the tumor-associated CA isoforms. The structures of the CA IX and XII-dual-target inhibitor complexes were determined through the X-ray crystallography technique to rationally improve the efficacy of these promising compounds as anticancer agents. A new crystal form in cubic space group I 41 3 2 has been found for the CA IX isoform with unit cell dimension a=157.96 Å, due to the presence of the His-tag residues at the enzyme N-terminal.

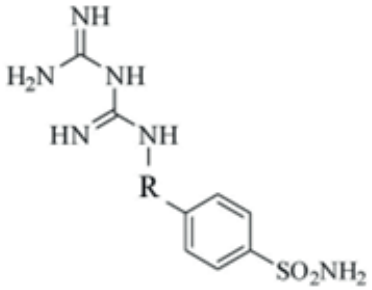

Figure 1. Structure of the metformin derivative dual-target CAIs.

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P39 Structural Characterization of the modular protein Spr1875 from *S. pneumoniae*

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Spr1875 is a *Streptococcus pneumoniae* protein regulated within the WalkR regulon. This regulon is associated with homeostasis and response to cell wall stress.

Spr1875 structure prediction using sequence-based bioinformatic tools reveals a protein formed by two domains, a LysM domain in N-terminal, and a mt3 metallopeptidase domain separated by a long and disordered linker. The C-terminal domain was previously solved by our group revealing a surprising three-dimensional structure with two different conformations. The inactive conformation shows an unprecedented capacity to bind up to 4 Zinc atoms, while the active conformation have only one Zn cation and presents muropeptidase activity.

Now, AlphaFold2 predicts the possibility that the N-terminal domain is not a LysM domain, thus we intend structural determination of the Spr1875-Ntem domain. This domain was expressed, overproduced and purified. Extensive crystallization experiments through the high-throughput platform provided different crystallization hits that were improved and tested at the ALBA synchrotron radiation facility (Barcelona, Spain). Best data were used to solve the 3D structure of the Spr1875-Ntem domain by the molecular replacement method and using the predicted AlphaFold2 structure as initial model. In the presentation details of the structure and potential role will be detailed

P40 Structural determinants behind the improved thermostability of a DyP-type peroxidase variant obtained by computational methods

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Dye-decolorizing peroxidases (DyPs) are microbial enzymes that can efficiently oxidize many substrates like anthraquinone and azo dyes, phenolic and nonphenolic lignin units, and metals, among others. This makes DyPs interesting candidates for biotechnological purposes. However, DyPs usually lack the robust stability properties required for industrial processes.

The in silico method PROSS1 was used to design a variant of the DyP from Pseudomonas putida (*Pp*DyP), with increased thermostability. This variant, *Pp*DyP PR, included 29 mutations, displayed an upshift of its optimum temperature from 15-30ºC to 60-70ºC and exhibited a melting temperature of 88ºC, a 25ºC increase from the wild type (WT; unpublished data). Furthermore, *Pp*DyP PR shows a 3-fold higher yield production and 5-fold higher catalytic efficiency (k_{cat}/K_m) for H_2O_2 and ABTS compared to the WT.

In this work, we used X-ray crystallography to solve the structure of this thermostable variant. *Pp*DyP PR orange thin needle-shaped crystals reached maximum dimensions of 10 x 10 x 10 µm. Diffraction data was collected at ALBA Synchrotron Light Facility (Barcelona, Spain) in the BL13-XALOC beamline and was processed with a resolution of 2.1 Å. *Pp*DyP PR crystals belong to P $2_12_12_1$ space group with cell dimensions of a = 49.97 Å, b = 84.81 Å, c = 128.28 Å, α = β = 90º, γ = 120º and two protein molecules per asymmetric unit, corresponding to 43% of solvent content. The structure was determined using *Pp*DyP WT structure (7QYQ)[2] as a search model. The structure of PpDyP PR shows R-free and R-work values of 23.6% and 19.0%, respectively.

