XIVth INTERNATIONAL CONFERENCE

NMR: a tool for biology

MAY 26-28, 2025

INSTITUT PASTEUR, PARIS, FRANCE

PROGRAMME BOOK

CONTACT AND INFORMATION
www.nmr2025.conferences-pasteur.org
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WELCOME ADDRESS

We are very pleased to announce the 14th "NMR: a tool for Biology" conference. This event, jointly organized by Institut Pasteur and Bruker Biospin, will be held at the Institut Pasteur in Paris from May 26th-28th, 2025.

This conference brings together internationally recognized experts with interests in the application of NMR to address biologically important questions.

The meeting represents an exceptional networking opportunity for our younger colleagues (Post-doctoral fellows and students) who are encouraged to present posters.

The conference will be held on site in order to promote exchanges between scientists.

ORGANIZING COMMITTEE

Institut Pasteur, Paris, France

Nadia Izadi-Pruneyre
Michael Nilges

Bruker Biospin, France

Eric Leonardis
Alain Belguise

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Biologie structurale et chimie

Silantes

Stable Isotope Labeled Biomolecules

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Frederic Allain
ETH, Zürich, Switzerland

Clemens Anklin
Bruker Biospin, United States

Haribabu Arthanari
Harvard Medical School, Boston, United States

Marc Baldus
Utrecht University, Utrecht, The Netherlands

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CEITEC, Brno, Czech Republic

Ana Paula Valente
Federal University of Rio de Janeiro, Brazil

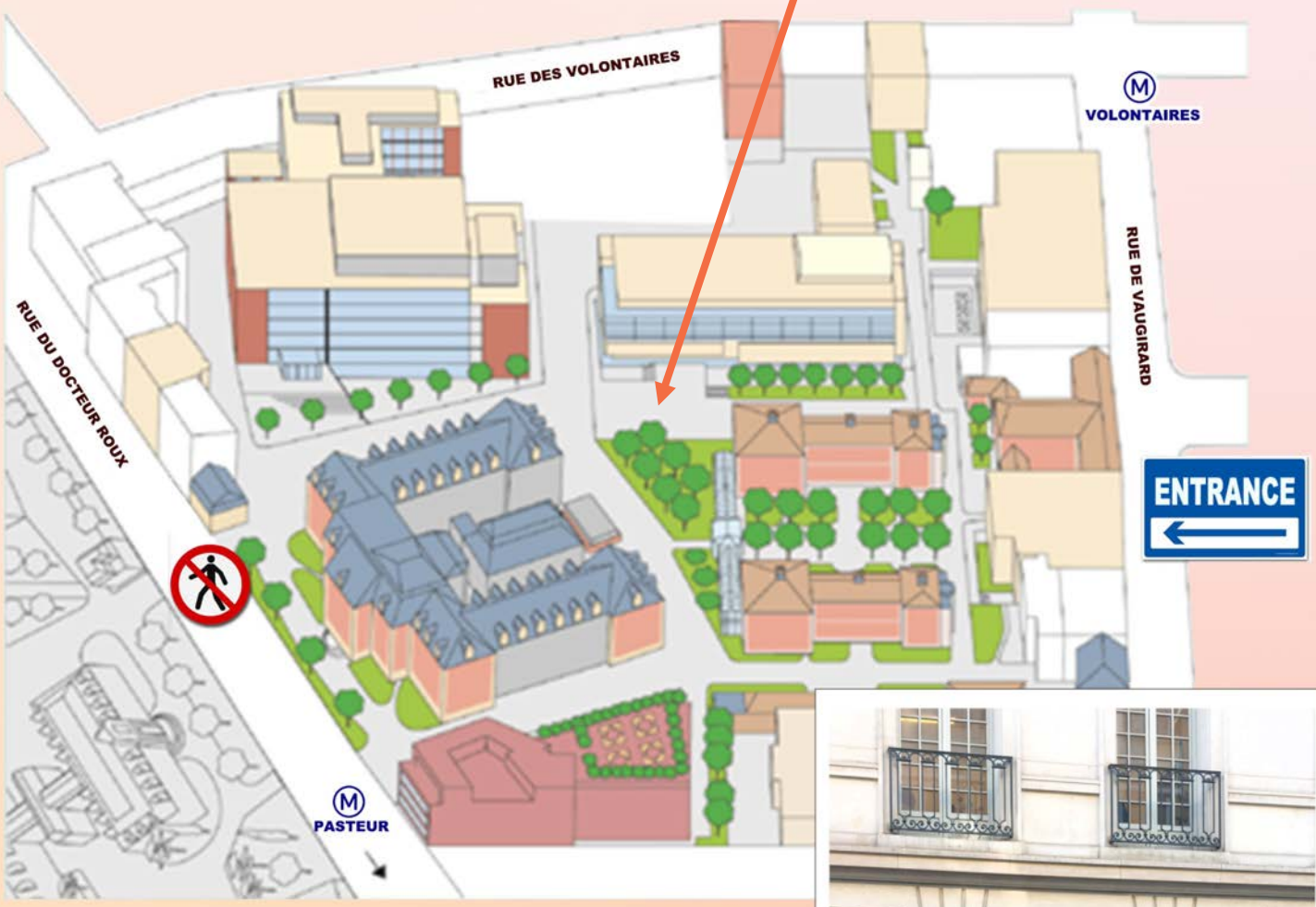
Sophie Zinn
CEA, Saclay, France

Markus Zweckstetter
Max Planck Institute, Göttingen, Germany

MAP OF THE CAMPUS

The entrance during the three days of the congress will be located
205, rue de Vaugirard, 75015 Paris

VENUE
Welcome Desk, Lecture hall, Posters, Cocktail, Lunches and Coffee breaks



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INSTITUT PASTEUR

May 26th, 2025

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Chair: Tatyana Polenova, *University of Delaware, United States*

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Rainer Kuemmerle
Bruker Biospin, Switzerland

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Rasmus Linser
Dortmund University, Dortmund, Germany

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ENS - PSL, Paris, France

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Ludwig-Maximilians University, München, Germany

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Four decades of using NMR as a tool for Biology and much more
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Bruker Biospin, United States

7 5.15 pm
Fluorine NMR isn't just flying – it's soaring!
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27th May 2025

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Harvard Medical School, Boston, United States

9 9.30 am
Assembly of DNA repair complexes mediated by intrinsically disordered regions
Sophie Zinn
CEA, Saclay, France

10 10 am
The fuzzy logic of the alarmin HMGB1: how disorder drives the interaction with the chemokine CXCL12
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San Raffaele Hospital, Milan, Italy

11 10.30 am
Hierarchical folding-upon-binding of an intrinsically disordered protein
Malene Jensen
IBS-CNRS, Grenoble, France

Coffee break — 11 pm - 11.45 pm

FLASH POSTER PRESENTATION (Odd numbers) — 11.45 am - 12.45 pm

Chair: Benjamin Bardiaux, *Institut Pasteur, CNRS, France*

Lunch buffet and poster session (Odd numbers) — 12.45 pm - 3 pm

IN-CELL AND IN-SITU NMR — 3 pm - 5 pm

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12 — 3 pm
In-situ solid-state NMR applied to complex biomolecules
Marc Baldus
Utrecht University, Utrecht, The Netherlands

13 — 3.30 pm
***In-cell* NMR spectroscopy of nucleic acids: Progress, Challenges, and Opportunities**
Lukas Trantirek
CEITEC, Brno, Czech Republic

14 — 4 pm
Searching for metabolic profiles of saliva in health and disease states using NMR
Ana Paula Valente
Federal University of Rio de Janeiro, Brazil

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University of Gothenburg, Sweden

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IBPC, CNRS, Paris, France

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Columbia University, New York, United States

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Helmholtz Munich, Technical University of Munich, Garching, Germany

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Max Planck Institute, Göttingen, Germany

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ORAL COMMUNICATIONS

May 26th-28th, 2025

INSTITUT PASTEUR

Increasing magnetic field strength for NMR: high temperature superconductors, force management & additional challenges

R. Kuemmerle
Bruker Biospin, Fällanden, Switzerland

Understanding protein dynamics – a new concept and a new application

R. Linser
Dortmund University, Dortmund, Germany

In my talk I will address two different aspects of dynamics, a more technical one and a rather applied one. In the first half, I will present a new solid-state NMR concept for accessing µs timescale motion of protein-bound ligands. Proton relaxation dispersion, in spite of the unique advantage of being applicable for molecules that evade isotope labeling, has been associated with conceptual and practical hurdles. I will present our latest view on this topic, which in my eyes is a highly important field to better explore.

In the second half, I will focus on a combined application of solution NMR and MD simulations to mechanistically understand how the properties of the three extended loops in a trimeric secondary-messenger receptor are orchestrated allosterically by messenger binding within the protein core. With its flexibility, the loop, which is responsible for binding downstream interaction partners and hence the key player in the signaling cascade, long escaped structural biology attempts. The core of the protein, conversely, showed absolutely no impact of messenger binding, which left the mechanism of activation a conundrum for more than a decade. We can resolve the question by demonstrating that the association of the messenger changes the network of transient interactions between core and loop, eventually redirecting the loop in such a way that it becomes differentially competent for its role as a molecular glue.

Dynamics of Biomolecules by Ultrafast High-Resolution Relaxometry

F. Ferrage
École normale supérieure, PSL University, Sorbonne Université, Paris, France

NMR relaxation is a fascinating phenomenon to probe the dynamics of biomolecules. Fast field-cycling relaxometry measure relaxation rates over several orders of magnitude of magnetic field, providing information on dynamics over many timescales, alas, with no spectral resolution. High-resolution relaxometry combines the analytical power of high-resolution NMR and the information of relaxometry, by using a sample shuttle on a high-field magnet, exploiting the stray field as a variable field. Yet, physically moving the sample leads to strong limitations: very low fields are too distant and fast relaxation cannot be probed as polarization would not survive shuttle transfers. Yet, accessing motions slower than ~10 ns requires measuring fast relaxation at very low fields. Our objective is to design a new type of instrument to overcome the limitations of high-resolution relaxometry and obtain information from relaxation on dynamics from picoseconds to microseconds, while preserving high-resolution and sensitivity.

We introduce ultrafast high-resolution relaxometry (UHRR) on an instrument built by Bruker Biospin, in collaboration with CERM, in Florence. A commercial high-field magnet is equipped with a sample shuttle to move at high speed the sample to a field-cycling coil. To preserve magnetization during the sample-shuttle transfer, the magnetic field is maintained above 1 T by a magnetic tunnel positioned inside the bore of the high-field magnet. Field cycling from 0.8 T down to 100 mT and back is achieved in about 2 ms, allowing the measurement of fast relaxation. Detection is performed at high field with high sensitivity and resolution.

We show the first application of UHRR to a series of biomolecular systems. Transient binding of tryptophan to serum albumin is probed and analyzed quantitatively from proton longitudinal relaxation. Site-specific nitrogen-15 relaxation in a disordered protein enables the determination of pico-to-nanosecond backbone dynamics. Critically, we measure and analyze carbon-13 relaxation in methyl groups in a 42 kDa kinase, p38g, between 100 mT and 28 T, and analyze the multiscale dynamics of these sidechains.

UHRR is a powerful approach to determine molecular motions over orders of magnitude of timescales in a broad range of biomolecular systems.

The dynamic chaperone network in the endoplasmic reticulum

S. Hiller
Biozentrum, University of Basel, Basel, Switzerland

The endoplasmic reticulum (ER) serves as the major protein folding compartment of the eukaryotic cell. Efficient ER functioning relies on a network of chaperones, calcium level and redox state, wheras variation in these cellular parameters can lead to ER stress and diseases. Here, we present structural and functional studies to resolve key mechanisms underlying the dynamic chaperone network in the ER.

In a first step, we resolve the complete functional cycle of the ER chaperone BiP Hsp70 at atomic level. We create a non-equilibrium steady-state under turnover conditions inside the NMR tube, resolving all significantly populated states, their structures, mechanisms and kinetics connecting them, and the dynamic interplay with co-chaperones. The data resolve for the first time that Hsp70 chaperone BiP undergoes a functional cycle comprised of five states that is regulated by two autoinhibition switches.

In a second step, we characterize the disulfide isomerase PDIA6. We find that PDIA6 forms biomolecular condensates, both in vitro and in the ER lumen during protein folding homeostasis. Two specific interfaces in PDIA6 create the multivalency required for the condensate formation, which are dynamically regulated by calcium concentration. PDIA6 condensates recruit Hsp70 BiP and a number of further chaperones specifcially into the condensates to form functional “folding factories” inside the ER. Together, our data establish the existence of a functional chaperone condensate that subcompartmentalizes the ER.

Tales of a Temperamental Protein - Taming the Non-Structural Protein of the Hepatitis B Virus

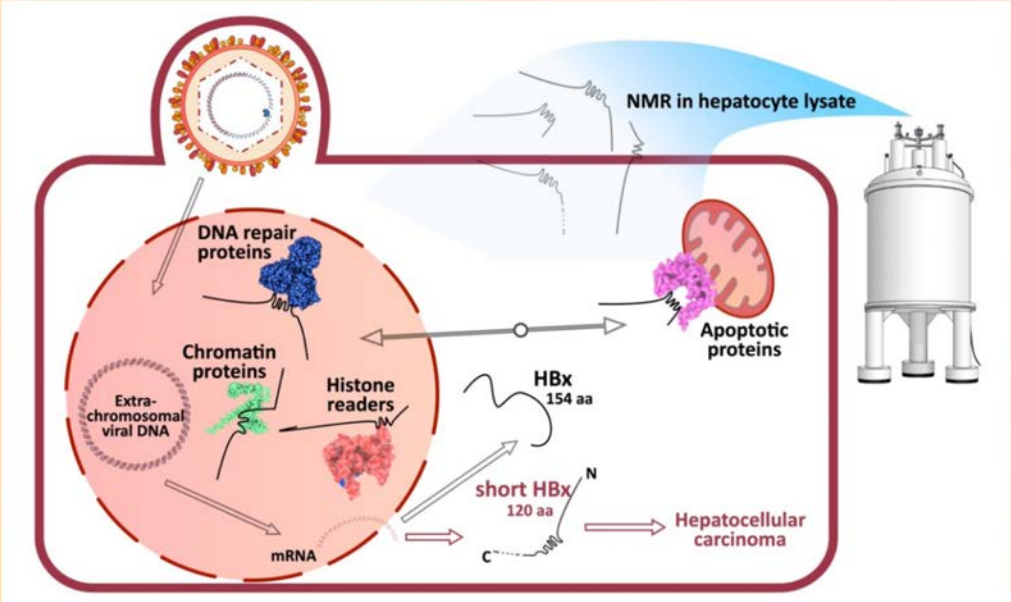
A. Clavier, A. Schütz
Ludwig-Maximilians University, München, Germany

To evade host defence mechanisms, many viruses encode proteins that disturb or hijack cellular signalling pathways. The small, non-structural HBx protein of the human Hepatitis B virus (HBV) is essential for viral replication [1]. It promotes the transcription of the extrachromosomal viral DNA. A truncated isoform of HBx [2], found in chronically infected patients, is linked to the carcinogenicity of HBV, which afflicts millions globally.

The ‘x’ in HBx refers to its unique amino acid sequence, which is not homologous to proteins of known fold, despite being conserved across mammalian hepadnaviruses. Isolated HBx is notoriously unstable in vitro; there is no model of a hypothetical structure. Proteomics, bioinformatics, and immunoprecipitation studies have identified a wide range of host proteins that HBx potentially engages [3], but few of these interactions have been confirmed as stable binary complexes.

Towards understanding the exclusivity, hierarchy and regulation of HBx interactions, we present an analysis of its conformational dynamics using solution NMR. We examine HBx in isolation, in complex with partner proteins and in hepatocyte lysate. These findings are discussed in the context of HBx sequence evolution, structure predictions, and current hypotheses on how HBx subverts viral antagonists, creating a cellular environment that is conducive to viral replication [4].

Our work demonstrates how a carefully optimised NMR setup can address even the most challenging protein targets that would not otherwise be stable in isolation.



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1. Tiollais, P.; Charnay, P.; Vyas, G. N. Science 1981, 213 (4506), 406–411.
2. Ma, N.-F.; Lau, S. H. et al. Clin. Cancer Res. 2008, 14 (16), 5061–5068.
3. Damme, E. V.; Vanhove, J.; Severyn, B.; Verschueren, L.; Pauwels, F. Front Microbiol 2021, 12, 724877.
4. Ramakrishnan, D.; Xing, W. et al. J. Virol. 2019. Decorsière, A.; Mueller, H. et al. Nature 2016, 531 (7594), 386–389. Liu, W., et al. Nat. Commun. 2023, 14, 4663.

Four decades of using NMR as a tool for Biology and much more

C. Anklin
Bruker Biospin, United States

After using NMR mostly for transition metal coordination chemistry during my studies I quickly began to accumulate an ever increasing amount of knowledge about biomolecular NMR. The involvement included hardware development and methods development but always in support of the community. From the early days when we discovered how to build probes that were more suitable for work in aqueous solutions or were able to publish a paper with a 15N HMQC on all 4 amino acids of met-Enkephalin, to some early triple and quadruple resonance experiment involving 31P the collaborations always helped me expand my horizons. Later on I worked on novel experiments where I first had to convince my collaborators that ~ 200 Hz is not the upper limit for coupling constants but they can go higher, much higher. I will present a number of examples where I could serve as the intermediary between you the researchers and my colleagues in our R&D groups. It has been a wild ride. P.S. I am not planning to quit just yet.

Fluorine NMR isn’t just flying – it’s soaring!

A. Gronenborn
University of Pittsburgh School of Medicine, Pittsburgh, United States

Fluorine-19 NMR has emerged as a powerful technique in the analytical chemist’s toolbox, thanks to its high sensitivity, 100% natural abundance of the fluorine-19 nucleus, and wide chemical shift range. With the rapid expansion of fluorinated drugs and ever-increasing ways to introduce fluorine into biomaterials and other advanced materials, fluorine NMR is no longer a niche methodology. Fluorine’s sensitivity to its conformational and electronic environment makes it a powerful probe for conformational analysis, ligand binding, and studying molecular interactions. In addition,given its virtual absence in biological systems, background free data can be obtained, even in complicated mixtures and complex environments.In my lecture I will explore the factors fueling the surge in fluorine NMR applications, from the growing prevalence of fluorinated compounds in pharmaceuticals to biomolecules to their expanding roles in reaction monitoring, ligand-binding studies, and structural analysis.

Beyond the Fold: Role of NMR in Deciphering the Functions of Disordered Protein Domains

H. Arthanari
Harvard Medical School, Boston, United States

Despite accounting for at least one-third of the human proteome, intrinsically disordered regions (IDRs) remain largely opaque to traditional structural biology and even evade state-of-the-art structure prediction tools. Yet these dynamic, unstructured segments encode critical regulatory functions that shape cellular decision-making at multiple levels. NMR spectroscopy stands out as a uniquely powerful and underexploited tool for probing the conformational landscapes and mechanistic roles of IDRs at atomic resolution. This talk will challenge the prevailing “structure equals function” paradigm, demonstrating how NMR-driven insights into disorder are transforming our understanding of enzyme regulation, transcriptional control, and complex biomolecular assemblies. I will also highlight recent methodological advances that are expanding the frontier of what is measurable in disordered systems, and argue that embracing NMR is essential for tackling the next generation of questions in protein science.

Assembly of DNA repair complexes mediated by intrinsically disordered regions

S. Zinn
CEA, Saclay, France

To mitigate the threat of DNA double strand breaks (DSBs), human cells rely on the activity of multiple DNA repair machineries, that are tightly regulated throughout the cell cycle. In G1-phase, DSBs are mainly repaired by the non-homologous end joining (NHEJ) pathway, whereas in S-phase, when the cell duplicates its genome to get prepared for mitosis, DSBs are repaired by the homologous recombination (HR) pathway. In mitosis, several kinases are expressed and activated, which inactivate the NHEJ and HR pathways, and activate an alternative end-joining pathway. Here, we will show how NMR can contribute to pinpoint the phosphorylation-dependent mechanisms that are activated in mitosis (Alik et al., Angew Chem 2020; Gelot et al., Nature 2023). Inhibiting these mechanisms is a new therapeutical approach to kill tumor cells deficient for HR. We will also show how NMR can be used to elucidate new mechanisms involving the HR protein BRCA2 in mitosis (Ehlen et al., Nat Commun 2021; Julien et al., Biomolecules 2021). We will focus on the interaction between BRCA2 and KIF2C, a microtubule depolymerase found at the centromere and centrosome in mitotic cells (Skobelkina et al., BioRXiv 2024). We will describe how KIF2C assembles into biomolecular condensates that concentrate both phosphorylated BRCA2 and the mitotic kinase PLK1 at kinetochores. We will also present how KIF2C might recruit other proteins, including DNA repair proteins, through its newly characterized N-terminal domain binding to phosphorylated proteins. The functions of these mitotic interactions will be discussed.

