ICGEB International Centre for Genetic Engineering and Biotechnology

NMR for combatting diseases: from cancer to SARS-CoV-2


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Lectures and Practicals

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NMR spectra as fingerprints of functional processes and of biological drugs and vaccines

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NMR spectroscopy is a unique tool for characterizing the structure and dynamics of biomolecules and, even more importantly, for describing transient interactions and functional events with atomic resolution, possibly in a cellular context.

Among the various functional cellular processes, the characterization of those involving metal homeostasis, metal transport and metal cofactor biogenesis requires the development of suitable approaches, as their description should be both at system (e.g. a cell) and at molecular level (e.g. atomic-resolution characterization of biomolecules).

Suitable methodologies should be capable of addressing multiple, specific, and sometimes non-conventional, aspects and amenable to describe the processes in living cells.

Indeed, metal transfer processes occur through a series of protein-protein transient interactions, with kinetic factors contributing to the selectivity of the processes. This implies the study of dynamical, multicomponent systems. Furthermore, the presence of paramagnetic centers, such as iron-sulfur clusters, which dramatically affects the NMR spectra, requires tailored experiments, possibly integrated with EPR spectra.

NMR is also essential for the optimization of biological drugs and of vaccines. Furthermore the availability of Ultra High Magnetic fields allows the analysis of biological drugs without the need of isotope labelling.

A few examples of these applications will be presented and discussed.

New aspects of fragment-based drug discovery by NMR

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Due to technological developments and hardware availability as well as triggered by work in Covid19-NMR to screen the proteome and transcriptome of SARS-CoV-2, there is an increased interest in fragment-based drug discovery by NMR spectroscopy. In this contribution, we will discuss technical aspects of such approaches and focus on the downstream follow-up to generate insight from these screening campaigns for follow-up medicinal chemistry studies.

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Protein screening: Angew Chem Int Ed Engl. 2022 61:e202205858. doi: 10.1002/anie.202205858.
RNA screening: Angew Chem Int Ed Engl. 2021 60:19191-19200. doi: 10.1002/anie.202103693.
RNA screening: Chembiochem. 2021 22:423-433. doi: 10.1002/cbic.202000476
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RNA resources: Nucleic Acids Res. 2020 Dec 16;48(22):12415-12435. doi: 10.1093/nar/gkaa1013.

From NMR basics to multidimensional biomolecular NMR

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A brief overview of the basic NMR concepts is given to provide non-NMR users with a survey of the opportunities offered by NMR for analytical and structural studies of complex mixtures, molecular interactions and protein structures. In particular, I will review how chemical shifts, scalar coupling, dipolar coupling and nuclear relaxation can be exploited into one and two (and three….) dimensional experiments and how this information is used for structure calculation and for the study of protein dynamics and protein interactions.

NMR titrations/monitoring interactions

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The characterization of intermolecular interactions is crucial to structural biology, proteomics, and pharmaceutical research. Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique for studying intermolecular interactions involving proteins and small ligands. This lecture will provide an overview of the NMR strategies used to characterize protein-ligand and protein-protein interactions. Particular attention will be placed on the experimental strategy needed to obtain a detailed characterization of the interaction, on the theoretical basis related to the determination of the affinity, and on the experimental issues that must be taken into account when planning the experiments.

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Solving 3D puzzles of biomolecular complexes by integrative modelling

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The prediction of the quaternary structure of biomolecular macromolecules is of paramount importance for fundamental understanding of cellular processes and drug design. In the era of integrative structural biology, one way of increasing the accuracy of modelling methods used to predict the structure of biomolecular complexes is to include as much experimental or predictive information as possible in the process [1,2].

We have developed for this purpose a versatile information-driven docking approach HADDOCK [3] (https://www.bonvinlab.org/software) available as a web portal from https://wenmr.science.uu.nl [4,5]. HADDOCK can integrate information derived from biochemical, biophysical or bioinformatics methods to enhance sampling, scoring, or both. The information that can be integrated is quite diverse: Interface restraints from e.g. NMR, mutagenesis experiments, or bioinformatics predictions; shape data from small-angle X-ray scattering and cryo-electron microscopy experiments.

In my talk, I will discuss general aspects of docking and describe in particular HADDOCK. I will illustrate its capabilities with various examples.

References:

[1] Koukos P.I. and A.M.J.J. Bonvin (2020) *J. Mol. Biol. 432,* 2861-2881
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HADDOCK tutorial on modelling antibody-antigen complexes

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This computer tutorial demonstrates the use of HADDOCK2.4 for predicting the structure of an antibody-antigen complex using information about the hypervariable loops of the antibody and NMR data identifying the epitope. This tutorial does not require any Linux expertise and only makes use of our web servers and PyMol for visualisation/analysis. The complex will also be modelled using AlphaFold2 and the results compared those obtained with HADDOCK.

The tutorial is available online at:
https://www.bonvinlab.org/education/HADDOCK24/HADDOCK24-antibody-antigen-basic/

References:

[1] Ambrosetti F., Jandova Z. and Bonvin A.M.J.J. (2023). *Methods Mol. Biol., 2552*, 267-282
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My first 1D and 2D NMR spectra: initial set-up, data acquisition and processing

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This practical course will be focused on the first fundamental steps and concepts that a spectroscopist must take into account when sitting in front of an NMR instrument to properly acquire and process the first, fundamental, spectra. These spectra will represent the fingerprint of a protein in solution and can provide plenty of information with atomic resolution.

The practical will start with the standard procedures needed to set up the optimal instrumental conditions for the acquisition of NMR spectra such as the choice of temperature, the tuning and matching process and the shimming routine. Examples of bad Vs optimal shimming conditions will be provided.

The importance of setting the carrier in an accurate position will be shown together with the effect on the calibration of ^{1}H hard pulses. The demonstration of possible ways to properly calibrate hard pulses will be shown eventually on different nuclei.

A series of monodimensional ^{1}H-detected experiments exploiting different solvent suppression schemes will be described and acquired pointing the attention also to the key pulse program parameters.

The main principles of bidimensional experiments will be described and a few examples of 2D experiments will be acquired. A brief description of the information that can be grasped from different kinds of 2D spectra will be provided.

During the practical, the basic processing procedures will be illustrated mainly focusing on the phasing procedure and different processing parameters.

Protein structure and dynamics through NMR

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NMR, with its broad range of applications, i.e. from solution to solid state, from structural to dynamic characterization of macromolecules, from the study of single molecules to macromolecular complexes, can provide a complete characterization of bio-macromolecules, contributing to the description of the mechanisms linked to the function of a biological system at the atomic level.
Despite recent advances in solution and solid-state NMR and the major progress in computational methods, obtaining the 3D structural and dynamic characterization of a biomolecule from experimental NMR data remains a challenge. An overview on resonance assignments and structure determination protocols will be presented with case studies. NMR studies of protein dynamics will be also presented.

Integration of NMR and X-ray for Covid19 research

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Drug discovery and the development of biologics are important for combating any disease, and the process needs to be more efficient, high-throughput, and affordable during pandemics. This was also observed during the Covid19 pandemic when interdisciplinary research became more focused. For rational drug design and discovery and quality assessment, the structural biology approach is most useful. X-ray crystallography and cryo-Electron Microscopy are mostly used for high-throughput structure determination of folded proteins, while NMR spectroscopy is unparalleled in providing structural and dynamics information on flexible proteins. Integrating both techniques help us understand the structure and dynamics of proteins, nucleic acids, and their complexes at atomic resolution. During the pandemic, a similar approach became very useful in the rapid screening of inhibitors, identification of new targets, and antibody screening.

