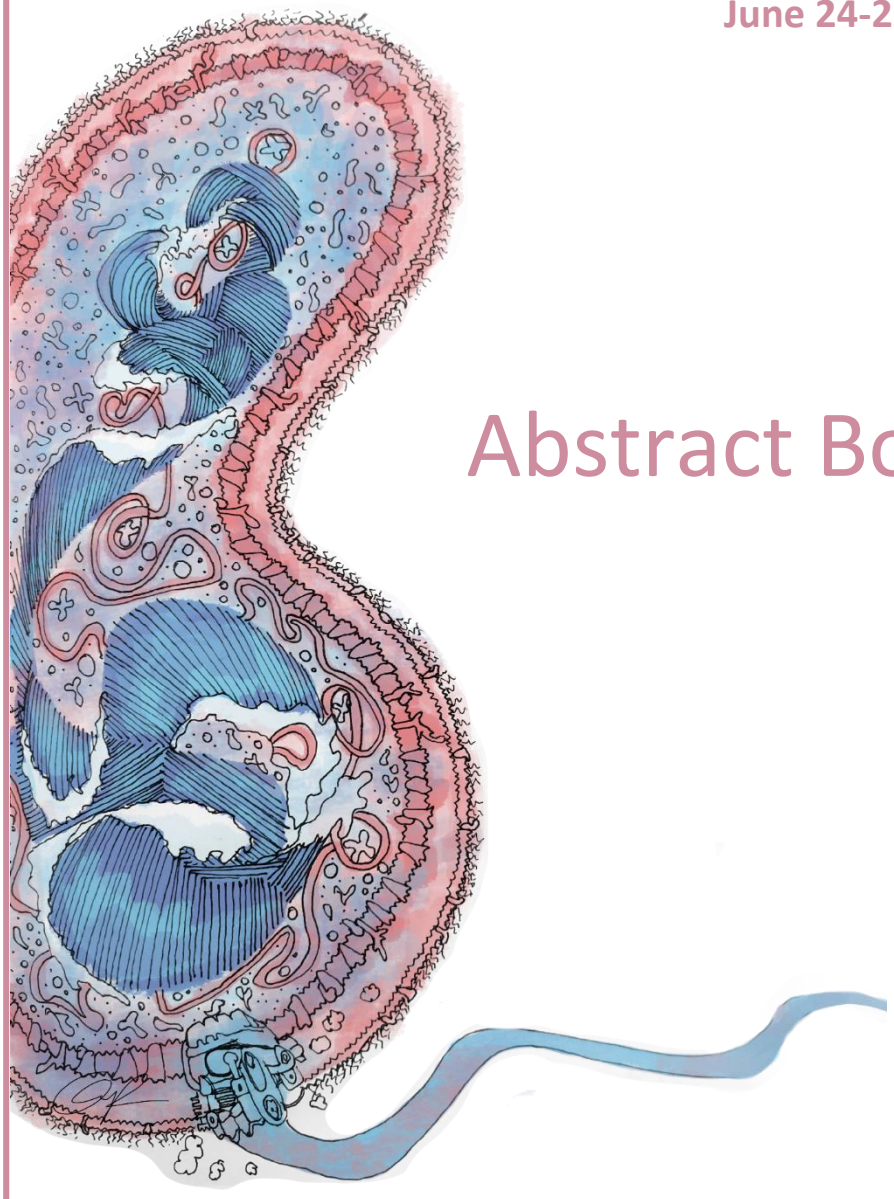
MESSAGE FROM THE CELL

A workshop on biophysical studies for structural biology inside cells

June 24-25, 2021, ON LINE



Abstract Book

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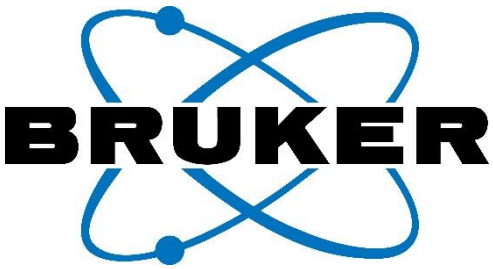
BRUKER

The Organizing Committee in Marseille

Bioénergétique et Ingénierie des Protéines (BIP) and Laboratoire d'Ingénierie des Systèmes Macromoléculaires (LISM), Institut de Microbiologie de la Méditerranée (IMM), CNRS and Aix-Marseille Université, Marseille, France.

From the left:

Hélène LAUNAY (BIP), Alessio BONUCCI (BIP), Annalisa PIERRO (BIP), Elisabetta MILEO (BIP), Valérie BELLE (BIP), Bruno GUIGLIARELLI (BIP), Latifa ELANTAK (LISM) and some giant microbes.

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10:25-10:40	Talk 2 - **Sophie Combet,** LLB, Université Paris-Saclay, CEA-Saclay, France. *Structural changes of human dystrophin upon binding to the muscle membrane.*
10:40-11:10 Coffee break avatar & Meet the Speakers	
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11:50-12:05	Talk 3 - **Marie Erard,** CNRS, Univ. Paris-Sud, Université Paris-Saclay, Orsay, France. *Combining quantitative imaging methods with 3D structures to characterize the cytosolic complex of the NADPH oxidase from phagocytes in living cells.*
12:05-12:20	Talk 4 - **Olav Schiemann,** University of Bonn, Germany. *Trytil spin labels: Studying Biomolecular Conformations within Cells by Pulsed Dipolar EPR.*
12:20-12:35	**Olivier Zelphati**, OZ – Biosciences, Marseille, France. *Magnetofection: Magnetically assisted and targeted nucleic acids delivery.*
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14:40-14:55	Talk 5 – **Annalisa Pierro**, BIP, CNRS, Marseille, France. *Studying protein dynamics in living cells by Nitroxide-based SDSL-EPR.*
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15:10-15:30	**Poster session 1** – *Flash presentations* Laura Galazzo, Janet Lovett, Ylenia Beniamino, Shari L. Meichsner, Dominik Gendreizig, Jonathan Revol-Tissot
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16:40-16:55	Talk 7 – **Hélène Launay, BIP**, CNRS, Marseille, France. *Redox regulation of photosynthesis in micro-algae monitored by NMR.*
16:55-17:35	Keynote 5 – **Marc Tramier**, CNRS/Université de Rennes 1, France. *Quantitative Fluorescence Microscopy for Spatiotemporal Study of Protein-Protein Interactions and Biochemical Activities: FRET by FLIM and 2c-FCS.*
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Chair: François-Xavier Theillet	
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16:20-16:35	Talk 10 - **Müge Kasanmascheff**, TU Dortmund University, Germany. *Tracing Protein Native Radicals in Living Cells with EPR.*
16:35-17:15	*Keynote 8* – **Enrico Luchinat,** *CERM, University of Florence, Italy. Protein-drug interactions monitored by time-resolved NMR in human cells.*
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17:45-18:00 Concluding remarks.	

Oral Contributions

Session 1

A Blueprint for the Structural Biology Department of the Future

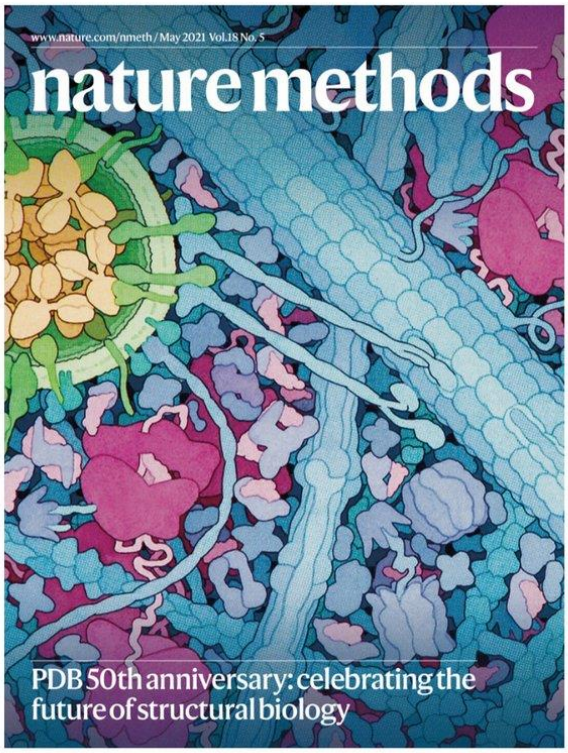
Phil Selenko

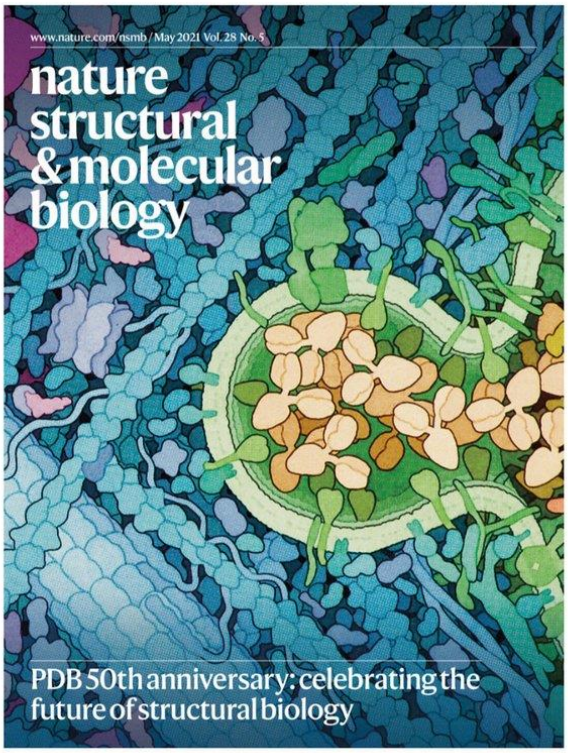
Weizmann Institute of Science, Department of Biological Regulation, Rehovot, Israel

The past decade has seen tremendous advancements in multiple areas of *in situ* Structural Biology. That is in methods and technologies that enable direct structural investigations of biological macromolecules in their native cellular settings[1]. In these applications, spectroscopic techniques such as nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) methods, together with single- and ensemble-molecule Förster resonance energy transfer (FRET) spectroscopy play fundamental roles that provide unique and often complementary insights. Together with cellular cryo-electron tomography (cryoET), in-cell cross-linking mass spectrometry (XMS), computational modeling and high-resolution optical imaging techniques, they define the toolkit that will shape the face of Structural Biology in the years to come.