1. Sensitivity-Enhanced ^{13}C-NMR Spectroscopy for Monitoring Multisite Phosphorylation at Physiological Temperature and pH. Alik A, Bouguechtouli C, Julien M, Bermel W, Ghouil R, Zinn-Justin S, Theillet FX. Angew Chem Int Ed Engl. 2020; 59(26):10411-10415. doi: https://doi.org/10.1002/anie.202002288
2. Polθ is phosphorylated by Polo-like kinase 1 (PLK1) to enable repair of DNA double strand breaks in mitosis. Camille Gelot, Marton Tibor Kovacs, Simona Miron, Emilie Mylne, Rania Ghouil, Tatiana Popova, FlorentDingli, Damarys Loew, Josée Guirouilh-Barbat, Elaine DelNery, Sophie Zinn-Justin, Raphael Ceccaldi. bioRxiv 2023.03.17.533134; doi: https://doi.org/10.1101/2023.03.17.533134

3. Proper chromosome alignment depends on BRCA2 phosphorylation by PLK1. Ehlén Å, Martin C, Miron S, Julien M, Theillet FX, Ropars V, Sessa G, Beaurepere R, Boucherit V, Duchambon P, El Marjou A, Zinn-Justin S, Carreira A. Nat Commun. 2020; 11(1):1819. doi: https://doi.org/10.1038/s41467-020-15689-9
4. Intrinsic Disorder and Phosphorylation in BRCA2 Facilitate Tight Regulation of Multiple Conserved Binding Events. Julien M, Ghouil R, Petitalot A, Caputo SM, Carreira A, Zinn-Justin S. Biomolecules. 2021; 11(7):1060. doi: https://doi/org/10.3390/biom11071060
5. KIF2C-induced nuclear condensation concentrates PLK1 and phosphorylated BRCA2 at the kinetochore microtubules in mitosis. Skobelkina, A., Julien, M., Jeannin, S., Miron, S., Egger, T., Chaaban, R., Bouvignies, G., Ghouil, R., Friel, C., Busso, D., Theillet, F.-X., Le Bars, R., Carreira, A., Constantinou, A., Basbous, J., & Zinn-Justin, S. (2024). *bioRxiv*, 2024.2004.2013.589357. https://doi.org/10.1101/2024.04.13.589357

The fuzzy logic of the alarmin HMGB1: how disorder drives the interaction with the chemokine CXCL12

G. Musco
San Raffaele Hospital, Milan, Italy

Chemokines heterodimers activate or dampen their cognate receptors during inflammation. CXCL12 chemokine forms with the alarmin HMGB1 (High Mobility Group B1) a crucial heterocomplex (HMGB1•CXCL12) that synergically promotes the inflammatory response elicited by the G-protein coupled receptor CXCR4. As such HMGB1•CXCL12 has become a pharmacological target for inflammation related malignancies (e.g. cancer) (1). However, the molecular details of complex formation were still elusive. By an integrated structural approach (NMR, ITC, MST, AUC, SAXS) we show that HMGB1•CXCL12 is a fuzzy heterocomplex, the first reported for chemokines (2). Unlike previous assumptions, HMGB1 and CXCL12 form a dynamic equimolar assembly, with structured and unstructured HMGB1 regions recognizing the CXCL12 dimerization surface.

Our findings shift the understanding of this complex from the traditional rigid heterophilic chemokine dimerization mechanism to a "fuzzy" model, where HMGB1's acidic intrinsically disordered region (IDR) plays a crucial role in complex assembly and in binding to CXCR4 on malignant mesothelioma cells surface. Simultaneous interference with multiple interactions within HMGB1•CXCL12 might offer novel pharmacological strategies against inflammatory conditions.

1. De Leo et al. Diflunisal targets the HMGB1/CXCL12 heterocomplex and blocks immune cell recruitment EMBO rep (2019) 20: e47788
2. Mantonico et al. The acidic intrinsically disordered region of the inflammatory mediator HMGB1 mediates fuzzy interactions with CXCL12 Nature Communications, 15, Article number: 1201 (2024)

Hierarchical folding-upon-binding of an intrinsically disordered protein

L. Kjær[1], F. Ielasi[2], T. Winbolt[1], E. Delaforge[1], M. Tengo[1], L. Bessa[1], E. Boeri Erba[1], G. Bouvignies[3], A. Palencia[2], M. Jensen*[1]
[1]Institut de Biologie Structurale, UGA, CNRS, CEA, Grenoble, France [2]Institute for Advanced Biosciences, UGA, CNRS, INSERM, Grenoble, France [3]Ecole Normale Supérieure (ENS), PSL Université, Sorbonne Université, Paris, France

Intrinsically disordered proteins (IDPs) make up approximately 40% of the human proteome and play essential roles in diverse biological processes such as signal transduction, transcriptional regulation and cell cycle control. Unlike structured proteins, IDPs do not adopt stable three-dimensional conformations, but instead exist as dynamic ensembles of interconverting states. Many IDPs interact with their binding partners through short linear motifs (SLiMs), which are sequence segments typically 5–15 amino acids in length. These motifs often undergo coupled folding and binding, adopting a defined structure upon partner association. However, a significant proportion of IDPs do not adhere to this “SLiM paradigm”. Some exploit much longer interaction segments spanning multiple molecular recognition elements. For these IDPs, the precise binding trajectory, the structural characteristics of potential folding intermediates and the thermodynamic forces driving such extensive interactions remain elusive.

Here we unveil hierarchical folding of an intrinsically disordered protein as it binds to its partner, exemplified by the signaling effector POSH and the small GTPase Rac1. We show that POSH lacks discernable secondary structure in its unbound state but adopts a unique effector fold upon interaction with Rac1. Using nuclear magnetic resonance (NMR) spectroscopy, we demonstrate that POSH undergoes extensive folding-upon-binding, initiated by specific contacts with Rac1, followed by the structuring of a first molecular recognition element. In this intermediate state, the remaining POSH sequence collapses while exploring binding-competent conformations, ultimately leading to the folding of a second molecular recognition element on the surface of Rac1. The folding of each element is contingent on the successful structuring of the preceding one, underscoring the hierarchical nature of the process. By dissecting the structural details of this folding trajectory at atomic resolution, our study offers new insight into binding-induced folding of long disordered sequences and highlights the potential of targeting folding intermediates and conformational transitions to unlock new therapeutic opportunities for IDPs.

In-situ solid-state NMR applied to complex biomolecules

S. Bahri, A. Bishoyi, I. Bodnariuc, N. Dwivedi, M. Krafcikova, A. Safeer, G.E. Folkers, M. Baldus*
NMR Spectroscopy Group, Utrecht University, Utrecht, The Netherlands

Increasing evidence suggests that a full understanding of biomolecular function and disease requires in-situ approaches that probe molecular structural and dynamics in a native setting. NMR is a non-invasive method that has made significant progress to study biomolecular systems in a native-like environment including bacterial, fungal or human cells. In our contribution, we will discuss tailored solid-state NMR (ssNMR) approaches to study complex biomolecular systems in a bacterial[1,2], fungal[3,4] and human[5,6] cell setting. These methods maximize spectral resolution and sensitivity and are geared towards elucidating complex molecular systems including membrane proteins[6,7] or microtubular protein complexes[8,9]. With these approaches we aim at deciphering the dynamic landscape of proteins as well as nucleic acids [10] at the surface and inside bacterial, fungal and human cells and cell organelles. Examples of such applications will be shown.

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1. Narasimhan, S. et al. *Nature Pro*tocols **2021,** *16*, 893-918 ().
2. Xiang, S., Pinto, C. & Baldus, M. *Angewandte Chemie International Edition* **2022,** *61*, e202203319 ().
3. Safeer, A et al., *Chemistry* **2023** 29, e202202616.
4. Pylkkänen, R. et al., *Science Adv.* **2023**, eade5417
5. Damman, R. et al. *Journal of Biomolecular NMR* **2020** *74*, 401-412 ().
6. Kaplan, M. et al. *Cell* **2016,** *167*, 1241-1251.e11
7. Xiang et al., *Angewandte Chemie International Edition* **2022**, 61:e202203319.
8. Luo, Y. et al. *Nature Communications* **2020.** *11*, 18.
9. Adler, A. et al. *Nature Communications* **2024** *15*, 1948.
10. Krafčíková, MD et al., *Angewandte Chemie International Edition* **2025,** e202424131.

In-cell NMR spectroscopy of nucleic acids: Progress, Challenges, and Opportunities

L. Trantirek
Central European Institute of Technology, Masaryk University, Brno, Czech Republic

In-cell NMR spectroscopy has emerged as a powerful method for studying the structure, dynamics, and interactions of nucleic acids directly within living cells. In contrast to traditional in vitro techniques, this approach enables the examination of nucleic acids at atomic resolution in their native intracellular environment, capturing biologically relevant conformations and interactions that are often missed outside the cell. Recent technological advances—including optimized nucleic acid labeling strategies that improve sensitivity and spectral resolution, automated sample preparation workflows, and bioreactor-assisted data acquisition—have significantly expanded the scope of in-cell NMR. These developments now make it possible to investigate nucleic acids across various physiologically defined cellular states.

Despite this progress, several challenges remain. These include inherently low signal intensity, potential metabolic interference caused by exogenous nucleic acids, and limitations in temporal resolution.

In this lecture, I will present recent advances in in-cell NMR spectroscopy of nucleic acids, discuss current technical and conceptual obstacles, and outline future opportunities for expanding our understanding of nucleic acid function in the cellular context.

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Searching for metabolic profiles of saliva in health and disease states using NMR

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Saliva is a complex biofluid containing numerous biological compounds with significant diagnostic potential for various clinical conditions. Our research group has extensive experience characterizing salivary metabolomic profiles using Nuclear Magnetic Resonance (NMR) spectroscopy combined with robust statistical analysis. In recent years, we have analyzed saliva samples from individuals of all ages, from infants to adults, both in healthy states and in the presence of oral or systemic diseases.

Our findings revealed notable differences in metabolite profiles between children before and after tooth eruption. Interestingly, more pronounced changes were observed in children over 30 months old, with elevated levels of alanine, choline, ethanol, lactate, and sugars before this age.

Furthermore, after eruption, the differences among children with deciduous, mixed, or permanent dentitions were minimal, suggesting that the most significant shifts in salivary profiles are linked to the development of salivary glands. Establishing a baseline profile for healthy individuals is essential for early detection and intervention in various diseases.

We also identified alterations in children with dental caries, where bacterial metabolites in saliva decreased significantly following treatment. However, the profile did not fully return to its initial state, and these metabolites were also characterized in biofilm and isolated *Streptococcus mutans* samples.

Our research highlights that saliva stimulation markedly influences the profile of metabolites, emphasizing the importance of choosing the appropriate collection method. For example, levels of acetate, glucose, propionate, and lysine are higher in unstimulated whole saliva, whereas isoleucine, N-acetyl sugars, hydroxybutyrate, glutamate, leucine, propionate, butyrate, valine, succinate, saturated fatty acids, and histidine are more abundant in stimulated saliva.

Additionally, we have successfully identified metabolic differences associated with various pathological conditions, including Type 1 diabetes, hemodialysis, obesity, inflammatory bowel diseases (such as ulcerative colitis and Crohn’s disease), juvenile lupus, and COVID-19.

Recently, we have been exploring how the circadian cycle influences the salivary profile, aiming to understand daily variations and age-related changes.

Overall, our studies contribute valuable insights into the characterization of specific physiological states, as reflected in the salivary profiles of the Brazilian population.

Deciphering the dynamic nature of essential cellular machineries – lessons learned from *in vitro* and *in situ* NMR spectroscopy

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Protein quality control is an essential cellular function mainly executed by a vast array of different proteases and molecular chaperones. My team has a long-standing interest to understand the underlying functional principles of the systems residing in the bacterial periplams as a model system. I will present our latest insight into the underlying allosteric regulation of ubiquitous bacterial DegP serine protease, where we could reveal a couple of years ago the underlying temperature dependent activation as well as our latest work on its mitochondrial counterpart HtrA2 [1,2].

I will demonstrate how protein-enriched OMVs [3] can be applied to characterize the structure and dynamics of periplasmic as well as outer membrane proteins *in-situ.* Using solution NMR spectroscopy, we utilize methyl TROSY methods in combination with ^{19}F-NMR of aromatic residues, to unravel the dynamics of periplasmic proteins within this crowded cellular environment. Likewise, using solid state NMR spectroscopy allows us to characterize transmembrane domains of outer membrane proteins embedded in the native asymmetric membrane of OMVs. I will present the first structure determination of the 8-stranded b-barrel protein OmpA in its native membrane environment, revealing direct contributions from the lipopolysaccharides on structure and dynamics.

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Monitoring tRNA splicing and nucleotide chemical modification by nuclear magnetic resonance

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Transfer RNA (tRNA) maturation is a complex and essential process involving intron splicing, if any, and the incorporation of numerous post-transcriptional modifications. Although the biological importance of these modifications in gene expression is well established, methods to directly monitor their introduction during RNA biosynthesis remain scarce and rarely provide insights into the temporal nature of events. We have introduced the application of NMR spectroscopy to observe the maturation of tRNAs in cell extracts. By following the maturation of yeast $tRNA^{Phe}$ with time-resolved NMR measurements, we show that modifications are introduced in a defined sequential order, and that the chronology is controlled by cross-talk between modification events. In particular, a strong hierarchy controls the introduction of the T54, Ψ55 and m^1A58 modifications in the T-arm, this modification circuits identified in yeast extract with NMR also impact the tRNA modification process in living cells. Furthermore, by studying intron-containing $tRNA^{Phe}$, we show that most modifications in the tRNA core occur prior intron splicing. The presence of an intron extends the maturation time, allows incorporation of the m^5C40 and $m^2{}_2G26$ modifications, and changes the order of incorporation of the m^2G10 and $m^2{}_2G26$ modifications into the D-arm (unpublished data). The chronology of the maturation events is consistent with the localisation of maturation enzymes in yeast. The NMR-based methodology presented here paves the way for future investigation on tRNA maturation processes in eukaryotes.

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Quantitative and predictive modeling of RNA cellular activity using conformational ensembles

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Major advances have been made in predicting the 3D structures of biomolecules from sequence. Yet, predicting cellular activity from sequence requires conformational ensembles describing the distribution of alternative structures and their energetics on the free-energy landscape. Ensembles are critical for understanding and modeling cellular processes but remain difficult to characterize and predict, with experimentally informed ensembles available for only a limited number of biomolecules. To address this data gap, we leveraged 1H CEST NMR experiments, which eliminates the need for 13C/15N isotopic enrichment, and quantitatively measured the thermodynamic preferences of HIV-1 TAR RNA to adopt two alternative secondary structures for a designed library of sequence variants. The thermodynamic propensity to form the native secondary structure quantitatively predicted the extent of HIV-1 Tat-dependent transactivation in cells. These results highlight a critical limitation of current models that aim to predict only the dominant structure from sequence and demonstrate that ensemble-level descriptions are needed to model and predict cellular function quantitatively.

Dynamic RNA-protein interactions in A-to-I editing and biomolecular condensates in non-coding RNA pathways

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We employ integrative structural biology, combining solution NMR with complementary techniques and molecular dynamics to study the dynamics of RNAs and RNA-protein interactions. Two studies will be presented: (1) We discovered extensive dynamics associated with A-to-I hyper-edited dsRNAs, which exhibit unique conformational features. These features are specifically associated with hyper-editing and are important for the specific recognition of hyper-edited RNA by inosine binding proteins such as Endonuclease V. (2) We are studying molecular interactions, dynamics and mechanism, that are involved in the formation of biomolecular condensates implicated in the separation of small non-coding RNA pathways in *Drososphila*, combining NMR, biophysical techniques and cell microscopy.

NMR of Protein-RNA and RNA-only condensates studied in a biphasic context

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Biomolecular condensates have now being implicated in many biological functions and in almost all nuclear gene expression processes in eukaryotes from transcription, splicing, RNA maturation and degradation and RNA export. Hallmarks of these activities are the presence of membrane-less organelles like nuclear speckles, the nucleolus or the nucleopore, but also the presence of nuclear foci that can form upon stress or upon high expression of the biomolecules. Protein-only biomolecular condensates have been studied extensively by solid-state and liquid-state NMR in order to understand the molecular basis of condensate formation, yet biomolecular condensates are often a dynamic and transient state that is in constant exchange with its environment. It is therefore important to study condensates in their droplet form in exchange with the dilute phase and under various solution conditions.

In recent years, we used the N-terminal region of FUS as a model system to study condensate in a biphasic form with NMR spectroscopy. Using low percentage agarose hydrogels (Emmanouilidis et al, Nat. Chem. Bio, 2021), we could stabilize the droplets and study by NMR their biophysical properties (diffusion rates in both phases, partitioning between the two phases, average radius, exchange rates between the two phases, Novakovic et al, Nature Com, 2025) and their maturation (Emmanouilidis et al, Nat. Chem. Bio, 2024). We could further detect the level of hydration in the condensate and how FUS interact and exchange with RNA within condensates (Novakovic et al, Nature Com, 2025, De Vries et al, Science Adv, 2024). More recently, we also investigated RNA-only condensates that unlike protein or protein-RNA condensates are semi-solid and can only be studied by NMR indirectly using exchange with water (Schmoll et al, submitted).

Solid-state NMR-based structure and dynamics in filamentous phage

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Filamentous bacteriophages (phages) infect bacterial cells that possess the F-pili organelle. Ff family are one-micron long viruses containing a single-stranded DNA (ssDNA) with ~8000 bases wrapped by approximately 3500 copies of a major coat protein, gVIIIp. During replication, the non-structural gene 5 protein (gVp) attaches to a ssDNA creating a pre-mature virus thus preventing further replication and signaling the assembly of a new viral particle.

Upon viral assembly, gVIIIp changes from a membrane protein with two separated helical parts to a single long curved helix, and multiple units assemble to form the elongated mature particle. We have shown, using solid-state NMR and Rosetta modeling, that the structure of the M13 phage assembles by forming strong hydrophobic stacking interactions and by pointing the positively-charge C-terminal lysines towards the DNA lumen. In order to assess the dynamic properties of the intact viral capsid, we combined several pseudo-3D REDOR-based experiments to measure ^{13}C and ^{15}N CSA, as well as backbone and sidechain C-N dipolar order parameters. The rigid-limit CSA interactions (6-12 kHz) were almost fully recoupled suggesting a rigid capsid on this time scale. On the other hand, the REDOR curves, which were resolved using either single-quantum (DARR, RFDR) or double-quantum J-based spectroscopy, report on capsid motions on a time scale of ~1 kHz and on the highly dynamic N-terminus. C-N dipolar order parameters are also shown to be an excellent tool to characterize the dynamics of residues involved in electrostatic interactions with the DNA.

During viral replication, gVp, a homodimer in solution, attaches to the ssDNA and signals the onset of assembly. We determined the structure of gVp in the gVp-ssDNA complex by magic-angle spinning NMR, obtaining a backbone RMSD of 0.37Å for the well-defined regions of the ten lowest energy structures, and good convergence criteria. Distance and torsion angle restraints were obtained from multi-dimensional experiments (on fully and sparsely labeled samples) and then given as an input to Xplor-NIH. Convergence to a successful structure was achieved by using (i) weak restraints from X-ray DNA-free dimeric form structure; (ii) avoiding structure deformation due to the homodimer interaction; (iii) repeated steps of ambiguity and violation analysis, and (iv) refinement. Free and DNA-bound g5p show significant changes in key locations having an RMSD of 6.4Å, which is accompanied also by loss of several secondary structure elements but with preservation of the main core of the beta barrel. The changes point to a more compact DNA binding pocket and flattening of the protein side facing the dimer-

dimer interface explains the cooperative binding which promotes the cellular assembly of this pre-mature virus.

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Amyloid structural biology: beginning of life, in life and end of life

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Amyloids are usually associated with diseases such as Parkinson's disease (alpha-synuclein), but there are many more functional amyloids such as the storage of the hormone beta-endorphin. In contrast to the functional amyloids with an evolutionary-optimized single polymorph structure that is related to its function disease-associated amyloids are polymorphic in nature as exemplified with alpha-synuclein amyloids. Furthermore, the aggregation kinetics can be dominated by secondary nucleation, which can be interfered with by the chaperone Brichos for example. In addition, alpha-synuclein amyloids can by catalytic active (i.e. ATP -> ADP hydrolysis).

Since also short peptide amyloids show catalytic activity such as ATP synthesis, can replicate themselves by template assistance, can grow inside membrane mimicking vesicles, and bind RNA with specificity their potential role in the origin of life is indicated.

Biomolecular condensates in neurodegeneration

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Biomolecular condensation through liquid-liquid phase separation plays a fundamental role in cellular organization but can become dysregulated in neurodegenerative diseases. In Alzheimer's disease, tau undergoes aberrant phase separation, leading to pathological aggregation and the formation of disease-associated filaments. Post-translational modifications and multivalent interactions further modulate these processes, influencing both toxic aggregation and physiological functions such as synaptic organization. Advanced biophysical approaches, including NMR and imaging techniques, provide key insights into the molecular mechanisms underlying condensation. Understanding these processes is crucial for developing strategies to target pathological protein assemblies and restore cellular homeostasis in neurodegeneration.

Protein dynamics and kinetics with applications to neurodegeneration studied by NMR spectroscopy

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NMR spectroscopy is a powerful tool to study dynamics and kinetics of conformational ensembles. While pico-second to one digit nano-seconds are well covered by relaxation measurements and several 10 micro-seconds to millisecond by relaxation dispersion, relying on the variation of isotropic chemical shifts, the region between one digit nano-seconds and several 10 mikro-seconds is difficult to access. High power relaxation dispersion can assess the amount and kinetics of motion in this region. This will be discussed in the context of protein motion (1) with approaches to get information about the time region between ns and µs. The importance of optimal control pulses for high field NMR [2] of proteins will be emphasized.

In the 2nd project, we have studied the process of aggregation of α-synuclein on membranes in vitro and identified key time points in the aggregation process, that enable targeted isolation of a so called intermediate 1 and the fibrillar endpoint (3). Intermediate 1 has the functional characteristics of a toxic oligomer and the structure of the tetramer will be presented (4). In addition, we determined the structure of anle138b, a clinical drug candidate for the treatment of various neurodegenerative diseases including Parkinson's, Alzheimer's and Huntington's disease, Multiple System Atrophy, and type II diabetes mellitus (5), bound to lipidic fibrils (6). The small molecule binds in a cavity of the lipidic fibril (7). The same is found for the PET candidate MODAG-005 when bound to the lipidic fibrils (8). Comparison with binding of this molecule to lipidic Aβ fibrils will be discussed (9) searching for commonalities. Toxicity mechanisms by the comparison of the aSyn tetramer in the presence and absence of anle138b will be discussed that provide first insight into detoxification of oligomers.