Biological Magnetic Resonance Data Bank

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The Biological Magnetic Resonance Data Bank (BMRB) is the open, international repository for biomolecular NMR data. Founded by John Markley and Eldon Ulrich more than 40 years ago, it currently contains more than 9 million assigned ^{1}H, ^{13}C, and ^{15}N chemical shifts for biological macromolecules, and more than 10,000 reference spectra for more than 1,000 small biological molecules. BMRB enabled the development of tools that are used every day by NMR structural biologists, including TALOS, SPARTA, PINE, PECAN, HIFI, DANGLE, and CSI. Reference spectra are used in academia and the biotech industry to identify metabolites and biomarkers. With growth of BMRB, extreme values of chemical shifts that were once ignored as outliers have gained statistical significance, able to reveal insights not available from overall trends. Much latent knowledge remains to be extracted from BMRB, for example insights into dynamics from shift equivalence and relaxation data, secondary isotope shifts, and shifts accompanying post-translational modification. The remain areas where additional data is needed, for example on disordered proteins and empirical (time-domain) data sets. A high priority for BMRB is to lower the effort required to deposit data, and to expand the amount and scope of the NMR data in BMRB. Resources currently available from BMRB will be described, including tools on the web site, the NMRbox platform, and the applications programming interface (API) for software developers.

Drug protein titration studies using NMR

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1. Prepare 10 mM cyclosporin stock solution in 100% methanol.

2. Prepare 1 mM *U*-^{15}N labeled human Cyclophilin A (hCypA) in NMR buffer.

3. Measure 2D [^{15}N,^{1}H] HSQC spectrum of 500 µl *U*-^{15}N labeled hCypA in 5 mm NMR tube.

4. Add 10 µl cyclosporin stock solution to 500 µl *U*-^{15}N labeled hCypA and mix thoroughly.

5. Repeat the above step till no further changes in the 2D [^{15}N,^{1}H] HSQC spectra are observed upon the addition of cyclosporine.

6. Tabulate the ^{15}N and ^{1}H chemical shifts of hCypA in the free and bound state (as shown below) from respective 2D [^{15}N,^{1}H] HSQC spectra.

7. Calculate chemical shift perturbations (CSP) using formula;

$$\Delta\delta^{15}N,\ ^{1}H^{N} = \sqrt{\left(\frac{\Delta\delta_{^{15}N}}{5}\right)^{2} + \left(\Delta\delta\ _{^{1}H^{N}}\right)^{2}}$$

where $\Delta\delta(^{1}H^{N})$ and $\Delta\delta(^{15}N)$ are the changes in backbone amide chemical shifts for $^{1}H^{N}$ and $^{15}N^{H}$.

8. Plot the CSP v/s residue number.

9. Download the structural coordinate of hCypA from PDB and the color the residues showing CSP higher than 1σ above mean on the hCypA structure using CHIMERA or PyMol.

10. Use HADDOCK to dock the molecule to model the structure of the complex.

STD, water LOGSY to monitor interactions

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Interactions of biomolecules with small molecules can be observed by NMR on the target molecule or on the binding ligands. The different methods for NMR-based fragment screening are presented [1]. It is explained in detail which parameters can be optimized and where the advantages and disadvantages are [2]. The experiments will be explained on the example of different target molecules like RNA [3] and proteins [4], especially the of SARS-Cov2 proteins [5] and RNA [6] will be discussed. The hardware required to establish a screening workflow is also shown. Sample data sets will be provided to practice the analysis.

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[1] A.D. Gossert, W. Jahnke: NMR in drug discovery: A practical guide to identification and validation of ligands interacting with biological macromolecules Progress in Nuclear Magnetic Resonance Spectroscopy 97 (2016) 82–125

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Sample quality/properties – inspection of different cases

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The high-throughput production of "good-quality" protein samples is an essential prerequisite of successful Nuclear Magnetic Resonance (NMR) structural and functional investigation of a protein, in order to ensure data reproducibility and reliability. The main requirements for a good protein sample are purity, solubility and structural and functional integrity. Additionally, it is important for the protein sample to be homogeneous and reproducible. Compared to other spectroscopic techniques NMR spectroscopy is a relatively insensitive method, requiring the production of recombinant proteins in high yields and their characterization at relatively high concentrations (i.e., in the 10^{-5}-10^{-3} M range), conditions that may lead to non-native folding or aggregation. Adding further complexity, NMR spectroscopy requires ^{15}N, ^{13}C and, depending on the protein size, ^{2}H isotopic labeling of the protein of interest. These requirements often increase the stress on the recombinant organism over-expressing the protein, thus potentially decreasing the yield and/or leading to misfolding and to protein insolubility. Moreover, since NMR spectroscopy involves long experimental times and a wide range of temperatures (generally from 10 to 40 °C), thermal stability of the sample is also required. It is essential therefore to carefully design the purification strategy from the molecular level. The wide range of proteins' size and biophysical properties makes high-throughput production and sample purification significantly challenging and not always allows for the application of "standard" strategies, each system requiring its own conditions.

Here, with the principle for the production of isotopically-enriched recombinant proteins in *Escherichia coli* and for a rational design of suitable purification strategies will be illustrated. Furthermore, the strategies to improve the quality of a protein sample in term of solubility and stability will be described.

Protein dynamics by NMR spectroscopy

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At room temperature, a protein does not adopt a single native structure, but rather fluctuates among different conformations. The lowest-energy conformation corresponds to the ground-state structure, whereas conformations with higher energies, and hence lower populations, are termed excited-state or transient structures. Each molecule in solution interconverts among these conformations, which constitutes protein dynamics. Protein dynamics is crucial for biological function, including ligand binding, catalysis, and allosteric regulation. For example, the formation of a stable protein-ligand complex is dynamical, with the receptor protein searching through multiple transient interactions through conformational change [1]. The connections between structure, dynamics, and function have practical importance e.g., for the design of efficient artificial enzymes. NMR spectroscopy is uniquely suited to characterize protein dynamics in solution at different timescales [2]. In a widely used approach, motions on the picosecond-nanosecond timescale are characterized through the quantification of spin relaxation of ^{1}H, ^{13}C or ^{15}N nuclei, with ^{15}N the most commonly utilized. Spin relaxation data are informative also on motions taking place on longer timescales, up to the millisecond. Even slower motions are addressed with different approaches, such as $^{1}H/^{2}H$ exchange measurements. Overall, NMR spectroscopy can provide information on protein dynamics occurring on multiple timescales and at multiple sites within a given protein, allowing researchers to obtain an extensive overview of the processes taking place. Finally, NMR data and the atomic-level data provided by molecular dynamics simulations may be compared effectively [3]. This permits the validation of simulations, revealing the structural changes underlying the experimentally observed dynamics.

References:

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Tips and tricks for paramagnetic NMR acquisition

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In this lecture I will provide guidelines to acquire simple NMR spectra of paramagnetic molecules. Inexperienced users often lose signals, or have serious baseline issues, or may observe artifacts, and then the whole approach may have serious problems. On the other hand, when performed properly, NMR experiments on paramagnetic molecules can reveal a wealth of structural and dynamical information, and to inform on the electronic properties of the system.

References:

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Setting up triple resonance experiments

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Signal overlap in biomacromolecules such as RNA or proteins can be overcome by using 3 dimensional triple resonance experiments. Different triple resonance experiments particularly for protein assignment are introduced and possible modifications to tailor these experiments to the needs of the investigated protein are mentioned. Required parameters for setting up these experiments ranging from chemical shifts and coupling constants to selective pulses are discussed. We will set up an HNCO triple resonance experiment using remote NMR. Sample data sets will be provided to practice data processing and to get an idea of the analysis of the data.

References:

[1] M. Sattler M., Schleucher J, Griesinger C. Heteronuclear multidimensional NMR experiments for thestructure determination of proteins in solution employing pulsed field gradients (1997) Progress in Nuclear Magnetic Resonance Spectroscopy *34* 93–158.