Here, I present my vision for the Structural Biology Department of the future. I outline how these techniques and technologies may be best integrated to arrive at a comprehensive understanding of biological processes in their cellular settings. Because the future of Structural Biology is in the cell.

1. *Structural Biology outside the box – inside the cell.*
Plitzko JM, Schuler B, Selenko P. Current Opinion in Structural Biology *2017* 46 110-121





Time-resolved NMR monitoring of tRNA maturation

Pierre Barraud[1], Marjorie Catala[1], Alexandre Gato[1], **Carine Tisné[1]**

[1]*Institut de Biologie Physico-chimique, Laboratoire d'expression génique microbienne, UMR8261, CNRS, Univeristé de Paris, Paris, France.*

Over 170 nucleotide modifications are currently reported in RNAs from the three domains of life, the vast majority being found in tRNAs. This family not only displays the largest variety of post-transcriptional decorations among RNA molecules, but also the highest density of modifications per RNA transcript[1]. The introduction of post-transcriptional chemical modifications is central in the maturation process to generate functional tRNA molecules. Although the biological importance of post-transcriptional RNA modifications in gene expression is widely appreciated, methods to directly detect their introduction during RNA biosynthesis are rare and do not easily provide information on the temporal nature of events[2].

In the past 10 years, NMR spectroscopy became a pre-eminent method to investigate post-translational modifications (PTMs) in proteins. Time-resolved NMR measurements provided the means to monitor the establishment of PTMs in vitro, in cellular extracts and in living cells. In addition, monitoring the introduction of PTMs with NMR has provided mechanistic insights into modification hierarchies, with initial modifications exhibiting stimulatory or inhibitory effects on subsequent modification events. Inspired by the NMR monitoring of PTMs in cellular environments, we have devised an original methodology to monitor RNA modifications in cellular extracts with NMR[3]. Using yeast $tRNA^{Phe}$ as a model system, we demonstrate that multiple modification events can be monitored in yeast extract with NMR in a time-resolved fashion. Using continuous NMR measurements to measure a series of snapshots of the tRNA along the maturation route, we observe a sequential order in the introduction of several modifications. This suggests that modification circuits could control the $tRNA^{Phe}$ maturation process in yeast. We next adopted a reverse genetic approach and investigated the interplay between the different modifications in yeast $tRNA^{Phe}$ with both NMR and mass spectrometry and show that modification circuits identified in the yeast extract on $tRNA^{Phe}$ also influence the process of tRNA modification in living cells[3].

1. P. Barraud & C. Tisné, To be or not to be modified: miscellaneous aspects influencing nucleotide modifications in tRNAs, **IUBMB life** 71 (2019), p. 1126-1140. doi : 10.1002/iub.2041

2. Y. Yoluç, G. Ammann, P. Barraud, Manasses Jorac, P. A. Limbach, Y. Motorin, V. Marchand, C. Tisné, K. Borlan & S. Kellner, Instrumental analysis of RNA modifications, **Crit Rev Biochem Mol Biol.** 56 (2021), p. 178-204. doi: 10.1080/10409238.2021.1887807

3. P. Barraud, A. Gato, M. Heiss, M. Catala, S. Kellner & C. Tisné, Time-resolved NMR monitoring of RNA maturation, **Nature Communications** 10 (2019), p. 3373-3387. doi: 10.1038/s41467-019-11356-w

Structural changes of human dystrophin upon binding to the muscle membrane

S. Combet[1], R. Dos Santos Morais[1,2,3], O. Delalande[2], J. Pérez[3], and JF. Hubert[2]

[1]Laboratoire Léon-Brillouin (LLB), UMR 12 CEA-CNRS, Université Paris-Saclay, CEA-Saclay, F-91191 Gif-sur-Yvette CEDEX, France, [2]IGDR CNRS UMR 6290, Université de Rennes 1, F-35043 Rennes, France. [3]Synchrotron SOLEIL, L'Orme des Merisiers, BP 48, Saint-Aubin, F-91192 Gif-sur-Yvette, France.

Dystrophin[1] is a filamentous peripheral membrane protein supporting the plasma membrane of muscular cells. Its absence due to gene mutations leads to severe Duchenne muscular dystrophy (DMD). Most part of the dystrophin consists in a central domain, made of 24 coiled-coil repeats (R1 to R24), whose 3D-structure is not accessible by classical high resolution methods[2]. We specifically probed by SANS the solution structure of R1-3 protein fragment of the dystrophin central domain, known to interact with the membrane lipids[3-5]. Its structure alone in solution was compared to that adopted in the presence of phospholipid-based membrano-mimetic bicelles whose SANS signal was "switched off"[6]. SANS data of the protein/lipid complexes were obtained with, either zwitterionic, or anionic bicelles, at two different temperatures, in order to probe the role of electrostatic interactions and membrane fluidity. Our results highlight that, when bound to zwitterionic bicelles, no important conformational modification of the protein fragment is detected. On the contrary, when R1-3 is bound to anionic bicelles, SANS data demonstrate large modifications of its 3D-structure. The R1-3/anionic bicelle complex was further analyzed by classical coarse-grained molecular dynamic simulations (CG-MD) and interactive CG-MD. The final models proposed for R1-3 bound to membrane lipids are totally in adequacy with the experimental SANS data (Fig. 1). Finally, we report an accurate mapping of the protein/lipid interactions obtained by coupling click-chemistry with mass spectrometry, in line with the *in silico* data. We highlight that the coiled-coil in repeat 1 specifically opens upon binding to anionic bicelles. Such opening could occur during the contraction/elongation process of muscles and ensure protein membrane anchoring. Moreover, using the same methodology combined with docking simulations, we showed recently how the R11-15 fragment of the central domain of dystrophin acts to bridge F-actin to the lipids of the muscle membrane[8]. Understanding these structural changes may help for the design of rationalized efficient shortened dystrophins devoted to gene therapy on DMD patients. Last but not least, our approach opens up new possibilities for structure determination of peripheral and integral membrane proteins not compatible with different high resolution structural methods.

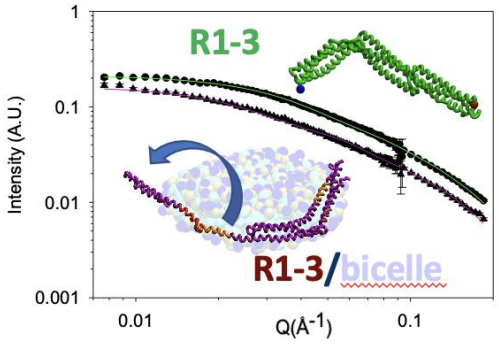


***Figure 1**. Experimental SANS intensities (circles and triangles for R1-3 alone or bound to contrast-matched anionic bicelles, respectively) fitted with the theoretical CRYSON[9] curves generated from the R1-3 model free in solution (green) and the R1-3 model bound to anionic bicelles (purple), showing an opening of repeat 1. The corresponding all-atom models are represented with the same color code. From [7].*

1. Le Rumeur E. *et al.*, Biochim. Biophys. Acta 1804 (2010) 1713. **2.** Molza AE. *et al.*, Faraday Discuss. 169 (2014) 45. **3.** Legardinier S. *et al.*, J. Mol. Biol. 389 (2009) 546. **4.** Vié V. *et al.*, Biochim. Biophys. Acta 1798 (2010) 1503. **5.** Sarkis J. *et al.*, J. Biol. Chem. 286 (2011) 30481. **6.** Dos Santos Morais R. *et al.*, Langmuir 33 (2017) 6572. **7.** Dos Santos Morais R. *et al.*, Biophys J 115 (2018) 1231. **8.** Dias-Lucquin D *et al.*, Journal of Structural Biology 209 (2020) 107411. **9.** Svergun D.I.*et al.*, Proc. Natl. Acad. Sci. USA 95 (1998) 768.