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Posters session 1

Posters with an even number

Structure and functional insights of SARS-CoV2 Nucleoprotein and non-structural protein 3

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Human coronavirus associated with severe acute respiratory syndrome (SARS) is responsible for the COVID-19 pandemic. The nucleoprotein (N) in SARS-CoV-2 encapsidates the viral genome and serves as a cofactor in the replication-transcription complex (RTC), whose function relies on an essential interaction with non-structural protein 3 (nsp3). The full-length nucleoprotein exhibits a flexible domain architecture, and the flexible linker region adjacent to the RNA-binding domain is crucial for its interactions with nsp3. As new variants of SARS-CoV-2 emerge, it is vital to investigate the structural complexity and functional dynamics of the nucleoprotein, along with its interactions with 5' UTR RNA and nsp3, as these may influence viral pathogenicity. Our study has shown that the dimeric nucleoprotein interacts with the N-terminal Ubl1 (Ubiquitin-like domain 1) of nsp3 through two linear motifs located in the intrinsically disordered region between its two folded domains. These bipartite interactions form a highly compact dimeric nucleoprotein that regulates its binding to RNA, suggesting that nsp3 plays a key role in facilitating the association between the nucleoprotein and the RTC. Additionally, the nucleoprotein comprises a serine-arginine-rich (SR) region reported to be hyperphosphorylated in infected cells. Our study revealed that the phosphorylated SR region binds to the same interface as single-stranded RNA, resulting in the complete abrogation of RNA binding. This suggests a potential role for phosphorylation in the unpackaging of the viral genome. In summary, this study emphasizes the structure and function of the nucleoprotein and nsp3 in SARS-CoV-2.

Flash poster 1

High-Resolution Relaxometry (HRR) for probing multiscale dynamics in complex systems

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Understanding biomolecular dynamics across multiple timescales is essential for uncovering the mechanisms behind recognition, regulation, and flexibility in biological systems—and, more broadly, in complex materials such as food. While high-field NMR spectroscopy offers atomic-level resolution of structure and fast motions, accessing slower or field-dependent processes remains a challenge.

We present a new methodology that bridges this gap by combining high-field resolution with low-field sensitivity to spin dynamics, unlocking new ways to study biological systems. At the core of this approach is a newly engineered high-speed hybrid pneumatic/mechanical shuttle capable of transferring samples at velocities up to ~27 $m \cdot s^{-1}$ between high (up to 16.4 T) and low magnetic fields (down to 36 mT), all while remaining compatible with standard NMR probes and hardware, without compromising spectral quality or experimental throughput.

This innovation powers **High-Resolution Relaxometry (HRR)**—a technique that leverages the stray field of a high-field spectrometer for variable-field relaxation measurements, overcoming the limitations of FFC in resolution and access to fast motions. As part of the Horizon 2020 *HIRES-MULTIDYN* project, we contributed to the development, validation, and exploitation of two Fast Shuttle System (FSS) prototypes installed in Paris and Florence. These systems enable HRR with unprecedented resolution and were successfully applied across a wide range of biological scenarios: protein-ligand interactions (e.g., MMP12), metabolite–macromolecule interactions in complex mixtures (e.g., urine), and intrinsically disordered proteins (e.g., α-synuclein), where nitrogen relaxation at low fields revealed key features of IDP dynamics.

Combined with FFC, HRR enables integrative relaxometry, extending the accessible spectral density range and capturing multiscale motions with unmatched detail. This synergistic approach has also proven powerful for studying highly viscous systems like olive oil and for evaluating the dynamic behavior of candidate MRI contrast agents.

This poster introduces the new FSS system and HRR methodology, its experimental robustness, and key biological applications. Together, these advances expand NMR's role as a unique tool to probe biological systems at the molecular level, offering new insights into their interactions and dynamics.

From Fold to Function: Key Structural Features of the HDV-like Ribozyme drz-Mtgn-1

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The Hepatitis Delta Virus (HDV) ribozyme[1] is a self-cleaving RNA with a nested double pseudoknot structure, essential for viral replication. Numerous HDV-like ribozymes have been discovered across diverse organisms[2], and despite substantial variation in sequence and length, they share a common overall fold to catalyze the same reaction, relying on only a few conserved nucleotides and motifs located within or near the catalytic core. Among them, the minimal HDV-like ribozyme drz-Mtgn-1, which lacks the P4 stem loop, was identified in a human intestinal metagenomic phage genome and exhibits a distinct bell-shaped self-cleavage activity in response to divalent metal ions.[3] While the biological role of drz-Mtgn-1 remains unclear, we have recently shown that other minimal HDV-like ribozymes - referred to as theta ribozymes - likely function in tRNA processing within the phage genomes.[4] However, until now, no structural information has been available for any minimal HDV-like ribozyme.

To investigate the structural basis of catalysis in these minimal systems, we employed NMR spectroscopy with a sophisticated $^{2}H/^{13}C/^{15}N$ isotope labelling strategy to study drz-Mtgn-1. We assigned the exchangeable protons and confirmed the formation of the predicted nested double pseudoknot structure in drz-Mtgn-1. Comprehensive resonance assignments via 2D NOESY and ^{1}H-$^{13}C/^{15}N$ HSQC experiments enabled us to characterize most of the non-exchangeable protons within key structural motifs forming the catalytic core. Titration experiments varying pH and metal ion concentrations revealed chemical shift perturbations and line broadening within these motifs, indicating underlying conformational dynamics that may be important for catalytic activity.

Our findings demonstrate that even a highly reduced structural scaffold can achieve the nucleotide arrangement necessary for catalysis, offering new insight into the adaptability of HDV-like ribozymes and the principles of RNA folding and function.

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Flash poster 2

Selective labeling strategies for In-cell NMR studies of intrinsically disordered proteins

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Selective isotopic labelling is a powerful tool to investigate protein structure and interactions inside living cells. As we previously demonstrated on folded proteins, the right selective labelling can result in more sensitive NMR experiments allowing for the detection of proteins at lower concentrations, closer to the endogenous levels, and can greatly simplify complex and crowded spectra arising from large proteins[1]. Similar strategies can be also applied to investigate intrinsically disordered proteins. In order to detect chemical shift and intensity changes in highly crowded 2D NMR spectra, we introduced an excess of ^{13}C-labelled amino acid precursor in a uniformly-^{15}N labelled culture medium which, once supplied to transiently transfected cells, overexpressing the protein of interest, allows for the incorporation of the corresponding double labelled amino acid. Exploiting this labelling scheme we recorded 2D H-N planes of triple-resonance experiments on intact HEK293T cells and cell lysates. The resulting spectra contain only a fraction of the signals, which are relatively well dispersed in the spectrum and provide relevant information on chemical shift and intensity changes due to interactions with the cellular environment. Other labelling strategies involving double labelled ^{15}N,^{13}C amino acids are currently being tested.

We were also able to optimize the experimental conditions to achieve coexpression of an intrinsically disordered protein and an interacting partner in HEK293T cells. Furthermore, in order to perform experiments observing one protein at a time during interaction, we produced a stably-transfected inducible monoclonal cell lines expressing the intrinsically disordered protein. To achieve this, we used a recently reported approach, developed together with Lukas Trantirek's group[2]. The approach exploits the Piggy-Bac method, which allows for multiple random integration of the protein gene in highly expressed regions of the cell genome. This method grants a leak-free, inducible protein expression system and allows for highly homogeneous protein expression levels across the cell population.

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Flash poster 3

A Deeper Understanding of APLF's Role in the NHEJ Pathway Through Intramolecular Interactions Revealed by NMR

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Non-homologous end-joining (NHEJ) is the primary pathway for repairing DNA double-strand breaks (DSBs) in eukaryotes. NHEJ involves numerous proteins, many of which contain intrinsically disordered regions (IDRs), making NMR an ideal tool for their study. Aprataxin and PNKP-Like Factor (APLF) is an NHEJ protein characterized by extensive disorder and comprising four functional regions: the fork-head-associated domain (FHA), the middle domain (MID), and the C-terminal acidic domain (CTAD). The FHA binds to the X4L4 ligase complex via XRCC4, while the MID interacts with the Ku heterodimer, the initial responder to DSBs. APLF is described as a scaffolding protein that stabilizes the NHEJ complex, tethers DNA synapsis, and promotes ligation. However, the exact mechanisms and underlying dynamics remain unclear.

We investigated the interactions of APLF with other NHEJ proteins using NMR titrations. We further characterized the exposed interactions using advanced NMR techniques such as CPMG relaxation dispersion and CEST experiments. Leveraging NMR's atomic resolution, we identified some binding sites and designed specific mutants. To complement our NMR approach, we employed single-molecule magnetic tweezers experiments.

Our findings describe two key intramolecular interactions: the binding of the CTAD to the FHA, and the homodimerization of the FHA. The CTAD binds to the FHA near its XRCC4 binding site, suggesting a potential competition between these partners. Additionnally, the CTAD competes with DNA for binding to the Ku complex. Point mutations introduced in the FHA revealed a putative allosteric regulation mechanism between the CTAD binding site and the FHA dimerization interface.

This network of weak interactions may help better understand the role of APLF in the NHEJ pathway. While some findings may suggest how APLF can stabilise DNA synapsis with only Ku, others could provide insights into the dynamics and regulations of APLF in the assembly of the NHEJ complex.

Flash poster 4

Unraveling the dynamics of the kinase p38γ through Ultrafast High-Resolution Relaxometry (UHRR): Insights into Molecular Motion

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Introduction: Relaxation is a unique tool to determine protein dynamics. Traditionally, relaxation experiments on proteins are performed at high fields (>10 T). Yet, such measurements are not sufficient to fully characterize nanosecond motions [1]. In this work, we introduce ultrafast high-resolution relaxometry as a new method to probe low ps to high ms motions in the protein kinase p38γ. The regulation of p38γ is expected to rely on allosteric pathways [2].

Aims: Our objective is to better understand allosteric pathways in p38γ and, in particular, the role of nanosecond dynamics of its side-chains with NMR relaxation and molecular dynamics simulations.

Methods: We combine extensive high-field relaxation measurements on ^{13}C methyl-bearing side chains with a new method, Ultrafast High-Resolution Relaxometry (UHRR). UHRR allows us to measure relaxation rates down to very low fields on a high-field spectrometer and get a full coverage of the spectral density function in a site-specific manner. A new prototype was used to accelerate field cycling from 14 T down to 100 mT in as little as 80 ms.

Results: With these experiments, we obtain and analyze Nuclear Magnetic Relaxation Dispersion (NMRD) profiles to get a further understanding of the dynamics in p38γ. We have assigned methyl resonances in p38γ and have measured relaxation rates at over 28 magnetic fields covering nearly 6 orders of magnitude. Our analysis of relaxation with the extended model-free approach allows the determination of nanosecond motions even in the presence of chemical exchange.

Conclusion: UHRR is a new technique which allows to study the dynamics of large proteins over a broad range of motions (low ps to high ms). In this work, we successfully apply this technique to fully characterize the dynamics of p38γ.

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CDK5-Driven Phosphorylation and Interaction Mapping of the Scaffold Protein JIP1

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The scaffold protein JIP1 plays a central role in the c-Jun N-terminal kinase (JNK) pathway by organizing signaling components, such as kinases, into highly regulated complexes[1]. As an intrinsically disordered protein, JIP1 also serves as a platform for post-translational modifications, most notably phosphorylation, which modulates the assembly of the signaling complexes[2]. Recent work has highlighted the *in vivo* interaction between JIP1 and the activator protein p25 of the cyclin-dependent kinase 5 (CDK5) leading to CDK5-mediated phosphorylation of JIP1 and enhanced neuroaxonal outgrowth[3]. However, the structural basis of this interaction and the CDK5-dependent phosphorylation landscape of JIP1 remain largely elusive.

In this work, we study the CDK5-dependent phosphorylation kinetics of the 450-amino acid disordered N-terminal tail of JIP1. Using real-time NMR, we obtain a quantitative, temporal and site-specific atomic resolution view of the CDK5 phosphorylation landscape. Our work reveals that CDK5 performs multi-site phosphorylation of JIP1 with markedly different kinetics, including of sites previously linked to biological function.

To further uncover the interaction mechanism of JIP1 with the CDK5-p25 complex, we performed AlphaFold3 predictions of the complex, highlighting a potential folding-upon-binding mechanism of JIP1. Following the expression of the CDK5–p25 complex in insect cells, we experimentally characterized its interaction with the disordered tail of JIP1. Chemical shift titrations and ^{15}N R_{1r} relaxation experiments reveal the precise interaction site on JIP1, consistent with the AlphaFold3 model. Ongoing work will focus on structure determination of the JIP1-CDK5-p25 complex and investigating how kinase docking influences the phosphorylation dynamics of JIP1.

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NMR structure calculation with ARIA and ARIAweb

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Nuclear Magnetic Resonance (NMR) spectroscopy allows the determination of protein structures, using a collection of distance information from Nuclear Overhauser Effects (NOE). The Ambiguous Restraints for Iterative Assignment (ARIA) program allows the determination of high-resolution structures trough automated assignment of NOE cross-peaks and iterative calculation of 3D conformations[1]. To improve the user experience and generate more reliable NMR structure, new developments are required. We will present the most recent features that we added to the ARIA program, such as the possibility to run calculation online via the *ARIAweb* server[2], fully compatible with the new NMR Exchange Format (NEF)[4] for streamline data import and deposition. Recent developments also include the possibility to run *consensus* calculations, that allows to achieve better accuracy and more objective structure ensembles[3], and symmetry parameters adapted to amyloid fibrils studied by MAS solid-sate NMR[5].

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Evolutionary and Structural Insights into the Ros/MucR Protein Family Across Prokaryotes and Eukaryotes

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The discovery of the ROS protein in *Agrobacterium tumefaciens*, a Gram-negative α-proteobacterium, challenged the assumption that zinc finger (ZF) domains are exclusive to eukaryotes. Genetic and biochemical studies have since revealed that ROS homologues, classified within the Ros/MucR family, are widely distributed across bacterial taxa, regulating genes involved in symbiosis, stress responses, and virulence. These proteins bind DNA by assembling into oligomeric structures that can adopt open or closed circular conformations, modulating DNA topology and silencing gene expression. By targeting AT-rich DNA, Ros/MucR proteins act as H-NS-like repressors in bacteria lacking canonical H-NS proteins.

We conducted a comprehensive computational analysis and identified 1,840 Ros/MucR homologues across diverse taxa. Most were found in α-proteobacteria, followed by γ- and β-proteobacteria, with fewer sequences detected in δ-proteobacteria and Gram-positive bacteria. Interestingly, 17 homologues were identified in eukaryotic lineages, including Chromista, Plantae, Fungi, and Animalia. Sequence identity was highest among α-proteobacteria, whereas greater divergence was observed in β- and γ-proteobacteria, and moderate similarity was found in eukaryotes compared to the original Ros protein. Moreover, zinc coordination pattern analysis revealed considerable structural diversity, particularly within β- and γ-proteobacteria, with numerous distinct coordination motifs. Distinct zinc-coordinating patterns were also observed among eukaryotic homologues, emphasizing the structural flexibility of the Ros/MucR family, although the zinc-binding capability of most of the coordinating prototypes needs to be investigated.

Among Ros/MucR family members, we selected MucR2 from *Sinorhizobium meliloti* for detailed structural characterization due to its unusual zinc-coordinating residues—Cys, Asp, His, Lys, and Tyr (CDHKY). Using NMR spectroscopy, we confirmed that the DNA-binding domain of MucR2 adopts a monomeric state in solution and binds zinc under near-physiological conditions.

These preliminary results expand our understanding of Ros/MucR evolution and function, opening new avenues for exploring the roles of this family of zinc fingers in both prokaryotic and eukaryotic systems.

Direct NMR evidence for weak binding of the vesicular SNARE protein synaptobrevin-2 to the α-synuclein C-terminus

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The protein α-synuclein is a hallmark of Parkinson's disease. While its pathological involvement in neurodegeneration is clearly established, its functional role under physiological conditions is less clear. There are several suggestions about the physiological function of α-synuclein in the presynapse. One is the interaction with the SNARE protein synaptobrevin-2. Synaptobrevin-2 (also called VAMP2) plays a crucial role in forming the SNARE complex, which is essential for the fusion of synaptic vesicles with the plasma membrane and the resulting release of neurotransmitters. Here, we provide direct evidence by nuclear magnetic resonance (NMR) spectroscopy and other biophysical methods that the α-synuclein C-terminus weakly binds to the vesicular SNARE protein synaptobrevin-2 , driving synaptic vesicle fusion at the neuronal synapse. Both proteins are intrinsically disordered in their monomeric pre-fusion state. Multiscale thermophoresis (MST) data, as well as biolayer interferometry (BLI), consistently find a dissociation constant K_D in the low µM range. NMR titration and paramagnetic relaxation enhancement (PRE) experiments map the binding site with residue-specific resolution to the α-synuclein C-terminus and the synaptobrevin-2 linker domain. Titration data also indicate an involvement of the synaptobrevin-2 N-terminus. NMR relaxation rate constants of both proteins indicate an increasing rigidity of the α-synuclein C-terminus and the synaptobrevin-2 C-terminal SNARE motif and linker domain upon binding.

The provided NMR residue-specific mapping of the binding region, together with the MST and BLI data on the binding affinity, establishes clearly a binding interaction between α-synuclein and the vesicular SNARE protein synaptobrevin-2. We propose that the weak affinity binding enables α-synuclein to, for example, attract and guide synaptobrevin-2 (and the attached vesicle) to SNARE complex formation. Once a synaptobrevin-2 binder of higher affinity, one of the other SNARE proteins, is approached, α-synuclein can, however, easily release synaptobrevin-2 towards its final destination, which is SNARE complex formation. Our finding links neurodegeneration to neuronal exocytosis. In the pathological case of α-synuclein aggregation, the lack of free, monomeric α-synuclein would eventually reduce the rate of synaptic vesicle fusion, suggesting an additional loss-of-function mechanism in the context of neurodegenerative processes.

Uncovering the structural logic of CNN autoinhibition: an integrative NMR, SAXS, and AlphaFold approach

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Drosophila Centrosomin (Cnn) is a key centrosomal protein that functions in centrosome assembly and microtubule nucleation. The N-terminal region of Cnn contains a highly conserved CM1 domain that binds and activates microtubule nucleating multi-protein γ-tubulin ring complexes (γ-TuRCs) specifically at the centrosome during mitosis. This CM1 domain is auto-inhibited by an adjacent CM1 autoinhibition (CAI) domain, something that is essential to avoid ectopic γ-TuRC binding and activation in the cytoplasm that would otherwise lead to severe mitotic and developmental defects (Tovey et al., 2021). Nevertheless, the structural basis of this auto-inhibition remains unknown, particularly as crystallographic approaches have failed.

To overcome this, we employed an integrative strategy combining solution-state NMR, small-angle X-ray scattering (SAXS), and structure prediction via AlphaFold. Alphafold predictions showed that the CM1 domain is dimeric (something we confirm via SEC-MALS) and split into 2 subdomains joined by a linker – a short dimeric helix (helix), which contains the highly conserved F115 essential for γ-TuRC binding, and a downstream longer coiled-coil (CC). AlphaFold models provided two initial structural hypotheses: in both models, the helix and CC form a hairpin due to direct interactions, but the CAI interacts either with the helix or the CC. We therefore purified a series of truncated constructs covering different combinations of individual sub-domains and performed ^{1}H-^{15}N HSQC NMR to detect intramolecular interactions based on spectral shifts. Our results reveal specific contacts between the CAI and the helix and between the helix and the CC. We used SAXS to examine the structure of the full-length N-terminal region, which confirmed a folded and dimeric state. However, current SAXS profiles do not fully validate the precise arrangements predicted by Alphafold, underscoring the potential structural heterogeneity of Cnn in solution. While the SAXS data remain preliminary, they help define boundaries for future modelling and guide ongoing work.

This study highlights the complexity of Cnn's autoinhibition and the strength of integrative approaches to tackle flexible, multi-domain proteins beyond the reach of classical structural methods.

Flash poster 5

Membrane binding properties of the intrinsically disordered regions in FCHO1 and FCHO2

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FCHO1 and FCHO2, two evolutionarily related members of the F-BAR domain-only (FCHO) protein family, are critical initiators of clathrin-mediated endocytosis (CME), the primary endocytic pathway in mammalian cells. Their folded F-BAR domain interacts with the cell membrane while their intrinsically disordered region (IDR) and folded µ-homology domain recognize several proteins at different interaction sites simultaneously, leading to an intracellular protein recruitment after cargo-receptor binding, initiating vesicle budding and cargo internalization. While the structures of their folded domains are already well characterized, the functional role of their IDRs remains elusive. In this study, we investigate the structural and functional differences between the IDRs of FCHO1 and FCHO2, with a focus on their membrane interaction potential beyond the established role of the F-BAR domain. Using nuclear magnetic resonance (NMR) spectroscopy and fluorescence microscopy, we analyzed the interactions between purified FCHO-IDRs and model lipid vesicles of different sizes and compositions. Our findings support the hypothesis that the IDRs contribute to membrane sensing and possibly assist in F-BAR domain-mediated membrane remodeling in a distinct fashion for FCHO1 and FCHO2. These results highlight a previously underappreciated role of IDRs in endocytic initiation and raise new questions about their interaction with other cellular partners. Ongoing and future work will explore their contribution to protein recruitment dynamics and competitive interactions during CME.

NMR study of the structure and interactions of the nucleosome-bound human BCL7A protein

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The SWI/SNF complex is a highly conserved ATP-dependent chromatin-remodeling complex that plays a crucial role in regulating gene expression by altering chromatin structure and enabling or restricting access to DNA for transcription factors and other regulatory proteins. This 2MDa complex is composed of at least 11 subunits and exists in three forms: canonical BAF (BAF or cBAF) complexes, polybromo-associated BAF (PBAF) complexes and non-canoncial BAF (ncBAF) complexes, which are distinguished by the presence of different subunits. In mammals, the SWI/SNF complex is essential for cellular differentiation, development, and response to environmental cues. Mutations and alterations in its subunits are associated with a wide range of diseases, which motivates the better understanding of the SWI/SNF complex at a molecular level.

The BCL7 (BCL7A, BCL7B and BCL7C) family proteins are among the newest identified subunits of the mSWI/SNF complex. Although their role within the complex remains unknown and structural information is limited, mutations in these proteins are implicated in several cancers, particularly blood cancers. In this study, the structure of the mammalian SWI/SNF complex is unraveled using cryo-electron microscopy (cryo-EM), showing that BCL7A forms a stable, high-affinity complex with the nucleosome core particle (NCP) through its interaction with the acidic patch of the nucleosome. However, several studies showed that another well-known SWI/SNF associated protein called BAF47 interacts with the same part of the nucleosome.

In this study, we use ^{15}N-NMR to prove a potential competition between BAF47 and BCL7, in order to understand their interaction with the nucleosome and with each other. ^{15}N-labeled BCL7 is used in this study and several constructs of BAF47 are tested.