How flexible is my protein? - Local mobility from ^{15}N relaxation

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Protein dynamics is directly linked to protein function, both for globular and intrinsically disordered proteins, as well as for multi-domains proteins with flexible linkers. Information about the protein aggregation state (monomeric, dimeric, etc.) and its internal flexibility can be obtained through NMR, by measuring 15 N relaxation rates (i.e. longitudinal, R1, transverse, R2, relaxation rates, and heteronuclear 15 N- 1 H NOE). [1] In this practical, we will see how to acquire NMR spectra for the determination of R1, R2 and 15 N- 1 H NOE values on a small globular protein, like Ubiquitin, using a Bruker's Avance™ NEO 900
MHz spectrometer, equipped with triple resonance TCI cryo-probe. 15 N Longitudinal relaxation rates will be measured using a pulse sequence modified to remove cross-correlation effects during the relaxation delay. [2] 15 N transverse relaxation rates will be measured using a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence. [2,3] The crucial parameters, to be carefully checked in the set-up of the experiments, will be pointed out; deeper details will be provided to more expert students. The change of 15 N relaxation values in higher molecular weight proteins and intrinsically disordered protein will be also discussed. Then, the use of Bruker Dynamic Center software will be shown to analyze the NMR spectra for the determination of relaxation parameters and the protein correlation time (τ_c). The analysis will be performed on two different sample cases: 1) globular protein, the catalytic domain of matrix metalloproteinase-12 [4] (for non-NMR expert students); 2) multidomain protein with tendency to aggregate, HuR (for NMR expert students). [5]
In details, in the first 10-15 minutes, the practical will be briefly introduced; 30/40 minutes will be dedicated to show how to acquire NMR spectra for the determination of 15 N relaxation; 30/40 minutes will be dedicated to the data analysis.

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Un-structural biology by NMR spectroscopy

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Intrinsically disordered regions (IDRs) of complex proteins have long been thought to be just passive linkers connecting functional globular domains and thus frequently ignored in structural biology studies. However, in many cases, they constitute a significant portion of a protein's primary sequence and are likely to play a role in protein function.

The characterization of highly flexible regions of large proteins as well as entire proteins characterized by the lack of a 3D structure, now generally referred to as intrinsically disordered proteins (IDPs), lies well behind that of their folded counterparts and is nowadays pursued by an increasingly large number of studies to fill this knowledge gap.

Nuclear magnetic resonance (NMR) spectroscopy plays a crucial role in IDPs and IDRs investigation, being the only method that allows a high-resolution description of their structural and dynamic features in solution. The high flexibility has several consequences on the NMR spectroscopic parameters that, if properly handled, can give precious information.

We will present recent results suggesting that more complex functions than expected can be ascribed to the long disordered chains connecting well-structured protein domains.

NMR spectroscopy for studies of structural dynamics in IDPs and protein drug interactions

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The classical structure-function paradigm that protein functionality relies on the existence of a stably folded protein scaffold has been put in question and it is now acknowledged that an increasing number of proteins are lacking stably folded tertiary structures and that this intrinsic flexibility has significant impact on biological functionality. This structural polymorphism is intriguing from a biophysical point of view and calls for new approaches, combining appropriate theoretical concepts and experimental technologies. In the talk novel NMR spin relaxation experiments are described to characterize the structural dynamics of IDPs.

The design of small molecules that bind target proteins with high specificity and affinity is based on the optimization of complementarity in terms of shape, electrostatics and H-bond donor/acceptor matching. Another crucial, often overlooked type of complementarity involves interactions between the π-electrons of aromatic ring systems with aromatic or aliphatic hydrocarbons. NMR spectroscopy together with suitable isotope-labeling schemes is ideally suited to probe the details of this important interaction.

In-cell NMR for real-time studies of protein-ligand interactions in human cells

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In-cell NMR provides insights on biological macromolecules in their native cellular environment at atomic resolution [1]. At CERM, we have developed an approach for expressing and labelling proteins directly in human cells, which is ideally applied to monitor functional processes such as protein folding and maturation, metal binding, chemical modifications, and interactions with ligands or with specific partners [2]. The development of an NMR bioreactor has made possible to study the above processes in real time [3]. The approach provides insights on intracellular ligand binding kinetics and thermodynamics, which are critical to optimize drug penetrance and potency [4,5]. Recently, we have developed ^{19}F in-cell NMR methodologies, which allow protein-observed [6] and ligand-observed screenings on targets that would be otherwise invisible by conventional ^{1}H in-cell NMR. Such approaches hold great potential in the development of more effective drugs towards pharmacologically relevant targets.

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Flash talks


Credits: Linda Cerofolini, Ph.D.

ICGEB International Centre for Genetic Engineering and Biotechnology

8-Oxoguanine can form quartets with a large central ion cavity

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Abundance of reactive oxygen species (ROS) leads to widespread oxidative damage to nucleic acids, among other biomolecules. Of the four nucleobases, guanine has the lowest redox potential and is the most susceptible to oxidation. The primary and predominant product of guanine oxidation is 8-oxoguanine. Susceptibility to oxidative damage is even higher when several guanine residues are positioned concurrently in the primary nucleotide sequence.[1] Such guanine-rich sequences are also able to form noncanonical four-stranded structures called G-quadruplexes. These sequences are not distributed randomly in the genome but are enriched in regulatory regions such as gene promoters and telomeres.[2] Incorporation of 8-oxoguanine into G-quadruplexes can lead to structural rearrangements, thermal (de)stabilization and affect cellular mechanisms.[3,4]

We have residue-specifically incorporated 8-oxoguanine residues into a short model oligonucleotide d(TG4T), which natively folds into a tetrameric parallel G-quadruplex. Using NMR spectroscopy, we determined that 8-oxoguanine residues do not hinder G-quadruplex formation. Furthermore, 8-oxoguanine residues form an 8-oxoguanine quartet with a modified hydrogen bonding scheme. The G-quadruplexes retain a parallel topology, as determined by through-bond and through-space NMR methods. The integration of 8-oxoguanine moieties into a quartet was investigated using molecular dynamics simulations and DFT geometry optimization. The oxidized quartets exhibit a larger central cavity with respect to G-quartets. Furthermore, the thermal stability was diminished when 8-oxoguanine residues were positioned inside the core of the formed G-quadruplexes. We conclude that 8-oxoguanine is not a taxing modification with respect to tetrameric G-quadruplex formation.[5] Further insights into structural changes of guanine-rich sequences as a consequence of oxidative damage will expand the knowledge of oxidative stress effects on nucleic acids and possible outsets of pathological states.

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Metabolic reprogramming in immune cells: TGFβ1 signaling in primary human macrophages

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The diversity of metabolic reprogramming mechanisms in immune cells results from the flexibility of these cells to respond to heterogeneous pathogenic/nonpathogenic stimuli. Recently, we have demonstrated how this diversity may characterize the stimulus type or effect (1, 2). In this session, we show the glycolytic pattern monitored in monocytes/macrophages under different stimuli. Results show specific glycolysis/stimulus patterns and suspect a role for TGFβ1 signaling under different glucose concentrations in human monocytes/macrophages. In conclusion, hyperglycemia is an essential factor to be considered when understanding strategies of metabolic reprogramming in immune cells and related disease progression, diagnosis and treatment.

Keywords:
Metabolic reprogramming; monocytes; glucose; TGFβ1

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Biochemical and structural study of a zinc efflux pump involved in the adaptation of *M. tuberculosis* within infected macrophages

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Although essential as traces, metals such as copper and zinc are also toxic at high concentrations. This property is naturally used in context of host/pathogen interaction: copper and zinc released within macrophages during Mycobacterium tuberculosis (Mtb) infection accumulates within the mycobacterial phagosome. Thus, the bacteria developed export system at the plasma membrane. The main zinc efflux system found in Mtb H37RV consists in CtpC (Rv3269), which is a membrane PIB-ATPase contributing to Mtb intracellular survival within macrophages.

CtpC expression at the membrane confers zinc resistance [1], which further requires the presence of a small chaperone-like protein, PacL1 (Rv3269) [2]. PacL1 is encoded together in operon with CtpC under control of a zinc-inducible promoter. This protein has a zinc-specific metal binding motif (MBM) only required for resistance to high zinc concentrations. PacL1 contains critical Glu/Ala repeats for complexes formation and resistance to zinc intoxication, even at low concentration [2]. Therefore, PacL1 both acts as an essential chaperone for CtpC activity through its Glu/Ala repeats, and as a metallochaperone through its MBM. Our work aims to decipher the molecular mechanism behind CtpC-PacL1 zinc efflux, and to discriminate interactions required for chaperone activity or for metallochaperone activity.