Session 2

In-cell dimer stablity by DEER using Gd(III) spin labeling

Yin Yang[1], Angeliki Giannoulis [1], Akiva Feintuch, Xun-Cheng-Su[2], **Daniella Goldfarb** [1]

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Observing proteins structural changes of proteins during function in-side the cell is a challenge yet to be met. The motivation for such studies is the notion the complex cellular environment affects both the conformational equilibrium and the stability of proteins. In this context, distance measurements between two spin labels attached at specific, well defined positions in a protein, by double electron-electron resonance (DEER) is as an attractive method to probe protein's conformations in cells. To realize the potential of such measurements the spin labels' properties in terms of chemical stability, EPR sensitivity and distance resolution have to be optimized along with increasing measurement sensitivity, allowing measurements at physiologically relevant concentrations. We have been using Gd(III) chelates as spin labels for in-cell measurements because of their high chemical stability and the high sensitivity they exhibit at high EPR frequencies. A number of Gd(III) tags will be presented and their in-cell performance in terms of stability, sensitivity and distance resolution will be compared, showing that by tuning the chemical structure of the Gd(III) chelate all these properties can be optimized. Then the feasibility of the methodology will be demonstrated on the dimeric BIR1 domain of the X-linked inhibitor of apoptosis protein (XIAP), which shows a significant difference between in the dissociation constant of the dimer measured in frozen solution and in frozen human Hela cells. Next, we turn to Hsp90, which is a molecular chaperone important in all organisms facilitating the folding and maturation of proteins called clients. It is homo-dimeric and each monomer is consisted of three successive domains, the N-terminal domain, NTD, where the ATPase site is; the middle domain, MD, important for ATP hydrolysis and binding of clients; and the C-terminal domain, CTD, responsible for dimerization of the two monomers[1]. Using Gd(III) spin labeling on the CTD and DEER we quantitatively studied the dimerization of the CTDs for full length Hsp90 and isolated CTD in vitro. Last, we exploited the Gd(III) labeling in the CTD to 'look' at Hsp90 in Hela cells.

[1] C. Prodromou *et al., Embo J.* 19, 4383-4392 (2000).

Combining quantitative imaging methods with 3D structures to characterize the cytosolic complex of the NADPH oxidase from phagocytes in living cells

Cornelia S. Ziegler[1], Leila Bouchab[1], Marc Tramier[2], Dominique Durand[3], Franck Fieschi[4] Sophie Dupré-Crochet[1], Fabienne Mérola[1], Oliver Nüße[1], **Marie Erard[1]**

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[2] *Univ Rennes, CNRS, IGDR [(Institut de génétique et développement de Rennes)] – UMR 6290, BIOSIT – UMS 3480, F-35000 RENNES, France*
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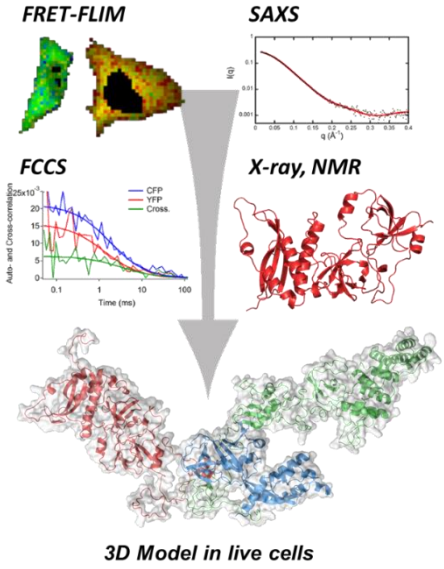
[4] *Univ. Grenoble Alpes, CNRS, CEA, Institut de Biologie Structurale, F-38044 Grenoble, France*

Many proteins contain structured domains separated by intrinsically disordered regions (IDR). These modular proteins adopt numerous conformations, which make their characterization *in vitro* difficult with conventional techniques such as X-ray diffraction, NMR, CryoEM. The two cytosolic subunits of phagocyte NADPH oxidase, p47 and p67, fall into this category. Their structural study requires the development of alternative strategies. They are associated in their resting state with a third subunit, p40.

We have combined different labeling of p40, p47 and p67 subunits with fluorescent proteins and FRET-FLIM or FCCS spectro-microscopy strategies in live cell to characterize the p40-p47-p67 complex in its resting state. Our studies demonstrated a 1: 1: 1 stoichiometry. We also estimated the affinity and analyzed the spatial organization of the subunits. Using our results and all the structural data available (RX, NMR and SAXS), we developed a new three-dimensional model of the entire cytosolic complex valid in cells. This model has an elongated topology featuring two modules separated by a flexible hinge, which is fully compatible with the multiple stages of the oxidase activation.



Topological model of the cytosolic complex: p67 (green), p47 (red), p40 (blue)

Reference: Ziegler et al, Quantitative live-cell imaging and 3D modeling reveal critical functional features in the cytosolic complex of phagocyte NADPH oxidase J. Biol. Chem. (2019) 294(11) 3824–3836

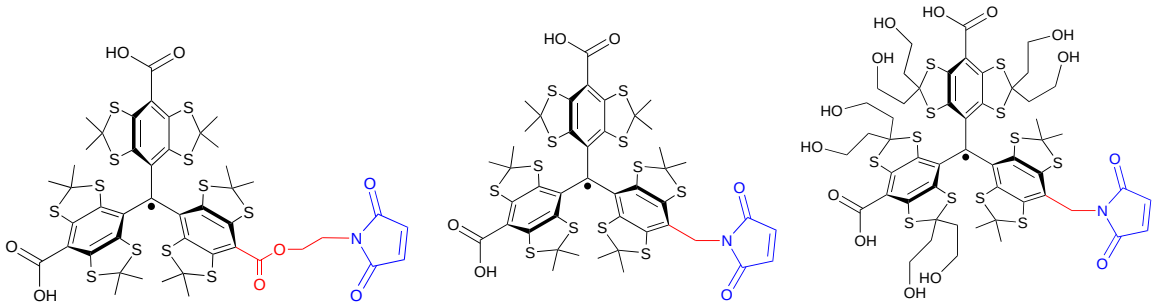
Trityl Spin Labels: Studying Biomolecular Conformations within Cells by Pulsed Dipolar EPR

Olav Schiemann

Institute of Physical and Theoretical Chemistry, University of Bonn, Wegelerstr. 12, 53115 Bonn, Germany

The understanding of biomolecular function is coupled to knowledge about the structure and dynamics of these biomolecules, preferably acquired under native conditions. In this regard, pulsed dipolar EPR spectroscopy (PDS) in conjunction with site-directed spin labeling (SDSL) is an important method in the toolbox of biophysical chemistry. However, the currently available spin labels have diverse deficiencies for in-cell applications, for example, low radical stability or long bioconjugation linkers.

In the talk, synthesis strategies are introduced for the derivatization of trityl radicals with a maleimide-group, which enables bioconjugation of the trityl labels **1 – 3** to cysteins.[1-4] Especially, the resulting trityl spin labels **2** (**SLIM**)[3] and **3** (**oxSLIM**)[4] yield narrow distance distributions, enable highly sensitive distance measurements down to concentrations of 45 nM, and show high stability against in cell reduction. Using these labels in combination with the Double Quantum Coherence experiment (DQC), the guanine-nucleotide dissociation inhibitor (GDI) domain of *Yersinia* outer protein O (YopO) is shown to change its conformation within eukaryotic cells.



1 **2 (SLIM)** **3 (oxSLIM)**

1. J.J. Jassoy, A. Berndhäuser, F. Duthie, S.P. Kühn, G. Hagelueken, O. Schiemann “Versatile Trityl Spin Labels for Nanometer Distance Measurements on Biomolecules in vitro and within cells" *Angew. Chem. Int. Ed.* **2017**, 56, 177.

2. J.J. Jassoy, C.A. Heubach, T. Hett, F. Bernhard, F.R. Haege, G. Hagelueken, O. Schiemann “Site Selective and Efficient Spin Labeling of Proteins with a Maleimide-Functionalized Trityl Radical for Pulsed Dipolar EPR Spectroscopy” *Molecules* **2019**, 24, 2735.

3. N. Fleck, C.A. Heubach, T. Hett, F.R. Haege, P.P. Bawol, H. Baltruschat, O. Schiemann “SLIM: A short-linked, highly redox-stable trityl label for high sensitivity in cell EPR distance measurements“ *Angew. Chem. Int. Ed.* **2020**, 59, 9767.

4. N. Fleck, C. Heubach, T. Hett, S. Spicher, S. Grimme, O. Schiemann „Ox-SLIM: Synthesis of and Site-Specific Labelling with a Highly Hydrophilic Trityl Spin Label” *Chem. Eur. J.* **2021**, 27, 5292.

Magnetofection: Magnetically assisted and targeted nucleic acids delivery

Olivier Zelphati, PhD, CEO OZ Biosciences, Marseille, France

The premise of this gene therapy approach is to shut down the expression of genes or to introduce a nucleic acid sequence encoding a protein of interest, altering or repairing the expression of an endogenous gene, or possessing the capacity to cure or prevent the development of a disease. During the last decade the development of nanotechnologies to deliver and target nucleic acids have been widely studied. Magnetofection™ technology is defined as the delivery of nucleic acids, either "naked" " (not complexed) or packaged (as complexes with lipids or polymers, and viruses) using magnetic nanoparticles (MNPs) under the guidance of an external magnetic field. Magnetic nanoparticles, are used to self-assemble with nucleic acids through non covalent hydrophobic and electrostatic interactions, before a magnetic force drives them onto the cell surface within minutes. Targeting and accelerating the delivery process using Magnetofection™ presents many advantages including high efficiencies and confinement of the nucleic acid dose to a magnetically targeted site. Principle, mechanisms and applications (transduction and transfection) of Magnetofection™ will be reviewed.

Session 3

Nucleic Acids *in-cell* NMR: Progress, Challenges, and Opportunities

Lukas Trantirek

Central European Institute of Technology, Masaryk University, Kamenice 753/5, 625 00 Brno, Czech Republic

In-cell NMR spectroscopy is a unique tool for high-resolution characterization of biomolecular structure and interactions under close-to-physiologically relevant conditions. For nucleic acids (NA), the state-of-the-art in-cell NMR applications include validating physiologically relevant conformations of drug targets and characterization of NA-ligand interactions in the intracellular space of living human cells[1–3]. While protein in-cell NMR applications generally exploit multidimensional NMR spectroscopy, the NA in-cell NMR applications exclusively rely upon interpreting one-dimensional NMR spectra. The limitation to 1D NMR detection is due to the inherent NAs instability and low intracellular concentrations. As a result, compared to protein in-cell NMR data, the in-cell NMR data have only a qualitative or semi-quantitative character.