To better understand the role of the mutations suggested by PR and the structural determinants behind the improved thermostability, we used the webserver Protein Interactions Calculator (PIC; http://pic.mbu.iisc.ernet.in/)[3] that identifies interactions in proteins such as hydrophobic interactions, H-bonds, and salt bridges which are known to contribute for the higher stability of enzymes.

Comparative analysis of *Pp*DyP WT and PR variant structures showed an increase in the PR variant packing due to decreased internal cavities number and volume. The analysis performed at PIC revealed an increase of 14 new hydrophobic interactions in PR relative to the WT, where some involve mutations (6) present in this variant. Moreover, we found that PR has 34 new H-bonds, and 15 mutations are involved in the appearance of these interactions in the variant. Regarding salt bridges, we did not find significant changes between the two enzymes.

The structural analysis of the PR will help to understand the variety of stabilization mechanisms that enzymes acquire to become more thermostable. It is essential to study their potential applications in biotechnology further.

P41 Structural studies of bacterial PHA synthases

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Polyhydroxyalkanoates (PHAs) are natural polyesters synthesized by bacteria that can be used in different applications, being a promising alternative for industrial use as a replacement of petroleum-based plastics. Due to remarkable characteristics, such as, biodegradability and biocompatibility, PHAs are also a suitable material for a wide variety of applications as fabrication of resorbable medical devices or drug encapsulation.

Bacterial PhaCs enzymes catalyse the PHA polymerization step and are key in defining the biopolymer composition and consequently its thermo-mechanical properties. Nonetheless, PhaCs still require optimization to produce PHAs with the desired mechano-chemical characteristics at a competitive price.

We heterologously expressed and characterized class I and II PhaCs from Pseudomonas mandelii. These enzymes are homodimers in solution and present two domains (N-terminal and catalytic domains). The N-termini domains seam to play a key role in dimerization and truncated forms of the enzyme do not present synthase activity. We aim at crystallizing and solving the crystal structure of PhaCs, that will enable identifying the molecular determinants for substrate specificity and mechanism in order to optimize efficiency and allow tailor made PHA production.

Keywords: Polyhydroxyalkanoates, Polyhydroxyalkanoate synthase (PhaC), X-ray crystallography, Bioplastics, Crystallization

P42 Structure-driven changes in the behavior of ABA receptors inside and outside of the crystal

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The binding of the plant phytohormone Abscisic acid (ABA) to the family of ABA receptors (PYR/PYL/RCAR) triggers plant responses to abiotic stress. Therefore, the implementation of genetic strategies to modulate their activity might be biotechnologically relevant.

We have employed the available structural information on the PYR/PYL receptors to design new ABA receptors, that show enhanced ABA activity, not only ABAdependent but also ABA-independent. Crystallographic studies show that the performed changes, even when point mutations, make the receptors display different conformations within the crystal. A point mutation is able to generate a displaced linking of the dimerization interface, and also a different interaction with the phosphatase.

This knowledge might lead to the identification of specific residues involved in the receptor activity, and also to the design of ecofriendly agricultural products that can help us face the increasing climate change effects on our crops.

P43 Sulfide:Quinone Oxidoreductase (SQR): a potential target to impair the H_2S-mediated antibiotic resistance in *Pseudomonas aeruginosa*

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Antibiotic resistance is currently recognized as one of the biggest threats to global health with serious social and economic burden. As available antibiotics become less effective, a growing number of infections are becoming progressively harder to treat thus requiring a radical approach to improve healthcare worldwide. *Pseudomonas aeruginosa* (Pa) is one of the most threatening bacteria ("ESKAPE" pathogens) and the main cause of death in patients with cystic fibrosis. Treatments against Pa infections are limited and generally ineffective (1).