Currently, recombinant PacL1 and CtpC expression is tested in appropriate E. coli strains with aim to obtain soon a functional membrane complex. Expression of a soluble form of PacL1 is carried out in routine while the purification protocol for a soluble form of CtpC' MBD is under optimization processes.

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Molecular imaging of FLASH radiation effects on cells

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Background: Monitoring radiation toxicity on relevant time scales (hours to days after radiation delivery) via the detection of free-radical and metabolite concentrations can afford an improved radiation scheme: higher doses delivered to the targeted tumor and reduced healing times. The goal of this project is pinpointing bimolecular markers via new magnetic resonance methods to monitor radiobiological effects on free-radical and metabolite concentrations at cell level.

Methods: Improved efficiency in cancer radiotherapy is expected with the advent of a new technique: 'FLASH' radiation delivery for radiotherapy ('FLASH RT') [1]. The delivery of large doses of radiation in a 'FLASH' manner (typically Gy to tens of Gy within ms) needs to be accompanied by molecular diagnostic on relevant time scales. Sensitivity-enhanced magnetic resonance using dissolution–Dynamic Nuclear Polarization (d-DNP MR) has been proposed [2-3]. The project includes experiments with different biomarkers as choline, creatine, ratio of choline-to-creatine ([Cho]/[Cr]), [13C]-pyruvate, alanine ([Ala]) or lactate ([Lac]) [4-5].

Expected Results: One of the expected outcomes is a contribution to diagnose radiation efficiency and toxicity within a timely hours-to-days interval via new Magnetic Resonance biomarkers. The results of the project are anticipated to help in the identification of the appropriate radiation dose-rates for an optimal FLASH Radiation Therapy to the cancer type via in-cell and ex-vivo studies conducted prior to and in parallel to in-vivo diagnostic and therapy.

Conclusions: This project is expected to improve the effectiveness of both conventional and FLASH Radiation Therapy. In a similar manner, ways to optimize therapeutic strategies combining chemotherapy and immunotherapy with radiotherapy can be evaluated via in-cell quantification of metabolites.

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Ruthenium(II) polypyridyl complexes with long-lived Nile Red-localised triplet states as potential candidates for use in photodynamic therapy

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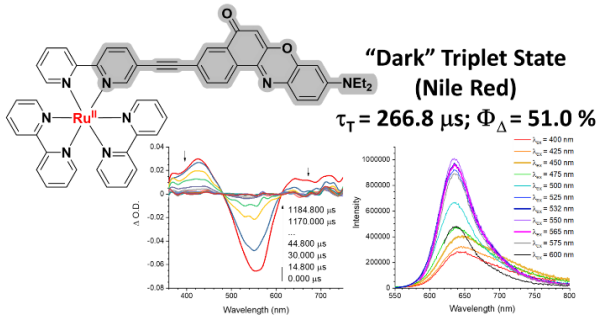
Nile Red, a common lipophilic dye, has an intense absorption in the visible region (λabs = 537 nm, ε = 20,250 M-1cm-1) and exhibits remarkable solvatochromism. Yet despite these properties, current literature examples of transition metal (TM) complexes containing Nile Red are rare or their photophysical properties poorly studied.[1] While the triplet state of Nile Red is typically inaccessible due to the absence of heavy atoms, recent work has achieved modest triplet state population, and O2 sensitisation in a thiocarbonyl version of Nile Red.[2] Building on the learnings from previous work in our group, which involved incorporating coumarin-6, triphenylamine, and BODIPY into the structure of TM complexes for applications in triplet-triplet annihilation (TTA) upconversion and photodynamic therapy (PDT),[3] the realisation of a number of Ru(II) photosensitizers bearing ethynyl-Nile Red derivatives as chromophoric appendages has been achieved through “chemistry-on-the-complex” methods.

Here, we present detailed spectroscopic and photophysical characterisations of our most promising candidate, Ru-3ENR.[4] This complex absorbs strongly in the visible region, and shows weak emission from two distinct states (1ILCT* and 3MLCT*). A further long-lived but non-emissive 3ILCT* state is identified, with the aid of transient absorption spectroscopy and comprehensive molecular calculations, demonstrating the utility of Nile Red localised triplet states for the first time. Preliminary biological studies have been carried out to determine if this complex would be a good candidate for use in PDT.



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Metabolites and lipoproteins profiles with associated immune responses of a pilot cohort of Pancreatic Ductal Adenocarcinoma patients

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Introduction: Pancreatic Ductal Adenocarcinoma (PDAC) is an aggressive cancer with a 5-year survival rate of only 11% [1]. Studies have shown that metabolites and lipoproteins alterations can affect immune response influencing tumour progression and response to therapy [2]. Furthermore, the immuno-inflammatory response plays a vital role in tumour growth and progression because PDAC patients exhibit immune dysfunction [3]. Thus, this study aimed to determine the levels of metabolites and lipoproteins as well as the expression of key immune cell markers in South African PDAC patients. Methods: Blood samples were collected from 34 PDAC (22 resectable, 8 locally advanced, and 4 metastatic), 6 chronic pancreatitis, and 6 healthy volunteers (M190681). Spectrophotometric assays were conducted to evaluate levels of metabolites, lipoproteins, and Reactive Oxidative Species (ROS). Flow cytometric immunophenotyping and gene expression analyses were used to assess the immune cell markers. Data analysis was done using the R software (v 3.6.1). Wilcoxon and Kruskal–Wallis rank-sum tests were used to compare differences in numerical covariates. Spearman's rank test was used to calculate the correlation coefficient (rho) between variables. Results: This study showed elevated levels of glycine and lactate with a worsening tumour stage. Decreased ethanol and 3-hydroxybutyrate concentrations were independently associated with longer survival time, irrespective of tumour stage. Glucose, lactate, and some lipoproteins were positively correlated to inflammatory markers (GlycA and GlycB). PDAC patients with CD4/CD8 ratio <1 survived longer (HR = 3.572; p-value = 0.049); with CD8 T-cells showing a negative correlation with most liver function parameters. Furthermore, PDAC severity was associated with elevated levels of ROS. Conclusion: This study has demonstrated dysregulated levels of metabolites, lipoproteins, and immune markers in a cohort of South African PDAC patients. The correlations observed between these parameters could hint at the possible relationship between them in tumour progression and patient prognosis. Ongoing studies would aim to validate these findings in a larger patient cohort.

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Study of biomarkers of radiation using magnetic resonance methods and Glioblastoma cells

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Radiotherapy mechanism need to be understood, being used in combination with other treatment for different types of cancer (more than 50% cases). What happens at the molecular level during irradiation? High dose-rate are thought to minimize toxicity in healthy tissue. In order to find out more about the high dose-rate ("FLASH" effect) is important to understand first, the processes that are taking place during the interaction of ionizing radiation with the biological samples. [1] [2]

We studied the effects of radiation at the metabolic level using the U251 Glioblastoma cell line irradiated with different doses. The cells were grown in cultures in our laboratory using a specific protocol and via nuclear magnetic resonance spectroscopy we quantitated the biomarkers (such as choline, lactate, creatine). [3]

Metabolites from NMR spectra were assigned using data bases and differences in concentrations were found for different irradiation doses. [4]

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A comprehensive assessment of the conformational ensembles through biophysical experimental data

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The study of Intrinsically Disordered Proteins (IDPs) has posed several challenges as they do not follow the conventional rules governing modular proteins (1). IDPs exhibit multiple interconverting structures called conformational ensembles, which altogether contribute to their function. This project proposes a stepwise workflow for studying IDPs and Intrinsically Disordered Regions (IDRs) and validating their conformational ensembles. Three proteins, including Nipha Virus Phosphoprotein, SMAD-4, and SMAD-2, were selected for this purpose.