There has been an outgoing effort to improve in-cell NMR readout on nucleic acids. The effort includes attempts to extend the time window for the acquisition of in-cell NMR data, adjustments to the sample preparation allowing in-cell NMR measurements in defined cellular states, and development of novel probes of NA structure/interactions to increase resolution and sensitivity of in-cell NMR spectra.

In this lecture, I will introduce the basic principles of in-cell NMR spectroscopy of nucleic acids. I will discuss the main technical problems limiting the application of the method. In parallel, I will overview recent developments and highlight the main future research directions in the field.

1. Dzatko et al. Evaluation of the Stability of DNA i-Motifs in the Nuclei of Living Mammalian Cells. *Angew Chem Int Ed Engl*. **2018**, 57(8):2165-2169.
2. Krafcikova et al. Monitoring DNA-Ligand Interactions in Living Human Cells Using NMR Spectroscopy. *J Am Chem Soc*. **2019**, 141(34):13281-13285.
3. Bao et al. Hybrid-type and two-tetrad antiparallel telomere DNA G-quadruplex structures in living human cells. *Nucleic Acids Res*. **2019**, 47(10):4940-4947.

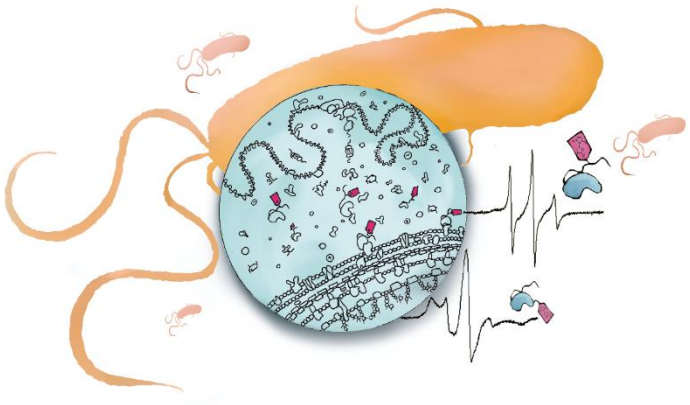
Studying protein dynamics in living cells by Nitroxide-based SDSL-EPR

Annalisa Pierro[1], A. Bonucci[1], A. Magalon[3], B. Guigliarelli[1], G. Gerbaud[1], E. Etienne[1], F. Vibert[2], G. Karthikeyan[2], O. Ouari[2], V. Belle[1], E. Mileo[1]

[1]Aix Marseille Univ, CNRS, BIP (UMR7281), IMM, IM2B, Marseille, France, [2]Aix Marseille Univ, CNRS, ICR, Marseille, France, [3]Aix Marseille Univ, CNRS, LCB (UMR7283), IMM, IM2B, Marseille, France

During the last two decades, the structural biology community has shown great interest in studying proteins in their native environment. With this aim, remarkable progress has been made in the development of new "*in-cell*" approaches. Among these, Site-Directed Spin Labeling (SDSL) coupled to Electron Paramagnetic Resonance (EPR) Spectroscopy presents competitive and advantageous features to capture protein dynamics inside cells[1-3]. In particular, nitroxide-based SDSL-EPR combines the advantages of high sensitivity and the lack of size constraints for the biomolecule of interest with the ability to study protein structural transitions and interactions at physiological temperature.

This work uses our efficient delivery protocol to study the structural dynamics of a flexible chaperone protein, NarJ, directly within its endogenous host, the gut bacterium *Escherichia coli*. The NarJ chaperone is involved in the acquisition of metal cofactors by the nitrate reductase respiratory complex[4-5]. Using cw-EPR and pulsed dipolar spectroscopy (DEER experiments), we revealed the site-specific structural behaviour of NarJ directly in *E. coli* and compared these data with those obtained *in vitro*. Finally, we demonstrated that delivered NarJ was active inside the cells by restoring nitrate reductase activity in a *narJ* strain. This work represents a new step in developing bio-structural *in-cell* EPR and opens new perspectives to address structural biological questions directly inside the cell.



1. Bonucci, A., et al; ChemBioChem 21, (2020).

2. Yang, Y. et al. ; Proc. Natl. Acad. Sci. U. S. A. 117, 20566–20575 (2020).

3. Kugele, A., et al.; ChemBioChem 20, 2479–2484 (2019).

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Compaction of RNA duplexes in the cell probed by PELDOR spectroscopy and molecular dynamics simulations

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The biological function of RNA is related to its structure and flexibility, which in turn depend on the environment. The aim of the present work is to gain information about the structure of a short RNA duplex, one of the basic constitutive elements of RNA structure, in the intracellular environment and to compare this information with the well-known A-helical structure reported in diluted buffered solutions.

Pulsed electron-electron double resonance (PELDOR)/double electron-electron resonance (DEER) spectroscopy [1–2], a technique that reports reliably on distances in the 2 – 8 nm range between site-specifically introduced paramagnetic tags, was used for this investigation. PELDOR data, converted by a model-free approach into a discrete set of distance probability distributions, has already been shown to be able to resolve even subtle differences in nucleic acids conformational ensembles [3].

In the current study, an isoindoline-derived, nitroxide-based paramagnetic tag was connected to the nucleic acid using a tether with limited flexibility [4], resulting in high accuracy of the measured distances; the required stability of the nitroxide moiety against reduction in the intracellular environment was achieved by means of steric shielding [5].
Our measurements show a clear and reproducible reduction of the distance between labels upon internalization of a 20-mer RNA duplex inside the cytoplasm of *Xenopus lævis* oocytes with respect to a diluted buffered solution [4]. These findings were confirmed by measurements performed on a duplex having a different sequence and labelled using a different strategy, which excludes an explicit role of the label and/or of the specific sequence.

To identify the origin of these changes, the effect of macromolecular crowders was also investigated. The incubation of the RNA duplex with highly concentrated lysozyme, a positively charged protein at neutral pH, resulted in distance changes similar to the ones observed in the cells, likely reflecting the role of charge-based interactions.

Single long-range distance measurements do not give sufficient insight into the full atomic-level picture, an information that is required for a deeper understanding of the rearrangements underlying the experimentally observed distance changes. For this purpose, with the aim of gaining microscopic understanding at least on a qualitative level, microsecond-scale molecular dynamics (MD) simulations of the RNA duplex in a diluted buffered solution and in the presence of lysozyme were performed. The MD simulations support the idea of an overall subtle structural compaction of the dsRNA in lysozyme with respect to the diluted solution.

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Session 4

In-cell EPR techniques for Studying Intrinsically disordered proteins (IDPs)

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Intrinsically disordered proteins (IDPs) play important physiological, but also disease-related roles. Here, we will focus on on a prominent example of IDPs, the 'Parkinson protein' α-synuclein (αS).

In order to understand the function and malfunction of IDPs, electron paramagnetic resonance (EPR) spectroscopy has proven to be a valuable tool, allowing investigation of the protein structural ensembles *in vitro.*

In order to produce biologically meaningful results in the context of human diseases, it is crucial to perform EPR experiments with IDPs under the most relevant environmental conditions, i.e., inside the cell. IDPs are highly regulated proteins, which are subject to numerous post-translational modifications (PTMs) influencing the IDP energy landscapes in the cell. The cellular environment is characterized by molecular crowding and features a huge variety of interaction partners that may modulate the protein structural ensemble and processes of structural reorganization, as well as the oligomerization behavior, resulting fibril conformers and the interaction kinetics with partners like other proteins. As a result, in cellula experiments may deliver different findings than the in vitro complement.[1]

However, the application of in-cell EPR is still demanding. Here, we report on effective ways of in-cell spin labelling strategies, e.g., via unnatural amino acids that allow in vivo expression of spin-labelled proteins.[2]

Recent studies showed that the signal-to-noise ratio in in-cell EPR experiments is crucial for the detection of all relevant protein subpopulations. Promising high-sensitivity in-cell EPR spectroscopic approaches are laser-induced dipolar spectroscopy[3] as well as rapid-scan (RS) EPR spectroscopy.

Using RS EPR spectroscopy to study αS interactions with negatively charged vesicles in vitro and upon transfection of the protein and lipid vesicles into model cells, we show that protein−vesicle interactions are reflected in RS spectra in vitro and in cells, which enables time-resolved monitoring of protein−membrane interaction upon transfection into cells. Our data suggest binding of a small fraction of αS to endogenous membranes.[4]

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Redox regulation of photosynthesis in micro-algae monitored by Nuclear Magnetic Resonance

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CO_2 assimilation by photosynthetic organisms in the Calvin-Benson-Bassham (CBB) metabolic cycle to produce sugar molecules corresponds to the transfer of 4 electrons. The reducing power that fuel the CBB cycle is provided by the photochemical phase of photosynthesis, in particular by the production of the reducing co-factor (NADPH) that is used by the glyceraldehyde phosphate dehydrogenase (GAPDH). In the dark, this enzyme in inhibited and clustered in a ternary complex mediated by a small intrinsically disordered protein CP12[1]. The third partner is another CBB enzyme phosphoribulokinase (PRK) that is also inhibited. In the light, GAPDH and PRK are the only enzymes that catalyze endothermic reactions of the CBB cycle. In the dark, when oxidizing conditions prevail, the inhibition of the CBB cycle avoids futile cycling between neoglucogenesis and glycolysis, and this inhibition is dependent on the interaction of GAPDH and PRK on two distinct regions of CP12, that is the "hub" protein.