Insights into the Substrate Uptake Mechanism of *Mycobacterium tuberculosis* Ribose 5-Phosphate Isomerase and Perspectives on Drug Development

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Mycobacterium tuberculosis ribose 5-phosphate isomerase B (MtRpiB) is a key enzyme in the pentose phosphate pathway and represents a promising target for tuberculosis drug development by the lack of homologues in humans. The active site of MtRpiB, located at the dimer interface, is partially shielded by a barrier formed by residues H12, R113, R137, and R141, requiring ribose 5-phosphate (R5P) to cross this barrier to access the catalytic site and undergo isomerization to ribulose 5-phosphate (Ru5P). Here, we combined high-resolution NMR spectroscopy and molecular dynamics simulations to elucidate the substrate recognition and binding mechanism of MtRpiB. Our results reveal that MtRpiB does not employ a conventional open/close-lid mechanism. Instead, it utilizes an uncommon "flipping" mechanism in which the phosphate group of the incoming ligand initially docks at the enzyme's surface, triggering a conformational flip that repositions the ribose and nitrogenous base into the internal cavity. NMR chemical shift perturbation (CSP) and saturation transfer difference (STD) experiments show that nucleotide derivatives such as AMP and ADP interact with the active site, whereas ATP also interacts but with lower affinity, and ribose alone exhibits negligible binding. The phosphate moiety anchors the ligand, while hydrophobic and van der Waals interactions stabilize both the ribose group and the nitrogenous base within the cavity. Molecular dynamics simulations further confirm that the enzyme-ligand complex undergoes dynamic conformational changes, with ligands alternating between solvent-exposed and buried states, while the phosphate group remains stably bound. This particular flipping mechanism highlights MtRpiB's selectivity for phosphorylated substrates and provides new structural insights into how different ligands are recognized. Our findings may help guide the design of MtRpiB inhibitors, suggesting that both the importance of phosphate binding for ligand internalization and the ability of the enzyme to accommodate different ligand sizes should be considered. Overall, these results contribute to the understanding of MtRpiB and may support future efforts to develop new tuberculosis therapies.

Flash poster 6

PI3K SH3 Domain - Amyloid Model Protein (Un)folding investigated by NMR

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The misfolding and aggregation of proteins is associated with neurodegenerative diseases like Alzheimer's or Parkinson's disease. To get a better understanding of the underlying processes, the PI3K SH3 domain is used as a model system. At acidic pH the well folded native structure [1] of the protein unfolds and aggregates into well-folded fibrils [2]. The structure of these PI3K SH3 fibrils was determined recently using cryo-EM [3].

We use diverse NMR experiments to get insights into the unfolding process of the PI3K SH3 domain itself and its dependencies [2]. To this end, we apply solution-state NMR experiments, perform secondary chemical shift analyses and relaxation experiments - hence obtaining information about the secondary structure propensities and the dynamics of the investigated protein - to follow the unfolding process with regard to its temperature dependency at residue-type resolution.

At acidic pH and lower temperatures an equilibrium between two conformations is present, that at higher temperature shifts towards the unfolded conformation. Moreover, the SH3 PI3K domain exhibits different aggregation behaviour at low and high temperatures. 3D and 2D experiments, as well as the conducted relaxation experiments and zz-exchange experiments allowed us to obtain site specific resonance assignments for both conformations.

These assignments form a basis for uncovering the cause of the different behaviour of the conformational states. In addition, it is a starting point for ultrafast-MAS-measurements using proton detection, that we want to execute to get further insights into the complexity of the (un)folding process of the PI3K SH3 domain.

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Deep-Learning: suppression of vibration artefacts in High Resolution Relaxometry experiments

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High Resolution Relaxometry couples low-field relaxation with polarisation and detection in a homogeneous high-field magnet by rapidly shuttling (~60 ms transfer times) the sample to the stray field or a fast field cycling coil. Combining high-field polarisation and detection with low-field relaxation.

In these experiments, fast shuttling times are critical to prevent polarisation loss during transfers. In mechanical shuttle systems, motorvibrations can propagate through the bore of the Dewar, magnet and detection coils, leading to modulations of the free induction decay and producing vibration artefacts in the recorded spectra. These peak distortions make quantifying intensities and relaxation rates challenging.

We developed a FidNet-based model to (1) efficiently remove the vibration artefacts while maintaining the correct line widths (2) produce well calibrated errors for the transformed spectra. This model was trained with on-the-fly data generation where simulated spectra were modulated by a point spread function yielding anti phase artefacts. The performance of the modelwas evaluated on Hilbert space simulations of the molecules found in the GISSMO database and on experimental spectra recorded on mixture of small molecules.

This work highlights the significant potential of deep learning for processing high-resolution relaxometry experiments and aiding in the extraction of information from complex spectra.

A novel phospho-binding domain at the N-terminus of the microtubule depolymerase KIF2C is essential for its condensation in mitosis

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KIF2C (also known as Mitotic Centromere-Associated Kinesin) is a microtubule depolymerase, essential for ensuring proper kinetochore-microtubule attachment and chromosome alignment during mitosis. It is overexpressed in various cancer types, including breast cancer. Both depletion and overexpression of KIF2C cause genomic instability. Therefore, KIF2C is tightly regulated by mitotic kinases, including PLK1 (Polo-Like Kinase 1). Our recent study show that KIF2C forms biomolecular condensates near the extremities of microtubules during prometaphase (Skobelkina et al., BioRXiV 2024). This serves as a hub for recruitment of various mitotic regulators like BRCA2 to ensure accurate chromosome alignment. Treatment with PLK1 inhibitors impairs the formation of KIF2C condensates which underscores its phosphorylation dependent control. Using NMR chemical shift mapping, we identified a conserved, positively charged surface of the N-terminal domain of KIF2C structure, which binds specifically to phosphorylated peptides. Mutational analysis indicates that K52 and K54 are essential KIF2C residues for binding to phosphorylated peptides; substitution of these sites disrupts the interaction. In addition, the KIF2C variant K52E + K54E is not able to form biomolecular condensates during mitosis. We searched for phosphorylated proteins binding to this N-terminal domain of KIF2C. Using a TurboID-KIF2C-mCherry-CRY2 construct and the optogenetic tool developed by the team of J. Basbous (IGH Montpellier), we identified by mass spectrometry the components of mitotic KIF2C condensates, including CEP170, CEP170B and Sgo2. We further checked experimentally, using NMR and ITC, for the presence of phosphorylated KIF2C binding motifs in these proteins. Based on these analyses, we propose that the KIF2C N-terminal domain selectively binds to a motif defined as [pS/T]-X-Φ-I, 'Φ' being a hydrophobic residue. From the knowledge of this KIF2C interaction motif (KIM), we will now search for new partners within the proteins detected by mass spectrometry in the KIF2C condensates. Finally, we found that these phospho-dependent interactions are conserved in KIF2A, which is an homologue of KIF2C also involved in the assembly of the mitotic spindle, underlining the importance of the KIM motif for a proper progression of mitosis.

Flash poster 7

Conformational Dynamics at the RNA 3' End in the Transcription Bubble of *E.coli* RNA Polymerase Studied by Solid-State NMR

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The transcription bubble formed by RNA polymerase (RNAP) serves as a structural pivot in regulating elongation, pausing, and termination of transcription. In intrinsic termination, both the structure and the sequence of the nascent RNA regulate the transcription efficiency. While the recent studies by cryo-EM have simplified the process, little is known about conformational variability at the RNA 3' end and how it is influenced by sequence composition.

Here, we present the Dynamic Nuclear Polarisation (DNP) enhanced solid-state NMR (ssNMR) investigation of the transcription bubble inside the elongation complex (~400 kDa) of *Escherichia coli* RNAP with a focus on conformational changes at the RNA 3′ terminus. The samples mimicking the termination-prone sequence with increasing uridine residues at the 3' terminus and stem loop at the 5' terminus were prepared using the *in vitro* reconstitution. The DNP-enhanced ssNMR allowed atomic-resolution detection of sugar pucker conformations, enabling the observation of the subtle differences in the hybrid architecture.

Our results reveal sequence-dependent shifts in ribose sugar pucker equilibrium, with increased uridine content promoting transitions from the canonical C3′-endo to C2′-endo conformations. These transitions were evident from characteristic chemical shift patterns associated with distinct puckering modes. While the C2' endo conformation is rare, it is found in specialized, important RNA structures, e.g., small RNA motifs, spliceosomes, and ribosomes.

These findings provide new structural insights into how uridine-rich RNA sequences may act as regulatory elements during termination. Through this study, we also explored the application of ssNMR in investigating the complex regions of the transcription process under near-native conditions and set the stage for adapting this methodology for broader biological assemblies

Flash poster 8

NMR-based secondary structure characterisation of the West Nile Virus Frameshift Element RNA and investigation of small molecule binding

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Programmed ribosomal frameshift is a regulatory mechanism for protein translation that occurs in many positive-stranded RNA viruses, including many human pathogens.[1][2] It enables the expression of different proteins encoded within a single open reading frame. Programmed ribosomal frameshift is primarily controlled by cis-acting elements in the mRNA sequence, such as pseudoknots or stable stem-loop structures. In West Nile Virus (WNV), the frameshift element (FSE) is predicted to form a pseudoknot structure, which is conserved within the Japanese Encephalitis Virus serogroup.[3] The -1 frameshift induces the translation of the NS1' protein, which enhances neuro-invasiveness[4] and plays a role in viral replication.[5]

The structural characterization and understanding of the function of the frameshift element in WNV and related viruses and the identification of frameshift-inhibiting ligands can contribute to the development of new antiviral pharmaceuticals. [6][7]

Solution-state NMR spectroscopy was used to investigate the secondary structure within the frameshift element of the WNV. We confirmed the predicted structure of this FSE as a pseudoknot structure, forming two base pairing stems and possible additional base pairs in the loop regions.

The base pairing of the two pseudoknot forming stems was confirmed by the assignment of the imino protons in the TROSY and NOESY spectra. The addition of Mg^{2+} showed a stabilising effect on the pseudoknot formation, especially on the formation of the second stem. Using CD spectroscopy in the addition to NMR methods, a Mg^{2+}-level and temperature induced conformational heterogeneity of the RNA was investigated. In addition, the formation of the second stem was confirmed by titration of a complementary oligonucleotide to a hairpin forming part of the pseudoknot. The assignment of aromatic and sugar resonances will allow high-resolution structural characterisation, opening up the possibilities for structure based drug design.

High-throughput fragment-based NMR screening was carried out to investigate the binding of small ligand molecules to the WNV frameshift element RNA. 1D-^{1}H, waterLOGSY and T_2-CPMG experiments were used to identify potential binders from a poised library of 607 compounds, which were also evaluated as binders on a Mg^{2+}-stabilised RNA structure.

Exploration of the stability of the HSP90 N-terminal domain at various Pressures and Temperatures by NMR spectroscopy

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HSP90 is a chaperone responsible for the correct folding of client proteins. When this protein dysfunctions it can cause various illnesses, therefore, it is a therapeutic target for possible treatments. This protein is highly flexible and undergoes large-scale structural rearrangements to perform its functional cycle. It is composed of three domains, and it is the ATP binding N-terminal domain (NTD, 25 kDa) that is the most flexible due to the presence of a long loop named ATP-lid that covers the nucleotide binding site. It was previously demonstrated that the HSP90-NTD possesses one major fundamental state in exchange with one minor excited state [1] characterized respectively by the ATP-lid in an open and closed conformation. The behavior of this flexible loop has been extensively described at various temperatures but never at higher pressures. However, pressure, like temperature, is a fundamental thermodynamic variable that can be used to modulate the free energy landscape of biomolecules, therefore it is a very interesting tool to study conformational fluctuations. Here, we investigated and optimized by NMR and thermal shift assays (TSA) the pressure/temperature conditions ensuring protein stability. We notably revealed at high pressure a new HSP90-NTD largely disordered state that is largely reversible. This preliminary study paves the way for the complete structural comprehension and analysis of HSP90-NTD.

1. Henot et al. Visualizing the transiently populated closed-state of human HSP90 ATP binding domain. Nature Communications, 2022

NMR Assessment of the High Order Structure of Biological Therapeutics in Erythrocytes Provides a New Tool for Drug Delivery Design

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An effective delivery system is crucial for ensuring the therapeutic efficacy of a drug. This is especially true for biological drugs, which possess unique physicochemical properties and complex pharmacokinetic profiles, and thus require a dedicate design. Whole erythrocytes, and more recently nanoparticles derived from red blood cells (RBCs), have been used in preclinical studies to deliver biological therapeutics: their biocompatibility and extended circulation time help to prevent immunogenicity, and reduce clearance and toxicity. However, characterizing such complex systems poses challenges that complicate their development and optimization. We argue that NMR spectroscopy enables the monitoring of the preservation of the high order structure of the encapsulated proteins, as well as their concentration, thereby assisting in formulation design, development, and manufacturing.

Specificity-Enhancing Mutations Remodel Allosteric Signaling and RNA-DNA Interactions in CRISPR-Cas9

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CRISPR-Cas9 is an innovative genome editing tool with broad applications in bioengineering. However, the occurrence of off-target effects has limited its use as a precision therapeutic. To address this limitation, engineering of the Cas9 enzyme has generated variants with increased specificity towards target DNA cleavage. However, the underlying mechanisms that confer specificity are not well understood, and characterization of specificity-enhancing properties is critical for the rational design of Cas9 variants that effectively mitigate off-target cleavage.

Biophysical studies and molecular dynamics (MD) simulations of *Streptococcus pyogenes* Cas9 have indicated that Cas9 function is mediated by multi-domain communication via allosteric signaling. It has been suggested that Rec3, a subdomain of the recognition lobe, allosterically activates the spatially distant HNH nuclease domain, though the molecular details of this Rec3-mediated activation remain elusive. Intriguingly, many notable high-specificity Cas9 variants house the majority of their specificity-conferring mutations within Rec3, including “high fidelity” HF1-Cas9, the “hyper accurate” HypaCas9, and the “evolved” evoCas9. How disparate mutations in Rec3 contribute to the specificity of Cas9, especially considering their distance from the catalytic sites, is unknown.

To provide insight into the mechanistic contributions of Rec3 to Cas9 specificity, we employed solution NMR spectroscopy and MD simulations to characterize the structural and dynamic effects of high-specificity mutations on the Rec3 domain, and more broadly, Cas9. We engineered an isolated Rec3 domain to experimentally probe its biophysical properties with atomic resolution, and generated high-specificity variants of Rec3, including HF1 Rec3, Hypa Rec3, and Evo Rec3. Our findings reveal that a universal high-specificity structure of Rec3 is induced by the specificity-enhancing mutations, which shift the dynamic profile of Rec3 to slower (ms) timescale motions. MD simulations suggest that the specificity-enhancing mutations also alter Rec3 contacts with the RNA:DNA hybrid, remodeling an important route of allosteric crosstalk to the HNH and RuvC catalytic domains. In a follow-up study, we are experimentally probing the structural and dynamic implications of guide RNA and RNA:DNA hybrid binding to Rec3 and full-length Cas9 to comprehensively define the biophysical mechanisms that drive Cas9 specificity.

Flash poster 9

Molecular basis of the activation of Type IV Filament machineries in bacteria

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Bacteria have evolved numerous specialised multiprotein membrane nanomachines to interact with their environment. Among them, Type IV Filaments (Tff) form a widespread family of nanomachines that span the bacterial envelope and mediate the assembly and disassembly of extracellular pilin fibres via cytoplasmic ATPases. Tff machines have been adapted to perform complex cellular functions, including protein secretion, DNA uptake, surface sensing and attachment, biofilm formation, prey killing and cell motility. These processes require the coordinated action of multiple machines and the precise control of their activity. While the molecular mechanism of pilin assembly is now well characterized, the factors that trigger the activity of Tff machines remain poorly understood. This project aims to decipher the molecular and structural basis of Tff machinery activation using a multi-scale approach, ranging from the structural investigation of protein complexes to the cellular investigation of protein dynamics using high-resolution microscopy techniques.

To achieve this, we studied two Tff subclasses: the TfaP machinery from the Gram-negative bacterium *Myxococcus xanthus,* and the MSHA pili machinery from the pathogenic bacterium *Vibrio cholerae.* In both systems, we identified key interactions targeting the same core components of the machinery, leading to the insertion of the assembly ATPase into the inner membrane platform. Our findings uncover a conserved molecular mechanism that regulates pilin fiber assembly across different Tff subfamilies.

Flash poster 10

NMR reveals the binding mode of three different full-length clinically approved monoclonal antibodies for the same target

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The Higher Order Structure (HOS) of multispecific biologics and conventional monoclonal antibodies can be inspected by mapping the fingerprint "left" on the target using solid-state NMR (SSNMR).[1] The chemical shift perturbation (CSP) experienced by the target in the complex is, indeed, sensitive to the HOS of the binding region of antibodies, and it can be used as an "indirect" reporter of the mAb structure. Here we show that SSNMR can be used to investigate the binding mechanism of different monoclonal antibodies against the same target. The pattern of the residues of the programmed cell death ligand 1 (PD-L1) ectodomain that are involved in the interaction with three different monoclonal antibodies allows us to monitor the preservation of the HOS. In particular, the analysis of CSP and signal intensities obtained for the three mAbs provides the fingerprint that each mAb "imprints" on the structure of PD-L1. While the preservation of the HOS can be used to develop mAb formulations and to monitor the antibody stability, the epitope mapping provides key information to improve the affinity by reshaping the binding site.

1. D. Rizzo, L. Cerofolini, S. Giuntini, L. Iozzino, C. Pergola, F. Sacco, A. Palmese, E. Ravera, C. Luchinat, F. Baroni, M. Fragai, *J. Am. Chem. Soc.* **2022**, *144*, 10006–10016.

Flash poster 11

Secondary structure motifs for high affinity binding to the human Y_4 receptor unravelled via $^{13}C,^{13}C$ DARR, T_1 and $T_{1\rho}$ relaxation solid-state NMR

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Achieving good selectivity towards the desired biological target is widely recognised as one of the major challenges in the field of drug design for therapeutic purposes. This is particularly challenging when the intended biological target is a G-protein coupled receptor (GPCR).[1,2] There are about 800 known GPCRs in the human body, which modulate a plethora of fundamental physiological functions, and are thus very attractive targets for drug-based therapeutic approaches. However, the complex energy landscape of their life cycle, *i.e.* (in)activation and downstream signalling pathways, and their many common structural motifs, *e.g.* the conserved disulphide bridge on the extracellular site, the "toggle switch" in helix 6 ($Trp^{6.48}$), the G-protein DRY binding motif, etc., strongly hinder the systematic development of effective therapies. Reaching receptor sub-type selectivity of a drug within the same phylogenetic GPCR family is still often rather elusive, as for example in the case of neuropeptide Y receptors (Y_xR).[3]

We have focused our attention on a specific member of the Y_xR family, namely the Y_4 receptor (Y_4R), a rhodopsin-like peptide-binding GPCR, primarily involved in the regulation of anorexigenic effects. We use a novel approach where we investigate the local changes of the receptor´s secondary structure depending on the specific activating ligand, via introduction of isotopically labelled tryptophan residues. Combining $^{13}C,^{13}C$ DARR, T1 and T1ρ relaxation NMR data with computational methods (molecular dynamics simulations, noise and detector analysis), we identified *unique secondary structure motifs* of the Y_4R necessary for high affinity binding to the receptor.

This approach has the enormous potential to bring us one step closer to understanding the specific receptor–ligand interactions, which give rise to particular biological functions, and thus to the development of subtype-specific therapeutics.

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3. X. Pedragosa-Badia *et al.*, *Front. Endocrinol.* **2013**, *4*, 1–13.

Flash poster 12

Probing the Role of Conformational Dynamics of Nuclease Domain in Cas12a Catalysis Using NMR spectroscopy

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CRISPR-Cas systems have revolutionized molecular biology, diagnostics, and gene editing through RNA-guided recognition and cleavage of nucleic acids. Cas12a, a multi-domain CRISPR-associated nuclease, contains a catalytic RuvC domain in the nuclease lobe, which sequentially cleaves the non-target strand (NTS) and target strand (TS) of double-stranded DNA. In addition to RuvC, the nuclease lobe also hosts a non-catalytic nuclease domain, which primarily guides the TS to the catalytic site after NTS cleavage and may also play a role in DNA binding and R-loop formation. Given the multifaceted role of the nuclease domain, its conformational flexibility may be crucial for regulating Cas12a's catalytic activity by ensuring the R-loop formation required for RuvC activation and coordinating the DNA cleavage process. However, the extent and the role of this flexibility remain poorly understood. In this work, we aim to characterize the structural dynamics of the nuclease domain in Cas12a from Francisella novicida and further investigate its mechanistic role in the catalytic activity of Cas12a. Methyl-CPMG experiments on the apo-nuclease lobe (50 kDa) at 25°C revealed the presence of conformational exchange in residues from the nuclease domain. We observed the same conformational exchange in methyl CPMG experiments on isolated nuclease domain (20 kDa). Upon reducing the temperature to 15°C, the two interconverting conformers, A and B, exhibited comparable populations and appeared as distinct peaks in the 1H-13C methyl-HMQC spectrum; the exchange between these conformers was confirmed by cross-peaks in the methyl ZZ-exchange experiments. Detailed thermodynamic and kinetic analysis, by fitting 15N-CEST and CPMG profiles of A and B states, showed that their population ratio and exchange rate are 3:2 and 40 s-1, respectively, at 15°C. We have performed triple resonance experiments to obtain backbone chemical shifts of A and B conformers, which will be used to determine their structural differences. We will further validate the presence of the conformational exchange of nuclease domain in full-length Cas12a by methyl CPMG and ZZ-exchange experiments. Characterization of this conformational exchange will provide the basis for understanding the dynamic regulation of Cas12a activity, which will contribute towards the rational design of gene-editing tools.

