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Warsaw, 5-8 May 2014

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Book of Abstracts

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Biological and Chemical Research Centre

The University of Warsaw Biological and Chemical Research Centre (CNBCh UW) is a leading-edge research and development centre located on the Ochota Campus. It gathers the most talented young scientists, as well as experienced academic staff. Thanks to European Union funds, the CNBCh UW will become one of the most modern research institutions in Europe, with capacity for carrying out advanced studies in life sciences, at the interface of chemistry and biology, contributing to the development of science and economy.

Establishment of CNBCh UW is a part of the program of modernisation and extension of life science faculties and infrastructure on the Ochota Campus. As a centre of innovative technologies, it enables research and development activities in the field of modern technology, strategically important for the country's progress, such as: biotechnology, nanotechnology, chemical, biochemical and energy technologies, environmental monitoring, protection and rehabilitation, modern technologies in informatics, pharmacy and medicine.

Laboratories of the CNBCh UW are provided with cutting-edge research equipment, among others: an animal PET scanner, sets for high performance chromatography, mass spectrometers, diffractometers, spectroscopes: CD, Raman-CD, IR, UV, PES, XPS and NanoSIMS, 600 MHz & 800 MHz NMR spectrometers, microscopes (including scanning electron microscopes), sequencers, cyclers and fluorimeters.

The CNBCh UW project is financed by the Operational Programme Innovative Economy, Priority II. R&D Infrastructure. Measure 2.1. Support for development of research infrastructure of scientific entities. The total cost of this project is 294 million Polish zlotys (€70 million).

INNOVATIVE ECONOMY
NATIONAL COHESION STRATEGY

EUROPEAN UNION
EUROPEAN REGIONAL
DEVELOPMENT FUND

UNIVERSITY
OF WARSAW

University of Warsaw
Biological and Chemical
Research Centre

Dear Colleagues,

Welcome to the 4th Bio-NMR Annual User Meeting in Warsaw, Poland. Bio-NMR is a project of the 7th Framework Program of EU, aiming at the advancement of nuclear magnetic resonance spectroscopy and its applications. The Bio-NMR project is the successor of a series of successful EU-funded projects of this type, such as EU and East-NMR.

The conference will feature presentations dedicated to the highlights in the field of NMR, and defining the state of the art in the specific areas of joint research activities. Users of Bio-NMR facilities will have an opportunity to present their achievements.

The primary aim of the conference is to provide an effective forum for discussions, at both formal and informal levels. The high number of registered participants should guarantee a very stimulating environment, and the program maximizes the time the participants spend together.

This is the final conference organized as part of the Bio-NMR project. The previous, exceptionally successful meetings have proven to be at the forefront of biomolecular NMR research. We hope that the present meeting will continue this tradition.

During the past four years, the Bio-NMR project has allowed us to develop and expand the scientific knowledge in the field of nuclear magnetic resonance spectroscopy and its applications. The project facilitated the acquisition of important results in biological NMR, and we hope that this meeting will once again allow us to exchange scientific experience and help find innovative solutions for all users from the Bio-NMR group.

Best wishes,

Wiktor Koźmiński

General Information

Internet Access

Free access to high-speed wireless internet is provided on the entire conference area (network name "SoundGarden WiFi", no password required). Individual 8-digit password for the WiFi in your rooms can be found on TV in the Internet/Language tab. The network name is your room number.

Check-in / Check-out

Check-in starts at 15:00. Rooms may be available earlier, you are encouraged to ask at the hotel reception. In the meantime, you can store your luggage in the safe room, if needed. Please check out no later than 11 am on the day of your departure.

Lectures

Oral presentations will take place in the Symphony Hall on the ground floor. Speakers should contact the technical assistant in the conference room before the session to ensure proper display of their presentations. During the lecture, the elapsed time will be displayed in front of the speaker. The chairpersons are asked to ensure that presentations do not exceed the allotted time limit. Five-minute slots should be reserved for discussion and switching of presentations.

Meetings

The *User Group Meeting* will take place in the Symphony Hall on the ground floor on Monday, May 5, 21:00. The *Local Operator Meeting* will take place in the Concerto 1 Hall on the 1st floor on Monday, May 5, 21:00. The *Bio-NMR General Assembly* will take place in the Symphony Hall on Tuesday, May 6, 21:00 – all participants are invited to attend.

Posters

Please pin your posters to the boards in the foyer to Symphony Hall upon arrival, using the provided pins. Posters are numbered by the page they appear in the abstract book (in alphabetical order). Posters should remain available during the entire conference. There are two poster sessions for *odd* (Wednesday, 16:55–19:00) and *even-numbered* posters (Thursday, 14:30–16:30). Presenting authors are asked to be present in front of their posters during the session. Posters should be removed before the dinner on Thursday, May 8. After this time, the remaining posters will be discarded.

Reimbursement of Travel Expenses

Those of you who bought tickets on your own are eligible to receive reimbursement of your travel expenses. Please contact us at the registration desk to fill and sign a form. Prepare IBAN/Swift code of your bank and the full number of your account. Travel costs are reimbursed up to €350. Electronic tickets and/or invoice is required. If in doubt, contact us by e-mail: `kmadrak@chem.uw.edu.pl`

Meals

Breakfast is served between from 7:00 and 9:30 in the hotel restaurant on the ground floor. Lunches and dinners (on Monday, Tuesday and Thursday) will be served in the foyer to Symphony Hall. The Conference Banquet (on Wednesday evening) will take place in the Symphony Hall. Coffee breaks will take place in the foyer to Symphony Hall. Since many

attendees may leave on Thursday evening, we will carry out a survey for the dinner on that day.

Emergency Numbers

Police	**997** or **112**
Ambulance	**999** or **112**
Hotel Sound Garden reception desk	+48 22 279 14 00
AUM Organizers' hotline	+48 505 807 699 (Jan Stanek)
	+48 509 908 760 (Krzysztof Kazimierczuk)

Travelling

You have been given a 20-minute ticket to get back to the airport. Please return it at the registration desk if you don't need it.

Public Transport

`www.ztm.waw.pl`	Public Transport Authority: timetables, tariff and travel planner, infoline 19 115 (24h)
`warszawa.jakdojade.pl`	a good online travel planner

Taxis (selection)

You are advised to confirm the tariff when ordering the taxi. It should also be clearly displayed on the passenger's window.

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+48 22 191 91	MPT	+48 22 644 44 44	Sawa Taxi
+48 22 196 25	Volfra Taxi	+48 22 196 44	Wawa Taxi

Weather Forecast

`www.meteo.pl`	numerical short-term forecast (select MODEL UW and Warszawa)
`www.pogodynka.pl`	official service of the Institute of Meteorology and Water Management (numerical and synoptic forecast)

Sightseeing

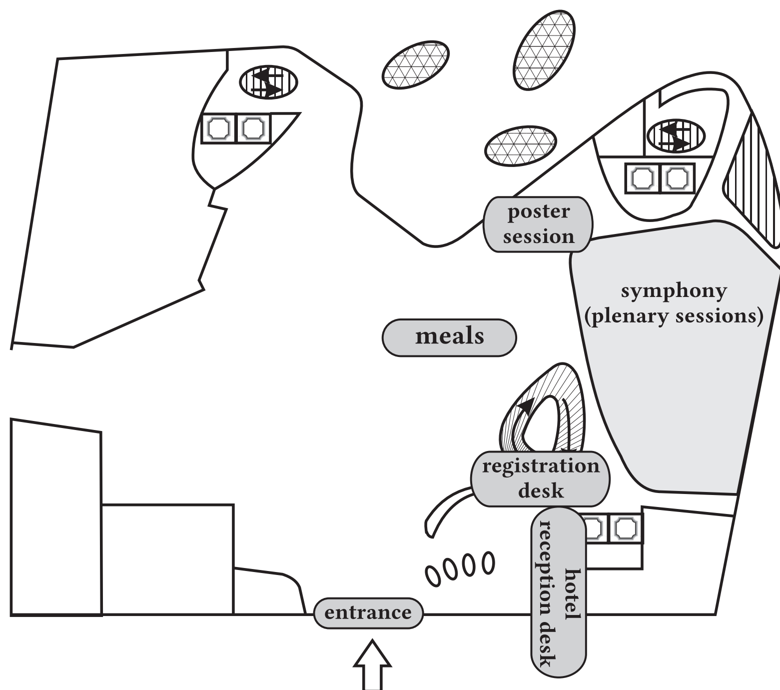
For some suggestions, see: `www.warsawtour.pl/en` and `www.um.warszawa.pl/en`
Tourist information: +48 22 194 31, `info@warsawtour.pl`
You can also find a city map and some brochures in your bag.