Under reducing condition (in the light) isolated purified recombinant CP12 is intrinsically disordered, and we have used Nuclear Magnetic Resonance (NMR) to confirm that it is also disordered in the presence of cell extract[2], that is in the presence of all its putative interacting partners. Under oxidizing conditions (in the dark), two intramolecular disulfides bridges associate in CP12: between the cysteine residues C23-C31 and C66-C75. The C-terminal disulfide bridge is in the GAPDH binding region, and oxidation results in the formation of a highly stable helical turn that is the same as that found in the GAPDH-CP12 interface[3,4]. We have confirmed that this redox-dependent structural transition also occurs in the presence of cell extracts[2]. The N-terminal disulfide bridge is in the PRK binding region, and the helical hairpin fold found in the PRK-CP12 complex is only partially stabilized in the isolated oxidized protein and in the presence of cell extract[2].

We aim at characterizing the redox-dependent structural transitions of the regulatory protein CP12 that are light dependent inside micro-algae cell *Chlamydomonas reinhardtii*. Preliminary NMR results on CP12 in the presence of cell extract showed that the redox-transition is active as long as the photochemical phase of photosynthesis is active, and is inhibited in the presence of a photosystem inhibitor[2]. We aim at pursuing these studies by monitoring the presence of other regulatory co-factors of the CBB cycle in *C. reinhardtii* by in-cell NMR under dark or light conditions.

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Quantitative Fluorescence Microscopy for Spatiotemporal Study of Protein-Protein Interactions and Biochemical Activities: FRET by FLIM and 2c-FCS

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Fluorescence based methods encompass different disciplines and a vast number of technical approaches. Advances in different photonic imaging techniques and the development of fluorescent probes, and particularly fluorescent proteins, have raised fluorescence microscopy to the level of dominance in the field of biology. For example, it is possible to use FRET imaging to monitor protein-protein interactions or protein conformational dynamics. Methods based on fluorescence correlation techniques (for example two-colour FCS) are also used for protein-protein interaction studies. Biological results based on methods to measure quantitative FRET imaging by FLIM or two-colour FCS will be presented. These approaches are critical to improve our understanding of different processes occurring in vivo (biochemical protein cascades) and if it is done quantitatively, to build or improve biological mathematical models.

Session 5

Conformations of ABC transporters in vitro and in living cells studied by nanobody-assisted DEER.

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Nanobodies (i.e. single-domain antibodies) are promising new tools for in-cell applications due to their low molecular weight, protein- and state- specificity, nano- or sub-nano-molar affinity to their target and the possibility to be inserted into cells. We show here how spin-labeled nanobodies can be used as conformational reporters of wild type ABC transporters via Double Double Electron Resonance (DEER), a pulsed EPR technique that accurately measures inter-spin distances. First, we show a proof of principle of the use of gadolinium-labeled nanobodies against the heterodimeric exporter TM287/288[1]. Caveats regarding the nanobody state specificity at micromolar concentrations will be discussed. Second, we present a systematic study on the homodimeric ABC exporter MsbA using non-state-specific nanobodies targeting the two nucleotide binding domains. We gathered structural information in detergent, proteoliposomes, nanodiscs, inside-out vesicles from *E. coli* and in living cells. We found that there is a remarkable modification of the conformational landscape of the transporter in specific membrane-mimicking environments, proving the need of in-cell structural studies[2]. Advantages and challenges of using biocompatible Gd-labels down to nanomolar concentrations in cells will be also addressed[3].

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Characterizing the in-cell ensemble of the intrinsically disordered α-synuclein using DNP-enhanced MAS NMR spectroscopy

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The conformation of intrinsically disordered proteins (IDPs) is strongly influenced by the environment. Therefore, it is important to develop approaches that allow us to study the conformation of IDPs in their native environment within cells. IDPs can form oligomers or bind to much larger cellular components that are difficult to characterize with solution-state NMR. Therefore, there is a need for an NMR technique that can provide us with information about high molecular weight species inside cells. We investigated a model IDP α-synuclein inside the HEK293 cell line using MAS NMR spectroscopy in combination with dynamic nuclear polarization (DNP). The DNP increases the sensitivity of MAS NMR and makes it possible to detect α-synuclein within cells at physiological concentrations. To characterize α-synuclein inside cells, the biradicals necessary for DNP-enhancement must be introduced into the cells, cell viability must be maintained during measurements, and computational tools must be developed to analyze the spectra and interpret them in terms of conformational sampling. We introduced isotopically labeled α-synuclein and the biradical AMUPol into cells via electroporation. We maintained cell viability throughout the process of rotor freezing, measurements, and thawing by freezing the cells in a controlled manner and cryogenically inserting the rotor with the cells into the spectrometer probe. We developed computational tools that allow us to analyze the DNP MAS NMR spectra of IDPs in terms of detailed conformational sampling. Our results are consistent with previous observations and show that α-synuclein is intrinsically disordered inside cells. We show that DNP-enhanced MAS NMR spectroscopy can be used for detailed characterization of IDPs in intact, viable cells.

Multiscale NMR for deciphering the lipidic metabolism of microalgae

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Microalgae are photosynthetic cells with a high biochemical diversity and a great potential to produce valuable biomass. Only a minor part of the millions of species living on earth are currently known, therefore microalgae form a subject with a strong investigation potential. Some microalgae are well known to naturaly accumulate lipids under nitrogen starving stress conditions [1]. This behavior is essential because it forms the basis for the production of third generation biofuel, a promising alternative energy. Microalgae are generally detected by cytometry at the cell scale, or by Infra-red and Raman spectroscopies but these techniques unfortunately fail to probe intra-cellular biochemicals due to the interfering signal of the culture water.

In this context, we are exploring the potential of multiscale NMR as a non destructive for the analysis of entire microalgae cells and their lipidic extracts. In order to better understand the lipidic metabolism, it is essential to identify and quantify lipids with advanced techniques in microalgae aqueous cultures. To reach this goal, we will show that high field NMR provides the best sensitivity and resolution to decipher complex lipidic extracts, in particular through quantitative conventional 1D and 2D tools that we recently developed [2].
Moreover, it becomes essential to optimize the culture conditions and processes for improving the production of valuable biomass. In the context of following bioprocesses by low field NMR [3], a 43 MHz apparatus is used for the first time for non-invasive monitoring of entire microalgae in their cultivation medium. Thanks to the implementation of a gradient coil in the hardware, the water peak representing more than 95% of the culture could efficiently be removed - 27,000 times less than in ^{1}H - with a WATERGATE W5 scheme [4]. The main peak from *in vivo* lipids is then observable after 1h of W5 acquisition and can be assigned and quantified as the total lipids [5]. In order to monitor lipid in-cell accumulation, a benchtop spectrometer was coupled to a photobioreactor, an automated device for microalgae cultivation. For the first time, the real-time online and *in vivo* access to lipid production kinetics during 3 weeks was monitored with this NMR hyphenated apparatus, providing a very similar profile to GC off-line reference method [6]. Our recent results with compact flow NMR monitoring open the avenue for any *in vivo* intracellular bioprocesses involving the detection and quantification of metabolites overtime. Works for controlling microalgae bioprocesses assisted by compact NMR are underway.

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Session 6

In situ observation of membrane proteins using electron spin resonance spectroscopy

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The properties of the biological membrane can significantly modulate the structure and function of the embedded membrane proteins. The lipid phase, raft/domain formation, curvature, and the lateral pressure etc., of the membrane is determined by the lipid composition. On the other side, membrane proteins can induce hydrophobic mismatch, lipid disorder, or clustering of specific lipids within the bilayer. Thus, the structure and function of a membrane protein is fine-tuned through the mutual interaction between the membrane and the protein, and it is crucial to observe membrane proteins within their native environments. Spectroscopically, this is a challenging task owing to their low expression levels and the difficulty for specific labeling in the complex membrane environment. The outer membrane of Gram-negative bacteria is an asymmetric bilayer consisting of phospholipids and lipopolysaccharides, which could not yet be experimentally reconstructed. Here we show that pulsed electron-electron double resonance (PELDOR/DEER) spectroscopy is a versatile tool for *in situ* observation of outer membrane proteins. Using the cobalamin transport complex as a model system, we demonstrate the potential of this approach to observe protein-ligand and protein-protein interactions as well as the conformational changes in isolated native outer membranes or intact *E. coli*[1-3]. An orthogonal labeling strategy further enhances the selectivity and sensitivity of such experiments[4-5]. We extended these methodical developments for observing the conformational space of BamA, which is the insertase of the β-barrel assembly machinery (BAM) complex[7]. BAM consists of BamA and four other interacting lipoproteins (BamB-E). It is responsible for the folding and insertion of the majority of outer membrane proteins in Gram-negative bacteria and is one of the most promising targets for rationally designed antibiotics. We show that the native membrane considerably modulates the conformational space of the BamA and it can transverse through multiple conformations in the absence of any of the lipoporteins[6].