In several bacteria and in Pa hydrogen sulfide (H_2S) was shown to play a central role in antibiotic tolerance and oxidative stress resistance (2). Therefore, H_2S synthesis and catabolism are finely tuned but not fully understood yet. We focused our attention on the Sulfide:Quinone oxidoreductase (SQR), one of the enzymes involved in the detoxification of H_2S in Pa. SQR oxidizes H_2S to soluble polysulfur using CoQ as electron acceptor (3,4). PAO1 strain genome encodes two isoforms of this enzyme. In silico analysis revealed that both isoforms are membrane-bounded proteins, but their structures have not been resolved so far. Recently we successfully co-expressed one of the SQR gene as his-tagged recombinant protein with the microbial molecular chaperones system genes. Purification of this membrane protein using detergent remains challenging.

In the next months we plan to purify both the isoforms and unveil the structure via crystallographic or Cryo-EM approaches. Structural details of these membrane proteins could lead to new insights about mechanisms this pathogen uses to detoxify H_2S and thus to design new antibacterial drugs to prevent infection from Pa.

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P44 The anti-influenza effect of flavonoids

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Keywords: Influenza, Viral endonuclease inhibitors, RNA-dependent-RNA-polymerase

Influenza virus cause infection of upper respiratory tract which is responsible for 290 000-650 000 deaths annually [1]. Due to its high virulence and mutation rate, the influenza virus remains a major threat to public health even though vaccines are available. There are many targets for drug development within virus particle, one of them is RNA-polymerase (RdRp). Influenza RdRp is a heterotrimeric enzyme composed of three subunits PA (polymerase acidic protein), PB1 and PB2 (polymerase basic protein 1 and 2). Influenza RdRp is unable of synthesize the 5'mRNA cap required for translation and is forced to obtain it from host mRNA by "capsnatching"mechanism. PA subunits play major role during this process as it has endonuclease activity. PA is binuclear proteinsubdivided into C-terminal and N-terminal domain (PA-Nter) connected by a long peptide chain. The active site localized at PANter is negatively charged pocket with two divalent ions either Mn^{2+} or $Mg^{2+,}$ which are critical for endonuclease activity. Inhibitors with possibility to tightly chelate these ions can block active site and thus interfere with its activity. Here, we report summary of possibility usage of diverse flavonoids and its analogs as inhibitors of PA endonuclease activity. IC50 of these compounds were characterized by AlphaScreen assay and Gel-based endonuclease assay. Also, three structures accompanying this summary of PANter with orientin (PDB ID 7NUG) at 1.9 Å, quambalirine B (PDB ID 6YEM) at 2.5 Å and luteolin (PDB ID 6YA5) at 2.0 Å were solved [2,3].

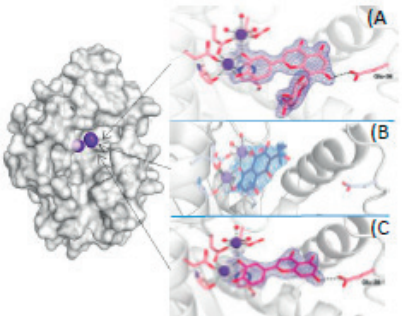

Figure 1. Crystal structures of PA-Nter in complex with orientin (A; PDB ID 7NUG), quambalirine B (B; PDB ID 6YEM) and luteolin (C; PDB ID 6YA5). The active site pocket containing two metal ions (purple spheres) is presented as a grey surface.
Interacting residues and ligands are in stick representation. Hydrogen bonds are shown as black dashes. Coordinating water molecules are presented as red spheres [2,3].

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P45 The crystallization of zebrafish dUTPase with its interaction partners

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In addition to the four canonical bases within DNA, uracil is one of the most frequently occurring non-orthodox constituent in the genome. Its appearance can be traced back to several reasons, on the one hand, it can happen due to the oxidative deamination of cytosines or due to an inappropriate dTTP:dUTP ratio. The reason for the latter case is that DNA polymerases cannot distinguish between the two building blocks, so it depends on their cellular concentration which one is incorporated into the DNA during polymerization [1]. Among other things, the dUTPase enzyme helps to maintain the proper ratio. It catalyses the cleavage of dUTP into dUMP and inorganic pyrophosphate, and by its effect effectively prevents the misincorporation of uracil into DNA, at the same time providing a dUMP substrate for de novo thymidylate biosynthesis [2]. We found dUTPase interacting partners from two yeast hybrid experiments. In the future we want to investigate these interactions and determine these structures. As a first step for this we crystallized the zebrafish dUTPase with a substrate analogue and then define the structure with x-ray crystallography.