Conference WWW Page

`nmr.cent3.uw.edu.pl/bionmr2014`

Venue

Sound Garden Hotel
Żwirki i Wigury 18
02-092 Warszawa
`info@soundgardenhotel.pl`


Ground floor
poster session
symphony (plenary sessions)
meals
registration desk
hotel reception desk
entrance

Program Outline

Monday, May 5	
11:00	*Arrival* *Registration*
14:10	Wiktor Koźmiński Paweł Kulesza Ewa Bulska Claudio Luchinat
15:00	Harald Schwalbe
15:35	Lukáš Žídek
16:10	*Coffee Break*
16:40	Mikael Akke
17:15	Natalia Kulminskaya
17:50	Bernhard Brutscher
18:25	Alexandre Bonvin
19:30	*Dinner*
21:00	User Group Meeting Local Operator Meeting

Tuesday, May 6	
9:00	Robert Konrat
9:35	Steve Matthews
10:10	Monika Baumann
10:30	*Coffee Break*
11:00	Lucia Banci
11:35	Bahareh Eftekharzadeh
11:55	Ago Samoson
12:30	Ivan Corbeski
12:50	*Lunch*
14:30	Isabella Felli
15:00	Vladislav Orekhov
15:30	Ēriks Kupče
16:00	Krzysztof Kazimierczuk
16:30	Jiří Nováček
17:00	*Coffee Break*
17:30	*Introduction*
18:00	Ronald Frank
18:30	Chris Morris
19:30	*Dinner*
21:00	BioNMR General Assembly

Wednesday, May 7	
9:00	Marek Potrzebowski
9:35	Ronald Melki
9:55	Jens Danielsson
10:30	*Coffee Break*
11:00	Ildefonso Marin-Montesinos
11:20	András Perczel
11:55	David Dias
12:15	Lieven Buts
12:50	*Lunch*
14:30	Joeri Veransdock
14:50	Kristina Haslinger
15:10	Michael Assfalg
15:30	Angeles Canales
15:50	Ulrich Günther
16:25	*Coffee Break*
16:55	**Poster Session** (odd)
19:30	*Banquet*

Thursday, May 8	
9:00	Hartmut Oschkinat
9:35	Edward Hookway
9:55	Stefano Gustincich
10:30	*Coffee Break*
11:00	Federico Rastrelli
11:20	Massimo Bellanda
11:55	Carl Diehl
12:15	Kurt Zatloukal
12:50	*Lunch*
14:30	**Poster Session** (even)
16:30	*Coffee Break*
17:00	Nicholas Dixon
17:35	Kristaps Jaudzems
17:55	Hartmut Schäfer
18:30	*Closing Remarks*

Detailed Program

	Monday, May 5
11:00	Arrival Registration
	Chairperson: Wiktor Koźmiński
14:10	Wiktor Koźmiński CNBCh, University of Warsaw, Poland Paweł Kulesza Faculty of Chemistry, University of Warsaw, Poland Ewa Bulska CNBCh, University of Warsaw, Poland Claudio Luchinat University of Florence, Italy
15:00	Harald Schwalbe Goethe University Frankfurt, Germany Solution and solid state NMR studies of rhodopsins
15:35	Lukáš Žídek Masaryk University, Czech Republic Spectral density mapping for disordered proteins
16:10	*Coffee Break*
	Chairperson: Wiktor Koźmiński
16:40	Mikael Akke Lund University, Sweden ^{13}C relaxation dispersion experiments for aromatic side chains in proteins
17:15	Natalia Kulminskaya Aarhus University, Denmark *In situ* atomic-resolution structure of the baseplate antenna complex in *Chlorobaculum tepidum* obtained by combining solid-state NMR spectroscopy and cryo-EM
17:50	Bernhard Brutscher Institut de Biologie Structurale, France Real-time multidimensional NMR studies of the folding of an amyloidogenic protein
18:25	Alexandre M. J. J. Bonvin Utrecht University, The Netherlands Integrative modelling of biomolecular interactions
19:10	*Dinner*
21:00	User Group Meeting (Symphony Hall) Local Operator Meeting (Concerto 1 Hall, 1st floor)

	Tuesday, May 6
	Chairperson: Miquel Pons
9:00	Robert Konrat University of Vienna, Austria NMR contributions to structural dynamics studies of IDPs
9:35	Steve Matthews Imperial College London, United Kingdom NMR wrestling with SUMO chains
10:10	Monika Baumann Martin Luther University Halle-Wittenberg, Germany Resonance assignment of amyloid forming proteins for hydrogen-deuterium exchange experiments
10:30	*Coffee Break*
	Chairperson: Miquel Pons
11:00	Lucia Banci University of Florence, Italy (to be announced)
11:35	Bahareh Eftekharzadeh Institute for Research in Biomedicine, Spain Atomic resolution description of aggregation initiation of intrinsically disordered domain of androgen receptor in SBMA
11:55	Ago Samoson Tallinn University of Technology & NMR Institute, Estonia Some impressions of 100 kHz magic angle spinning
12:30	Ivan Corbeski Goethe University Frankfurt, Germany Enzyme dynamics under macromolecular crowding and in a cellular environment by CPMG relaxation dispersion NMR spectroscopy
12:50	*Lunch* — Managing Committee Meeting
	Special Session: *Non-linear sampling methods and their applications in liquid and* in vivo *NMR* Chairperson: Vladimír Sklenář
14:30	Isabella C. Felli University of Florence, Italy Novel methods based on ^{13}C detection to study IDPs
15:00	Vladislav Orekhov University of Gothenburg, Sweden Fast NMR tools for intrinsically disordered proteins
15:30	Ēriks Kupče Bruker BioSpin GmbH, Germany New ways to record multi-dimensional NMR spectra
16:00	Krzysztof Kazimierczuk University of Warsaw, Poland Sparse sampling in non-frequency dimensions
16:30	Jiří Nováček Masaryk University, Czech Republic Towards optimal resolution NMR of intrinsically disordered proteins
17:00	*Coffee Break*
	Special Session: *Links of Bio-NMR with other BMS Research Infrastructures* Chairperson: Vladimír Sklenář
17:30	*Introduction*
18:00	Ronald Frank Leibniz-Institut für Molekulare Pharmakologie, Germany EU-OPENSCREEN: chemical tools for the life sciences
18:30	Chris Morris Science and Technology Facilities Council, United Kingdom BioMedBridges: constructing data and service bridges in the life sciences
19:30	*Dinner*
21:00	BioNMR General Assembly

Wednesday, May 7

	Chairperson: Peter Tompa
9:00	Marek J. Potrzebowski — Polish Academy of Sciences, Poland Simple and robust method for analysis of local molecular motion of side-groups in crystalline peptides and proteins by 2D and 3D NMR spectroscopy under fast magic angle spinning
9:35	Ronald Melki — National Center for Scientific Research, France Structural and functional characterizarion of two α-synuclein strains
9:55	Jens Danielsson — Stockholm University, Sweden Protein stability in living human cells – implications for SOD1 aggregation and ALS?
10:30	*Coffee Break*
	Chairperson: Peter Tompa
11:00	Ildefonso Marin-Montesinos — University of Barcelona, Spain Self-assembled trityl radical capsules. Implications for dynamic nuclear polarization
11:20	András Perczel — Eötvös Loránd University, Hungary Structure and dynamics driven rational design of Exendin-4 analogues
11:55	David M. Dias — University of Cambridge, United Kingdom BAZ2B bromodomain binding hotspots revealed by solution NMR and HADDOCK
12:15	Lieven Buts — Vrije Universiteit Brussel, Belgium High-resolution NMR studies of prokaryotic toxin-antitoxin systems reveal intricate regulatory networks
12:50	*Lunch*
	Chairperson: Janez Plavec
14:30	Joeri Veransdock — ETH Zurich, Switzerland Type III secretion system needles: a comparison between native and *in vitro* reassembled needles from *Shigella*
14:50	Kristina Haslinger — Max Planck Institute for Medical Research, Germany Structural characterisation of the peptidyl carrier protein domain from teicoplanin biosynthesis – a vital “piece in the puzzle” of biosynthetic glycopeptide formation
15:10	Michael Assfalg — University of Verona, Italy Equilibrium partitioning of fatty acid binding proteins to phospholipid membranes
15:30	Angeles Canales — Complutense University of Madrid, Spain Characterization of N-terminal domain of CHOP protein by using ^{13}C direct-detected NMR experiments
15:50	Ulrich Günther — University of Birmingham, United Kingdom (to be announced)
16:25	*Coffee Break*
16:55	**Poster Session** Odd-numbered posters
19:30	*Official Conference Banquet*

Thursday, May 8

	Chairperson: Lucio Frydman
9:00	Hartmut Oschkinat Leibniz-Institut für Molekulare Pharmakologie, Germany (to be announced)
9:35	Edward Hookway University of Oxford, United Kingdom Selective jumonji demethylase inhibitor alters glutamine and glucose metabolism in JJN-3 multiple myeloma cells
9:55	Stefano Gustincich International School for Advanced Studies (SISSA), Italy SINEUPs: a new functional class of natural and synthetic antisense non-coding RNAs that activate translation
10:30	*Coffee Break*
	Chairperson: Lucio Frydman
11:00	Federico Rastrelli University of Padua, Italy Functionalized gold nanoparticles for biological applications: a NMR viewpoint on the dynamics and organization of protecting monolayers
11:20	Massimo Bellanda University of Padua, Italy Structure of *Trypanosoma brucei* 1-C-Grx1, a mitochondrial monothiol glutaredoxin with an unusual intrinsically disordered N-terminal extension
11:55	Carl Diehl Copenhagen University, Denmark Structure and dynamics governing the binding mechanisms in the interactions between SUMO-binding motifs and SUMO
12:15	Kurt Zatloukal BBMRI, Medical University of Graz, Austria (to be announced)
12:50	*Lunch*
14:30	**Poster Session** Even-numbered posters
16:30	*Coffee Break*
	Chairperson: Christina Redfield
17:00	Nicholas Dixon University of Wollongong, Australia Design principles for large dynamic multiprotein machines: the bacterial replisome
17:35	Kristaps Jaudzems Latvian Institute of Organic Synthesis, Latvia Mechanism of spider silk formation studied by NMR
17:55	Hartmut Schäfer Bruker BioSpin GmbH, Germany (to be announced)
18:30	*Closing Remarks*

Oral Presentations

Solution and solid state NMR studies of rhodopsins

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In the presentation, we will present the NMR characterization of two rhodopsins: (i) the mammalian visual dim-light photoreceptor rhodopsin that is considered a prototype G protein-coupled receptor, and (ii) channel rhodopsin.