The workflow begins with creating a pool of conformers based on the primary sequence of the proteins using various software and methods. The conformers are then fixed, and multiple predictors are used to predict SAXS and NMR observables for each conformer (Forward Models). The goodness of fit is then evaluated by calculating the reduced Chi squared score between predicted and experimental observables, which reflects how well each ensemble can represent the average experimental observables like SAXS curve and NMR chemical shift.

The study also involved conducting DSSP analysis of all created conformers against different conformational ensembles, including AlphaFold predicted models, randomly selected ensembles, and ensembles derived from the Protein Ensemble Database (PED) (2). The results showed that AlphaFold predictions are mostly biased towards structured proteins, and caution should be taken in exploiting AlphaFold's potentials for predicting unstructured proteins. (3)

Furthermore, the distribution of the Radius of gyration (Rg) among all the groups of ensembles was considered as an additional index for validating conformational ensembles. The Rgs were visualized using raincloud plots and compared with experimental Rg calculated with SAXS instrument to shed light on the average dimension of conformers in the protein solution. The results suggested that experimental Rgs tend to be more extended than the predicted Rgs for proteins whose structures are completely disordered, while for intrinsically disordered regions placed between modular proteins, the experimental Rgs are more compacted than the predicted Rgs.

Future challenges for validating conformational ensembles were also highlighted, including determining a comprehensive pool of conformers, considering the error of measurement in experiments and predictions, and addressing problems due to sampling the best conformers among the pool of conformers. This study's workflow provides a comprehensive method for validating conformational ensembles, which can aid in understanding the function of IDPs and IDRs.

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Phen-DC_3 induces refolding of human telomeric DNA into a chair-type antiparallel G-Quadruplex through ligand intercalation

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Human telomeric G-quadruplex DNA structures are attractive anticancer drug targets, but the target's polymorphism complicates the drug design: different ligands prefer different folds, and very few complexes have been solved at high resolution. Here we report that Phen-DC_3 , one of the most prominent G-quadruplex ligands in terms of high binding affinity and selectivity, causes $dTAGGG(TTAGGG)_3$ to completely change its fold in KCl solution from a hybrid-1 to an antiparallel chair-type structure, wherein the ligand intercalates between a two-quartet unit and a pseudo-quartet, thereby ejecting one potassium ion.[1] This unprecedented high-resolution NMR structure shows for the first time a true ligand intercalation into an intramolecular G-quadruplex.

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Multicore vs core-shell PFCE/PLGA NPs: impact of ultrastructure on particle stiffness and its influence on cell uptake mechanisms

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INTRODUCTION: Two PFCE encapsulating PLGA nanosystems were developed for imaging applications. Composed of the same materials, multicore ultrastructured nanoparticles (NPs) show an in vivo a clearance rate of 15 times faster than the alternative core-shell structured nanocapsules (NCs) [1]. Further analysis with heteronuclear Overhauser enhancement spectroscopy (HOESY) revealed a high interaction between fluorine atoms of encapsulated PFCE and water molecules in NPs, which was very low in NCs [2]. These findings expose strong differences in structural characteristics that can highly impact biological activity. Nonetheless, it is known from literature that particle internalization mechanisms are influenced by the elastic properties of the biomaterials, therefore affecting the uptake capacity of different types of cells [3]. **METHODS:** In this study, we further characterize the mechanical properties of these two types of particles, using particles of similar size, Z-potential and PFCE content. To determine the entire particle composition and study how it can impact mechanical properties, we also quantify amount of surfactant (PVA) included in both particles. Particle elasticity is measured in air and solution by means of Atomic Force Microscopy (AFM). For the cell uptake experiments, NPs and NCs at 2 mg/million cells were incubated with different cell lines for 24h. Cell uptake was measured by flow cytometry, and confocal imaging is performed for particle localization. To explore the uptake mechanisms chose for the particle uptake, RAW 267.7 cells are incubated with both particles for 2h with different uptake inhibitors. **RESULTS:** AFM analysis show the lower stiffness of NPs, which strongly decreases in the liquid state, compared to NCs. PVA composition was significantly higher in NPs, which matches with the multicore ultrastructure model. Cell uptake is generally greater with NPs than with NCs, but there are differences depending on incubation time and the used cell line. Differences in uptake efficiency with different inhibitors are also detected between NPs and NCs. **CONCLUSIONS:** The ultrastructure of the particles highly impacts their elastic properties. Differences in cell uptake between NPs and NCs depending on incubation time, cell line, and inhibitor used shows that different mechanisms might be involved in their uptake. Clatrin-mediated endocytosis seems to play a role in the uptake of both particles, and other inhibitors enhance alternative uptake pathways. Further studies are needed to determine all internalization pathways involved for each particle.

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Fighting entropy: enzyme active sites designed to minimize dynamics

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The focus of this project is how rigidification of the active site in enzymes is achieved. We propose that entropy reduction is critically influenced by the amino acids around the active site.1,2 As a model, the enzyme family of β-lactamases is used, because these enzymes can have either narrow or broad substrate specificities and evolve rapidly. Such evolution is the cause of the growing problem of antibiotic resistance. In this project, the role of residues in entropy reduction will be tested by large-scale mutagenesis and NMR spectroscopy to characterize the dynamics in enzyme variants. Also other biochemical (kinetics, stability), biophysical (crystallography), and computational approaches (molecular dynamics)3 will be employed to characterize variants in detail.

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Folding of a PDZ tandem repeat - a cryptic physiological role for a misfolded intermediate

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PDZ domains are small protein interaction modules that can bind to short amino acid sequences typically found at the C-terminus of target proteins. They play an important roles in the assembly and organization of protein complexes involved in various cellular processes, such as signal transduction, cell adhesion and synapse formation; for this reason, alterations of their functions are associated to diseases [1].

Several human proteins (~150) contain PDZs, both as single domains and often as a part of structural motifs composed of two or more PDZ domains called tandem repeat systems [2]. During the years, the PDZ characterization in term of folding and binding occurred considering these domains as independent structural and functional entities [3], despite the specific arrangement and composition of the PDZ domain tandems can confer distinct functional properties, including different binding specificities and affinities [4].

To shed light on the relationship between structural environment and protein function of a PDZ domain, here we characterized a PDZ domain of sPDZD2. sPDZD2 is a protein composed by two PDZs, named PDZ5 and PDZ6, deriving from the cleavage of the full-length PDZD2 [5] and involved in the early-stage tumorigenesis in prostate cancer [6]. In detail, we performed kinetic studies of folding and binding on PDZ6 - alone and in presence of the neighboring PDZ5 - by using fluorescence and NMR spectroscopy as well as circular dichroism.

By carrying out double-jump kinetic experiments, we detected a misfolded intermediate; interestingly, this kinetic trap is generated only when both domains of sPDZD2 are denatured. We then investigated the binding activity of misfolded and the native states versus a peptide mimic a likely PDZ6-binding motif of CD4 protein. We surprisingly measured a higher affinity of the misfolded state (KD= 9 ± 2 μM) than the native state (KD range of mM) for the peptide thus individuating a scenario in which, for the first time, a cryptic misfolded intermediate can exert a physiological function independent from the native state [5].

These data show the need to increase the complexity of studying models in terms of structural elements to unveil conformational states pathophysiologically relevant, for elucidating the molecular mechanisms underlying various biological processes and for the development of new potential therapeutic targets and strategies.

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Unravelling the biological role of membrane interaction by α-synuclein

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α-synuclein (αS) is a 14kDa intrinsically disordered protein primarily expressed in neuronal cells, whose aggregation is strongly linked to Parkinson's Disease (PD). Both the physiological and pathological functions of αS remain poorly understood, as there is still the need to characterise the molecular mechanisms undergoing its transient membrane-bound and cytosolic states [1]. There are evidence showing that the membrane binding of αS modulates the physiological properties of the protein, the kinetics of amyloid formation and the toxicity of the aggregates [2]. Currently, characterising the nature of this binding as well as the structural and functional properties of αS at the biological membranes' surface is a significant challenge. We employ a multidisciplinary approach using solution and solid-state NMR together with other biophysical techniques to elucidate the structural details of αS balance between disordered solution state and ordered membrane-bound state. In detail, the study characterise kinetics, thermodynamics, structure and dynamics of αS at the surface of various synaptic membranes [3]. Our overall goal is to generate a deeper understanding of the functional and pathological role of the αS interaction with biological membranes.