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Tracing Protein Native Radicals in Living Cells with EPR

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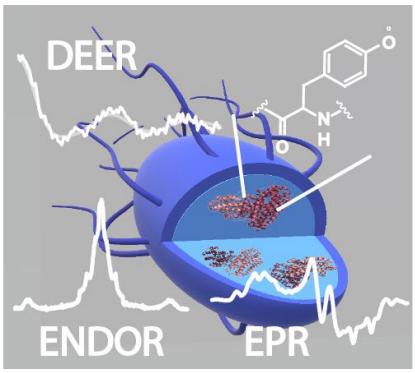

Determining the structure of biomolecules in their intracellular environment is fundamental to understand their function. Yet, achieving high resolution within the cell possesses a great challenge. In this work, we employed advanced electron paramagnetic resonance (EPR) spectroscopic methods to characterize the structure and dynamics of Y_{122}•, the stable tyrosyl radical in *E. coli* ribonucleotide reductase (RNR), in whole cells at atomic resolution. Y_{122}• initiates the unique radical transfer reaction over 35 Å in class Ia RNR that catalyses the biosynthesis of DNA building blocks in *E. coli* and humans.[1] Therefore, determining *in vivo* structure and distribution of Y_{122}• in RNR is essential to understand RNR function in its native intracellular environment. First, we employed multi-frequency EPR (9, 34 and 94 GHz) spectroscopy to characterize the generated Y_{122}• radical in whole *E. coli* cells. Our experimental data combined with spectral simulations demonstrated that the structure and environment of Y_{122}• in the cells are highly similar to those of Y_{122}• *in vitro*. Next, we performed orientation-selective ^{1}H ENDOR spectroscopy at 34 GHz to probe the H-bonding environment of Y_{122}• in living cells. The analysis of our ENDOR data displayed that the number and strength of H-bonds coupled to Y_{122}• in the cells and *in vitro* are same. At last, we employed DEER spectroscopy to measure the distance between Y_{122}•'s residing in each β monomer of *E. coli* RNR in whole cells. These measurements give insights into *in vivo* Y_{122}• per β2 distribution for the first time supporting the key role of Y• concentrations in regulating RNR activity.[2] Additionally, we have site-specifically incorporated 2,3,5-trifluorotyrosine (F_3Y) at residue 122, generated and identified its radical form F_3Y_{122}• in the cells, and obtained *in vivo* distances between F_3Y_{122}•s. This marks the first spectroscopic verification of generation of an unnatural amino acid radical in whole cells providing a new possibility for investigating the structure and role of tyrosyl radicals involved in fundamental processes such as photosynthesis, reduction of O_2 to water and DNA repair in living cells.

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Protein-drug interactions monitored by time-resolved NMR in human cells

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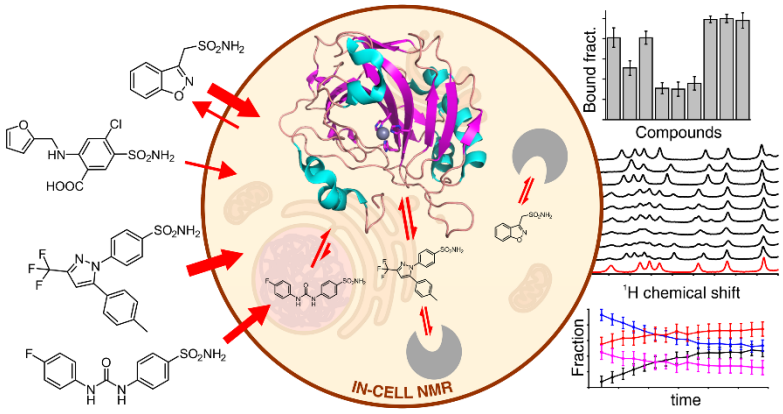
In-cell NMR can investigate protein conformational changes and chemical modifications at atomic resolution directly in living cells. The approach has recently shown great potential in the context of drug development, as it can investigate directly the interaction between a ligand and its target protein in its physiological environment. As such, in-cell NMR can provide precious insights on the intracellular drug-target interaction, that can be beneficial to assess the efficacy of active compounds at an early stage of drug development and to increase the chances of success in the subsequent pre-clinical and clinical studies. Furthermore, NMR bioreactors can greatly improve the cell sample stability over time and allow monitoring the evolution of intracellular processes, including protein-drug interactions, by time-resolved in-cell NMR. Here, the latest developments of in-cell NMR applied to protein-drug interactions in human cells are overviewed. First, drug screening in human cells is applied to investigate a set of novel carbonic anhydrase (CA) inhibitors, which are found to bind the intracellular target in a dose- and time-dependent manner.[1] The same approach is applied to study the off-target binding to CA of drugs originally developed to interact with other targets.[2] Then, an optimized design of in-cell NMR bioreactor is applied to measure drug diffusion and binding in real time.[3,4] Finally, a novel application of in-cell NMR is reported, where intracellular ligand binding affinities are measured in human cells by competition binding experiments.



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Poster Presentations

Day 1

Caught in the cell: the wide-open conformation of MsbA in *E.coli*

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ABC transporters are membrane proteins that couple the energy deriving from the binding and hydrolysis of ATP to large conformational changes that enable substrate translocation across membranes. MsbA is a homodimeric ABC exporter that flips lipid A - the precursor of lipopolysaccharide (LPS) - across the cytoplasmic membrane and is therefore essential for the survival of *E. coli*[1].
In this study, we use spin-labeled nanobodies (i.e. specific single-domain antibodies) as conformational reporters targeting MsbA, to investigate the degrees of separation of the nucleotide binding domains (NBDs) in apo conditions, an unresolved question in the field. The advantage of using spin-labeled nanobodies relies in the possibility of investigating membrane proteins in different environments, from in vitro to in situ without the need of site-directed mutagenesis, as previously shown by our groups[2]. Ideally, in fact, to understand structure and dynamics of membrane proteins, they should be investigated in situ, namely in the context of the living cell. However, structural elucidation of membrane proteins mostly requires their extraction from the native membrane environment with detergents. Membrane mimics such as liposomes or nanodiscs serve as surrogates of the native lipid bilayer and are widely used for structural and functional analyses of membrane proteins.
We make use of DEER (Double Electron Electron Resonance) to reliably measure distances between pairs of Gd(III) spin probes introduced in a non-state-specific nanobody targeting the two NBDs in the homodimer. Data obtained in detergents, proteoliposomes, nanodiscs and cellular membranes from *E. coli* during the catalytic cycle will be presented. Intriguingly, we found that nanodiscs, in stark contrast to all other environments, funnel one specific conformation with closed NBDs of the apo state of MsbA. The EPR-derived structural findings in nanodiscs are corroborated by cryo-EM structures obtained on the same spin-labeled samples. This funneling effect in the energy landscape of a protein might not be an isolated case, rather it may occur in proteins characterized by a large degree of heterogeneity and flexibility. Finally, this study represents the first *in-cell* investigation of an inner membrane protein in its native environment via DEER spectroscopy, which has been possible thanks to the use of the above-mentioned nanobody strategy.

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The use Gd(III) with a narrow ZFS for DEER

Janet E. Lovett[1], Anokhi Shah[1], Hassane EL Mkami[1], Robert I. Hunter[1], Paul A. S. Cruickshank[1], Michael J. Taylor[1], Graham M. Smith[1], Amandine Roux[2], Matthieu Stark[2], Jackie A. Mosely[2], David Parker[2], Michael Stevens[3], David G. Norman[3], Akiva Feintuch[4], Mian Qi[5], Adelheid Godt[5]

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Gadolinium spin labels are a promising approach for EPR detection of proteins in the cell. This contribution will demonstrate a Gd(III) spin label that binds to a cysteine sulfhydryl (Figure 1) retaining its very narrow central transition[1]. The use of this label, [Gd-sTPATCN]-SL, for double electron electron resonance (DEER) EPR measurements outside cells will be demonstrated. The contribution will then move on to show that Gd(III) may be optimally measured with DEER by avoiding the central transition altogether[2].

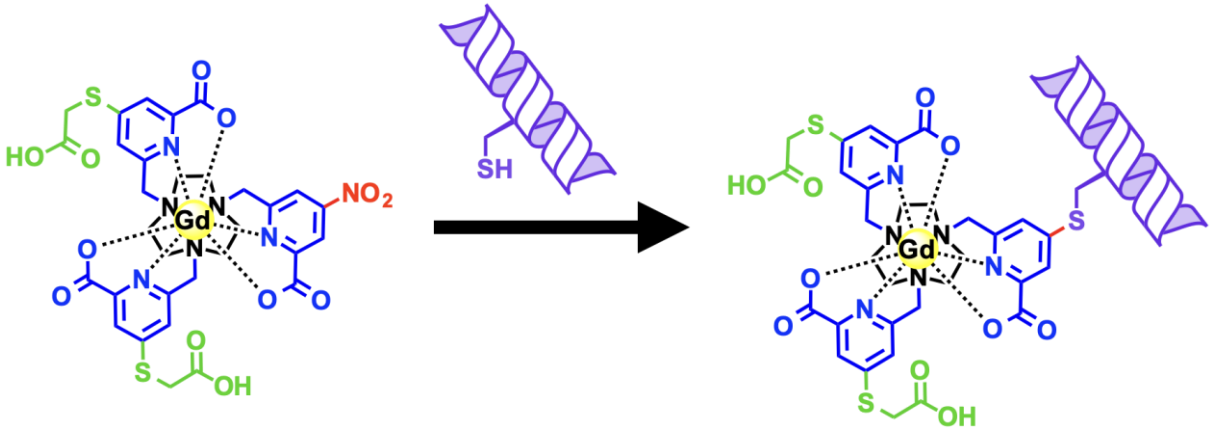

Figure 1: Labelling a cysteine residue on a protein with [Gd-sTPATCN]-SL.

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Insights into the intrinsically disordered region of the human protein N-myc downstream regulated gene 1 by NMR and EPR spectroscopy.