For bovine rhodopsin, we could characterize the kinetics of its light-activation process. Milligram quantities of α,ε-^{15}N-labeled tryptophan rhodopsin were produced in stably transfected HEK293 cells. Assignment of the chemical shifts of the indole signals was achieved by generating the single-point-tryptophan to phenylalanine mutants, and the kinetics of each of the five tryptophan residues were recorded. We find kinetic partitioning in rhodopsin decay, including three half-lives, that reveal two parallel processes subsequent to rhodopsin activation that are related to the photocycle. The meta II and meta III states emerge in parallel with a relative ratio of about 3:1. Transient formation of the meta III state was confirmed by flash photolysis experiments. From analysis of the site-resolved kinetic data we propose the involvement of the E2 loop in the formation of the meta III state.

For channel rhodopsin, we present a new labelling mechanism in *P. pastoris*, the successful reconstitution and initial DNP-assisted solid-state NMR results.

Spectral density mapping for disordered proteins

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Joint Research Activities of the Bio-NMR project involve improvement of the methodology for investigating large, functional systems. One of the directions of the research development is the effort to extend applicability of the existing methods to intrinsically disordered proteins (IDPs). Description of internal motions is particularly important for these highly dynamic molecules, present in multiple conformations in the sample. Traditionally, NMR relaxation of ^{15}N in peptide bonds is used as an optimal probe for monitoring the backbone dynamics. As the internal dynamics of IDPs is not well-separated from overall tumbling, the most popular model-free approach is not well applicable. Spectral density mapping represents a more suitable approach, but specific features of IDPs have to be taken into account.

Recently, we have examined the standard spectral density mapping protocols and their applicability to IDPs, and extended them by employing cross-correlated relaxation rates [1]. The results showed that highly accurate spectral density values are obtained by combining auto- and cross-correlated relaxation rates, or by using cross-correlated relaxation rates exclusively. Determination of the cross-correlated relaxation rates benefits for the high signal-to-noise ratio of the IDP NMR spectra, which allows one to evaluate relaxation dates using less sensitive experiments while keeping a sufficient precision. The developed protocols also help to separate effects of slow conformational (or chemical) exchange from fast dynamics. In the case of IDPs exhibiting extremely narrow chemical shift dispersion, peak overlap significantly reduces the number of residues whose relaxation rates can be determined. Therefore, 3D non-uniformly sampled experiments have been developed and applied to ^{13}C,^{15}N-labeled samples in order to improve resolution. Measurement of cross-correlated relaxation rates in such a manner is especially attractive, because analysis of the data is not influenced by the presence of additional ^{13}C nuclei. Our results show that data can be obtained for almost all residues of the tested IDP with a particularly poor chemical shift dispersion – the delta subunit of the RNA polymerase of *Bacillus subtilis* [2].

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^{13}C relaxation dispersion experiments for aromatic side chains in proteins

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Aromatic residues play a number of important roles in protein function. They are prevalent in protein binding interfaces, where especially Tyr and Trp contribute a significant fraction of the binding free energy. His and Tyr, and to some extent also Trp, are often found to be critical for enzyme catalysis. Aromatic residues contribute a significant part (roughly 25% of the volume) of the hydrophobic core, where they are typically involved in specific aromatic-aromatic pair interactions, or clusters of three or more aromatic residues Despite the dense packing of protein interiors, Phe and Tyr residues undergo frequent 180° rotations ('ring flips') of the χ_2 dihedral angle, i.e. around the C^{β}–C^{γ}–C^{ζ} axis, as was well established in landmark studies on protein dynamics by early pioneers in the field of protein NMR spectroscopy.

We have recently introduced a set of longitudinal- and transverse-relaxation optimized (L-TROSY) pulse sequences for ^{13}C relaxation experiments, including R_1, R_2, [^{1}H]-^{13}C NOE, as well as CPMG and $R_{1\rho}$ relaxation dispersion. Here we use the ^{13}C L-TROSY-CPMG and $R_{1\rho}$ experiments to study conformational exchange of aromatic side-chains in BPTI. We show that the relaxation of the 'TROSY spin state' can be affected by strong coupling between the ^{13}C-attached proton and its vicinal proton neighbor, leading to anomalous relaxation dispersion profiles. We show that the observed relaxation dispersions can be analyzed to determine the ring-flip rate for cases that are normally intractable because they are characterized by slow flip rates (on the order of 1–100 s^{-1} and the absence of chemical shift differences of the monitored ^{13}C spin between the two sides of the ring. Furthermore, the ^{13}C L-TROSY-CPMG experiment also detects conventional relaxation dispersions due to modulation of the ^{13}C chemical shift that arise as a consequence of either ring flips or reorientations of the aromatic ring relative to its surroundings. The finding of quite slow ring flips for aromatic residues that exhibit single and non-broadened peaks in both the ^{1}H and ^{13}C dimensions of the NMR spectrum raises the possibility that aromatic ring flips in many cases might be slower than previously anticipated.

In situ atomic-resolution structure of the baseplate antenna complex in *Chlorobaculum tepidum* obtained by combining solid-state NMR spectroscopy and cryo-EM

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Photosynthetic antenna systems enable organisms to sense and harvest light and transfer the energy to the photosynthetic reaction centre, where the conversion to chemical energy takes place [1]. Most photosynthetic antennae are complex entities composed of light sensitive pigments bound by specialised proteins, which are immersed in membrane compartments of the cells [2–9]. Due to complexity, structural characterization of these composite systems has so far been carried out only on isolated protein-pigment complexes.

One of the most heterogeneous systems, the organelle found in the photosynthetic green sulfur bacteria *Chlorobaculum tepidum*, contains a baseplate formed by the protein CsmA and bacteriochlorophylls [10]. This baseplate serves to facilitate attachment of the chlorosome to the cytoplasmic membrane in order to efficiently transfer the harvested energy out of the chlorosome. We present the first atomic-resolution structure of the CsmA baseplate using preparations of intact, fully functional, light-harvesting organelles [10], by combining three complementary methods: solid-state NMR spectroscopy, cryo electron microscopy, and circular dichroism. The structure reveals that CsmA with their bacteriochlorophyll A pigments form a fully symmetric two-dimensional lattice superstructure, where the basic building block is a dimer of the strongly amphipathic CsmA, with pigments sandwiched within the dimer at the hydrophobic side of the helix. The dimer is translated along two perpendicular directions to form parallel rods, which are bridged by oppositely charged side-chain interactions. The presented structural information provides the first detailed insight into the organization of a protein in a fully functional organelle *in situ*.

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Real-time multidimensional NMR studies of the folding of an amyloidogenic protein

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The characterization of both the structure and conformational dynamics of biological macromolecules – proteins and nucleic acids – is required for understanding the molecular mechanisms underlying physiological function and disease. Molecular dynamics involves the transient departure from the ground-state structure to populate short-lived excited state conformations that can play important functional roles. Real-time multi-dimensional NMR spectroscopy represents a unique tool for investigating dynamic molecular processes occurring on time scales of seconds or longer, providing atomic-resolution information about short-lived conformational states.

Real-time 2D and 3D NMR spectra can be obtained during a folding reaction using sensitivity enhanced fast-pulsing techniques such as SOFAST-HMQC, BEST-HSQC, and BEST-TROSY developed in our laboratory. Here we present an application of these techniques to investigate the refolding of the amyloidogenic human protein β_2 microglobulin from an acid-denatured to the native state. Our multidimensional NMR data revealed the presence of several intermediate species and provided sequential NMR assignments, as well as atom-resolved information on structural and dynamic features within the major folding intermediate with a half-life of only 20 minutes.

Integrative modelling of biomolecular interactions

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Biomolecular interactions underlie most cellular processes, including signal transduction and apoptosis. Understanding how the cell works requires describing these at molecular level, which is bound to have a dramatic impact on current and future structure-based drug design. Computational methods may assist this task, particularly when some experimental data can be obtained. For this purpose, we have developed HADDOCK, a versatile information-driven docking approach (`http://haddock.science.uu.nl`), which is also provided through the WeNMR portal (`http://www.wenmr.eu`), next to a large variety of other NMR and SAXS software tools.

In my talk I will give a short overview of the WeNMR services and present new developments around HADDOCK, describing in particular how we have now included cryo-EM data into the modelling process.

NMR contributions to structural dynamics studies of intrinsically disordered proteins

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Intrinsically disordered proteins (IDPs) are characterized by substantial conformational plasticity. Given their inherent structural flexibility, X-ray crystallography is not an useful method of studying these proteins. In contrast, NMR spectroscopy offers unique opportunities for structural and dynamic studies of IDPs. The past two decades have witnessed significant development of NMR spectroscopy that couples advances in spin physics and chemistry with a broad range of applications. The talk will summarize key advances in basic physical chemistry and NMR methodology, outline their limitations and envision future R&D directions.

NMR wrestling with SUMO chains

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The small ubiquitin-like modifier (SUMO) can form polymeric chains that are important signals in cellular processes such as meiosis, genome maintenance and stress response. The SUMO-targeted ubiquitin ligase RNF4 has a key role in the DNA damage response and in arsenic therapy for acute promyelocytic leukaemia. RNF4 engages with polySUMO chains of sumoylated substrates and catalyses their ubiquitination, which targets the substrate for proteasomal degradation. Structural investigation into the mechanisms polySUMO recognition has been hampered by the inherent flexibility and the production of suitable polySUMO chains. A segmental labelling approach combined with solution NMR spectroscopy and extensive biochemical characterisation reveals how RNF4 manipulates the conformation of the polySUMO chain thereby facilitating optimal delivery of the distal SUMO domain for ubiquitin transfer.