Structural dynamics in signal transduction by dectin-1

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Dendritic cell-associated C-type lectin (dectin-1) is a membrane C-type lectin receptor (CLR) expressed on myeloid cells and involved in immune response to pathogens and damaged host cells. Together with other CLRs, dectin-1 constitutes a potential target for the development of therapeutics able to tune the immune response to infectious pathogens or inflammatory diseases (Mata-Martínez al, Front. Immunol, 2022). It possesses an extracellular carbohydrate recognition domain (CRD), a single-transmembrane domain and an intracellular domain (ICD). The ICD displays a phosphorylable hemITAM motif able to trigger a variety of signaling pathways by recruiting kinase or phosphatase partner proteins. While structural insight into extracellular ligand recognition by dectin-1 has been obtained (Brown et al, Protein Sci, 2007), the mechanisms of signal transduction and intracellular partner protein recruitment remain elusive. Here, we performed NMR in solution to investigate the conformational dynamics of the ICD upon phosphorylation and its interaction with the Src homology 2 (SH2) domains of the spleen tyrosine kinase (SYK). Our experiments highlighted the disordered nature of the ICD and revealed that mono-phosphorylation of the hemITAM (pICD) did not alter its conformational state. We observed that a region of the ICD around the hemITAM is animated by more restricted conformational dynamics and displays a propensity to form a-helical structures. Furthermore, the residues within this region undergo large changes in chemical environment upon binding of the pICD with the tandem SH2 or with individual N- and C-terminal SH2s of SYK. Together with preliminary ^{15}N NMR relaxation measurements of pICD – SH2 complexes, these data point to pICD folding upon binding to the signaling partners of dectin-1. These findings enhance our understanding of the conformational dynamics that occur during the first steps of dectin-1 signalling and enable structural studies aiming to decipher the binding selectivity of the ICD in different phosphorylation states for its signalling partners. In longer-term perspectives, we have established a yeast-based expression strategy that enables the production of isotope-labelled full-length dectin-1 and pave the way to integrative studies in membrane-mimetic environments. This approach aims to unravel the molecular determinants of the functional coupling between extracellular ligand binding and intracellular partner protein recruitment.

Flash poster 13

The Avocado Effect: Targeted Inhibition of Acetyl-Coenzyme A Carboxylase and Lipid Metabolism

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The enzyme Acetyl-coenzyme A carboxylase (ACC) catalyzes the ATP-dependent carboxylation of an acetyl-CoA substrate into malonyl-CoA, the first step in *de novo* fatty acid biosynthesis. Understanding ACC function and regulation is of high interest because ACC is increasingly recognized as a nexus for integrating signals that balance energy metabolism, leading to its emergence as a potential therapeutic target for obesity and some cancers. This potential relevance conflicts with the complexity of the ACC catalysis mechanism to be tackled for drug experimental optimization. To provide a methodological contribution, a real-time kinetics method based on NMR and HPLC monitoring was developed and successfully applied for the quantitative assessment of *Caenorhabditis elegans* (*C. elegans*) ACC activity, in the absence and presence of the inhibitor avocadene acetate, a naturally occurring compound in avocado fruit pulp and seed. Whereas previous studies typically employed radioactivity counting of labeled substrate(s) and/or product(s), or UV estimation of chromatographically resolved components, to determine the initial reaction rates at different substrate concentrations on pure protein preparations, the presented work was conducted using partially purified protein extracts, at a single concentration of acetyl-CoA substrate, leading to consistent results with both monitoring techniques and kinetic parameters (K_M and V_{max}) which compared well to the previously reported values and gave valuable insights into the inhibition. Therefore the described approach represents a significant advance over previous methods to assess ACC activity.

Flash poster 14

Phenol Probe Exploration to Reveal Local Druggable Hotspots Through an Integrated NMR and Computational Simulation Approach

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Phenol molecules are well-established drug-like fragments, capable of engaging in both aromatic and polar interactions—via their hydroxyl groups—with well-defined local regions of biomolecular targets. In this communication, we demonstrate how the main hot spots relevant for drug binding in the NTD module of ClpC1 can be dissected through an integrated approach combining phenol NMR titration and molecular dynamics simulations in water/phenol solutions.

Interestingly, comparison of NMR chemical shift patterns from phenol titrations and our recently identified NTD-ClpC1 binder—discovered through an integrated structure-based virtual screening and NMR approach—reveals an outstanding correlation (see Weinhäupl K. et al., *Scientific Reports*, 2025). Furthermore, combining experimental NMR data with *In-Silico* results reduces the number of false positives typically generated by each method alone, and pinpoints the most relevant residues involved in protein–ligand interactions.

In conclusion, our results show that MD simulations and NMR provide complementary local conformational and thermodynamic insights, offering a powerful strategy to guide fragment-based drug design campaigns.

Flash poster 15

A Tale of the Tail: Understanding the Structural Basis of Phosphorylation-Mediated PERIOD-2 Destabilization

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PERIOD-2 (PER2) is a key clock protein that regulates circadian rhythms through a transcription-translational feedback loop. It is largely disordered, except for the tandem PAS (PER-ARNT-SIM) domains. PER2 stability is controlled by site-specific phosphorylation. Casein kinase-1 (CK1) phosphorylates two key regions: the Familial Advanced Sleep Phase (FASP) site (S659), which stabilizes PER2, and the degron site (S478), which promotes proteasomal degradation of PER2. Interestingly, the degron serine lies in a disordered region close to the ordered PAS domains.

Our study investigates the structural basis of PER2 degron-site phosphorylation. We revealed that PER2 forms a 32-subunit oligomer (~653 kDa). Interactions from C-terminal residues and LARK motifs within the disordered tail, harbouring the degron site, are essential for oligomerisation. Although these sequence determinants reside in the disordered region, we showed that the PAS domains provide critical synergistic stabilization of the oligomer.

Next, we elucidated how this PER2 oligomer influences phosphorylation-mediated degradation. Using SEC-MALS, we detected a minor dimeric population co-existing with the oligomer. Truncation of the previously identified C-terminal residues in the disordered tail, led to dimerization of PER2, though a minor oligomer population persisted. We then used ^{15}N-DEST experiments on the PER2 dimer and demonstrated an equilibrium between the dimer and the NMR –'invisible' oligomer. We found that CK1 phosphorylates the dimer only and not the oligomer, possibly from differential accessibility of the degron site to CK1. Fluorescence quenching experiments revealed that the disordered tail is buried in the oligomer but exposed in the dimer, thereby affecting degron-site phosphorylation. Furthermore, ^{1}H-^{15}N HSQC spectra of the phosphorylated dimer indicated multiple serine residues within the disordered tail being phosphorylated, apart from the degron-site and were identified using triple resonance NMR experiments. Phospho-mimetic mutations of these serine residues progressively reduced oligomer size from 32-mer to 10-mer, suggesting that phosphorylation significantly destabilises the oligomer. Our findings suggest a mechanism where oligomerization regulates PER2 stability and cellular abundance by sequestering the degron site. We aim to resolve the PER2 oligomer structure using cryo-EM to identify PAS domain residues at the oligomer interface. We also seek to determine how oligomerization influences circadian oscillations in a cellular context.

Investigate the dynamics and molecular mechanism of a bacterial molecular motor involved in Iron Transport

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Molecular motor formed by ExbB-ExbD protein complex is located in the inner membrane of bacteria. This molecular motor energizes the import of scarce nutrients (metals, vitamins, sugars) through the bacterial envelope via a specific outer membrane transporter. The energy is generated by the proton-motive force or the proton gradient of the inner membrane, and is transferred to the transporter via a third protein TonB/HasB [1]. The energy is used to open a channel allowing the nutrient entry through the transporter.

The complex formed by ExbB-ExbD and a TonB paralog HasB is involved in the heme acquisition system (Has). To import external heme, which is the major source of iron for bacteria such as *S. marcescens*, this system transfers energy by a coupled physical motion between ExbB-ExbD, HasB and transporter.

Recent studies have identified the interface between the periplasmic domain of the molecular motor and HasB, which overlaps with the region recognizing bacterial peptidoglycan. In addition, it has been shown that this interaction is mediated by glycan moiety of the peptidoglycan and is transient [2]. However, how the ExbB-ExbD-HasB complex energizes the whole process and the role of the peptidoglycan remain unknown.

Understanding these processes necessitate a scrupulous molecular and atomic-level investigation of the interactions between various proteins of the Has system and peptidoglycan, as well as their dynamics. To do so, we will use a combination of experimental approaches (NMR and Cryo-EM) and molecular dynamics (MD) simulations in a model envelope and in the presence of peptidoglycan.

As a first step, we study by MD the sequential binding of HasB and peptidoglycan to the molecular motor. Further experimental works will validate the results of the *in silico* prediction.

The resulting knowledge could shed light on the regulation of bacterial nutrient uptake and the development of novel antibacterial strategies.

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Exploring the influence of binding of RG-rich peptides on G-quadruplex structures by NMR

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Interactions between proteins and nucleic acids are crucial for the regulation of many cellular pathways. However, exact mechanisms at the atomic level are often still poorly understood due to difficulties *in vitro* mimicking of intracellular conditions that are needed for breakthrough structural studies. One example of such important biological interactions are the ones between non-canonical nucleic acid secondary structures called G-quadruplexes and the arginine/glycine-rich (RGG/RG) domains of DNA/RNA binding proteins.[1] G-quadruplexes are structurally diverse and capable of performing a broad range of cellular functions, most notably regulation of gene expression, which may be facilitated by the binding of various DNA or RNA processing proteins. Nucleolin, a multifunctional nucleolar protein, contains an intrinsically disordered C-terminal RG/RGG-rich domain. It plays a role in various cellular functions and is also capable of G-quadruplex binding.[2]

We investigated the interaction between the nucleolin-derived RG/RGG-rich peptides and two different G-quadruplexes, one being a well-studied anti-parallel TBA G-quadruplex and the other parallel M2 G-quadruplex. We also assessed binding of RG-rich peptides by the G-quadruplexes formed by oligonucleotide with four d(G_4C_2) hexanucleotide repeats, which are characteristic for the gene C9orf72 and the onset of ALS neurodegenerative disease.[3] We show that the investigated binding has a moderate strength and that the binding is influenced even by the smallest differences in the amino acid sequence of RG/RGG-peptides, while a specific amino acid sequence may be responsible for the major contribution towards the binding affinity. Folding of the oligonucleotide into the G-quadruplex during temperature annealing is also potentially affected by the presence of the peptides, resulting in altered G-quadruplex topology. Our results may become of greater interest considering the importance of the investigated interaction for the development of ALS and FTD diseases and other therapeutic purposes based on nucleolin recognition of G-quadruplexes.

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Flash poster 16

Identifying interactions between proteins and excipients using NMR, and their consequences on viscosity/injectability

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Higher concentrated forms of biopharmaceutical treatments allow for better injection deliveries and patients' quality of life. However, increasing the concentration have consequences on the stability and properties of the treatment. It comes with increased viscosities, which impacts the injection. The knowledge is very limited on how the most common excipients stabilize and fluidify highly concentrated therapeutic proteins.

In this context, we sought to examine the correlation between the strength of excipients' interactions with concentrated proteins and the resulting viscosity. NMR spectroscopy appears as an excellent technique in this context, because of its ability to report low affinity interactions. Different NMR methods exist for this, based on non-redundant physical mechanisms related to weak interactions. These approaches are commonly used for structural biology purposes, but pharmaceutical formulations are conspicuously different from the standard solutions used in academic labs. Here, we present the first results of our work. We tested the adaptation of various standard pulse sequences to fit our need and biopharmaceutical samples. These NMR methods were developed to report on protein/ligand interactions. We will show how they can complement each other and their adaptability to our protein/excipient samples. We will finally discuss the use of other NMR experiments and the complementarity with other techniques, using size-exclusion chromatography and light-scattering methods.

A novel natural siderophore antibiotic conjugate reveals a chemical approach to molecule coupling

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Inspired by natural sideromycins, the conjugation of antibiotics to siderophores is an attractive strategy to facilitate “Trojan horse” delivery of antibiotics into bacteria. Genome analysis of a soil bacterium, *Dactylosporangium fulvum,* found a “hybrid” biosynthetic gene cluster responsible for the production of both an antibiotic, pyridomycin, and a novel chlorocatechol-containing siderophore named chlorodactyloferrin which was studied in its free and gallium(III) forms by solution NMR. While both of these natural products were synthesized independently, analysis of the culture supernatant also identified a conjugate of both molecules. We then found that the addition of ferric iron to purified chlorodactyloferrin and pyridomycin instigated their conjugation, leading to the formation of a covalent bond between the siderophore-catechol and the pyridomycin-pyridine groups. Using model reactants, the positions in catechol and pyridine involved in the new bond were defined by solution proton/carbon-13/nitrogen-15 NMR. This iron-based reaction was found to proceed through a Michael-type addition reaction, where ferric iron oxidizes the siderophore-catechol group into its quinone form, which is then attacked by the antibiotic pyridyl-nitrogen to form the catechol−pyridinium linkage.

These findings prompted us to explore if other “cargo” molecules could be attached to chlorodactyloferrin in a similar manner, and this was indeed confirmed with a pyridine-substituted TAMRA fluorophore as well as with pyridine-substituted penicillin, rifampicin, and norfloxacin antibiotic analogues; the conjugates structures were checked by solution NMR. The resultant biomimetic conjugates were demonstrated to effectively enter a number of bacteria, with TAMRA−chlorodactyloferrin conjugates causing fluorescent labelling of the bacteria, and with penicillin and rifampicin conjugates eliciting antibiotic activity.

These findings open up new opportunities for the design and facile synthesis of a novel class of biomimetic siderophore conjugates with antibiotic activity.

Caradec *et al.* (2023) A novel natural siderophore antibiotic conjugate reveals a chemical approach to macromolecule coupling. *ACS Central Science* **9**, 2138-2149. DOI: 10.1021/acscentsci.3c00965

Characterization of the phospho-dependent interactions between Mdm2 and p53

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The E3-ubiquitin ligase Mdm2 is the main negative regulator of the "tumor suppressor" p53. This Mdm2-p53 axis is altered in nearly all cancers and is thus an attractive anti-cancer target, but the current inhibitors have clinical limitations.

Mdm2 is phosphorylated by two classes of kinases: i) under normal conditions, Ck1δ phosphorylates the Intrinsic Disorder Region 1 (IDR1) of Mdm2, whereas ii) in response to DNA damage, ATM/ATR/DNAPK kinases are activated and phosphorylate the IDR2 of Mdm2. So far, all the biochemical/biophysical works on Mdm2-p53 have been achieved using non-phospho, i.e. non-native, fragments of these proteins.

We sought to characterize these hyperphosphorylated forms of Mdm2, which would be representative of those present in cells. We have produced recombinantly a number of fragments containing IDR1 and/or IDR2, and the full-length protein, and used NMR spectroscopy to monitor their phosphorylation in a site-specific fashion [1]. We identified 14 phosphorylation sites on IDR1, 8 on IDR2, and determined the corresponding phosphorylation mechanisms [2,3]. Then, we have characterized the NMR relaxation and the intramolecular interactions between the 3 folded domains of Mdm2 and IDR1/2, in the non-modified and in the phosphorylated forms. These revealed weak interdomain interactions, which might be exploited to tune Mdm2 activity: although IDRs establish supplementary binding to p53, their intramolecular interactions compete with p53 and diminish the affinity for p53 by a factor ~30 as measured by ITC.

Hence, we present a pioneer structural study on hyperphosphorylated IDRs in a protein made of multiple folded/unfolded domain. Such proteins are very common in eukaryotic cells but much less structurally characterized in the literature. Our results are thus of interest for the axis p53/Mdm2 and may motivate similar studies on many drug targets with similar multidomain compositions.

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Flash poster 17

Structural dynamics and long-range interactions controlling timing of the Neurospora circadian clock

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The circadian clock in *Neurospora crassa* operates through a feedback loop that incorporates both negative and positive regulatory elements, which together regulate the oscillating rhythm with a period of around 24 hours. A crucial component of the negative feedback mechanism is the protein FREQUENCY (FRQ), which is dimeric and intrinsically disordered. FRQ is subject to post-transcriptional modification, specifically hyperphosphorylation by casein kinase 1a (CK1a). Thereby, phosphorylation of clock proteins is a conserved feature across species, extending from fungi to mammals, with the human PERIOD (PER) protein as a notable example. However, the specific role of hyperphosphorylation in regulating these proteins remains not fully understood. We hypothesize that the time-dependent hyperphosphorylation of FRQ at multiple sites enables a conformational shift from a closed to an open state, thereby modulating its interactions with partner proteins. To explore this, we are using nuclear magnetic resonance (NMR) spectroscopy to study the structural dynamics of FRQ phosphorylation by recombinant CK1a. The research aims to create a multi-conformational model of unphosphorylated FRQ, examine its protein interactions, and understand how phosphorylation-induced changes influence the circadian feedback loop. By combining NMR with molecular modeling, we aim to gain insights into how phosphorylation affects the conformation of FRQ and triggers a switch in its behavior. This research has broader implications, enhancing our understanding of circadian mechanisms and providing insights into human clock proteins like PER, which are linked to sleep disorders, metabolic issues, and even cancer.

Influence of stereochemistry in a local approach for calculating protein conformations

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Protein structure prediction is generally based on the use of local conformational information coupled with long-range distance restraints. Such restraints can be derived from the knowledge of a template structure or the analysis of protein sequence alignment in the framework of models arising from the physics of disordered systems. The accuracy of approaches based on sequence alignment, however, is limited in the case where the number of aligned sequences is small. Here, we derive protein conformations using only local conformations knowledge by means of the interval Branch-and-Prune algorithm. The computation efficiency is directly related to the knowledge of stereochemistry (bond angle and ω values) along the protein sequence and, in particular, to the variations of the torsion angle ω. The impact of stereochemistry variations is particularly strong in the case of protein topologies defined from numerous long-range restraints, as in the case of protein of β secondary structures. The systematic enumeration of the conformations improves the efficiency of the calculations. The analysis of DNA codons permits to connect the variations of torsion angle ω to the positions of rare DNA codons.

Flash poster 18

Quantitative Analysis of Fast Ligand Dissociation Kinetics Using ^{19}F $R_{1\rho}$ Relaxation Dispersion

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Fragment-based drug discovery (FBDD) is a powerful approach for generating small-molecule lead compounds from weak-binding fragments. However, FBDD faces a challenge in detecting and characterising weak interactions (high µM to low mM binding affinities) associated with typical fragment hits, which can push detection methods to their limits, resulting in false positives and false negatives that often go unrecognized.[1] Ligand-observed (LO) NMR is a key tool in this discovery process, and several experiments have been developed, including saturation transfer difference (STD), waterLOGSY, and R_2 relaxation (through CPMG measurements).[2,3] The latter can also be applied to fluorine-containing ligands using ^{19}F spectroscopy. However, translating 'hits' from these experiments into affinity measurements, even only in relative terms, is challenging and inhibits ranking of different compounds. In this work, we propose an alternative approach using ^{19}F ligand-observed NMR to detect binding-induced changes in the rotating-frame relaxation rate, $R_{1\rho}$. We have applied our approach to diverse ligands binding to three protein targets and have successfully extracted dissociation rate constants for both isolated compounds and fragments in mixtures of up to 30 compounds. We have shown that our method can quantify dissociation rates between 5,000 s^{-1} and 60,000 s^{-1}. Importantly, by measuring dissociation kinetics, we provide medicinal chemists with a simple physical quantity free from complex NMR parameters such as tumbling times, cross-relaxation rates, or chemical shift anisotropies. Furthermore, we have developed easy-to-use software with a simple graphical interface for efficient data analysis. We anticipate this approach may help accelerate early stages of FBDD, facilitating the characterisation, screening, and ranking of fragment libraries.

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Structural analysis of the interaction between TopBP1 and PLK1 in mitosis

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Topoisomerase IIβ-binding protein (TopBP1), a prototype of 'scaffold' protein, orchestrates DNA repair throughout the cell cycle, yet its regulation during mitosis remains unclear. It consists of nine well-folded BRCA1 C-terminal (BRCT) domains and several intrinsically disordered regions (IDRs) that are post-translationally modified. TopBP1 interacts with the mitotic kinase PLK1 to suppress aberrant mitotic DNA repair synthesis and binucleation. We want to understand which regions of TopBP1 regions interact with PLK1 and how these interactions are regulated during the cell cycle and after DNA damage.

We demonstrated by NMR that the disordered region between BRCT2 and BRCT3 (IDR2-3) containing a predicted PLK1 docking site directly interacts with the Polo-Box Domain of PLK1 (PLK1_PBD). In this region, T298 can be phosphorylated by a proline-directed kinase, which enhances binding to PLK1_PBD and promotes further phosphorylation of TopBP1 by PLK1. We also identified by NMR a novel interaction between the disordered region between BRCT6 and BRCT7 (IDR6-7) and PLK1. This region contains three highly conserved motifs exhibiting partial structural ordering based on 2D ^{1}H-^{15}N NMR heteronuclear NOE analysis. PLK1_PBD directly interacts with all three conserved motifs of IDR6-7. We now study how phosphorylation strengthens this interaction. Finally, we found that TopBP1 forms biomolecular condensates that concentrate proteins, including PLK1. We will discuss the molecular events involved in the localization of PLK1 in TopBP1 condensates, and the impact of TopBP1 condensation on the phosphorylation of PLK1 targets in mitosis.

Characterising the intrinsically disordered C-terminal domain of calreticulin by solution-state NMR spectroscopy

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Proteins that are destined for the secretory pathway are translocated co-translationally into the ER lumen for further processing prior to acquiring their native structure. The lumen provides an oxidising and calcium-rich environment with an abundance of molecular chaperones. In parallel with translocation, nascent polypeptides undergo N-linked glycosylation, which mediates progression along the glycan quality control (GQC) pathway. Proteins that ultimately fail to fold are directed to the ER-associated degradation (ERAD) pathway for proteasomal degradation[1]. Calreticulin (CRT) is a bifunctional chaperone protein that assists protein folding along the GQC pathway and has been reported to bind calcium[1]. CRT is comprised of three domains: a split N- and C-terminal lectin domain; an extended, proline-rich 'P' domain; and an acidic C- terminal domain (CTD), predicted to be intrinsically disorded[1,2,3]. Structural studies have been performed on the lectin and P domains of CRT individually, but little experimental information on the CTD is known[1,2,3]. Here, we have generated constructs of the human CRT CTD and successfully established the first protocols for its expression and purification. We will report our progress for complete assignment of the challenging low-complexity sequence using combination of standard triple-resonance and HNN/HNH experiments. This further permits the analysis of the residual structure and backbone dynamics using ^{15}N relaxation measurements. Additionally, we investigate its interactions with divalent cations to provide mapping binding sites and affinities and examining their impact on the residual structure and dynamics. Together, this prepares the way to a complete characterisation of the internal dynamics and interactions of the domain.