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Immunoinformatics design of multi-epitope vaccine candidate to combat *Leptospirosis*

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Leptospirosis is one of the seriously neglected tropical diseases, despite its apparent burden on human health and livestock productivity. The disease is endemic in Southeast Asia, with incidence rate of 5.40 and mortality rate of 0.08 per 100,000 population in Malaysia in the year 2021 (data from Ministry of Health Malaysia, moh.gov.my). However, this figure is known to be misleading as it may be an understatement due to misdiagnosis and inaccuracies of diagnostic tools. Infection may lead to fatal systemic complications and multiorgan dysfunction, where broad-spectrum antibiotic is the main choice of treatment for severe form of leptospirosis. Overuse of these antibiotics significantly hastens the development of antimicrobial resistance. Therefore, development of vaccine strategies is crucial in preventing leptospirosis. Proteins embedded in the outer membrane are thought to play key roles as receptors for cellular communication and gatekeepers for iron and substrate transport across cell membranes [1]. Additionally, other key reasons why outer membrane proteins (OMP) have been chosen as target in vaccine development is mainly due to the conserved protein showing wide cross-reactivity, besides they are not serovar specific [2]. One of the strategies employed in our study to develop vaccine candidates for leptospirosis is to design multi-epitope chimeric protein containing sequences of the OMPs of *Leptospira interrogans.* The B-cell lymphocyte and T-cell lymphocyte epitopes were evaluated using immunoinformatics approaches. Potential epitopes were selected to design a chimeric vaccine construct with incorporation of linkers and an adjuvant. A three-dimensional (3D) model of the vaccine construct were modelled and docked to Toll-like receptor 2 (TLR2). The 3D structure of the multi-epitope protein will be validated by NMR analysis. Additional experiments on immunogenicity will be carried out to ratify the development of epitope specific chimeric vaccine approaches applied in this study.

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Structural characterization of a novel antimicrobial peptide lactolisterin BU using NMR spectroscopy

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In the age where the spread of antimicrobial resistance is bringing us dangerously close to the dark predictions of the *"post-antibiotic era"*, coming up with novel alternatives to antibiotic therapy has become more important than ever. The four-helix-bundle bacteriocins, a largely uncharacterized group of antimicrobial peptides that exhibit bactericidal effects, are one of the promising, yet underutilized alternatives.
Here we report the early beginnings of structural studies on lactolisterin BU. This novel bacteriocin acts on Gram-positive pathogens in micromolar concentrations and has the potential to be used both for treating infections and for food preservation.
Spectral properties of data obtained from 1D ^{1}H NMR have given us reason to believe that the combination of different methods in NMR spectroscopy will provide us with a high-resolution structure of lactolisterin BU and its variants, and even more importantly - the answer to *how* this peptide acts. Structural characterization is an essential prerequisite for further studies that will be dedicated to deciphering the mechanism of bactericidal action - the implications of which could reach importance far beyond the example of lactolisterin.

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Phytochemical assessment of critically endangered Himalayan medicinal plants using multi-analytical platforms

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Phytochemical analysis of rare and endangered medicinal plants of Himalayan flora will help in validating their bioactive potential against a range of disease targets. This study aims to investigate the phytochemicals of endangered plants *Gentiana* and *Swertia* using ^{1}H Nuclear Magnetic Resonance Spectroscopy and Gas Chromatography-Mass Spectrometry. The plant samples of *Gentiana* and *Swertia* were collected from Chamoli district of Uttarakhand. Hot methanolic extracts of root and leaf samples were further subjected to ^{1}H NMR and GC-MS analysis in AMRC (Advanced Materials Research Centre) and MSBL Lab at IIT Mandi, respectively. The ^{1}H NMR spectra were recorded on JEOL–ECX 500 NMR instrument for 128 scans [1]. The raw GC-MS data was pre-processed for baseline correction. Metabolic features in total ion chromatograms (TIC) were further annotated against available commercial standards and match score of minimum 70% against the libraries NIST17 and Fiehn13 [2].The ^{1}H NMR spectra of the extracts from *Gentiana* and *Swertia* highlighted chemical shifts that match with phytochemicals like Swertiamarin, Gentiopicroside and few others. The identities were confirmed based on phytochemical analysis reported in different species [3]. The GC-MS profiles of hot methanolic extracts confirmed the presence of a variety of compounds like Sugars (Sucrose, Glucopyranose etc.); Organic acids (Malic acid, Myristic acid, etc.); Alcohols (Glycerol, Myo-Inositol etc.). Phytochemicals analysis using ^{1}H NMR points to the presence of Swertiamarin and Gentiopicroside in the root and leaf of endangered plants *Gentiana* and *Swertia.* The GC-MS profiles also confirmed the presence of different types of compounds like sugars, organic acids, alcohols, etc. Further extractions using different solvents and fractionation along with phytochemical analysis using multi-analytical platforms (2D-NMR, HPLC, Mass spectroscopy) will help in comprehensive analysis.

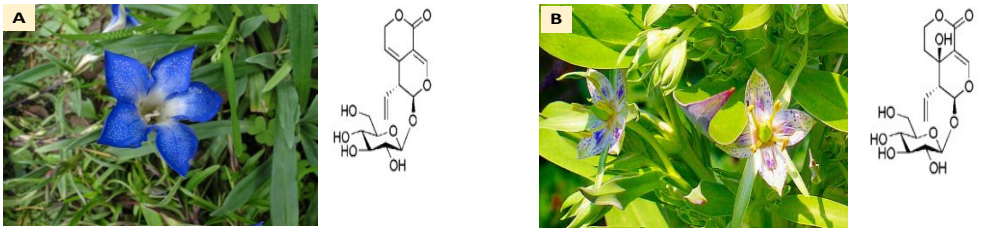

Figure: Plant in flowering stage and phytochemicals (Gentiopicroside and Swertiamarin) of *Gentiana* (A) and *Swertia* (B).

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Stability of PLGA-PFCE nanoparticles

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Introduction: Poly(lactic-co-glycolic acid) (PLGA) encapsulated perfluoro-15-crown-5-ether (PFCE) nanoparticles (NPs) are used for unambiguous identification and quantification of labelled cells through ^{19}F Magnetic Resonance Imaging (MRI)[1]. ^{19}F MRI has high specificity due to the natural lack of fluorine in biological tissues[2]. PLGA-PFCE NPs have been developed in our group for about 10 years[3], and can be produced at GMP grade[4] for clinical cell tracking. These NPs are stored at -20˚C and freezing may lead to increase in diameter and polydispersity index (PDI) because of aggregation. PFCE content in the particles is used to quantify the number of ^{19}F atoms. Hence, we aim to study stability of PLGA–PFCE NPs in terms of PFCE content, diameter and PDI.

Methods: PLGA-PFCE NPs were prepared by a miniemulsion method[3]. Briefly, PLGA and PFCE together constitute the organic phase which was added to the water phase [polyvinyl alcohol (PVA)] and emulsified by probe sonicator. Subsequently, after overnight solvent evaporation, NPs were washed several times by ultracentrifuge and lastly, freeze dried. Post-preparation, diameter and polydispersity index (PDI) were determined by Dynamic Light Scattering (DLS) and PFCE content was established using ^{19}F Nuclear Magnetic Resonance (NMR). NPs formulated 2016 onwards were re-characterized recently using the same methods to study the stability in terms of PFCE content, diameter and PDI. For freeze thaw experiments, NPs were subjected to freeze thaw cycles as powder and as suspension in water before characterization using DLS and ^{19}F NMR. NPs suspended in water and serum free media were characterized using DLS for 30 days in terms of diameter.