Resonance assignment of amyloid forming proteins for hydrogen-deuterium exchange experiments

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The pathogenic self-assembly of proteins and peptides into amyloid fibrils is characteristic for many neurodegenerative disorders, such as Alzheimer's disease. The combination of hydrogen-deuterium exchange experiments and nuclear magnetic resonance spectroscopy allows the identification of fibril core structures and the comparison of hydrogen bonding pattern for different fibril morphologies [1].

To obtain residue-specific information by NMR, resonance assignment needs to be performed under fibril denaturing conditions, typically in dimethylsuphoxide. These conditions lead to very low dispersion for all nuclei, hence resonance assignment is rather difficult by three dimensional NMR spectra. In contrast, 5D HN(CA)CONH and HabCabCONH [2] provided full assignment within short measurement and assignment time.

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Atomic resolution description of aggregation initiation of intrinsically disordered domain of androgen receptor in SBMA

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Spinal and bulbar muscular atrophy (SBMA), together with Huntington's disease, is a member of the family of polyglutamine (polyQ) expansion diseases. These disorders are associated with the deposition of aggregates of Androgen receptor proteins with an expanded polyQ tract of a larger size in neural cells. The androgen receptor (AR) is a nuclear receptor sensitive to testosterone, which is predicted to be intrinsically disordered in its N-terminal domain that carries the polyQ tract. Aggregates of this polyQ-expanded protein have been observed in the motor neurons of SBMA patients.

NMR spectroscopy is well suited for the study of intrinsically disordered proteins, which are in general highly flexible and have no well-defined secondary or tertiary structure. The crucial role of IDPs in many cellular processes, including transcription regulation, becomes more evident and therefore there is a growing demand for structural information on IDPs. The N-terminal domain of the androgen receptor contains regions essential for AR transcription activities. There is still no atomic resolution information of these regions and therefore, we investigated the last 135 N-terminal residues of AR with NMR. We have used NMR spectroscopy to study the structural consequences of an expanded repeat (25Q) on the structural properties of the AR-N-terminal domain. We utilize C-direct detected experiments to assign the protein and further, characterize the secondary structure propensities. NMR spectroscopy revealed that in aqueous solution, the AR-NTD has a relatively limited amount of stable secondary structure regions and expansion of the polyQ repeat resulted in a dramatic increase in α-helix structure in a region flanking to polyQ. Taken together, these findings support the view that the presence and length of the polyQ repeat and its flanking regions modulate the folding and structure of the AR-NTD and are involved in aggregation initiation of the androgen receptor in SBMA.

Some impressions of 100 kHz magic angle spinning

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One of the technical leaps in non-solvent bio-NMR has been magic angle spinning at frequencies beyond 40 kHz, which allowed to reduce decoupling power by orders of magnitude. 100 kHz seems to enable universal inverse detection as well as the study of dynamics in a range not attainable as easily by other methods. We shall present linewidth-sensitivity analyses for all-proton refinement of protein structures. The absolute sensitivity enhancement compares to current dynamic nuclear polarization (DNP) values, while considerations of resolution render DNP clearly inferior.

Enzyme dynamics under macromolecular crowding and in a cellular environment by CPMG relaxation dispersion NMR spectroscopy

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Since the beginnings of structural biology in the middle of the 20th century, enzyme function has been known to be governed by structure. But in the first decade of the new millennium, a series of studies have recognized intrinsic dynamics as another basic principle of enzyme function [1, 2]. At the same time, macromolecular crowding and the cellular environment have been shown to affect the energy landscape of biochemical processes [3]. Enzymatic catalysis and dynamics occur in the microsecond to millisecond time range. This is the right range for Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion (RD) NMR [4]. A CPMG RD NMR study of enzyme dynamics under cellular conditions could reveal whether and to what extent these dynamics play a role for enzyme function in the cell.

The peptidyl-prolyl *cis*/*trans* isomerase and immunophilin FKBP12 is a ubiquitous model enzyme [5]. It shows different backbone and side-chain dynamics upon binding substrates, inhibitors, and cellular targets [6, 7]. Furthermore, an NMR analysis of the visible substates of FKBP12 and its mutants has identified three distinct conformational transitions to underlie the exchange process of this enzyme [8].

A CPMG RD NMR study has revealed marked effects on the kinetics and thermodynamics of the dynamics of this multifunctional enzyme under macromolecular crowding conditions and in a cellular environment. A cellular environment generally slows down conformational exchange in FKBP12. Furthermore, the composition of the energetic barrier is changed. Whereas in dilute buffer solution, a positive transition enthalpy is the hurdle for enzyme dynamics, in a cellular environment the transition state is enthalpically favored and the barrier is entirely entropic. This may contribute to a higher specificity and effectivity of FKBP12 in the cell.

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Novel methods based on ^{13}C detection to study intrinsically disordered proteins

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Intrinsically disordered proteins (IDPs) are characterized by highly flexible solvent exposed backbones and can sample many different conformations. These properties confer them functional advantages, complementary to those of folded proteins, which need to be characterized to expand our view of how protein structural and dynamic features affect function beyond the static picture of a single well defined 3D structure that has influenced so much our way of thinking.

NMR spectroscopy provides a unique tool for the atomic resolution characterization of highly flexible macromolecules in general and of IDPs in particular. The peculiar properties of IDPs however have profound effects on spectroscopic parameters. It is thus worth thinking about these aspects to make the best use of the great potential of NMR spectroscopy to contribute to this fascinating field of research.

Recent progress in NMR instrumentation has stimulated the development of a variety of new NMR methods and, among them, exclusively heteronuclear NMR experiments based on ^{13}C direct detection now offer a valuable tool to address the peculiar features of IDPs [1], in particular approaching physiological conditions [2]. The experimental variants to improve the performance of ^{13}C detected NMR experiments to study IDPs include the design of multidimensional experiments, the exploitation of longitudinal relaxation enhancement, the design of experiments to alleviate the problem of extensive cross peaks overlap [2–6]. The new experiments are demonstrated on a paradigmatic IDP, α-synuclein.

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Fast NMR tools for intrinsically disordered proteins

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When used for studying intrinsically disordered proteins (IDP), NMR spectroscopy faces challenges and opportunities that are distinctly different from those in the case of globular proteins. Because of low dispersion of the chemical shifts and numerous repeats in the amino acid sequence, IDPs typically show very high overlap in their spectra. Thanks to high structural flexibility and hence favorable relaxation properties of IDPs, the problem of overlap can be tackled using spectra with high dimensionality and long acquisition times in combination with non-uniform data collection and novel signal processing methods. The latest advances in NUS processing algorithms and a number of applications to disordered cytosolic domains of T- and B- cell receptors are presented. We show that the use of available yet usually unexploited prior knowledge about the phase and the causality of the NMR signal is a general approach for a major quality improvement of the sparsely detected spectra. For the new approach, we present a theoretical framework and demonstrate notable improvement of the protein spectra reconstructed with two commonly used state-of-the-art signal processing algorithms, compressed sensing and SIFT [1].

Time-resolved experiments demand high resolution both in spectral dimensions and in time of the studied kinetic process. The latter requirement traditionally prohibits applications of the multidimensional experiments, which, although capable of providing invaluable information about structure and dynamics and almost unlimited spectral resolution, require too lengthy data collection unless NUS is used. A continuous fast pulsing three-dimensional experiment is acquired using non-uniform sampling during full time of the studied reaction. High sensitivity and time-resolution of a few minutes is achieved by simultaneous processing of the full data set with the multi-dimensional decomposition. We applied the method for characterizing the kinetics of in vitro phosphorylation of two tyrosine residues in B-cell receptor CD79b domain. Signals of many residues, including tyrosines, in both phosphorylated and unmodified forms of CD79b are found in heavily crowded region of 2D ^{1}H-^{15}N correlation spectrum and the spectral resolution provided by the 3D time-resolved approach was essential for the quantitative site-specific analysis [2].

Recently we presented a platform for characterizing IDPs, which is based on cell-free protein synthesis and fast NMR spectroscopy [3] Using the platform, we examine the secondary structure propensity of the cytosolic domains of CD79a and CD79b in vitro before and after phosphorylation [4]. The secondary structure propensities are calculated using the method of intrinsic referencing, where the reference random coil chemical shifts are measured for the same protein in denaturing conditions.

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New ways to record multi-dimensional NMR spectra

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The development of cryoprobes offers significant increases in sensitivity. On the other hand, the recent introduction of NMR spectrometers with multiple receivers permits spectra from several different nuclear species to be recorded in parallel, and several standard pulse sequences to be combined into a single entity. These developments offer an exciting possibility of changing the conventional paradigm of NMR data mining to better optimize the efficiency of spectrometer use. It is shown how these improvements in the flow and quality of spectral information can be significantly augmented by compressive sensing techniques [1–3] – controlled aliasing, Hadamard spectroscopy, random sampling, projection-reconstruction (PR), and ultra-fast (UF) NMR spectroscopy. Future developments of these techniques are confidently expected to mitigate one of the most serious limitations in multidimensional NMR – the excessive duration of the measurements.