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N-myc downstream regulated gene 1 is a human protein of 43 kDa, member of the NDRG family. As well as the other NDRG proteins, NDRG1 is characterized by a N-terminal domain presenting an α/β hydrolase fold and a flexible region of 32 residues [1]. Differently to the N-terminal domain, the 83 residues long C-termini of the protein is unique and is predicted to be intrinsically disordered. This region contains a 3 times repeated sequence of ten residues showing nickel binding activity [2] and undergoing functional serine and threonine phosphorylation by different kinases [3]. NDRG1 functions as regulator of tumor progression: its expression is up-regulated in lung cancer and associated to higher cancer aggressiveness and worse prognosis [4][5] . For this reason, it has been proposed as a new possible target in lung cancer therapy, the first cause of tumor-related death worldwide.

This work focused on the structural, biophysical and biochemical characterization of NDRG1, focusing the attention on its C-terminal domain, heterologously expressed and purified from *Escherichia coli.* The techniques of circular dichroism, light scattering and isothermal titration calorimetry were applied to study its structural and functional properties. In particular, the C-terminal tertiary structure was examined by NMR spectroscopy that showed an HSQC spectrum typical of an intrisincally disordered protein. Its structural dynamics is currently under investigation by site-directed spin labeling (SDLS) approach coupled to Electron Paramagnetic Resonance (EPR) in vitro and in cell.

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Unnatural Amino Acid Radicals within the Cells: *E. coli* Ribonucleotide Reductase as a Paradigm

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The *E. coli* ribonucleotide reductase (RNR) catalyzes the biosynthesis of DNA building blocks and represents a paradigm for class Ia enzymes including human RNR. The catalysis involves at least five redox active amino acid residues. The stable di-iron tyrosyl radical (Y_{122}•) cofactor is the only redox active amino acid characterized in the wild-type RNR. Spectroscopic detection of other redox-active amino acids in wild-type enzyme has not been achieved due to the rate-limiting steps in the catalytic reaction, which are the conformational changes upon substrate and allosteric effector binding.

The involvement of three other Y• intermediates in RNR catalytic reaction was evidenced by site-specific incorporation of unnatural amino acids (UAAs) with altered pK_as and reduction potentials such as DOPA, NH_2Y, NO_2Y, and F_nYs.[1,2] The radical formation on these amino acids allowed the characterization of the tight α2β2 complex and provided insights into proton-coupled electron transfer steps within RNR. The near-atomic-resolution cryo-EM structure of the active α2β2 complex was achieved by employing F_3Y_{122}•.[3] Through the help of this UAA-tool, the knowledge on *in vitro* protein structure has now accumulated over the years; yet, little information exists under *in vivo* conditions.

Very recently, we generated and characterized Y_{122}• in whole *E. coli* cells via advanced electron paramagnetic resonance (EPR) spectroscopic methods.[4] Here, we extend this technique to spectroscopically verify the generation of F_3Y_{122}• in whole cells for the first time. Multi-frequency EPR and DEER experiments hereby confirmed, that the structure of RNR does not change upon F_3Y incorporation into the cells. These studies provide first steps towards insights into RNR catalysis under physiological conditions. Furthermore, it provides the possibility of unraveling the *in vivo* structure and role of tyrosyl radicals involved in other fundamental processes such as photosynthesis, reduction of O_2 to water, and DNA repair.

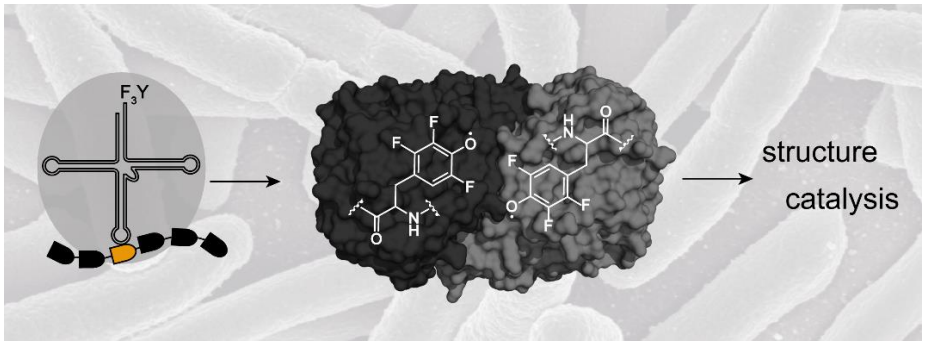

Figure 1: Incorporation of F_3Y into *E. coli* RNR delivers insights into *in vivo* protein structure and catalysis.

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Sensing pore formation in the mitochondrial outer membrane via EPR and UV/Vis spectroscopy

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Apoptosis is a necessary process for the healthy development of a multicellular organism. The Bcl-2 family of proteins is tightly associated with the mitochondrial pathway of apoptosis. Bax is the key pore former member of the family, cBid has been shown to be an activator of Bax, while Bcl-xL inhibits apoptosis interacting with cBid and Bax[1][2]. Here we use a minimal interaction network consisting of full length Bax, cBid and Bcl-xL to trigger and/or inhibit pore formation in the outer membranes of isolated mitochondria from rat liver. Membrane permeabilization is monitored with temporal resolution through the efflux of cytochrome c via UV-vis spectroscopy. In addition, using electron paramagnetic resonance (EPR) we found that there is a time-dependent release of metal cofactors from the mitochondria when permeabilization occurs, which is accelerated by cBid and inhibited by Bcl-xL. Therefore, we can monitor the effect of the Bcl-2 tripartite switch *in organelle*, which poses the basis for the EPR characterization of the structural transformation of the protein players at onset of apoptosis in physiological membranes.

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SYNTHESIS OF NEW FLUORESCENT CHEMICAL PROBES TARGETING BACTERIAL EFFLUX TO EARLY DETECT AND FIGHT THE FIRST BARRIER IN GRAM-NEGATIVE BACTERIA ANTIBIOTIC RESISTANCE

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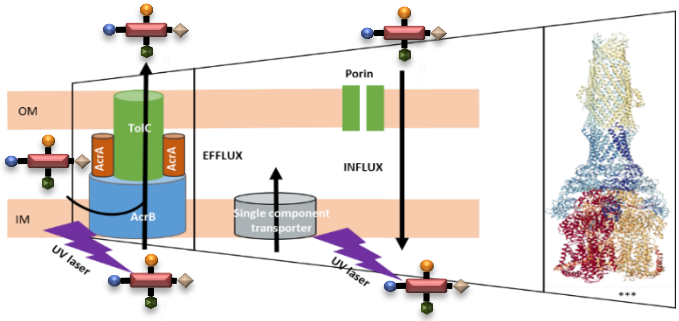
Antimicrobial resistance (AMR) is one of the more serious problem of Public Health. The 2014 WHO's global report[1] and the more recent one of J. O'Neal[2] outlined worrying levels of AMR worldwide involving therapeutic failure of large classes of antibiotics especially in Gram-negative bacterial diseases. Resistance is a natural response of microorganisms like bacteria, allowing them to counteract pharmacological effects of antibiotic agents. A lot of mechanisms as membrane permeability variations, enzymatic degradations and intracellular target modifications contribute to the Multi-Drug Resistance (MDR) phenotypes. Furthermore, efflux overexpression is a major early stage trigger in the MDR setting up[3]. Especially, Resistance- Nodulation-Cell-Division (RND) efflux pump superfamily constitutes a tripartite protein system which is the resistance first line in Gram-negative bacteria[4]. They can extrude structurally different substances outside the cell space; hence antibiotics struggle to reach effective intracellular concentrations. Thanks to the spectrofluorometric method developed in our laboratory[5] and by a drug design approach, we are working on the use of new natural product derivatives with high and linear fluorescent signal with intracellular concentration and cells number; not forgetting good efflux substrate properties useful to detect their capacity to cross membranes and to accumulate in bacteria according to pharmacomodulations. These compounds must not have cytotoxic effects on bacteria growth to minimize their resistance impact on new drug discovery and do not interfere significantly with the host's biochemistry. Starting from a screening of chemical libraries of natural products and by a medicinal chemistry approach, pharmacophores and their positions were identified on a fluorescent scaffold. Then, S.A.R. studies allowed us to highlight physicochemical properties responsible not only for a high fluorescence intensity but also for substrate efflux-pump features according to doses and time accumulation on *E.coli* and other Gram-negative bacteria. These results have guided targeted pharmacomodulations to improve the design of a real-time diagnosis trial allowing antibiotic drug accumulation monitoring, identification, minimization and prevention in the early appearance of efflux bacterial resistance in clinic while decreasing patient exposure to antibiotics.



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Poster Presentations

Day 2

Monitoring tRNA maturation and identifying modification circuits with NMR

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Although the biological importance of post-transcriptional RNA modifications in gene expression is widely appreciated[1], methods to directly detect the introduction of these modifications during RNA biosynthesis are rare and do not easily provide information on the temporal nature of events[2]. Here we introduce 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 found that modifications are introduced in a defined sequential order, and that the chronology is controlled by cross-talk between modification events[3]. In particular, we uncovered a strong hierarchy in the introduction of the T54, Ψ55 and m^1A58 modifications in the T-arm, and demonstrate that the modification circuits identified in yeast extract with NMR also impact the tRNA modification process in living cells. The NMR-based methodology presented here could be adapted to investigate different aspects of tRNA maturation and RNA modifications in general.

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In-cell Double Electron-Electron Resonance at nanomolar protein concentrations

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Double Electron-Electron Resonance (DEER or PELDOR) is an established Pulsed Dipolar Spectroscopy (PDS) technique to measure inter- and intra-spin distances within 1.5 to 8 nm in biomolecules in *vitro*[1]. Emerging in-cell PDS approaches aim to address protein conformations in their native environment at physiologically relevant protein concentrations[2]. Since different proteins exist in cells at different concentrations varying from high micromolar (e.g. α-synuclein) to low nanomolar (e.g. pro-apoptotic Bax), establishing the concentration limit for PDS techniques in cell is of high relevance. Here, we present Double Electron-Electron Resonance (DEER) study on T4 lysozyme electroporated into HEK cells demonstrating the feasibility to measure distances up to 4.5 nm at nanomolar concentration between protein's sites carrying commercial gadolinium spin labels.