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POSTERS SESSION 2

Posters with an odd number

Ingrilimine, a novel cytotoxic and apoptotic cyclic imine toxin of 369 Da, is a potent antagonist and a weak agonist of α7 nicotinic acetylcholine receptor

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Marine toxins are a fascinating group of complex organic compounds that have had a lasting impact on human society. They pose a risk to human health through contamination of fish and shellfish and at the same time, they are sources of life-changing medicines. According to their chemical structure and some criteria, marine phycotoxins of importance for human health are classified in 10 groups, among them are the cyclic imine toxins (CiTX) considered as emerging marine toxins in Europe, but they are not regulated (1,2). A common feature of CiTXs is the presence of a five-, six- or seven-carbon imine ring, responsible for their potent antagonism towards α7 nAChR.

CiTXs exhibit strong neurotoxicity to mice by oral or intraperitoneal administration at lethal doses provoking transient hyperactivity, followed by a decrease of the respiratory rate with prominent abdominal breathing leading to death by respiratory arrest within 3-5 minutes through blockade of the neurotransmission mediated by muscle α7 nAChR. Here we describe the discovery of a new cyclic imine named *ingrilimine,* the smallest cyclic imine described to date. *Ingrilimine* was purified from an extract of *Vulcanodinium rugosum* and was characterized by HR-Mass and its structure solved by NMR. The mode of action of *ingrilimine* was determined by two-electrodes voltage clamp on *Xenopus laevis* oocytes expressing the human α7 nAChR. *Ingrilimine* behaves as a potent antagonist of human α7 nAChR at nanomolar concentrations, while, at micromolar concentrations, it displays an agonistic activity. *Ingrilimine* also shows potent cytotoxic and apoptotic activities. The referred biological activities singularize *ingrilimine* from the other members of the cyclic imine toxin family of dinoflagellate origin. *Ingrilimine* can serve as a blueprint to conceive new drugs targeting α7 nAChR that are involved in a series of neurodegenerative disorders. *Patent accepted*.

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Acknowledgments:

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Flash poster 1

Shifting the mechanistic paradigm: the role of cholesterol in defining phorbol ester membrane binding modes as revealed by DNP-NMR of intact T cells

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Phorbol esters have long been demonstrated to both promote and inhibit inflammation and tumorigenesis through activation of Protein Kinase C (PKC) proteins. However protein interactions cannot account for the disparate biological effects of different phorbol ester analogues, implying a role for membrane interactions. Traditional *in vitro* approaches to membrane studies are limited by their inability to replicate the heterogeneity and architecture of cellular membranes, underscoring the critical need for *in situ* methodologies. By enhancing the NMR sensitivity 20–200-fold and cryogenically preserving cell samples, in-cell DNP-NMR enables structural studies of small molecules dissolved in cellular membranes with minimal perturbation to their native context. Using this approach, we investigated phorbol myristate acetate (PMA) in the context of intact cells, revealing distinct membrane topologies compared to model membrane systems which were dependent on the presence of cholesterol. We link these distinct states to hydrogen bonding that can be associated with the drugs membrane insertion depth using MD simulations combined with in-cell ^{13}C chemical shifts and ^{31}P dephased REDOR measurements. We identify cholesterol as an important mediator of drug topology and combine new trimodal cholesterol reagents for in cell DNP and fluorescence microscopy towards identification of the nature of cholesterols influence on phorbol ester membrane localization. These findings demonstrate how in-cell DNP-NMR can uncover previously inaccessible details of membrane-bound drug interactions, revealing new insights into phorbol ester-membrane interactions that may explain the distinct biological effects of different phorbol ester analogues.

A resolution of the large Grb2 conformational landscape in solution

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Grb2 is a central adaptor protein that connects activated Class I RTK to the RAS-MAPK signaling cascade. It regulates key cellular processes such as proliferation, differentiation, and survival. Dysregulation or sustained activation of this pathway is often associated with oncogenic transformation.

Structurally, Grb2 is composed of a central SH2 domain flanked by two SH3 domains, each exhibiting distinct binding specificities toward proline-rich motifs. Through its SH2 domain, Grb2 was shown to bind to phosphorylated tyrosines on the activated receptor, while its SH3 domains recruit Sos, thereby initiating RAS activation. Despite its well-established role, the molecular mechanism by which Grb2 coordinates these interactions remains incompletely understood.

In particular, the conformational dynamics of Grb2 in solution—and how it relates to its functional state—are still not well resolved. Existing structural and biophysical data yield conflicting interpretations, potentially due to artifacts introduced by protein modifications (e.g., tags, mutations) or far from physiological experimental conditions (e.g, ionic strength, pH, crystalline state) [Maignan, 1995; Tateno, 2024; Tedesco, 2023].

To address these discrepancies and provide a clearer understanding of Grb2's native conformational landscape, we investigated full-length Grb2 in solution, in the absence of ligand, tag, or mutations [Pinet, 2020].

By integrating NMR, SAXS, SEC and MD, we found that Grb2 is predominantly monomeric in solution and adopts an extended conformation in which its three domains behave largely as independent units, in contrast to the dimeric crystal structure [Maignan, 1995].

The data obtained on the dimeric form of Grb2 are compatible with an SH2-swapped dimer similar to that observed in the context of the isolated SH2 domain [Benfield, 2007; Hosoe, 2019; Schiering, 2000], as previously proposed by Sandouk et al [Sandouk, 2023]. We moreover show that the three Grb2 domains remain independent of each other in the dimer. This modular organization suggests a high degree of flexibility, which may be functionally relevant for mediating interactions with multiple partners within the signaling cascade. The structural change that occurs in the SH2-swapped dimer may influence the binding specificity of phosphorylated ligands, as it involves residues proximal to the SH2 domain binding pocket.

Structural and dynamics characterization of the Zika virus NS2B using nuclear magnetic resonance and RosettaMP

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Zika virus (ZIKV) is an emergent flavivirus that represents a global public health concern due to its association with severe neurological disorders such as microcephaly and Guillain-Barré syndrome. NS2B is a multifunctional viral membrane protein primarily used to regulate viral protease activity. NS2B has transmembrane domains critical for the localization of viral protease to the endoplasmic reticulum membrane and a hydrophilic domain essential for folding, recruitment, and protease activity. Therefore, NS2B is considered a cofactor of viral protease processes viral polyprotein and is crucial for virus replication, making it an appealing target for antiviral drugs. The objective of this work is the structural elucidation of full-length ZIKV NS2B in sodium dodecyl sulfate (SDS) micelles using solution nuclear magnetic resonance experimental data and RosettaMP. First, ZIKV NS2B was cloned, expressed in *E. coli* and purified by nickel affinity chromatography. Structural NMR studies of the target protein were performed in SDS micelles at 298K. The resonance assignment of 92.3% of the NS2B amino acids was obtained. The protein structure has four transmembrane *a*-helices, two amphipathic *a*-helices, and a *b*-hairpin in the hydrophilic region. NS2B presented secondary and tertiary stability in different concentrations of SDS. Furthermore, we studied the dynamics of NS2B in SDS micelles through relaxation parameters and paramagnetic relaxation enhancement experiments. These data showed that hydrophilic domain of NS2B is more flexible and exposed to the solvent than transmembrane domain. The findings were consistent with the structural calculations. Our work will be essential in understanding the role of NS2B in viral replication and screening for inhibitors against ZIKV.

Flash poster 2

Selective DNP combined with ^{13}C-methyl labeling for the study of specific sites in large proteins

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Dynamic Nuclear Polarization (DNP) has revolutionized the scope of solid-state NMR by enhancing sensitivity by several orders of magnitude, enabling experiments that were previously deemed infeasible, including for biomolecular systems. This technique typically involves doping the sample with paramagnetic molecules, known as polarising agents, and performing experiments at cryogenic temperature under high-frequency microwave irradiation. However, a major limitation of DNP, especially in biomolecular NMR, is the substantial loss of spectral resolution at low temperatures. This degradation arises from the freezing of local molecular motions and hampers the determination of site-specific information, in particular for uniformly ^{13}C,^{15}N-labeled biomolecular systems.

In this context, our group developed over the past years a methodology called *Selective* DNP (*Sel*DNP), which allows obtaining high-resolution DNP spectra of a specific site in a uniformly labeled protein. This approach is based on differential spectroscopy between the spectrum of a standard DNP sample, exhibiting uniformly signal enhancement, and that of the same sample modified with a paramagnetic spin label at the position of interest. The spin label induces localized bleaching of nearby resonances (similar to the PRE effect at room temperature). The spectral difference yields a set of sensitive, highly resolved spectra containing only resonances of residues at the targeted site, within a tunable radius around the spin label. So far, *Sel*DNP has only been demonstrated on the binding site of a small metalloprotein of 12 kDa.

Whereas *Sel*DNP can be applied to biomolecular systems of any size, the residue-type assignment of the sub-spectra may be significantly hindered when multiple sites share similar residue compositions – a situation that becomes increasingly likely as protein size increases. We therefore investigate here the possibility of combining *Sel*DNP with specific methyl labeling for overcoming this problem. We aim to apply this strategy on a 58 kDa sulfatase. Different approaches are envisaged for the introduction of the spin label and preliminary results are discussed. Once the methodology is developed, we plan to use it to investigate the binding site for chondroitin sulfate of this protein, contributing to the elucidation of mechanistic details of the interaction, which plays a major role in related pathologies.

Flash poster 3

Simultaneous acquisition strategies for probing charged residue side chains

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Acidic and basic residues such as arginine, lysine, glutamate, and aspartate play a central role in mediating protein–protein and protein–ligand interactions due to their delocalized charges. However, their high conformational flexibility poses challenges for traditional ^{1}H-detected NMR experiments, which often suffer from line broadening and spectral overlap. Heteronuclear direct detection, such as detection of ^{13}C nuclear spins, offers a valuable complementary strategy for probing these residues.

A common method to enhance signal sensitivity involves using ^{1}H spins as the initial source of magnetization. However, solvent-exposed residues like arginine often undergo rapid chemical exchange of amide protons with water, hindering magnetization transfer via INEPT-based techniques. In contrast, cross polarization (CP) leverages this exchange to increase signal sensitivity by utilizing water protons, making it a promising alternative.[1]

Arginine side chains introduce an additional challenge due to the conformational exchange of the guanidinium group, contributing to line broadening within the intermediate exchange regime. To address this, approaches based on heteronuclear double-quantum coherence and CP have been proposed. [2,3]

In this study, we present two novel NMR experiments designed to investigate the side chains of both positively and negatively charged amino acids. These experiments integrate established strategies with a multi-receiver acquisition setup, enabling simultaneous acquisition of complementary data within a single experiment.[4,5]Specifically, we introduce the MR $H^{\varepsilon}N^{\varepsilon}//C^{\zeta}N^{\varepsilon}$ experiment, which combines CP-HISQC and $C_{\zeta}N_{\varepsilon}$-HSQC for efficient observation of arginine side chains. Additionally, we propose an experiment combining $C_{\zeta}N_{\eta}$-HDQC with a CACO experiment, selectively targeting aspartate, asparagine, glutamate, and glutamine residues. Both methods offer time savings while maintaining same performance as individual acquisitions. We then tested the applicability of these approaches in monitoring the interaction between the N-terminal domain of the SARS-CoV-2 nucleocapsid protein and heparin (enoxoeparin).[6]

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Flash poster 4

Insights into SARS-CoV-2 nucleocapsid protein and its interaction with polyanions

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Among the structural proteins of SARS-CoV-2, the nucleocapsid (N) protein is notable for its pronounced flexibility and central role in the viral life cycle, including RNA packaging, genome replication, and immune modulation. It counts 419 amino acids divided in both folded domains, N-terminal (NTD) and C-terminal (CTD) domains, and extensive intrinsically disordered regions (IDR1, IDR2 and IDR3). The coexistence of ordered and disordered regions raises an important question: how does this heterogeneous structural organization influence its interaction with highly charged ligands such as RNA or heparins?

Extensive studies have investigated individual N protein constructs, particularly the N-terminal domain (NTD, residues 44–180) and the N-terminal region (NTR, residues 1–248) [1, 2]. Building on this knowledge, we aimed to do a step forward by exploring the interaction mechanisms comparing three constructs — NTD, NTR, and the full-length protein (FL) — and their binding to three heparin-based ligands of increasing length. Specifically, we assessed how ligand length affects binding to NTD, and how interaction profiles shift as protein complexity increases from NTD to NTR and FL.

Our NMR data suggest a correlation between ligand length and binding affinity, with longer oligosaccharides showing stronger interactions. Additionally, the presence of IDRs appears to enhance binding capacity, as the NTR construct including disordered segments exhibited more extensive chemical shift perturbations compared to NTD alone. Notably, the full-length protein displayed distinct spectral behavior, potentially reflecting additional binding contributions from IDR3 and altered dynamics due to its complex and dimeric structure.

These findings support the hypothesis that the IDRs play an active and modulatory role in mediating polyanion interaction, potentially acting as flexible and functionally relevant modules rather than passive linkers. While further studies are needed to fully define their mechanistic contributions, our results underscore the importance of analyzing full-length, multidomain proteins to capture the interaction complexity.

Acknowledgment: Professor Guerrini and his group at the Ronzoni Institute (Milan, Italy) is gratefully acknowledged for providing me the opportunity to work with the heparin ligands.

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Unraveling Protein-Protein Interactions with Complementary Isotope Labelling and Multi-Receiver NMR Spectroscopy

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Intrinsically disordered proteins are prevalent in eukaryotic systems but remain difficult to target pharmacologically. NMR spectroscopy is well suited to study these proteins and their interactions with one another or with drug candidates. Although NMR can provide detailed information about these interactions, spectral overlap and limited sample quantity and stability hinder its widespread application. The proposed approach involves simultaneously mapping the protein-protein binding sites of two interaction partners using complementary isotope labelling and multiple receiver detection. One binding partner, an IDP, was labelled ^{13}C,^{1}H and the other, a heterodimer, where one component was ^{15}N,^{2}H labelled, and the second protein was not isotopically enriched. Thus, proton and carbon detection were exploited for fast and simple output. This method was applied to the 50kDa ternary protein complex, consisting of the prominent oncogenic transcription factor complex Myc/MAX and the tumour suppressor BRCA1, to demonstrate its feasibility.

Flash poster 5

^{19}F NMR study of the interaction between an amino acid derived ligand and the transmembrane protein TSPO

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Translocator protein (TSPO, 18 kDa), previously known as peripheral-type benzodiazepine receptor, is an evolutionarily conserved transmembrane protein involved in various physiological processes and patho-physiological conditions. The endogeneous TSPO ligand is a polypeptide of 9 kDa, but dipeptides with biological activity have been previously synthesized and characterized. Herein, we synthesized a phenyl alanine derived ligand with a ^{19}F labelling which opens prospective for ^{19}F-MRI and potential ^{18}F-PET applications. We characterized the coexistence of two conformers and performed interaction studies with the recombinant mouse TSPO (mTSPO) in different membrane-mimicking environments using ^{19}F NMR hence enabling structure/function characterizations. A change in the mTSPO environment from pure detergent to lipid/detergent mixture reveals different exchange rates between bound and free ligand forms. Competition experiments with the high-affinity drug ligand (*R*)-PK 11195 suggest that phenyl alanine derived ligand binds in the same protein cavity.

Flash poster 6

Structural studies of FOXM1 regulation by β-catenin

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Forkhead box protein M1 (FOXM1) is a proliferation-related transcription factor overexpressed in human solid tumours, which gives it exceptional potency as a target for cancer treatment therapeutics. FOXM1 was shown to interact with β-catenin, a Wnt-signalling effector, in glioma and triple-negative breast cancer. Since FOXM1 and crucial parts of β-catenin are intrinsically disordered, NMR spectroscopy is the key technique to study their interactions with single-residue resolution. Thus, we aim to resolve the molecular details and regulation of FOXM1/β-catenin interaction *in vitro* using liquid-state biomolecular NMR.

So far, I have revealed that the C-terminal part of the overall disordered FOXM1 transactivation domain (TAD) contains a region exhibiting distinct rigidity from the rest of TAD sequence and adopting a transient α-helical conformation. I have shown that the interaction of FOXM1 with β-catenin is driven by multiple binding sites. The C-terminus of FOXM1 TAD directly interacts with β-catenin *via* the N-terminal part of the folded Armadillo repeat region, competing against β-catenin homotypic intermolecular interactions, which are mediated by β-catenin's disordered N/C-termini. FOXM1 TAD residues involved in this interaction are a part of the region identified to contain transient α-helical structure. Interestingly, binding sites of β-catenin and FOXM1 negative regulatory domain (NRD) overlap with each other at FOXM1 TAD. Furthermore, I discovered that the structured FOXM1 Forkhead domain (FH) contains a binding site for disordered β-catenin N-terminus. In addition to NRD/TAD interaction, FOXM1 TAD binds FOXM1 FH domain. The binding site of disordered β-catenin N-terminus overlaps with FOXM1 TAD binding site at FOXM1 FH. In the next phase, we aim to solve the structure of key complexes. Given the large molecular mass of β-catenin (85.5 kDa), localizing FOXM1 TAD binding site at β-catenin requires deuteration, methyl-specific labelling, and using high-field spectrometers. The elucidation of the β-catenin/FOXM1 complex structure will enhance our understanding of the molecular basis of their interactions and guide future experiments to uncover their functional relationship relevant to cancer context.

To summarize, NMR spectroscopy was essential for providing the first insight into the intricate network of intra- and intermolecular interactions within FOXM1 and with the pro-oncogenic co-factor β-catenin.

A Designed LptD Protein to guide the Development of Novel Antibiotics

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The rising dissemination of multidrug-resistant bacterial pathogens poses a great threat to our global health system and urges the development of novel classes of antibiotics.

In recent years, the lipopolysaccharide transport (Lpt) bridge has become an attractive antimicrobial target for the development of antibiotics with novel mechanisms of actions against Gram-negative bacteria. Several promising products have been put forth to target different components of the Lpt system, i.e. LptF (Zosurabalpin, *Pahil et al.*), LptA (Thanatin-derivatives, *Schuster et al.*) as well as LptD (Murepavadin, *Srinivas et al.*) that are currently in different stages of (pre-)clinical trials.

LptD is an outer membrane protein with a β-barrel fold and an N-terminal periplasmic extension that secures the interaction with its adjacent partner within the bridge, LptA. Although, LptD has been validated as novel antimicrobial target by drugs (2, 4), the characterization of these has remained challenging due to the intrinsic complexity and interdependency of the transmembrane domain and the periplasmic domain.

In this study, computational protein design was used to create a soluble version of the respective periplasmic domain of LptD to allow a detailed characterization of the LptD-Thanatin interaction.

To select the best design, first, thousands of constructs were calculated *in silico* using RFdiffusion and ProteinMPNN and further selections were made by application of filters and based on structure prediction. The best constructs were then experimentally tested and judged for quality by NMR spectroscopy.

Herein, we present an NMR solution structure of our designed LptD construct bound to Thanatin. This high-resolution structure provides insight into the binding mode of this antibiotic to LptD. Moreover, this stable and soluble construct can assist in screening peptide libraries to obtain an improved antibiotic with LptD as its target.

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Investigation of the structure and dynamics of an atypical aegerolysin

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Fusver belongs to the family of aegerolysin proteins and was originally found in a fungal species, *Fusarium verticillioides*. It has an unusually elongated N-terminal region. We aimed to investigate how this N-terminal extension affects the structure and function of aegerolysins. Bioinformatic analysis revealed that fusver has significant potential to form an N-terminal α-helix, while the protein core exhibits a β-sandwich fold, which is also found in actinoporins. The predicted structure suggests that actinoporins, which form pores in sphingomyelin-containing membranes via an N-terminal α-helix, may have evolved from aegerolysins.

Crystallization of the recombinant protein has not yet been successful, but the uniformly ^{15}N- and ^{15}N/^{13}C-labelled protein was expressed in *E. coli* in sufficient quantities to perform NMR studies of its structure and dynamics in solution. The dispersion of signals in the ^{1}H-^{15}N HSQC spectrum indicated that the core of the protein has a well-defined structure in solution. Therefore, we recorded a standard set of 3D heteronuclear experiments to preform backbone and side chain assignments and determine NOE connectivities. In parallel, the ^{15}N relaxation experiments were performed, which showed that the N-terminal extension of the protein is very flexible. Furthermore, no NOE connectivities were observed that would indicate the formation of an α-helix in this region. Therefore, we extended the relaxation studies with ^{15}N CPMG relaxation dispersion experiments to detect and characterize a possible formation of a transient α-helix. We will present and discuss the NMR results with respect to the search for a functionally important formation of an N-terminal α-helix in the ground state or higher energy states of this type of aegerolysins.

Flash poster 7

Large-scale conformational changes of the bacterial outer membrane transporter FhaC provide insights into two-partner secretion

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The two-partner secretion (Tps) pathway mediates protein transport across the outer membrane of Gram-negative bacteria. TpsB transporters belong to the Omp85 superfamily, whose members catalyze protein insertion into, or translocation across membranes without external energy sources. They are composed of a transmembrane β barrel preceded by two periplasmic POTRA domains that bind the incoming protein substrate.

Here we used an integrative approach combining in vivo assays, mass spectrometry, solid- and solution-state nuclear magnetic resonance and electron paramagnetic resonance techniques suitable to detect minor states in heterogeneous populations, to explore transient conformers of the TpsB transporter FhaC. This approach revealed substantial, spontaneous conformational changes on a slow time scale, with parts of the POTRA2 domain approaching the lipid bilayer and the protein's surface loops. Specifically, our data indicate that an amphipathic POTRA2 β hairpin can insert into the β barrel. We propose that these motions enlarge the channel and initiate substrate secretion. Notably, we find that these large-scale motions do not occur in a nanodisc environment, suggesting that this membrane mimic may be too constrained for analysis of larger conformational transitions.