We also propose [4] a new general form of two dimensional spectroscopy where the indirect "evolution" dimension is derived using the Radon transform. This idea is applicable to several types of spectroscopy, but is illustrated here for the case of NMR spectroscopy. This "projection spectroscopy" (PROSPECT) displays characteristic correlation peaks that highlight perturbations of chemical shifts caused by temperature, pressure, solvent, molecular binding, chemical exchange, hydrogen bonding, pH variations, conformational changes, or paramagnetic agents. The results are displayed in a convenient format that allows the chemist to see all of the chemical shift perturbations at a glance and assess their rates of change and directions. As a proof of principle, we present several simple, practical examples that display two-dimensional representations of the effects of temperature and solvent on NMR spectra.

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Sparse sampling in non-frequency dimensions

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Serial NMR measurements are often employed in chemical and physical analysis. Experiments in a given series can differ in temperature settings (monitoring phase transitions), pulsed field gradient value (diffusion-ordered spectroscopy) or simply can be performed at different moments of time (monitoring chemical reactions). The serial measurement of multidimensional spectra is time-consuming, because of costly sampling of indirect time dimensions. Non-uniform sampling (NUS) can help to shorten the experiment, but so far was limited to frequency dimensions. In this study we show how to extend it to non-frequency (pseudo)dimensions. We provide examples of applications: diffusion-ordered NMR [1] and temperature dependent studies of protein unfolding [2]. The latter is based on the idea of time-resolved NUS [3].

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Towards optimal resolution NMR of intrinsically disordered proteins

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It is now widely recognized that a large portion of eukaryotic genome encodes proteins, which sample variety of distinct conformations in their native state. Yet, these intrinsically disordered proteins (IDPs) were shown to take part in number of fundamental cellular processes. Among various biophysical techniques used to their characterization, only NMR spectroscopy supplies details of their structural variability at the atomic resolution. However, a number of specific issues needs to be considered when applying NMR to study IDPs. Compared to characterization of well-structured proteins, fast conformational averaging of a disordered polypeptide chain produces spectra with very low dispersion of the observed resonance signals. The signal overlap is further pronounced when studying systems of increasing molecular weight or in IDPs with high incidence of sequential repeats.

The lecture will present recent methodology developed in our lab aimed to significantly shorten time needed for thorough desription of unstructured or partially disordered proteins. The adopted concept incorporates increasing the dimensionality of measured spectra together with application of sparse sampling in the indirectly detected dimensions. Further, the direct detection of ^{13}C' is employed and suitable correlation schemes are used to remove the signal overlap from the spectra for the resonance assignment. Moreover, a set of sparsely sampled experiments for measurement of the fast motions in IDPs and residual dipolar couplings is presented for the cases when these parameters can not be reliably extracted from conventional spectra due to the high signal overlap.

EU-OPENSCREEN: chemical tools for the life sciences

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EU-OPENSCREEN is a pan-European research infrastructure initiative on the ESFRI roadmap (European Strategy Forum on Research Infrastructures). It aims at enabling academic chemical biology researchers to develop novel 'tools' (i.e. chemical inhibitors or activators of biological targets) for all areas of the life sciences, including molecular, cell, plant, structural and microbiology; synthetic and medicinal chemistry; pharmacology and early drug discovery, etc.

EU-OPENSCREEN supports all stages of a tool development project from assay development, high-throughput screening to chemical optimization of 'hit' compounds and bioprofiling of tool compounds. EU-OPENSCREEN offers to scientists open access to its shared resources, including latest screening technologies, follow-up chemistry services for hit optimization, a unique compound collection composed of commercial and proprietary compounds, and a database containing validated output from the screening centers in a public as well as pre-release environment.

EU-OPENSCREEN builds on existing networks and facilities in now 16 partner countries. It interacts with similar large consortia of other continents to advance mutual exchange of compound collections, linkage of databases, agreement on standards, and exchange of best practice. EU-OPENSCREEN is expected to start full operations in late 2015. It can already look back on a growing number of transnational activities: development of new design principles for its central compound collection; exchange of local compound libraries, joint screening projects, and creation of national interest groups.

EU-OPENSCREEN's mission, operational concept, and services will be presented and discussed in the light of joint interests, common services and synergies with Bio-NMR.

BioMedBridges: constructing data and service bridges in the life sciences

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BioMedBridges will form a cluster of the emerging biomedical sciences research infrastructures (BMS RIs) and construct the data and service bridges needed to connect them. The missions of the BMS RIs stretch from structural biology of specific biomolecules to clinical trials involving thousands of human patients. Most serve a specific part of the vast biological and medical research community, estimated to be at least two million scientists in Europe across more than 1000 institutions from more than 36 ESFRI Member States and Associated Countries.

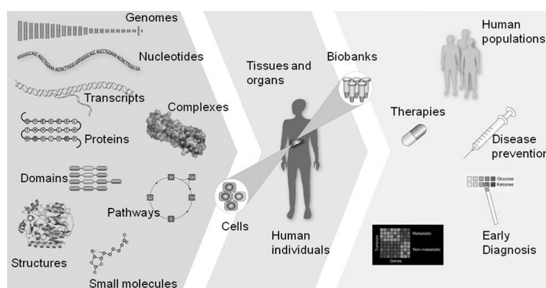

Figure 1

Three construction work packages are providing description and harmonisation of standards, technical integration, and secure access. Five use cases are providing interoperability of image data sets, reconciliation of phenotypic description of mouse and human, support for personalised treatment of acute myeloid ;eukemia, a database of electronic density shapes, and integration of disease related terms.

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Simple and robust method for analysis of local molecular motion of side-groups in crystalline peptides and proteins by 2D and 3D NMR spectroscopy under fast magic angle spinning

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We report a new multidimensional solid-state NMR methodology which permits one to analyze ^{1}H-^{13}C dipolar splittings in a simple and accurate way [1] and further scrutinize the molecular motions in side chains in peptides [2, 3] and nanocrystalline proteins. The power of the technique is demonstrated in 3D NMR CPVC-RFDR correlation experiments in two proteins, GB1 and DLC8. In this talk, we focus attention on probing the dynamics of aromatic residues: phenylalanine, tyrosine and tryptophan. Our results clearly show that this approach provides precise information about subtle differences in molecular dynamics of aromatic groups, which are related to their local environment in the crystal lattice (Figure 1). The presented methodology is general and can be extended to other systems.

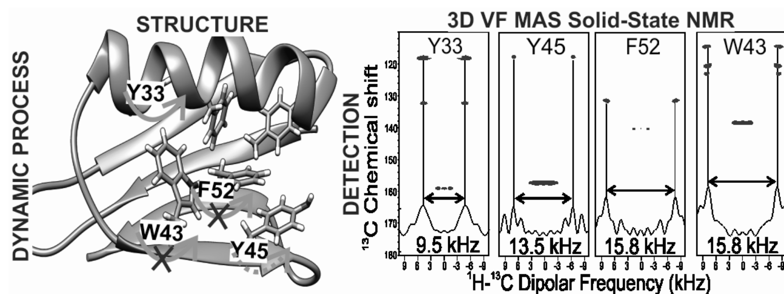

Figure 1: Left: visualization of GB1 protein with an indication of distances between selected aromatic residues. Right: dipolar doublets extracted from 3D CPVC-RFDR NMR spectrum

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Structural and functional characterizarion of two α-synuclein strains

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α-Synuclein aggregation is implicated in a variety of diseases, including Parkinson's disease, dementia with Lewy bodies, pure autonomic failure and multiple system atrophy. The association of protein aggregates made of a single protein with a variety of clinical phenotypes has been explained for prion diseases by the existence of different strains that propagate through the infection pathway.

We structurally and functionally characterize two polymorphs of α-synuclein we have generated. We show, in particular using solid-state NMR, that the two α-synuclein forms, which can each be produced as a pure polymorph, are greatly different in secondary structure. While β-sheets are the dominating secondary structure elements for both polymorphs, they are markedly divergent in terms of number of elements, as well as their distribution. We demonstrate that all identified β-sheets feature an in-register parallel stacking for both polymorphs. The two forms show a different molecular arrangement in the unit cell and distinct dynamic features. These differences in the structure are reflected in the functional characteristics of the two isoforms that exhibit distinct seeding and permeation properties as well as cellular toxicity, tropism, propagation and persistence.

Our data indicate that the two forms fulfill the molecular criteria that define distinct α-synuclein strains. Such strain differences perfectly account for differences in disease progression in different individuals, cell types and/or types of synucleinopathies.

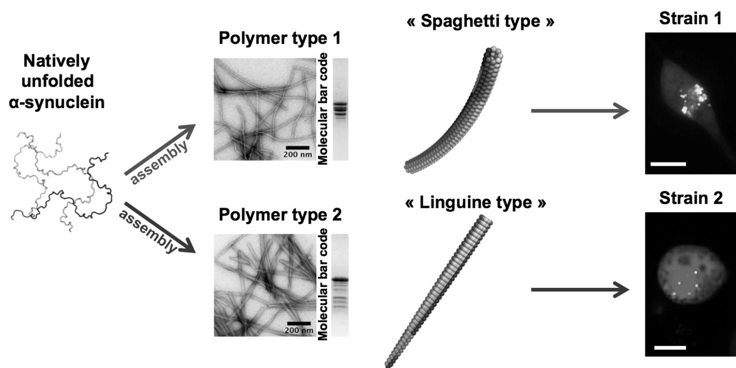

Figure 1

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Protein stability in living human cells – implications for SOD1 aggregation and ALS?