Results: Typical values of diameter, PDI and PFCE content of PLGA-PFCE NPs are 191 ± 31 nm, 0.12 and 30 ± 8 % respectively. All the conducted experiments showed that the diameter and PDI values NPs were within the range of typical values and the PFCE content reduction in these NPs were less than 10%.

Conclusions: PLGA-PFCE NPs stored at -20˚C are stable for a minimum of 6 years in terms of diameter, PDI and PFCE content. They show no significant change in diameter in water and medium suspensions for at least 30 days. Additionally, they are stable after being subjected to freeze-thaw cycles making them reliable imaging agents.

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Preliminary studies on the biophysical characterization of RelP through nuclear magnetic resonance spectroscopy

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The phenomenon of bacteria surviving antibiotic exposure despite being fully susceptible (phenotypic resistance) can manifest at a whole population level (tolerance) or involve just a small subfraction of the population (persistence). Persisters represent an infection reservoir that allows for the acquisition of (genetic) antimicrobial resistance (AMR), which has been classified as one of the current top 10 global public health threats to humanity [1]. However, the molecular mechanisms regulating the insurgence of persistence in bacteria are still relatively unknown [2]. Nevertheless, understanding the molecular regulation of these non-essential bacterial pathways is extremely important due to the severe repercussions that AMR poses to human health and agriculture, *etc.* [1-4]. Several studies have shown the central role of guanosine tetra- and pentaphosphate, collectivity called (p)ppGpp, in regulating the bacterial response to hostile conditions, such as nutrient starvation and antibiotic treatment [5]. Intracellular levels of (p)ppGpp are regulated by a superfamily of enzymes called RSH (RelA/SpoT homolog) widespread in the bacterial kingdom that promote its synthesis via pyrophosphate transfer from an ATP molecule to the 3' position of either GDP or GTP [6]. In our quest for (p)ppGpp synthesis inhibitors, we are pursuing the structural characterization of a small RSH protein, RelP (*S. aureus*), using nuclear magnetic resonance spectroscopy. We will describe our preliminary studies in this direction.

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Characterization of the major pseudocoelomic globin of *Dioctophyme renale*.

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The giant kidney worm Dioctophyme renale, is a debilitating and potentially lethal parasite that inhabits and destroys, typically host's right kidney, and may also be found in ectopic sites. It is circumglobally distributed, mainly in dogs, and is increasingly regarded as a threat to other domestic animals and humans. There is little information on the parasite's true incidence, or immune responses to it, and none on its biochemistry and molecular biology. We have characterised the soluble proteins of body wall, intestine, gonads and pseudocelomic fluid (PCF) of adult parasites [1]. Two proteins, P17 and P44, dominate the PCF of both male and females. P17 is of 16,622 Da by mass spectrometry, and accounts for the intense red colour of the adult parasites. It may be related to the 'nemoglobins' found in other nematode clades. Nematode globin-like genes are divided into class 1 globins, similar to vertebrate myoglobins, and a wide range of additional classes [2].
We have isolated P17 from the PCF of D.renale. We use Edman degradation sequencing of the N-terminal ,this sequence was used as ´query´ in a BLASTP search against the parasite proteome (data not published). We were able to obtain the sequence of P17 with and without the 17 amino acid secretion peptide signal at the N-terminal. P17 elutes from a size exclusion chromatography as a monomeric heme-binding protein, and it can be classified as a nematode class I globin. Heme can be extracted by HPLC in a C8 column. After incubation of apoP17 with heme, it binds the protein, obtaining holoP17. The fluorescence spectrum of apo and holoP17 shows that tryptophan (Trp) fluorescence is being quenched by heme group in the holo protein. We measured GnHCl-induced and urea-induced equilibrium unfolding by Trp fluorescence titration curves for apo and holoP17. We can conclude that holoP17 accounts for a more stable structure than apoP17. These studies will be complemented with circular dichroism and Soret absorbance measurements. Further studies to structural, functional and evolutionary characterize P17 are needed.
Nematode globins are extremely abundant in adult nematode tissues. The roles of class I globins appear to lie mainly in oxygen binding, though whether this reflects a role as a transport protein or as some other system (such as enzymatic catalysis or oxygen scavenging) remains unclear. Further investigation of P17 will reveal a diversity of both biochemical and cellular functions for this protein which may be useful for biotechnological applications or as targets for the control of D.renale parasitosis.

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Molecular insight into the PCNA-binding mode of FBH1

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Proliferating Cell Nuclear Antigen (PCNA) is a 90 kDa homotrimeric protein with a ring-shaped structure that embraces the DNA double helix. It acts as a binding platform for enzymes and regulatory proteins involved in the replication and repair of DNA in eukaryotes [1].

F-box DNA helicase 1 (FBH1) is involved in the regulation of cellular responses to replicative stress. FBH1 is recruited to stalled DNA replication fork by PCNA where it inhibits homologous recombination and catalyzes fork regression [2]. Cell-based assays indicate that two different sequence motifs of FBH1 interact with PCNA. The first one is in a long N-terminal region predicted to be disordered, while the second one is in a predicted ordered region with an α-helical structure (according to the AlphaFold model of FBH1).

He have studied the structural basis for the molecular recognition of two distinctly different motifs of FBH1 (FBH1PIP and FBH1APIM) by PCNA. The crystal structure of PCNA in complex with FBH1PIP and analysis of NMR resonance perturbations reveal overlapped FBH1PIP and FBH1APIM binding sites of PCNA and the dominant contribution of FBH1PIP in this interaction [3].

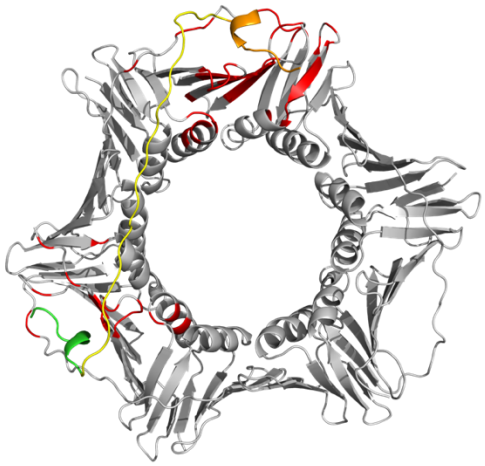

Fig.1 PCNA homotrimer (grey) with docked PIP (orange) and APIM (green) peptides connected by a 25 polyglycine (yellow). PCNA residues whose NMR signals are perturbed by peptide binding are colored in red.

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Insights into structural and dynamic properties of mitochondrial tRNA fragments

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Mitochondria serve as powerhouses of the cell. They contain their own circular and double-stranded genome, which in addition to respiratory chain complexes also codes for 22 different mitochondrial tRNAs (mt-tRNAs) needed for translation of previously mentioned proteins [1]. However, this is not their only function in the cells. mt-tRNAs, similarly to their nuclear-encoded counterparts, engage in biogenesis of tRNA fragments, which are usually 10-35 nucleotides long, depending on where in the mt-tRNA sequence the enzymatic cleavage occurs. Importantly, it has been shown that tRNA fragments participate in several crucial cellular processes, such as gene silencing, RNA processing, translation, and epigenetic regulation. Additionally, it was discovered that tRNA fragments are dysregulated in several types of cancer cells, where their increased expression has been linked to cancer cell proliferation, invasion, and metastasis, which indicates tRNA fragments' potential to be used as biomarkers and therapeutic targets in cancer treatment [2, 3].