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Structural Insights into Activation of a Human G Protein-Coupled Receptor by Membrane Phospholipids

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The 826 human G protein-coupled receptors (GPCRs) are involved in nearly every physiological process and comprise the largest class of "druggable" proteins. For over the past decade, crystal and cryo-EM structures have focused on revealing molecular details of GPCR interactions with small molecules and intracellular partner proteins, which are well-established regulators of GPCR function. More recently, literature data from biological and biophysical studies have demonstrated that phospholipids found in the endogenous membrane environment also control GPCR function and act as allosteric modulators. In particular, phospholipids with charged headgroups have been reported to be especially important regulators of GPCR function and facilitate the formation of signaling complexes.

Using nuclear magnetic resonance (NMR) spectroscopy in aqueous solutions, we investigated the structural basis for the regulation of GPCR activity by phospholipids in nanodiscs containing the human A_{2A} adenosine receptor (A_{2A}AR), a representative class A GPCR. Lipid nanodiscs containing mixtures of uncharged and charged phospholipids were produced, yielding homogeneous preparations over a wide range of lipid compositions. NMR data of uniformly stable-isotope labeled A_{2A}AR showed the receptor was properly folded in lipid nanodiscs and shared a similar global conformation to detergent micelle preparations of A_{2A}AR. Using ^{19}F NMR with conformationally-sensitive probes located at the A_{2A}AR intracellular surface, we systematically investigated the influence of lipid composition over a wide range of mixtures of charged and uncharged phospholipids. A_{2A}AR complexes with antagonists showed almost no response to variation in lipid composition. In striking contrast to this, A_{2A}AR complexes with agonists showed a clear response to changes in the relative proportion of charged phospholipids. NMR data of agonist complexes in nanodiscs containing lower proportions of charged lipids were highly similar to data of antagonist complexes, showing a higher proportion of neutral lipids negated the efficacy of bound agonists. As the proportion of charged phospholipids increased, the relative population of an A_{2A}AR active conformation also increased. The dependence of the receptor response to bound agonists on lipid composition suggested specific interactions between lipid headgroups and charged amino acids at the A_{2A}AR intracellular surface underlie the regulation of activity. Results from this study indicate that lipid composition must be carefully selected in studies of GPCRs in nanodiscs. Our data also provide a potential molecular mechanism for how changes in membrane composition, due to disease for example, can directly impact the efficacy of drugs.

Construction and characterization of micro reactors of Chlorocatechol 1,2-dioxygenase using low complexity domains as molecular adhesives

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Abstract

Low Complexity Domains (LCDs) are amino acid residues sequences with low diversity, often repetitive. These domains, when used as molecular adhesives at C- and N- terminals of proteins, are capable of forming Liquid-Liquid Phase Separation (LLPS). LLPS is a molecular condensate that undergoes phase transition from a dissolved phase to a protein-rich phase, with liquid like properties. In this work, we propose to use LCDs on Chlorocatechol 1,2-Dioxygenase (CCD), an enzyme that catalyze degradation reactions of polynuclear aromatic compounds, having great biotechnological applications as micro reactors *in vitro*.

Materials and Methods

The initial phase of this project was based on the construction of the DNA coding for the chimera protein, named LCD2-CCD. Firstly, the CCD DNA cloning was performed using the PCR (Polymerase Chain Reaction) process. Then, the enzyme and the previously purchased vector (pET28a, containing the LCD's code) underwent reaction with the enzymes BamHI-HF and HindIII-HF, creating cohesive ends , to perform cohesive binding reactions with T4 DNA Ligase. The purification steps used were 1% Agarose Gel Electrophoresis, which can also estimate the DNA size, and a liquid mini chromatography by commercial kit, separating the DNA and other compounds. To verify efficiency of the process, a bacterial transformation by heat shock was made in DH5-α competent cells in the presence of antibiotic Kanamycin. As positive control to LLPS, we made a construction of DNA coding (as described to CCD) for a chimera GFP (called LCD_2-GFP), expressed in E. coli and characterized by DIC microscopy, DLS, Spectrophotometry, Fluorimetry and Circular Dichroism.

Results

The gene coding for CCD is still in construction, where we obtained 750 pb fragments on PCR, observed in Agarose Gel Electrophoresis. The restriction reactions have been successful to the vector (pET 28a, containing LCD's code), and now, we are focused on optimizing the restriction reactions for CCD DNA coding and for binding reactions between vector and insert. The gene coding for LCD_2-GFP was obtained, so the expression and purification was initiated and concluded. The characterization process is in the final steps and we could determine the pH and thermal stability of their LLPS by DLS and DIC microscopy. The protein folding stability was determined by Spectrophotometry and Circular Dichroism, using native GFP as positive control.

Conclusions

The project is still in an initial stage, but the success in obtaining the DNA construction of the chimera GFP (and their characterization), and the progress on chimera CCD DNA coding allow us now to move forward to express, purify and characterize the LLPS formed by.

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Combined EPR and NMR study of a novel periplasmic protein involved in bacterial copper resistance.

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CopI is a periplasmic protein of 15kDa which is induced with high copper concentration and is directly involved in the copper resistance of the purple bacterium, Rubrivivax gelatinosus[6]. So far the 3D structure and the copper resistance mechanism are unkown.
EPR studies we have performed show that there are two specific Cu(II) binding sites: a green cupredoxin T1.5 site conferring its colour to the protein, and a square planar (T2) site located in the N-terminal region. The latter site is not well conserved among CopI analogs and not required for copper resistance. On the other hand, the green cupredoxin site, for which only few examples are known in the literature, is fully conserved and mandatory. To get insight into the mechanism of this novel protein, we are first performing a combined EPR and NMR study on the non labelled and the labelled N^{15} et C^{13} protein to better characterize these sites structurally.
The results we have obtained so far will be presented and discussed in the context of related copper proteins.

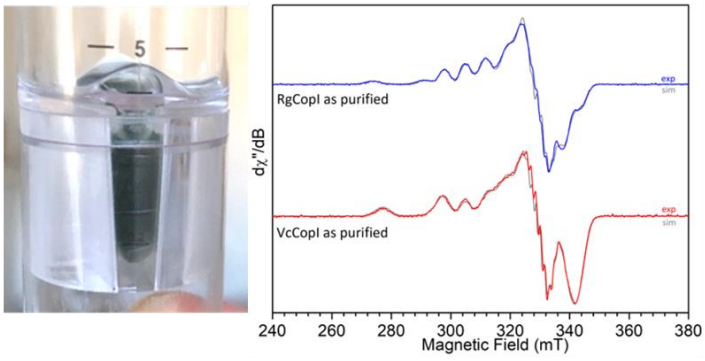

Figure 1. Left : purified soluble fraction containing CopI. Right : EPR spectrum of CopI from *R. gelatinosus* (blue) and CopI from *V. cholerae* (red).

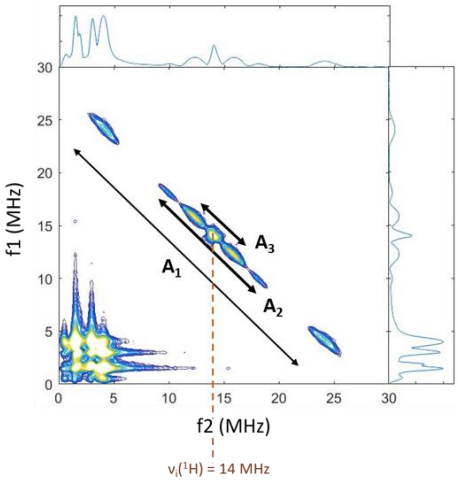

Figure 2. 6-pulse HYSCORE spectrum of the green cupredoxin site.

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The speciation of zinc complexes with chloroquine ligand

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Chloroquine (CQ) is a first choice drug against several diseases, such as malaria and lupus, and has recently been used for the emergency treatment of COVID-19[1]. Lysosomal targeting underlies the possible mechanism of action of CQ[2]. Considering that various pathogens, including SARS-CoV-2, use the endocytic pathway to invade host cells[3], CQ can inhibit pathogen entry through endosomal alkalinization. Other studies indicated that CQ can act as a Zn ionophore and that its cytotoxicity is enhanced by zinc[4]. Zn ions, which lie in lysosomes too, can block coronavirus replication by inhibiting RNA synthesis *in vitro* and Zn ionophores can stop the pH-dependent replication of these viruses in cell cultures[5]. Although the combination of CQ with Zn has shown some positive results for the treatment of COVID-19[6], the authorization for the emergency use of this drug and hydroxychloroquine was revoked by FDA due to the high risk/benefit ratio[7]. Moreover, the NIH recommended against the use of CQ for COVID-19 except as part of clinical trials[8]. Hence we deemed of importance to investigate the coordination chemistry of CQ with the aim of obtaining mechanistic insights into the impact of CQ on Zn binding and intracellular distribution. Based on previous findings by Navarro *et al.*[9-10], we studied the effect of Zn salt additions on both purified CQ and its diphosphate form. The Zn complexes were characterized by solution NMR, ESI-MS, and X-ray absorption methods. The results showed that, depending on the pH and other solution conditions, CQ can bind Zn through different N donor atoms. The findings may lead to the optimization of prophylactic and therapeutic strategies against microbial infections.

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