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Misfolding, aggregation and formation of intracellular inclusions of superoxide dismutase 1 (SOD1) is a hallmark of the neurodegenerative protein-misfolding disease ALS [1]. We have recently shown that *in vitro*, SOD1 aggregation and fibril elongation is driven by recruitment of globally unfolded protein [2]. Like all proteins, the structure of SOD1 undergoes constant dynamic transitions to unfolded or partially unfolded short-lived conformations *in vivo*. For yet unknown reasons, under some circumstances, misfolded SOD1 species can escape the cellular housekeeping system and cause neural damage. The neurons are progressively degenerated, which is accompanied by the deposition of macroscopic SOD1 aggregates. Consequently, large efforts have been focused on studies of early misfolding events and aggregates *in vitro*. From a molecular perspective, such extrapolation of *in vitro* data to complex cellular conditions is questionable. Protein aggregation is a multidimensional complex dynamical process that is critically sensitive to the starting point, i.e. the molecular environment. To circumvent this problem, our strategy is the direct analysis of the protein misfolding mechanism at atomistic resolution as it occurs inside living human cells.

Here we use electroporation, a recently developed method, to deliver isotope labelled SOD1 into living human cells to directly measure the effect of the in-cell environment on protein stability. As the exchange rate between the folded and unfolded states is slow on the NMR timescale, two easily distinguishable, quantifiable sets of NMR peaks arise from the unfolded and folded states of SOD1, respectively. We have recently shown that the stable β-barrel of SOD1 is fully folded in living cells [3]. So, in order to be able to measure minor affects on stability, we have constructed a destabilised variant of SOD1, tuned such that under physiological conditions, half of the population is unfolded *in vitro*. This variant, SOD150/50, is extremely sensitive to small changes in the external pressure and will respond by changes in folded and unfolded populations.

Interestingly, SOD1 is strongly destabilised in the cell, and the unfolded population increases from 50% to almost full occupancy at 37 ℃. At a lower temperature, 17 ℃, the protein stability is similar to *in vitro* controls. The crowding pressure and the high number of collisions and transient interactions exerted upon SOD1 in the living cell is expected to affect protein stability and structural integrity. However, In-vitro crowding experiments fail to accurately reproduce the in-cell results, underlining the importance of performing these experiments under in-vivo -like conditions. Thermodynamic analysis of the stability in-cell and in various mixed crowding agents indicates that the destabilisation found in cells may arise from an increase in ΔC_p between the folded and unfolded state, which in turn indicates a different unfolded state in cells than *in vitro*.

Wild-type SOD1 is, without its posttranslational modifications, a marginally stable protein, and further destabilisation caused by the in-cell environment has great impact on the concentration of unfolded aggregation-competent material. These studies on SOD1 in living cell systems may provide a unique insight into the molecular pathogenesis of ALS.

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Self-assembled trityl radical capsules. Implications for dynamic nuclear polarization

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The enhanced NMR sensibility provided by Dynamic Nuclear Polarization (DNP) experiments has opened the possibility of applications that would have not been otherwise possible. Examples include solid-state NMR experiments under magic angle conditions of biological sample [1, 2] and surfaces [3], as well as solution NMR of metabolites and imaging of hyperpolarized samples for medical diagnosis [4–6].

High NMR sensitivity is achieved by transferring the large electronic spin polarization present at low temperatures and moderate magnetic fields by microwave irradiation. For this purpose, samples are doped with paramagnetic species, or polarizers. The nature of the polarizers plays a key role in DNP enhancement. Dissolution DNP, used mainly in the context of metabolic and *in vivo* imaging studies, typically uses direct polarization of heteronuclei using trityl radicals at 15–30 mM concentrations at very low temperature (1–2 K). After nuclear polarization has been achieved, the sample is rapidly melted, dissolved and transferred to a standard NMR or MRI instrument by using pressurized hot solvent [7].

The widely used tetrathiatriarylmethyl radicals contain sulfur substituents at the ortho and meta positions linked by a substituted carbon forming a five membered ring [8] (Figure 1). Interactions between radicals have a strong effect on their efficiency as DNP polarizers and considerable synthetic effort has been devoted to the synthesis of covalently linked radicals [9]. Supramolecular interactions between the components of the polarization mixture (glassing solvent, polarizer and target molecule) may also have dramatic effects in the outcome of the DNP experiment. Supramolecular effects were suggested to explain the positive and negative polarizations observed using the same combination of chlorinated trityl radical and polarized target depending on the pH [10].

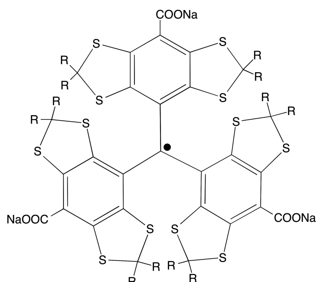

Figure 1: The structure of tetrathiatriarylmethyl radicals

In the present study, the combination of X-band CW-EPR, DNP, UV-Visible spectroscopy, ion-mobility mass spectrometry using mild electrospray ionization, SQUID measurements of electron magnetism, and DFT calculations, has been used to demonstrate the supramolecular interactions between molecules of Finland and OX63 trytil radicals.

Here we show that the Finland radical forms dimers and higher oligomers at micromolar concentrations in water. Self-association of the Finland radical involved the intermolecular interaction of methyl groups of one radical with the methyl groups and sulfur atoms of a second molecule and was favored by the propeller framework of the triaryl groups. We also show that, in the OX63 radical, the larger hydroxyethyl groups hinder the interaction and self-association is reduced in the absence of a suitable guest. OX63 forms

dimeric capsules with inclusion of TMA. The DNP efficiency of OX63 is negatively affected by dimerization. However, at the low temperatures used in dissolution DNP, dimerization is reduced and dramatic increases in polarization were achieved by temperature annealing the sample. Additional gains in polarization were achieved in the case of choline in the presence of tetramethylammonium, presumably by competition between the two tetraaklylammonium guests. These results highlight the importance of considering supramolecular effects in DNP and, in particular in the design of new polarizers.

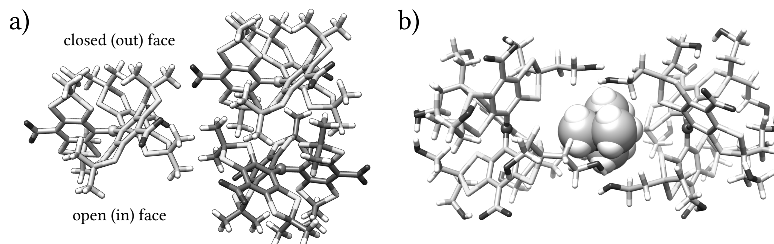

Figure 2: a) DFT minimum energy structure of the monomer Finland radical, showing the non-equivalent faces, and of the Finland dimer; b) DFT minimum energy of a OX63 dimer with encapsulated TMA

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Structure and dynamics driven rational design of exendin-4 analogues

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Exendin-4 (Ex4) is a potent, long-acting glucagon-like receptor-1 agonist, a drug which regulates the plasma glucose level of patients with type 2 diabetes. In this study, our aim was to design Ex4 analogues with improved helicity and compare their mechanism of action using both *in vitro* structural studies and by preliminary *in vivo* affinity measurements. Our starting molecule was the Ex4-derived 20 amino acid Trp-cage (TC) miniprotein [1, 2]. Here, we report how the step-by-step α-helical elongation of TC with the corresponding sequence of Ex4 affects the molecules' structure and stability.

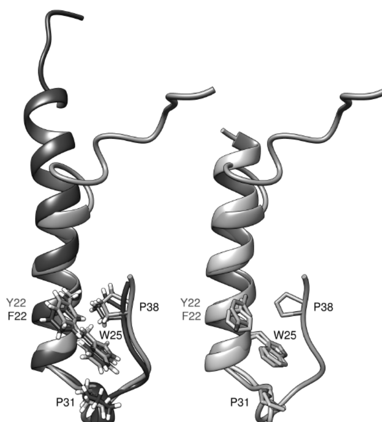

Figure 1: Ribbon representation of E19 (coral) with (a) Ex4 NMR structure, PDB: 1JRJ (blue) and (b) with Ex4 X-ray structure, PDB: 3C5T (orchid). Y22/F22, W25, P31 and P38 are displayed with all-atom representation. For clarity and better comparison, in panel (b) the H atoms are omitted from the E19 NMR structure

We applied CD and NMR spectroscopy to characterize the structural changes [3–5] among the studied 14 polypeptides with different length. The Asn → Arg mutation at the helix N-terminus of TC was highly destabilizing, but the gradual helix incrementation restored the fold stability, so that the 25 residue long E5 and TC have comparable stability. The 39 amino acid long construct E19, which differs from Ex4 only at four positions, is soluble in water, less prone to aggregation and more helical under all studied conditions than Ex4. In cellular assays, Ex4 and E19 show similar glucose lowering ability. We believe that the presented structural studies will help the development of more potent Ex4 related drug candidates or small-molecule agonists with even better pharmacokinetical properties.

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BAZ2B bromodomain binding hotspots revealed by solution NMR and HADDOCK

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Bromodomains are epigenetic reader domains, which have recently come under close scrutiny from both the academia and the industry [1]. Effective targeting of the BAZ2B bromodomain has been recently reported, but no information is yet available on its natural binding partner, acetylated lysines (Kac) in histones.

We have obtained the spectral assignment of BAZ2B bromodomain and determined its backbone dynamics. Once we have validated the backbone assignment as resource for screening small molecules [2], we then studied its interaction with H3 acetylated peptides by NMR. Since this interaction is mediated by water, we used both chemical shift perturbation (CSP) data and clean chemical exchange (CLEANEX) NMR experiments to characterise it for the first time. We identified the Kac binding site and the BC loop as the key hotspots for the interaction. This insight was used to create a data-driven model using HADDOCK. The models were further validated by site directed mutagenesis and isothermal titration calorimetry (ITC). The obtained information offers a new rationale to optimise current inhibitors for inspecting the function of the BAZ2B bromodomain.