The zebrafish as a model organism is very popular among researchers today, as it is easy to maintain and requires little cost, their development is quite fast, and they show good survival against different post-fertilization procedures. In addition to these, it has the additional advantage of being a vertebrate species that shows 70% homology with the human genome [3]. As a result of these benefits we use the zebrafish recombinant dUTPase.

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P46 The mechanism of the SorC protein family revealed

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SorC family is a family of bacterial transcription regulators involved in carbohydrate metabolism and quorum-sensing control [1, 2]. SorC protomers consist of a DNA-binding domain (DBD) and an effector-binding domain (EBD). Several SorC structures have been determined so far [3-6], but there was no structural information on their complex with the cognate DNA (operator).

We used an integrative approach of structural biology combining X-ray crystallography and cryogenic electron microscopy (cryo-EM) to structurally characterize two prototypical SorC members in the complex with their operators, *bs*DeoR and *bs*CggR. X-ray and cryo-EM studies of the full-length repressor-DNA complexes gave us low-resolution information revealing the general mechanism of binding. To gain insight into the repressor-DNA atomic interactions, we determined 2.3 and 2.1 Å resolution crystal structures of *bs*DeoR and bsCggR DBDs in complex with DNA duplexes representing halves of the operator sequences. Putting all the information together, we propose the SorC protein family mechanism of the function, which might be used for further basic and applied research.

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P47 Unravelling the function of a new protein modification enzyme

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The 5′ end caps protecting eukaryotic mRNAs from uncontrolled degradation is removed by the decapping holoenzyme composed of the Dcp2 catalytic subunit and its intrinsic partner Dcp1. Additional factors such as Pat1 or Edc3 interact with Dcp2 in Saccharomyces cerevisiae to stimulate the decapping activity of Dcp2. Recently, my host lab has characterized Pby1 as a new Dcp2 partner. A former PhD student from the lab has determined the crystal structure of the C-terminal Pby1 domain either alone or as a complex with the Dcp1-Dcp2-Edc3 complex [1]. Based on its crystal structure, Pby1 belongs to the ATP-grasp protein family, which is responsible for the ATP-dependent ligation of a nucleophilic group to an acid group. Yet, its function in mRNA decapping is still unknown.

Using a combination of enzymology and mass spectrometry analysed, our collaborators have identified a Pby1 substrate, revealing that Pby1 catalyzes the formation of a novel protein post-translational modification on a factor distantly related to mRNA decay. My PhD project aims at combining various biochemical (pull-down, enzymology, ITC, fluorescence…) and structural biology (X-ray crystallography, cryo-EM…) approaches to obtain molecular details about the recognition of its substrate by Pby1 and about Pby1 catalytic mechanism. This information is essential to help unravelling the function of this protein in mRNA metabolism.

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P48 Structural studies of human transferrin for its potential application as a drug delivery system

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The present work explores the proposal of using an endogenous transport molecule, human serum transferrin, as a highly specific drug delivery system, due to its receptor-mediated transcytosis mechanism [1]. Human serum transferrin is a ~80 kDa bilobular glycoprotein that transits the blood plasma, whose function is to transport iron from its absorption site towards every cell in the organism, by the coordination of the metallic ion with 4 residues located in the inter-domain region of each lobe. Both lobes are linked by an unstructured linker peptide, which makes the protein highly flexible [2-3]. Thus, this work is focused on studying the conformational changes of this protein throughout its transport mechanism, mainly by two structural resolution techniques, small angle X-ray scattering (SAXS), and protein X-ray crystallography, to explore potential binding sites. The results obtained so far have shown the coexistence of three main conformations in different physiologically relevant pH conditions, as well as a particular sensitivity to changes in the medium conditions, namely pH [4].