However, many aspects about the mechanisms of mt-tRNA processing into fragments and about their influence on other processes in cells still remain unknown [3, 4]. In addition, mitochondrial pathologies that arise from mutations in the mitochondrial genome seem to be highly tissue specific, which complicates matters even further [4, 5]. To start investigating how mt-tRNA fragment processing works, how these molecules influence cellular processes, and how mt-tRNA dysfunction is connected to mt-tRNA fragment dysregulation, we need to obtain their high-resolution structural and dynamic information. Since mt-tRNAs are quite unstable and structurally ''plastic'' they are notoriously hard to crystalize, which makes X-ray crystallography studies less suitable for elucidating structural properties of these molecules. Consequently there are barely any high-resolution structural and dynamic data available as of yet [6]. Additionally, data that are available suggest that many mt-tRNAs adopt unusual structural features, which differ greatly from nuclear tRNAs [7]. Therefore, high-resolution NMR spectroscopy could provide the solution to this problem, enabling us to structurally and dynamically characterize mt-tRNAs as well as their fragments, which would in turn allow us to identify which structural motifs are necessary for interactions with different maturation enzymes, translation machinery, and other possible targets.

Herein, we present the structure investigation of the mt-tRNA fragment A2 and its A-to-G mutant. Analyzing differences in correlations in 2D ^{1}H-^{1}H NOESY, 2D ^{1}H-^{1}H TOCSY and 2D ^{1}H-^{13}C HSQC spectra will help us understand the influence of mutation on structural characteristics of the mt-tRNA fragment A2. We are confident that this will allow us to start unraveling the mysteries surrounding mitochondrial tRNA fragments and their role in cellular environment as well as in pathology of mitochondrial diseases and cancers.

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Study of the impact of site-specific ubiquitination of the protein tau on its structural and aggregation propensities

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The microtubule associated protein tau is an intrinsically disordered protein whose main role is in the assembly and stabilization of microtubules. The damaged neurons of patients of Alzheimer's disease are characterized by the presence of paired helical filaments constituted by aggregates of hyperphosphorylated tau [1]. In these aggregates, tau is found mono- and polyubiquitinated in different positions, and it has been hypothesized that ubiquitin could mediate specific protofilament packing [2-4]. Ubiquitination is a post-translational modification involved in different cellular processes, ranging from protein degradation to regulation of gene expression [5]. In this modification, ubiquitin is covalently attached to target proteins through an isopeptide bond between the C-terminal glycine of ubiquitin and the ε-amino group of a target lysine of the substrate.

In this work, we optimized a method to synthesize single or double ubiquitinated tau at residues Lys311 and Lys317 which are strongly associated with pathological conditions. We exploited chemoselective disulphide coupling to bind ubiquitin at one or both of those positions and obtained the desired products at high purity. We then employed different techniques to characterize the aggregation behavior of the double ubiquitinated tau in comparison with singly ubiquitinated species. The data clearly indicate a site specific effect of ubiquitination on the conformational transitions leading to aggregates formation.

We also investigated the impact of this post-translational modification on the structural propensities of tau employing NMR spectroscopy. 1H- 15N HSQC spectra were acquired to perform a residue-by-residue analysis of the signals of tau, which suggests that the conformations of tau are unaffected by the presence of the ubiquitin molecules.

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Structural characterization of the postsynaptic Drebrin protein

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The postsynaptic density (PSD) of excitatory synapses is a complex network of nervous system proteins involved in postsynaptic signaling. It also modulates and regulates several functions of the nervous system, thereby responsible for a few molecular mechanisms involved in learning and memory [1].

The protein Drebrin is an essential component of the cytoskeleton, and its presence is required for actin polymerization of synapses and recruitment of CXCR4 chemokine receptors [2], as well as for the morphogenesis of the dendritic spike. Drebrin also plays an important role in synaptic plasticity associated with hippocampal memory and establishes several key interactions with other proteins present in PSD [3].

In this work we aim to characterize the structure of three different Drebrin domains, namely the ADFH (Actin-Depolymerizing Factor Homology) domain at the N-terminal, the SAH (Single Alpha Helix) domain which was earlier predicted with bioinformatic methods [4], and the HBMs (Homer Binding Motifs) near the C terminus. We have optimized the bacterial expression of the corresponding constructs and already performed initial structural analysis with CD (Circular Dichroism) and NMR (Nuclear Magnetic Resonance) spectroscopy. Molecular interactions with other PSD proteins and F-Actin will also be investigated with BLI (Biolayer Interferometry) and ITC (Isothermal Titration Calorimetry) measurements.

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Peptides targeting EphA2-Sam/Ship2-Sam interaction: a multidisciplinary study

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EphA2 is a receptor tyrosine kinase (from the Eph family receptors) that plays a crucial role in cell migration and proliferation. [1] EphA2 attracts attention in the anticancer drug discovery field as it is over-expressed in different types of tumors (e.g., breast, ovary, lung and prostate) and has a complex role in cancer, balancing anti-tumorigenic and pro-tumorigenic pathways. [2] EphA2 contains at the C-terminal side a cytosolic Sam (Sterile alpha motif) domain. EphA2-Sam is a protein module with a small five-helix bundle fold, that is involved in heterotopic Sam-Sam interactions with other protein regulators of receptor endocytosis and degradation. [3] [4] For example, the lipid phosphatase Ship2 contains a Sam domain (Ship2-Sam) able to interact with EphA2-Sam. The Sam-Sam interaction between Ship2-Sam and EphA2-Sam follows the canonical interaction topology called Mid Loop (ML) - End Helix (EH) model. Ship2-Sam provides a negatively charged ML interface to bind EphA2-Sam positively charged EH site, that is made up of the receptor C-terminal helix along with close loop regions. [5] The goal of my research is to design and evaluate peptide inhibitors of the EphA2-Sam/Ship2-Sam complex able to induce receptor endocytosis and degradation and thus playing the role of anticancer therapeutics. Our lab. previously identified the KRI3 peptide. [6] KRI3 sequence consists of the triple repetition of the positively charged “KRIAY” motif that is contained in EphA2-Sam EH area. KRI3 is able to inhibit Ship2-Sam/EphA2-Sam association by interacting with Ship2-Sam ML interface (dissociation constant KD ~100 μM). [6] In addition, in vitro studies show for KRI3 a higher cytotoxicity in the cancer cell line PC3 if compared to that found for normal dermal fibroblasts. [6] In order to improve binding affinity to Ship2-Sam and get novel insights into structural features, that could direct binding to Ship2-Sam, several KRI3 analogues, containing multiple mutations within the original peptide sequence or cyclization, were designed and evaluated. [7] The newly designed peptides were synthetized and analyzed by a multidisciplinary approach. First, conformational studies were conducted by CD (Circular Dichroism) and NMR to get information on peptide structural preferences. Next, peptide capacity to bind Ship2-Sam was investigated by different techniques (NMR, MST (MicroScale Thermophoresis) and SPR (Surface Plasmon Resonance)). The data reveal that KRI3 cyclization through a disulfide bridge or an increase of positive charge causes unspecific interactions without improving the binding affinity to Ship2-Sam. Tyrosine residues appear instead crucial for binding to Ship2-Sam. The collected data will be employed in the near future to design optimized Ship2-Sam ligands.

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Monitoring the inhibition of copper chaperone Atox1 by NMR spectroscopy and molecular docking studies

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The interaction between the metallochaperone Atox1 and the ion pump Atp7a (related to Menkes syndrome) is essential for the transport of copper in human cells and for its detoxification, but some anticancer drugs are able to interfere with this process [1]. In this work, a molecular study of the effects caused by the chemotherapeutic drug cisplatin [2] and the Atox1 inhibitor DC_AC50 [3] on copper transport was conducted. By NMR spectroscopy and molecular docking, we investigated the ability of cisplatin to stabilize the Atox1-Cu(I)-Mnk1 heterodimeric complex by forming interprotein cross-links [4] between Atox1 and Mnk1 (the N-terminal domain of Atp7a), which underlie a possible mechanism of drug resistance [5,6]. In addition, the inhibitor DC_AC50 was characterized, the activity of which is aimed at preventing the formation of the heterodimer and the exchange of copper between Atox1 and Mnk1. Finally, we studied the combined effect of the two small molecules, cisplatin and DC_AC50, which makes it possible to resensitize cancer cells to cisplatin treatment by promoting the intracellular accumulation of copper [7].

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