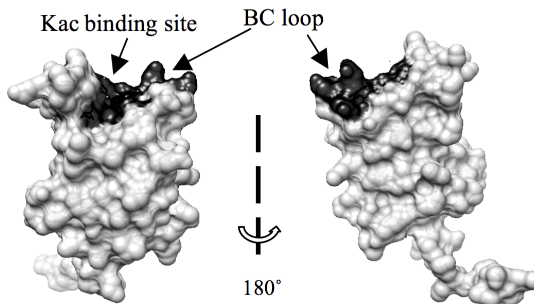

Figure 1: BAZ2B binding hotspots for the interaction with H3 acetylated lysines (Kac)

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High-resolution NMR studies of prokaryotic toxin-antitoxin systems reveal intricate regulatory networks

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Prokaryotic toxin-antitoxin (TA) systems are regulatory genetic elements combining a stable toxin and a specific, labile antitoxin [1]. Each toxin is capable of interfering with an essential cellular function, leading to a reduction in metabolic activity which has been associated with the response of bacterial cells to stress conditions. This response is of great medical interest in the case of the formation of persister cells which survive antibiotic therapy. The antitoxins are typically two-domain proteins, consisting of a well-structured DNA-binding domain and a highly dynamic toxin-binding domain. The latter domain is generally disordered in its free state, only adopting a specific conformation when it binds to its toxin partner. The DNA binding activity of the antitoxin has a central role in the autoregulation of the TA system.

Two representative TA systems were studied using high-resolution NMR methods. The first comprises the antitoxin CcdA and the toxin CcdB, which poisons the topoisomerase gyrase, thus inhibiting DNA replication and transcription. Simultaneous binding and folding of the CcdA neutralisation domain triggers an allosteric change in CcdB, releasing it from the target [2, 3]. The second prototypical system consists of the antitoxin MazE and the toxin MazF. The latter is a ribonuclease which degrades the majority of messenger RNA molecules in the cell, leading to a global shutdown of protein biosynthesis [4]. Sequence-specific resonance assignments and structure calculations were completed for MazE from *Escherichia coli*, CcdB from *Vibrio fischeri* and MazF from *Staphylococcus aureus*. The resulting structures were used as a foundation for an in-depth study of the antitoxin-DNA and toxin-antitoxin interactions, leading to a complete description of the intricate regulatory networks in TA systems.

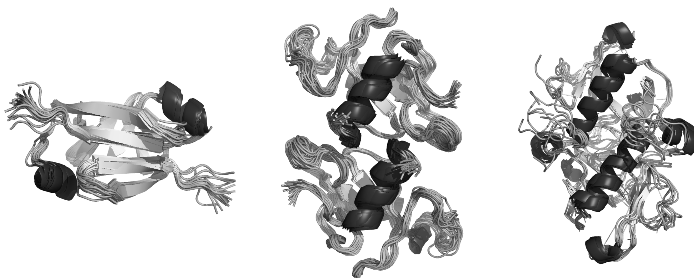

Figure 1: NMR ensembles of the *E. coli* MazE antitoxin (left), the *V. fischeri* CcdB toxin (middle) and the *S. aureus* MazF toxin (right)

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Type III secretion system needles: a comparison between native and *in vitro* reassembled needles from *Shigella*

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Type III secretion systems are used for the transportation of virulence factors from many Gram-negative bacteria into eukaryotic host cells [1, 2]. These systems consist of a cytoplasmic part, a trans-membrane part and a hollow extracellular needle, which is involved in host cell sensing and virulence factor injection [3]. The needle is made from a helical polymerization of a $\sim$9 kDa protein monomer [4, 5].

Recently two different models have been published that describe the needle part at atomic resolution [6–8]. The model of Fujii et al. was derived from a 7.7 Å electron cryomicroscopy (cryoEM) reconstruction into which a prior crystal structure of the monomer was docked. The model by Loquet et al. was derived by solid-state NMR (ssNMR). The needles used by Fujii et al. and Loquet et al. are formed by two different proteins, MxiH and PrgI respectively. However, a later study by Demers et al. on MxiH needles suggested a structure similar to Loquet et al. [9]

The two models might differ as a result of different sample preparation methods. The needles were natively and recombinantly derived in Fujii et al. and Demers et al., respectively. Here, we performed solid-state NMR on a sample prepared identically to the one used in Fujii et al. and compared the data to Demers et al. The results show that the two structures are strongly similar. This indicates that the substantial differences in monomer fold and packing between the two models are not due to differences in sample preparation. These findings rather suggest the inconsistencies derive from problems with data interpretation or analysis.

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Structural characterisation of the peptidyl carrier protein domain from teicoplanin biosynthesis – a vital "piece in the puzzle" of biosynthetic glycopeptide formation

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Non-ribosomal peptide synthesis is an important mechanism for the biosynthesis of clinically relevant yet synthetically unavailable compounds such as penicillins [1] and glycopeptide antibiotics [2]. Non-ribosomal peptide synthetases (NRPSs) produce a broad range of peptides composed of proteogenic and non-proteogenic amino acids that can be further diversified by the actions of tailoring enzymes. In most cases these additional modifications confer biological activity to the final compounds [3]. The generic layout of NRPSs – with multi-domain modules, each containing at least an adenylation (A), a condensation (C) and a peptidyl carrier protein (PCP) domain [4] – offers several potential platforms for interactions with tailoring enzymes. The nature of these interactions, however, remains elusive. In the case of the glycopeptide vancomycin, it has been shown that oxidative tailoring performed by cytochromes P450 occurs while the peptide is still attached to a PCP domain [5], thus bringing the function and dynamics of these small domains into the focus of scientific interest.

In this work we studied the PCP of the last NRPS module in teicoplanin biosynthesis, another clinically important glycopeptide antibiotic [6]. Structural characterisation of the isolated PCP domain by X-ray crystallography was attempted with several approaches but has not been successful. The small size and great stability of PCP, however, were proven prerequisites for solution NMR studies. In the course of a two month travel fellowship funded by BioNMR, we made backbone and side chain assignments for both the apo and the biologically relevant holo form of the protein, which bears a phospohopantetheinylation at a conserved serine residue. In 2D and 3D NOESY experiments with ^{13}C and ^{15}N labeled protein, through-space correlations were assigned and used as distance restraints in structure calculations by simulated annealing. Furthermore, titrations with a putative interaction partner and a putative magnesium cofactor were perfomed, showing that neither titrant perturbs the ^{15}N-HSQC pattern of the protein and thus does not bind to PCP, in contrast to previous studies on other carrier proteins [7, 8]. These results show that despite the highly conserved fold of carrier proteins, their functionality is highly dependent on their biological context. Having the solution structure of this PCP at hand will help to elucidate its interaction with other NRPS domains and taloring enzymes in the teicoplanin biosynthetic pathway.

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Equilibrium partitioning of fatty acid binding proteins to phospholipid membranes

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Fatty-acid trafficking in cells is a complex and dynamic process that affects many aspects of cellular function. Intracellular lipid chaperones known as fatty acid binding proteins (FABPs) are a group of 14–15 kDa polypeptides that coordinate lipid responses in cells and are also strongly linked to metabolic and inflammatory pathways [1]. Membrane binding by FABP appears to constitute a key step of intracellular lipid trafficking. Several members of the FABP protein family have been shown to interact with anionic phospholipid vesicles mimicking cell membranes [2].

The liver bile acid binding protein (L-BABP) present in non-mammalian vertebrates and the mammalian-type liver (L-) FABP belong to the same subfamily of FABPs. They share the same overall tertiary fold and are able to bind the same ligands, however with different relative affinities. The mechanisms by which they exchange their lipid cargo with cell membranes or membrane transporters are still not clarified. We have used NMR spectroscopy and ancillary techniques to show that L-BABP, at variance with other FABPs, is avidly attracted by negatively charged phospholipid vesicles and that the interaction is specific, involving the N-terminal protein region and the nearby turns [3, 4].

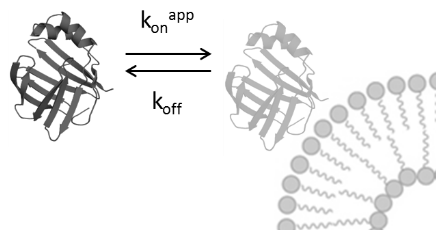

Figure 1: Equilibrium partitioning of FABP to a phospholipid vesicle

We are currently focusing on the possibility to describe the properties of the NMR-invisible, vesicle-bound FABPs based on saturation transfer experiments [5]. These investigations could help to understand the mechanism of membrane-mediated ligand uptake and release, a core process of lipid trafficking.

The present project has been developed within the Bio-NMR Network Activity, coordinated by Miquel Pons (University of Barcellona, Spain). The activity includes the transfer of new methods to interested users. The NMR laboratory of Verona is acting as the Italian reference site for the implementation of DEST and CEST type experiments, implemented by Dr. Moreno Lelli (RALF-NMR).

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Characterization of N-terminal domain of CHOP protein by using ^{13}C direct-detected NMR experiments

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CHOP is a transcription factor that induces growth arrest and apoptosis after endoplasmic reticulum stress or DNA damage. CHOP belongs to the C/EBP family of transcription factors. These proteins contain a leucine zipper domain at the C-terminal involved in DNA recognition that is highly conserved in the family. In contrast, the N-terminal half is less conserved and poorly characterized. The N-terminal segment contains transactivation domains that play a key role in transcriptional control. Despite its importance, the presence of intrinsically unstructured stretches in the N-terminal half of CHOP has hampered a structural characterization of this region using conventional methods.