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P49 Optimized Protein Purification and Crystallization of Photosystem ll

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Billions of years ago, Photosynthesis starts to provide the energy for all higher life on Earth to generate an anoxygenic environment with an oxygen-rich biosphere. Although most mechanisms of Photosystem ll(PSll) are already discovered, there are still some mysteries about the process. Our goal is to study the transient [S4] state during the catalytic process where oxygen is evolved. To investigate this state of PSll, time-resolved X-ray Emission spectroscopy, and serial fs crystallography are used to observe the structural and quantum changes to the Oxygen Evolving Complex inside PSII. Depending on the X-ray and instruments' current status, crystal size needs to be controlled, also crystal shapes are important because they can affect diffraction quality.

Two solubilization protocols for PSll purification are well established. The first one is to use beta-dodecylmaltoside for protein solubilization and the other one is to use LDAO and beta-dodecylmaltoside. The pure PSII proteins from both solubilization conditions are screened by a different concentration of PEG and are incubated at different temperatures. From the best growth condition, crystals show uniform size around 8-10 um. The best resolution we collect from those crystals was 2.4 Å.

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P50 Amino acids as heterogenous soft template nucleants in lysozyme crystallisation

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Macromolecular crystallization has been instrumental in characterizing the structure of many proteins of biological and has greatly contributed to our understanding of biological processes. (Su et al. 2015). Until recently the focus of macromolecular experiments has been on structure elucidation rather than understanding the physio mechanical processes governing protein crystallization. I aim to add to our fundamental knowledge of the processes governing protein crystallization. Possible applications of protein crystallization include biopharmaceutical drug purification and formulation, and biosensor production. This study investigates the use of amino acids as soft templates in lysozyme crystallization. A soft template is a small organic molecule which facilitates and controls nucleation (Link and Heng 2021). Soft templating behaviour of amino acids in insulin crystallization has been studied by Link and Heng (2021) who report an increase in % crystal occurrence of insulin crystals when leucine is present in the mother liquor. I studied the effect of addition of glycine, L-arginine and L-histidine to the crystallization liquor on lysozyme crystal number and size through sitting drop vapour diffusion experiments. L-histidine and L-arginine have been shown to respectively promote and inhibit lysozyme crystallisation at pH 4. Lysozyme solubility at the conditions studied will be investigated to determine if these effects are kinetic or thermodynamic in nature. Amino acids provide a suite of related molecules with varying degrees of hydrophobicity, charge, size and acidity/basicity. This allows for design of experiments in which soft templating behaviour on protein crystallisation can be studied in a parameter specific manner.

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P51 Structural characterization of avian orthoreovirus σNS

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Fusogenic avian orthoreoviruses of the family Reoviridae are important avian pathogens that can cause significant economic losses in the poultry industry and are associated with a variety of poultry diseases and exhibit icosahedral, non-enveloped virions and 10 dsRNA genomic segments (23.5 kb)[1,2.] RNA replication and reovirus morphogenesis occur exclusively in cytoplasmic inclusion bodies, also known as viral factories or viroplasms. Viroplasms are formed by the non-structural protein μNS in association with the non-structural protein σNS[1]. Each progeny of avian reovirus (ARV) must encapsidate a complete set of 10 different genomic segments. However, due to the low probability of selecting the complete genome by a random assortment, a specific RNA assembly mechanism is required. The RNA chaperone activity of the σNS protein, whose oligomeric state dynamically controls its activity, is thought to be responsible for this process through a series of sequence-specific RNA-RNA interactions[3,4.] In order to elucidate the molecular mechanism we have imaged σNS apoprotein using cryo-EM, after many unsuccessful attempts with X-ray crystallography, and reconstructed an electron density map and build an atomic model for octamers which are further stabilized by RNA binding. In addition, long RNAs induced the assembly of filamentous ribonucleoproteins (RNPs). Using a deletion mutant, we show that the N-terminal tail of σNS is required for filament and octamer formation. Structural analysis of the octameric σNS provides basis for mechanism of assembly of the dimeric building block as well as higher oligomeric species and RNPs.