Our data suggest a solution to the conundrum how TpsB transporters mediate protein secretion without the need for cofactors, namely by utilizing intrinsic protein dynamics.

Structural Insights into Covalent Inhibitors Targeting a Key Enzyme Linking Peptidoglycan to the Outer Membrane in Gram-Negative Bacteria

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The cell envelope of Gram-negative bacteria is a complex, multilayered structure comprising an inner membrane (IM), an outer membrane (OM), and a periplasmic space that contains a thin peptidoglycan (PG) layer responsible for maintaining cellular integrity. While PG crosslinking is normally mediated by D,D-transpeptidases, inhibition of these enzymes by β-lactam antibiotics triggers an alternative crosslinking pathway catalyzed by L,D-transpeptidases (LDTs), notably LdtD and LdtE in *E.coli*. In addition, three unconventional LDTs—LdtA, LdtB, and LdtC— have been identified in *E.coli*, playing a crucial role in covalently anchoring Braun's lipoprotein (Lpp) to the PG. As Lpp is also anchored to the OM, this reaction forms a vital structural bridge between the OM and PG, critical for maintaining periplasmic architecture, OM stability, and intermembrane communication. Disruption of this linkage, or absence of Lpp, results in increased OM permeability and enhanced antibiotic susceptibility. Given their conservation across Gram-negative pathogens such as *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, LDTs are emerging as attractive antibacterial targets for combinatorial therapies aimed at potentiating existing treatments and bypassing resistance.

Two classes of antibiotics—carbapenems and cephalosporins—have been tested in vitro on LdtB, the most abundant L,D-transpeptidase in *E. coli*, confirming the formation of covalent adducts. Promising results have also been obtained testing newly synthesized N-thio monocyclic beta-lactams and non-β-lactam compounds provided by collaborators within the doctoral network. An integrative approach, combining NMR spectroscopy, mass spectrometry, and X-ray crystallography, is being employed to characterize enzyme–inhibitor interactions at atomic resolution. To gain deeper insight into LdtB's mechanism of action, its interactions with physiological substrates, Lpp and peptidoglycan, are being characterized through a multidisciplinary workflow involving NMR, mass spectrometry, mutagenesis, and additional biophysical techniques, supported by computational docking simulations. Combined structural and mechanistic insights will aim to uncover the key catalytic determinants and identify novel druggable surfaces, ultimately guiding the rational design of next-generation LDT inhibitors.

Histone assembly mechanism coupled to DNA synthesis

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Most cancers or cellular aging processes result not only from the alteration of gene sequences but also from the loss of epigenetic information that regulates gene expression. Essential epigenetic marks carried by histones may be lost during DNA replication or DNA repair, processes that require dissociation and reassembly of nucleosomes. Chromatin Assembly Factor 1 (CAF-1) is a three subunits complex, conserved in all eukaryotes, that orchestrates the assembly of histones H3-H4 coupled to DNA synthesis in the context of DNA replication and repair [1]. This histone chaperone is thus particularly important for the maintenance of cell identity, but its action mechanism remains poorly understood. It has been established that its association with PCNA, is required for its functions during DNA replication, heterochromatin maintenance, and genome stability [2-3]. A model for the histone deposition mechanism has been proposed from studies of a truncated S. cerevisiae CAF-1 complex [4], but we lack a framework to demonstrate its generality and how histone deposition is coupled to PCNA interaction.

We have undertaken structure function studies of the CAF-1 complex from yeast S. pombe, using an integrative approach combining solution NMR spectroscopy, SAXS, biochemical assays, and bioinformatic modeling. In particular, we investigate how this complex (composed of pcf1, pcf2 and pcf3) and its constitutive domains interact with different partners to deliver histones into DNA. We focused on CAF-1 interactions with DNA, histones H3-H4 and PCNA. We have previously shown that the ED domain of Pcf1 mediates histone binding and promotes conformational changes in CAF-1 [5]. We are now exploring how additional interactions of histones H3-H4 with the two other subunits, Pcf2 and Pcf3 may trigger this conformational change. Regarding CAF-1 in full complex, we are studying the interplay between DNA and PCNA interactions with the aim of obtaining a high-resolution structure of this large complex. Here, we present the results we have obtained so far, revealing distinct binding interfaces and conformational adaptations within CAF-1 upon partner binding.

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Flash poster 8

Revealing the Dynamics of Intrinsically Disordered Protein Complexes Using Paramagnetic NMR

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IDPs often rely on linear motifs, i.e. short sequence segments, to mediate interactions with partner proteins. Due to their intrinsic flexibility, IDPs can exploit various interaction mechanisms, including folding-upon-binding, however, IDP complexes often remain dynamic in their bound states. For example, the IDP may significantly populate folding intermediates, or the final bound state may best be represented by an ensemble of conformations rather than a single, static, three-dimensional structure. Recent studies have shown that paramagnetic NMR can been used to gain insight into low-populated protein states. Here, we present new NMR methods, based on paramagnetic lanthanide tagging and chemical exchange saturation transfer (CEST) experiments, to study the structural dynamics of IDP complexes.

We apply our methods to the interaction between the GTPase Rac1 and its intrinsically disordered effector, POSH, that undergoes extensive folding-upon-binding to Rac1. The structural transition of POSH can be monitored by CEST experiments of ^{15}N-labeled POSH in the presence of sub-stoichiometric amounts of Rac1 revealing the presence of a folding intermediate. Lanthanide DOTA chelators loaded with a selection of lanthanide metals (Tb^{3+}, Dy^{3+}, Tm^{3+}) are optimum for inducing pseudo-contact shifts (PCS). Ten cysteine mutants of Rac1 were designed and tagged with Tm-M7Py-DOTA. Four mutants were kept for further studies as they show fast and reliable tagging (monitored using HRMS) and induce strong PCS in Rac1. Due to the complexity of the NMR spectra, the magnetic susceptibility tensors were determined for each tagging site using ^{15}N-Leu labelled samples of Rac1. ^{15}N Dante-CEST experiments acquired of ^{15}N POSH in the presence of 20% Tm-tagged Rac1 reveal that PCSs are successfully induced in POSH upon binding to Rac1. *In silico* methods such as conformational ensemble generation and molecular dynamics will be used to sample the conformational space of the intermediate species and the results leveraged with the experimental PCS data. The goal is to combine all the PCS data from different mutants to elucidate the structural dynamics of each of the folding intermediates of POSH.

Exploration of lipid phase transitions and lipid-scaffold protein coupling in nanodiscs using high-pressure NMR

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Biological membranes play essential roles in cellular function, mediating molecular transport and regulating processes such as signaling. Nanodiscs (NDs) are widely used models for mimicking biological membranes and provide a controlled lipid environment for the study of membrane proteins. These ~10 nm nanoparticles consist of hundreds of phospholipids surrounded by two lipoproteins. However, the dynamic interactions between lipids and lipoproteins in these systems remain poorly understood.

In this study, we use liquid-state ^{1}H, ^{13}C NMR combined with hydrostatic pressure and temperature variation to investigate the lipid fluid-to-gel phase transition in NDs of various sizes, aiming to assess potential dynamic coupling between lipids and lipoproteins.

We first compared NDs assembled with MSP1D1 lipoprotein and two lipid compositions, DMPC and 9-cis-PC. ^{1}H NMR revealed that only DMPC, but not 9-cis-PC undergoes a pressure-sensitive phase transition at 35 °C in the 1-2500 bar pressure range, in accordance with their known lipid phase transition properties in infinite bilayers. Analysis of ^{13}C NMR chemical shifts along the DMPC lipid chains revealed pressure-induced conformational changes, especially for carbons in the bilayer core, likely reflecting the increased conformational *trans-gauche* fraction in the acyl chain in the gel phase.

To assess the influence of ND size on lipid phase transition, we prepared DMPC-based NDs with three lipoproteins: D1 (reference, ~10 nm), E3 (larger, ~12 nm), and H5 (smaller, ~8 nm). Pressure and temperature-dependent ^{1}H NMR, supported by DSC data, confirmed the presence of one or more phase transitions in all three NDs. ^{13}C NMR spectra suggest that ND size does not significantly alter lipid conformations in the tested P/T range.

Finally, to probe lipoprotein behavior upon DMPC gelation, we analyzed a ^{15}N, ^{13}C, and partially ^{2}H-labeled D1 protein within a DMPC ND. The 2D ^{15}N and ^{13}C HSQC spectra revealed changes in side chain orientation for several tryptophan and asparagine/glutamine residues upon fluid-to-gel phase transition, suggesting potential partial exclusion from the gel phase for a subset of MSP1D1 sidechains.

These results provide new insight into lipid–lipoprotein interactions in nanodiscs and lay the groundwork for future integration of membrane proteins into nanodiscs.

Flash poster 9

Can Alphafold help us refine ensembles of Intrinsically Disordered Proteins?

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The rise of biomolecular structure prediction AI tools such as Alphafold has revolutionized the field of structural biology. Despite an extensive characterization of these AI tools, their ability to provide accurate information on disordered proteins remains unclear. Here, we use bAIes, a new Bayesian framework that combines AI information and molecular dynamics force fields to explore the capabilities of Alphafold2 to accurately describe structural ensembles of Intrinsically Disordered Proteins (IDPs). We derive structural ensembles of multiple different IDPs featuring distinct structural properties using bAIes on a random coil model and we methodically assess the agreement of calculated NMR and SAXS observables obtained from the simulations with experimental data. We show that the agreement with experiment is highly dependent on the accuracy of the AI model and find a relative improvement in the structural ensemble of IDPs when Alphafold2 accurately depicts the secondary structure elements of the disordered domains. We also highlight the fact that Alphafold alone cannot decipher environment-dependent structural changes and that in this case NMR remains crucial for high resolution structural ensemble refinement. Disorder is important in many biological processes and predicting atomistic-detailed ensemble properties is therefore valuable. This study paves the way to a better understanding on how to combine AI methods and experimental data for an accurate structural description of IDPs.

Solid-State and Solution NMR study of Tryptophan Synthase Enhanced by Perdeuterated Amino Acids

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Tryptophan synthase (TS) is a 140 kDa αββα heterotetrameric enzyme complex that catalyzes the final step in L-tryptophan biosynthesis by channeling intermediates between α and β subunits. Owing to its large size and structural complexity, TS remains a challenging system for high-resolution solution NMR spectroscopy, yet it is a system of considerable interest due to ongoing questions about its allosteric regulation and subunit communication.

To overcome spectral overlap and relaxation limitations in such a high molecular weight system, we applied multidimensional solid-state NMR experiments under magic-angle spinning (MAS) conditions. Whereas for perdeuterated samples, obtained well-resolved spectra and a large part of the backbone assignments were obtained, amide proton backexchange is impossible in the hydrophobic core of the protein. To overcome this bottleneck, we used $^{15}N/^{13}C/^{2}H$-labeled amino acids derived from ISOGRO algal amino acid mixes in H_2O. This circumvents the step of amide proton back-exchange altogether, while preserving the spectral advantages of perdeuteration.

To further advance towards assignments as complete as possible, we combine the solid-state spectra with solution-state spectra further simplified by implementing the SEA (solvent exposure attenuated) block experiment [1], which selectively filters for amide protons that exchange with water. This results in the preferential detection of solvent-accessible surface residues and significantly reduces spectral crowding. In addition, HD exchange experiments were pursued to identify those peaks that retain their protonated amides, together enabling elucidation of solvent interactions of the enzyme in particular for the catalytically important residues and the indole tunnel connecting the subunits.

Our integrated strategy demonstrates that perdeuteration based on deuterated amino acids [2] combined with MAS solid-state NMR and SEA block filtering enables detailed study of large, multimeric proteins. This methodology is broadly applicable for structural and dynamic insights even for water-inaccessible regions, opening new avenues for the analysis of high molecular weight systems.

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Exploring Mur Ligase Dynamics Across Timescales: Implications for Mechanism and Inhibitor Design

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The bacterial enzyme MurD, a 47 kDa multidomain muramyl ligase, plays a central role in peptidoglycan biosynthesis and is a promising target for the development of new antibacterial agents amid growing resistance. Despite being widely used in structure-based drug design, efforts to develop effective MurD inhibitors have so far shown limited success—largely due to the dynamic nature of this protein, which remains underexplored.

In our study, we investigated MurD backbone dynamics using ^{15}N NMR relaxation in both its apo form and ligand-bound complexes. Analysis of spectral density functions, derived from the relaxation data, revealed distinct mechanistic features of MurD motion and enabled residue-specific classification of dynamics across the pico- to millisecond timescale. To further explore micro- to millisecond conformational exchange, we performed CPMG-RD measurements, which we also extended to $^{13}CH_3$-bearing AILV^{proR} side chains**,** providing complementary insight into intermediate dynamics**.** The experimental results regarding the interplay of dynamics on different time scales were compared with data derived from MD simulations, providing further confirmation of the multiscale action of this enzyme.

Our results reveal new perspectives on MurD binding mechanism while suggesting the presence of multiple (pre-existing) conformations that may be relevant for ligand recognition. Moreover, AMP-PCP preserves C-terminal domain flexibility to allow downstream binding of substrate, while the inhibitor suppresses it. However, residual dynamics at binding sites in inhibitor-MurD complex suggest that the limited binding efficacy of inhibitor may result from suboptimal adaptability to local structural dynamics, highlighting the need for fine-tuning its moieties. Altogether, these findings deepen our understanding of MurD dynamics and offer a refined framework to support the design of structurally novel inhibitors with improved therapeutic potential.

Flash poster 10

NMR spectroscopy of self-hydrolysing DNA

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Since the 1990s, DNA enzymes (DNAzymes) have been gaining popularity as they are applicable in diverse fields of biotechnology and therapeutics. Albeit essential for deciphering their mode of action, acquiring high-resolution structural insights into DNAzymes remains highly challenging. Still, crystallographic studies have been successful for the RNA-cleaving 8-17 DNAzyme [1] and the RNA-ligating 8-17 DNAzyme [2]. Recently, the structure of the RNA-cleaving 10-23 DNAzyme was revealed using NMR spectroscopy [3]. However, so far, no high-resolution structural insights into an autohydrolytic DNAzymes could be obtained. Autohydrolytic DNAzymes are capable to catalytically cleave their own DNA strand with the help of metal-ion cofactors, offering a wide range of biotechnological and therapeutic applications.

Here, we report on our recent progress in characterized a set of autohydrolytic DNAzymes. To gather structural insights into metal-ion binding sites, the catalytic cleavage reaction and the influence of temperature on the structure, solution NMR spectroscopy was used. A potential metal ion binding site was identified by real-time NMR and titration experiments with stabilized DNAzyme constructs. Structure prediction tools, such as AlphaFold and RNAfold, predict a defined hairpin structure and NMR experiments at low temperatures are consistent with the predicted Watson-Crick base pairing of this arrangement. Interestingly, our data suggest that the defined hairpin structure is not the active form of the system and that instead conformational plasticity, activated at higher temperatures, is a central element of the catalytic capabilities of the investigated DNAzymes. The observed high level of dynamics could explain why crystallographic studies failed so far and suggest that a certain degree of flexibility is necessary to accomplish effective DNA autohydrolysis.

Our study reveals exciting new insights into autohydrolysing DNAzymes and confirm that NMR spectroscopy is an excellent tool to study DNA catalysts.

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Dimeric DNA structure(s) promoted by divalent cations

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Dynamic structural landscape of genomic DNA is the central feature of vital cellular processes, none of which are (fully) understood. Moreover, apart from the ubiquitous double-stranded helix, DNA (transiently) adopts also so called non-canonical structures acting as biosensors or switches, which roles are coupled to the (microenvironmental) variations in concentrations and nature of ions and (bio)molecules. In this regard, a particular non-canonical DNA structure may be perceived as a platform enabling peculiar interactions and/or the means to dynamically control features of the nearby or a distal part of the genome, e.g. by promoting unwinding of double-stranded helix or altering DNA compaction via supercoiling, respectively. In any case, the delicate susceptibility of DNA folding and the subtleness of the biological roles linked to the formation of non-canonical DNA structures are the key reasons why our understanding of even fundamental cellular mechanisms is still incomplete.

Majority of human genome consists of repetitive sequences, including short tandem repeats (STR). STR instabilities, i.e. their expansion and contractions, are increasingly recognized as critical drivers of (path)physiological cellular processes.(1,2) Herein we will discuss DNA folding in the presence of divalent cations, particularly focusing on the unique domain-swapped dimeric structure adopted by $d(ATTTC)_3$ in the presence of Mg^{2+} and Ca^{2+} ions. The results shed light on possible molecular mechanisms promoting the instability of d(ATTTC) repeats linked to neurodegenerative diseases.(3) Additionally, the obtained NMR-based high-resolution insights highlight the diverse ways of how STR can fold and interact, which is important for advancing our understanding of fundamental biological processes. In fact, one of the striking aspect of our study is the demonstrated power of NMR characterization of nucleic acids, which even if employing well-established and routine approaches, represents one of few strategies suitable to elucidate curious structures coupled to DNA interactions with divalent cations.

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NMR-based metabolomics as a tool for human milk research

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Breastfeeding is considered the standard for infant feeding in their early stages of life. Determining the metabolomic profile of human milk (HM) will help us understand how it is linked to infant health and nutrition.

Six different sample preparation procedures were tested, including a miniaturized protocol designed for low volumes of HM (100 µL), better suited for clinical study application.

The analyses were conducted on 33 HM samples using a Bruker 600 MHz Avance IVDr system equipped with a room temperature probe. The spectra were acquired using a NOESY 1D with water suppression, or by using a CPMG sequence to remove the macromolecular signal contribution in ^{1}H-NMR spectra.

For the identification, bidimensional NMR experiments, including COSY, HSQC, and HMBC, were performed on selected samples from each preparation method. More than 40 polar and apolar metabolites were identified and quantified, including human milk oligosaccharides (HMOs), amino acids, organic acids, and nucleotides. Furthermore, authentic standards of 3'-fucosyllactose (3'-FL), lacto-N-difucohexaose I (LNDFH I) and lacto-N-difucohexaose II (LNDFH II) were compared to the signals detected in HM.

The miniaturized extraction and analysis procedure was successfully applied to 111 HM samples from a cohort of 53 mothers (registered at ClinicalTrials.gov under NTC02052245), whose HM was collected longitudinally over 16 weeks postpartum at different time intervals. Concentrations of 10 HMOs determined by NMR compared well with the concentrations measured by liquid chromatography.

The performance of the NMR approach was good, as demonstrated by the significant Pearson correlations obtained between the two techniques, with most correlations exceeding $r > 0.80$. This underscores the potential of NMR as a reliable quantification tool for HM analysis.

Characterization of PGC-1α Molecular Mechanisms at single-residue Resolution by NMR Spectroscopy

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The peroxisome proliferator-activated receptor ɣ co-activator 1α (PGC-1α) is a eukaryotic transcriptional co-activator in energy metabolism. Interacting with a vast network of transcription factors and other coregulators, PGC-1α serves as a molecular docking platform to sense the metabolic state of the cell and elicit an appropriate transcriptional response (1). Thereby, PGC-1α is referred to as the 'master regulator of energy metabolism', allowing cells of high-energy demanding tissues to adapt to specific environmental stimuli that the organism is presented with. Misregulation of PGC-1α has implications on diseases such as diabetes, muscle dystrophy, obesity, cancer or neurodegenerative diseases (1).

PGC-1α comprises large intrinsically disordered regions (IDR) spread throughout its approximately 800 amino acid residues, rendering nuclear magnetic resonance (NMR) spectroscopy the method of choice for atomic resolution studies of PGC-1α *in vitro* in aqueous solution. We combine NMR spectroscopy with cellular assays and muscle (patho)-physiological mouse studies to obtain insights into PGC-1α molecular mechanisms at atomic resolution.

Here, we report initial data on the molecular mechanism of interaction of the N-terminal transactivation domain of PGC-1α with co-activators relevant for transcription initiation, in particular the CREB binding protein (CBP) and the Mediator subunit 15 (Med15). Moreover, we identify novel phosphorylation patterns of PGC-1α through Casein kinase II (CKIIα).

These findings pave the way to gain a deeper insight into the PGC-1α sequence-structure-function relationship.

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Analysis of conformation manifolds for intrinsically disordered proteins

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Determining a protein's functional conformation is one of the greatest challenges in structural biology. Although folded proteins easily interact with numerous partners, roughly 35–50% of the human proteome does not adopt stable three-dimensional structures[1]. Instead, many proteins are partially (IDRs) or entirely (IDPs) intrinsically disordered. Their inherent flexibility facilitates interactions with multiple partners and participation in various physiological and pathological processes, such as and neurodegenerative diseases and cancers[2]. However, standard structural techniques fail to capture these proteins' plasticity.

To address this limitation, we propose a computational strategy to explore the local folding of IDPs. First, secondary structure elements are assigned from experimentally derived backbone chemical shifts using δ2d[3]. These observations define probabilistic "boxes" on the Ramachandran map that reflect the likelihood of specific backbone torsion angles (φ and ψ). Due to the dynamic nature of IDPs, the protein is divided into intervals of residues. To manage the conformational complexity, we employ a threading-augmented interval branch and prune (TAiBP)[4] method to generate an ensemble of candidate conformations, which are clustered using a self-organizing map (SOM) to select representative structures. After fragment assembly, side chains are added via molecular dynamics simulations using NAMD[5].

For validation, we apply our method to a set of PED proteins, including the wild-type[6] C-terminal domains of the vasopressin receptor, growth hormone secretagogue receptor type 1a, and B2-adrenergic receptor, along with their phosphomimetic analogues[7]. Several analyses compare the conformations obtained from the PED proteins with those generated by TAiBP.

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Flash poster 11

Unveiling hidden intermediates in a metamorphic protein through disulfide locking and NMR spectroscopy

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Disulfide bonds play a crucial role in maintaining the structural integrity of the canonical αβ fold in chemokines. While chemokines generally contain two disulfide bonds, Lymphotactin (Ltn) is unique in having only one, enabling a remarkable exchange process between two native conformations: Ltn10, a canonical 3β+α monomer and Ltn40, an all-β dimer. Previous studies demonstrated that each of the two native states can be stabilised by incorporating a second disulfide bond. For instance, the addition of a cysteine bond using V21C/V59C (known as CC3) locks Ltn in the Ltn10 fold, eliminating the native-state metamorphism. However, how the disulfide-locked Ltn10 conformation of CC3 channels the thermal energy, by virtue of which the wild-type undergoes Ltn10-Ltn40 interconversion, remains unknown.