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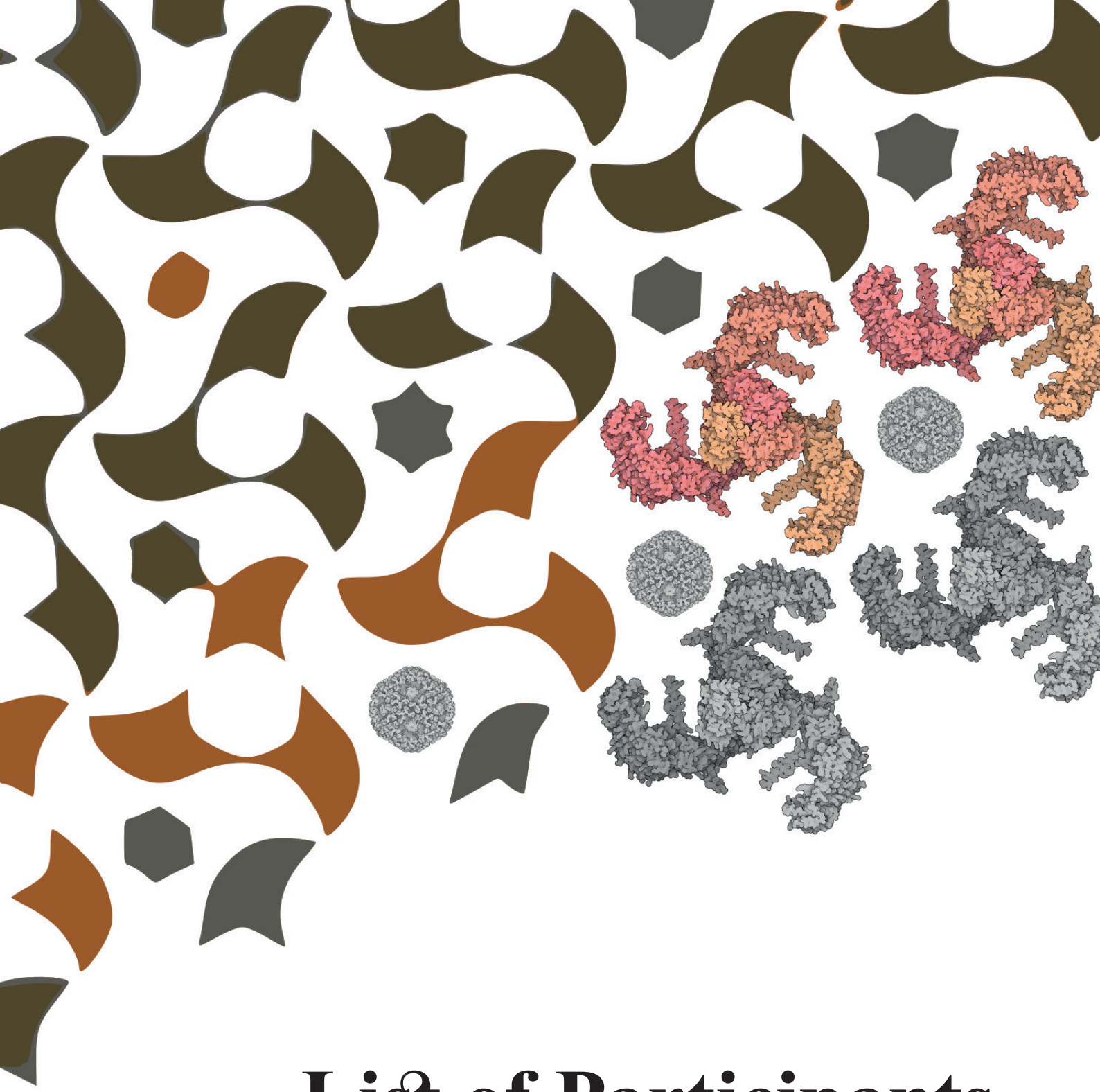
International School on Biological Crystallization. 2023 125



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