Here, we show that despite arresting Lymphotactin in Ltn10 conformation, the CC3 mutation reveals previously hidden local minima within the global free energy minimum of Ltn10, offering new insights into Lymphotactin's conformational landscape. A combination of 15N ZZ-exchange, CEST and CPMG experiments establish the presence of two exchange processes occurring at distinct timescales, $k_{ex,1}$ = 10s^{-1}(slow) and $k_{ex,2}$ = 400s^{-1}(fast).

Temperature-dependent 15N CEST data reveals that with an increase in temperature (10-30^{o}C), minor state population increases for the slow exchange residues, while the fast exchange residues show an opposite trend. Notably, the excited state chemical shifts for both the slow- and fast-exchange processes exhibit overlap for a significant number of residues, indicating shared structural features.

Chemokines, except Ltn, form dimers or oligomers with the β+α fold. Restricting the native-state dynamics in Ltn10 conformation could lead to structural frustration potentially driving oligomerization. The concentration-dependent 15N CEST confirms that both the slow and fast exchanging excited states are monomeric, ruling out canonical chemokine dimer or oligomer. Our NMR data suggest that CC3 unmasks hidden intermediates in the free-energy landscape of Ltn. We aim to determine the backbone chemical shifts of the two excited states using multinuclear-CEST experiments to obtain structural insights which will help us understand why these exchange events remain hidden in the WT. We also seek to investigate whether the intermediates play a role in Ltn10's interaction with cognate GPCRs.

Flash poster 12

Molecular Dynamics Alterations in the TEM β-Lactamase Family Induced by Nanobody Binding

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The most common and clinically significant mechanism of bacterial resistance to penicillin-based antibiotics is the production of β-lactamases, which hydrolyze and thereby inactivate the β-lactam ring of penicillin. Among these enzymes, TEM-1 is one of the most extensively studied and highly diversified β-lactamases [1]. To date, over 200 TEM-1 variants have been identified [1].

TEM-121 is one such variant, distinguished from TEM-1 by six amino acid substitutions: Gln39Lys, Glu104Lys, Arg164Ser, Ala237Thr, Glu240Lys, and Arg244Ser. Notably, four mutations Glu104Lys, Arg164Ser, Ala237Thr, and Glu240Lys confer extended-spectrum β-lactamase (ESBL) activity, while Arg244Ser is associated with inhibitor-resistant β-lactamase (IRT) activity.

Understanding the molecular dynamics of β-lactamases and developing tools to probe these dynamics offer promising avenues for designing diagnostic and therapeutic strategies. In this context, we have initiated a study to investigate the inhibition mechanisms of TEM-1 and TEM-121 upon binding to the nanobody cAb_{TEM-1}, originally isolated by Conrath and colleagues in 2001 [2].

To achieve this, we employed a combination of NMR relaxation techniques including chemical shift perturbation (CSP), DANTE chemical exchange saturation transfer (DCEST) [2-3], and ^{15}N CPMG relaxation dispersion [4] and alongside steady-state kinetic analyses.

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Investigating the Activation Mechanism of GHSR Using Solid-State NMR and Ligand Binding Assays

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Advancements in the structural biology of membrane proteins, particularly G protein-coupled receptors (GPCRs), have significantly improved our understanding of their complex architectures and ligand-induced activation mechanisms. One particularly interesting GPCR is the Growth Hormone Secretagogue Receptor (GHSR). Together with its natural ligand Ghrelin, GHSR plays a crucial role in food intake, glucose metabolism, and immune response.[1] Due to its high constitutive activity[2], the receptor has become a key target for pharmacological interventions aiming to reduce the basal activity of GHSR. We study the activation of GHSR using recombinant receptors and de novo designed peptide ligands. Recent structural investigations regarding the position of ECL2 involve a ghrelin analog in which the phenylalanine at position 4 is substituted with proline, a modification suggested by ProteinMPNN5-based on MD simulations. These simulations revealed two distinct receptor conformations characterized by ECL2 movement, indicating that the Phe-to-Pro transition could optimize ligand binding. Based on these findings, we design a ghrelin analog with the Phe-to-Pro substitution. For these site-specific studies, the GHSR receptor is selectively labeled with ^{13}C-histidine residues, expressed cell-free, folded into DMPC membranes, and analysed using fluorescence-based ligand binding assay and solid-state NMR spectroscopy. This approach allows for the observation of structural changes upon ligand binding. Changes in chemical shifts, induced by interaction with Ghrelin-Phe4Pro, provide valuable insights on receptor activation and ligand-induced conformational changes[3]. By integrating these findings, we deepen our understanding of ligand-induced GHSR dynamics, which could inform the rational design of modulators targeting its constitutive activity.

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Expression And NMR Characterization Of Labelled P-domains Of Emerging Norovirus

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Noroviruses are members of the Caliciviridae family and are non-enveloped, single-stranded, positive-sense RNA viruses. Norovirus is the major cause of acute gastroenteritis, particularly affecting vulnerable populations such as children, the elderly, and immunocompromised individuals. With millions of illnesses and thousands of deaths yearly, norovirus poses a significant global health burden. The economic impact is equally staggering, with billions of dollars in costs annually. Additionally, new virus strains emerge every 2-5 years, making outbreaks difficult to contain. Among the various norovirus strains, the GII.4 variant is the most prevalent globally.

The ability of norovirus to infect host cells depends on the interaction between the Protruding domain (P domain) of its capsid protein VP1 and Histo-Blood Group Antigens (HBGAs) on the surface of host cells. Furthermore, previous cell-based studies found, that Human Milk Oligosaccharides (HMOs) prevent norovirus infection to cells, by acting as a decoy receptor and blocking the interaction between HBGAs and human noroviruses. Therefore, understanding the structure of the P domain is essential for the development of effective antiviral drugs and vaccines.

In this study, we successfully expressed and purified the P domain from the GII.4. P16 norovirus strain in *E. coli*. To enhance NMR signal resolution, isotopically labeled samples (^{15}N, ^{2}H, and ^{13}C) were prepared, and multidimensional NMR experiments, along with chemical shift perturbation (CSP)-based NMR experiments, were performed. These experiments will help identify key residues involved in binding to HBGAs and other antiviral molecules, such as analogs of HMOs. By elucidating these interactions, our studies provide crucial insights into the viral infection mechanism and offer valuable information for the development of new therapeutic strategies.

Mediator MED25 subunit hijacking by RSV NS1 protein

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Respiratory syncytial virus (RSV) is a major cause of bronchiolitis in infants worldwide. RSV elicits a weak innate immune response and low type-I interferon (IFN-I) levels. IFN-I antagonism is partially mediated by the non-structural RSV NS1 protein. In the nucleus of infected cells NS1 interacts with Mediator, a transcriptional coactivator of RNA polymerase II, and modulates host transcription. We and others identified the MED25 Mediator subunit, in particular its ACID domain, which is targeted by transcription factors (TFs), as an NS1 interaction partner. However, since NS1 lacks a DNA binding domain, it cannot act like a TF, and its mechanism remains puzzling. We analyzed the high affinity NS1−MED25 complex by mutational analysis and a combination of biophysical techniques, including AlphaFold prediction, NMR, Isothermal Titration Calorimetry and BioLayer Interferomtery. The two subdomains of NS1 bind to a dual site, occluding the two TF-binding sites of MED25 ACID. We showed that NS1 disrupts the complex formed between MED25-ACID and ATF6α TAD, indicating how NS1 could outcompete MED25-mediated transcription. To investigate the impact of the NS1−MED25 interaction on viral infection, we engineered NS1 mutants of recombinant rRSV with decreased MED25 affinity. These rRSV mutants were attenuated and induced increased production of interferon-stimulated genes compared to WT rRSV. MED25 knock down further corroborated MED25 hijacking by RSV.

pH sensitivity of SERF1a conformational ensemble

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The MOAG-4/SERF class of proteins is a positive regulator of aggregate formation of amyloid proteins which plays an important role in the origin of numerous age related diseases. In this class of proteins, previous structural studies showed that SERF1a is a very flexible protein, containing at least one α helical region. The present work propose the first description of the conformational space of SERF1a at two pH values (6 and 6.8), using Nuclear Magnetic Resonance (NMR), and Small-angle X-ray scattering (SAXS). SERF1a conformations have been generated using the CYANA FLYA procedure, as well as the systematic enumeration of conformations using TAiBP, an original distance geometry approach. The generated conformations were filtered by fitting SAXS data. At pH=6.8, a good fit of SAXS curves was obtained for CYANA and TAiBP conformations whereas at pH=6, NMR and SAXS curves analysis indicated that there could be a conformational exchange between a compact conformation displaying a long-range NOE between residues LYS-13 and THR-32, and slightly more extended conformations. The shortening of the C terminal α helix as well as the destabilization of the N terminal α helix at acidic pH can been related to the physiological function of SERF1a in the nucleoli. The N terminal region of SERF1a displays numerous possible binding pockets, in agreement with the chemical shift perturbations observed for the protein during a titration by α-synuclein. Overall, the TAiBP procedure allows a more expanded exploration of the SERF1a conformational space as well as the better description of the SERF1a internal dynamics determined from NMR relaxation.

Flash poster 13

4D protein structures from solution NMR data

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The stunning success of AlphaFold2 (AF2) in the CASP14 protein structure prediction competition represented a watershed moment for structural biology. For NMR spectroscopy this has been seen an existential crisis, with AF2 models now widely regarded as more accurate than NMR structures, especially for the small proteins where NMR is usually applied[1]. However, it is now becoming clear that the two are complementary methods, with NMR able to provide data on proteins with distinct functional states where AF2 models may be less reliable[2,3]. We have previously described the CoMAND method, which uses a powerful R-factor expressing the match between experimental and back-calculated NOESY spectra to map protein conformational states[4]. We then extend this to a hybrid method coupling the accuracy of AF2 in terms of the global fold to detailed descriptions of local micro- sates and their populations. The central advantage of this method is the ability to select structural ensembles from unrestrained molecular dynamics trajectories, resulting in thermodynamically relevant ensembles, something that can not be achieved in conventional NMR structure determination where data is applied as restraints. Here we extend this method to include other NMR observables, such as RDCs and scalar couplings, making it possible to compile ensembles that explain all input data well and therefore approach a true ensemble representation of protein structure in solution.

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Flash poster 14

NMR insights into the thermostability of an improved PETase

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Thermophilic enzymes have become invaluable in biotechnology due to their exceptional robustness. Here, we demonstrate the power of NMR-derived parameters in characterizing the backbone hydrogen bond network and its role in protein stability at elevated temperatures. By analyzing three parameters: (1) peak presence in spectra recorded at increasing temperatures, (2) protection from H/D exchange, and (3) H^N temperature coefficients, we provide a comprehensive methodology for assessing structural stability beyond traditional model systems. Applying this approach to the high-Tm PETase, LCC^{ICCG}, across an extended thermal range up to 84°C, we identify a robust protein core primarily composed of the inner β-sheet and shielded regions of the surrounding helices. Notably, residues protected from H/D exchange for 10 hours exhibit consistency across all measured parameters, while the rest of the protein shows more variation, reflecting the intricate interplay between hydrogen bonding strength, structural orientation, and solvent accessibility. Our findings highlight this complexity, providing a framework for detailed residue-level analysis of thermostable proteins.

Integrating ^{19}F Restraints for Structural Biology by MAS NMR Spectroscopy

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I will present recent results establishing ^{19}F high-frequency (40-110 kHz) MAS NMR and DNP approaches that overcome sensitivity and resolution bottlenecks in atomic-level characterization of large biological assemblies, pharmaceutical formulations at natural abundance, and proteins in their natural cellular milieus.

Flash poster 15

Combining NMR and AlphaFold to identify new partner protein binding motifs in intrinsically disordered proteins

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Intrinsically disordered proteins (IDPs) constitute approximately 40% of the human proteome and play essential roles in various biological processes, including signal transduction, cell cycle regulation and transcription. Many IDPs engage with their partner proteins through short linear motifs, typically exhibiting moderate binding affinity. Recent advances in artificial intelligence-driven structure prediction have revolutionized structural biology. However, prediction of complexes involving IDPs remains a major challenge due to the lower quality of their multiple sequence alignments and the underrepresentation of IDP complexes in the Protein Data Bank (PDB).[1]

In this study, we investigate two scaffold proteins, JIP1 and JIP2, from the JNK signaling pathway, both of which possess long intrinsically disordered tails (450 and 510 amino acids, respectively). Using NMR spectroscopy, we obtain an atomic resolution description of these tails and study their interactions with the kinase JNK1 through a combination of chemical shift titrations, $R_{1\rho}$ relaxation rates and CPMG relaxation dispersion experiments. Our data reveal previously unrecognized kinase-binding motifs within the scaffold proteins, and we determine high-resolution crystal structures of JNK1 in complex with these motifs.[2] Using the experimental NMR and X-ray crystallography data, we evaluate the ability of AlphaFold to predict these interactions. While some motifs are accurately predicted, others are not captured by AlphaFold. To improve prediction accuracy, we introduce a sliding-window approach, which outperforms predictions based on the full-length disordered sequences.

Our work not only demonstrates the power of NMR spectroscopy in identifying novel binding motifs within long disordered regions but also highlights both the strength and limitations of AlphaFold in modeling complexes involving IDPs, even when using adapted prediction strategies.

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2. Bipartite binding of the intrinsically disordered scaffold protein JIP1 to the kinase JNK1 (2025) Orand T., Delaforge E., Lee A., Kragelj J., Tengo M., Tengo L., Blackledge M., Boeri Erba E., Davis R.J., Palencia A., Jensen M.R. *Proc. Natl. Acad. Sci. USA*

Elucidating the structural landscapes of mitochondrial tRNA^{ILE} derived fragments

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tRNA-derived fragments (tRFs) are a class of small non-coding RNAs originating from precise cleavage of mature or precursor tRNAs. Beyond their traditional role in translation, these fragments participate in gene regulation, ribosome biogenesis, chromatin remodeling, and cellular stress responses(1). Advancements in state-of-the-art research techniques over the past decade have identified clinically relevant tRFs as potential biomarkers for cancer diagnosis and prognosis. Despite their functional importance, the structural basis of tRF-mediated regulation remains largely unknown(2,3). To advance our understanding of this unresolved aspect of tRNA biology, we employ high-resolution solution-state nuclear magnetic resonance (NMR) spectroscopy to investigate the conformational dynamics of mitochondrial tRNA^{ILE} and its fragments, which have also been consistently linked to various mitochondrial dysfunctions. By analyzing their native structures, which can adopt multiple conformational states, and assessing the impact of mutations and post-transcriptional modifications, we aim to uncover key structural determinants that influence their biological activity. Utilizing different NMR experiments, we seek to provide detailed insights into their folding patterns, stability, and molecular interactions. Our study sheds light on the structural diversity of tRFs and their potential as regulators in cancer biology. Understanding their conformational landscape will offer new perspectives on their mechanisms of action and contribute to the development of tRF-based biomarkers and therapeutic strategies.

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Flash poster 16

Structural and interaction insights into translation initiation factors (eIFs) in *Trypanosoma cruzi*: Regulation of the cap-4 RNA binding network

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Chagas disease, caused by *Trypanosoma cruzi* and transmitted by Triatominae insects, remains a major health concern in the Americas, affecting approximately 6 million people (PAHO). Its spread to non-endemic regions, including Europe, Japan, and Australia, raises global concerns. Currently, no vaccines are available, and existing treatments, such as antiparasitic drugs and chemotherapy, have limited efficacy due to side effects and low selectivity[1].

To address this, identifying drug targets unique to trypanosomatids is crucial. Our research focuses on their distinct translation initiation machinery, particularly the cap-binding eukaryotic translation initiation factors (eIF4E). Unlike higher eukaryotes, which typically have a single eIF4E, trypanosomatids possess six homologues (eIF4E1–6), each interacting with cap-4 RNA. Cap-4 consists of a cap-0 structure—an N7-methylguanosine linked to the 5′ nucleotide via a 5′-to-5′ triphosphate bridge—followed by an AACU sequence with 2′-O-ribose and base methylations on nucleotides 1 and 4, a feature absent in mammalian hosts[2].

Using an integrative approach combining solution-state NMR, X-ray crystallography, and HDX-MS, we study eIF proteins and their interactions with a synthetic 5′ cap-4 mRNA motif mimicking *T. cruzi* modifications. We present results on eIF4E1-based translation initiation regulation, focusing on its interactions with the putative activator and suppressor—i.e., eIF3A and 4E-IP, respectively—of the pathway in the presence of cap-4 RNA fragments.

Based on structural insights and homology with *T. brucei*[3] and *L. major*[4], we propose models of eIF4E1 cap binding regulation. Complemented by *in vivo* assays across *T. cruzi* life stages, our study advances understanding of translation initiation in trypanosomatids and informs therapeutic strategies.

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Flash poster 17

Probing Protein-Ligand Interactions and Complex Formation with ^{19}F NMR

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^{19}F solution-state NMR spectroscopy is a powerful technique for probing protein structure, dynamics, and ligand interactions due to its high sensitivity, lack of background signals, and large chemical shift range [1]. We present the use of ^{19}F NMR in two case studies:

Firstly, incorporation of fluorinated tryptophan residues into Maltose-binding protein (MBP) in order to characterize its ligand binding. The MBP binding site contains three tryptophan residues, and we demonstrate that chemical shifts for these residues are highly sensitive to distinguish distinct ligands in the binding site. In cells, MBP is exported into the periplasmic space. Using fluorinated tryptophan residues allows us to obtain ^{19}F spectra of MBP encapsulated within outer membrane vesicles (OMVs) [2], facilitating the characterization of ligand binding in a biologically relevant environment. We then employ the growing toolkit of ^{19}F based relaxation experiments [2], including Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion measurements and Chemical Exchange Saturation Transfer (CEST) to characterize the dynamics of apo MBP along with MBP bound to several ligands.

Secondly, we use ^{19}F NMR to study the mechanism of mitochondrial transcription initiation. In human mitochondria, mtDNA is transcribed by the 140kDa single-subunit mitochondrial RNA polymerase (mtRNAP) [4]. Post-translational incorporation of ^{19}F to the full-length human mtRNAP via BTFA-labeling provides a sensitive probe for initiation complex formation, facilitating the characterization of interactions with mtDNA and initiation factors.

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Flash poster 18

Recognition and m6A Modification of the lncRNA MALAT1 by METTL3-METTL14

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The human METTL3-METTL14 methyltransferase complex is responsible for the m6A methylation of a large set of RNAs and plays a crucial role in tumorigenesis. It targets numerous long non-coding RNAs (lncRNAs), including MALAT1, the overexpression of which is implicated in cancer. To date, structural studies on METTL3-METTL14 have essentially considered single-stranded RNA, and it is unknown how structured regions, (e.g. in lncRNAs) are recognized and methylated. The structure of a minimal complex has been solved in its apo form, in which the METTL3 and METTL14 methyltransferase domains interact to form the catalytic core of the enzyme. However, this minimal complex lacks catalytic activity, as flexible regions that are essential for activity are not present, including the METTL3 tandem zinc-finger domains and the RGG region of METTL14. We employ biochemical assays and solution NMR spectroscopy to study the METTL3-METTL14 complex *in vitro*, and the interaction with the MALAT1 hairpin targets. We purified the full-length METTL3-METTL14 complex and performed some methylation assays on different MALAT1 constructs. The methylation efficiency of the enzyme varies depending on the target. To investigate this, we purified the flexible regions of METTL3 (the tandem zinc-finger domain) and METTL14 (the RGG region) and performed some interaction assays with MALAT1. For both METTL3 and METTL14, we observed different affinities depending on the RNA constructs. Those results highlight the key features for the recognition per METTL3-METTL14 of the MALAT1 lncRNA.

Flash poster 19

Exploring the versatility of NMR in drug discovery: focus on human pre-miR-21 RNA and SARS-CoV-2 M^{pro}

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NMR is a powerful technique for drug discovery, providing detailed structural and mechanistic insights that are essential for hit-to-lead optimization. Its versatility makes it well-suited for investigating both protein and nucleic acid targets. Here, we present NMR-based approaches applied to two therapeutically relevant targets: human pre-miR-21 RNA and SARS-CoV-2 Main Protease (M^{pro}).

Pre-miR-21 is a stem-loop RNA whose maturation produces the non-coding microRNA miR-21, a key player in oncogenesis and resistance to anticancer therapies [1]. Inhibiting pre-miR-21 maturation is therefore a promising anticancer strategy. We initially employed native mass spectrometry to screen a library of bicyclic aromatic small molecules for their ability to bind pre-miR-21, leading to the identification of a high-affinity hit compound. To validate its binding mode and guide hit optimization, we then applied both ligand- and target-observed ^{1}H NMR techniques. WaterLOGSY experiments enabled epitope mapping of the ligand, while RNA-observed NMR approaches were used to determine the binding site on pre-miR-21. One-dimensional NMR experiments monitoring the imino protons of guanine and uracil nucleobases, combined with TOCSY experiments targeting the aromatic protons of pyrimidines, revealed that the ligand binds to the stem-loop region of pre-miR-21. This region is critical for pre-miR-21 maturation, thereby supporting the anticancer potential of our promising hit. NOESY experiments provided atomistic insights, revealing specific interactions between aromatic protons of the ligand and nucleobases within the stem-loop of pre-miR-21, in good accordance with WaterLOGSY and TOCSY data.

M^{pro} is a key enzyme for SARS-CoV-2 replication and lacks human homologs, making it a highly attractive antiviral target [2]. Ebselen, an organoselenium compound, potently inhibits M^{pro} through covalent modification of the catalytic cysteine within the active site of the protease [3]. However, potential off-target interactions with additional cysteine residues of M^{pro} raise concerns about Ebselen selectivity. To investigate this, we employed heteronuclear ^{1}H-^{15}N HSQC analysis on the ^{15}N-labeled N-terminal domain of M^{pro}, aiming to identify other cysteines in proximity to the active site that may also be targeted by Ebselen.

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