In this context, we are applying ^{13}C direct detection experiments [1] to the study of the N-terminal domain of CHOP protein. First, backbone resonances were assigned by using the combination of CBCANCO and CBCACON spectra. Once the backbone resonances were assigned, we used that information to study the secondary structure propensities of the protein by comparing the experimental chemical shifts with the values expected for a random coil peptide. The obtained results point out that N-CHOP is highly disordered with some regions with α-helical propensity. It is described that other activation domains form alpha helices in complex with their activators [2]. In the next step of the project, we would like to see if this is also the case for N-CHOP protein.

Finally, ^{15}N relaxation experiments were carried out to further characterize the dynamic properties of the N-terminal domain of this transcription factor.

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Selective jumonji demethylase inhibitor alters glutamine and glucose metabolism in JJN-3 multiple myeloma cells

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Multiple myeloma (MM) is a malignancy of plasma cells, terminally differentiated antibody-producing B lymphocytes. The majority of cases occur in those over 65 and represents approximately 1% of cancer diagnoses. 5 year surivial has improved in recent years, but remains below 40%. The lysine demethylase (KDM) superfamily of enzymes are able to remove metyl marks from lysine residues of proteins, including histones. Different methylation states of lysine residues of histones alter local chromatin structure and can alter gene expression. Emerging evidence suggests over-expression of KDM5B is an independent poor-prognostic factor in MM. GSK-J4 is a cell-permeable, small molecule inhibitor of the lysine demethylases KDM5B, KDM6A and KDM6B [1]. GSK-J4 induces a unique transcriptional profile within MM cells, resulting in cell death via apoptosis. These studies examine the role of GSK-J4 on the metabolism of the MM cell line JJN-3.

7.5×10^6 cells were treated with either 5 µM GSK-J4 or vehicle control for 24 hours. For flux experiments, media was removed 3 hours prior to harvest and replaced with media containing either 10mM glucose-1,2-^{13}C or 4 mM glutamine-3-^{13}C. Metabolites were extracted using methanol, chloroform and water in a ratio of 2:2:1.8. The aqueous phase was dried, resuspended in 10% D_2O and analysed on a Bruker 600 MHz machine.

Under control conditions, little glucose enters into the TCA cycle with ^{13}C label derived from glucose seen in lactate, ribose and other products of the pentose phosphate pathway. Glutamine feeds the the TCA cycle with label seen strongly in 2-oxoglutarate, succinate, malate, fumerate and citrate, in addition to weak labeling of lactate and asparagine. On addition of GSK-J4, there is evidence of reduced glutaminolysis with a decrease in the level of glutamate and greatly reduced label from glutamine seen in 2-oxogluterate and other TCA cycle intermediates. Addition of GSK-J4 increases the label from glucose seen in glycerol-3-phosphate and does not increase entry of glucose-derived metabolites in to the TCA cycle.

JJN-3 MM cells use glutamine as the main carbon source for the TCA cycle. Inhibition of the lysine demethylase enzymes KDM5B, KDM6A and KDM6B using the small molecule GSK-J4 is able to prevent glutaminolysis, leading to decreased TCA cycle activity. It is unclear whether the observed changes in metabolism are the cause of the reduced profileration and apoptosis observed, or a further consequence of a common upstream event. The mechanism by which these changes occur is under active investigation.

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SINEUPs: a new functional class of natural and synthetic antisense non-coding RNAs that activate translation

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ENCODE and FANTOM projects have been proving that the majority of the mammalian genome is transcribed, generating a vast repertoire of transcripts that includes mRNAs, long non-coding RNA (lncRNA) and repetitive sequences, such as SINEs (short interspersed nuclear element) and LINE (long interspersed nuclear element).

Analyzing the non-coding part of the transcriptome, we have identified a group of natural and synthetic antisense non-coding RNAs that activate translation of their sense protein-encoding genes. These molecules have been named SINEUPs, since their function requires the activity of an embedded inverted SINEB2 sequence to UP-regulate translation. SINEUPs are thus the first example of gene-specific inducers adding an unexpected layer to post-transcriptional gene regulation and providing a versatile tool to increase protein synthesis of potentially any gene of interest.

The secondary structure of the SINEB2 element (183-nt) has been determined utilizing DMS and CMCT chemical footprinting. The SINEB2 RNA folds into a structure with mostly helical secondary structure elements. A high-resolution structure of the relevant hairpin has been derived utilizing NMR spectroscopy in solution.

Functionalized gold nanoparticles for biological applications: a NMR viewpoint on the dynamics and organization of protecting monolayers

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With respect to classic pharmacology, the emerging field of nanomedicine offers a groundbreaking approach to deal with core biomedical issues such as molecular targeting, biosensing, drug delivery and tissue or cell imaging. Indeed, nanomedicine relies on the use of nanotechnologies to trigger molecular responses, to interfere with specific biochemical pathways or to modify the distribution properties of active species. In particular, nanoparticles are considered as the most promising candidate for biomedical applications, since they provide a protein-sized template with countless possibilities of surface functionalization and distinct properties of the nanosized core [1].

In the context of nanomedicine applications, our project focuses on a NMR investigation of the surface dynamic processes occurring in monolayer-protected nanoparticles. The first results [2] support the idea that different functionalities on the nanoparticle surface can be arranged into specific motifs that promote the interaction towards selected biomolecules.

The investigation of functionalized nanoparticles has been carried out on a multi-level approach, both by solution and by solid-state NMR. Solution state NMR is useful to study the dynamic processes in the more flexible moieties of the monolayer, which are the most interesting as far as interactions with biomolecules are concerned. On the other hand, solid-state (SS) NMR experiments can reveal those signals that in solution are broadened beyond detection, thus providing complementary information about slow dynamic processes. Accordingly, we recorded solid-state ^{13}C NMR spectra and performed relaxation times measurements under fast-MAS conditions (60 kHz of MAS frequency) for samples of nanoparticles functionalized with different monolayers. Both the solution and the solid-state NMR data highlight a different mobility of the chains grafted on the nanoparticles surface, depending both on their distance from the gold core and on the functional groups inserted. The analysis of experimental relaxation rates will be then compared with predictions obtained from a stochastic model built on a likely description of internal hydrodynamics and on the features of the torsional free-energy landscape. Relying on a novel computational non-equilibrium tool [3], we expect this analysis to provide criteria for designing nanoparticles targeted at the interaction with biological partners.

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Structure of *Trypanosoma brucei* 1-C-Grx1, a mitochondrial monothiol glutaredoxin with an unusual intrinsically disordered N-terminal extension

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Glutaredoxins (Grx) are small proteins conserved throughout all the kingdoms of life that are engaged in a wide variety of biological processes. According to the number of cysteines in their active site, Grx are classified as dithiolic or monothiolic (1-C-Grx) [1]. In most organisms, 1-C-Grx are implicated in iron-sulfur cluster (FeS) metabolism and utilize glutathione as cofactor.

Trypanosomatids are parasitic protozoa of the order *Kinetoplastida* that cause severe diseases in human and domestic animals. These parasites exploit a unique thiol-dependent redox system based on bis(glutathionyl)spermidine (trypanothione) rather than on glutathione [2]. Mitochondrial 1-C-Grx1 from trypanosomes differs from orthologues in several features including the use of trypanothione as ligand for FeS binding and the presence of a parasite-specific N-terminal extension whose role has yet to be elucidated.

We have recently shown that 1-C-Grx1 from *Trypanosoma brucei* (Tb) is indispensable for parasite survival in mouse, making this protein a potential drug target candidate against trypanosomiasis [3]. We will present here the high resolution structure of Tb1-C-Grx1 and discuss possible implication of this structure on the protein function. Although the conserved domain presents a well defined global fold very similar to other glutaredoxins, it reveals subtle structural peculiarities compared to orthologues. Furthermore, our analysis proved that the N-terminal extension is highly disordered and paved the way to disclose the role of this intrinsically disordered element.

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Structure and dynamics governing the binding mechanisms in the interactions between SUMO-binding motifs and SUMO

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DNA double stranded breaks (DSBs) are highly cytotoxic, and if left unrepaired, can lead to mutations, genomic instability and the subsequent development of various cancers. Cells respond to a DSB by recruiting numerous DNA damage signaling and repair proteins, many which are post-translationally modified, to the chromatin surrounding the break. As part of the DNA DSBs repair process, the chromatin surround the break is ubiquitylated by the ubiquitin E3 ligases RNF8, RNF168 and HERC2. Recently it has been shown that HERC2 binds SUMO1 non-covalently [1], where further investigations have identified a zinc-finger ZZ-domain of HERC2 responsible for the interaction. Hence, the ZZ-domain represents a new SUMO interacting motif (SIM). There are ZZ-domains present in 20 different proteins in the human genome, where at least 2 additional ZZ-domains (ZZ_CBP and ZZ_MIB1) bind SUMO1 as shown by ITC and SPR.

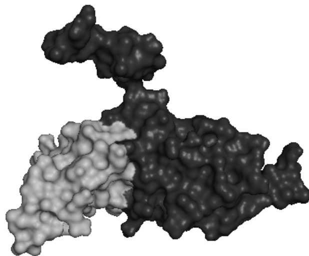

Figure 1: ZZ_CBP-SUMO1 complex

Chemical shift changes upon formation of the ZZ_CBP-SUMO1 complex allow us to map the interaction surface for both ZZ_CBP and SUMO1. These measurements show that ZZ_CBP binds SUMO1 through a novel binding mode, which is significantly different from other known interaction surfaces previously identified on SUMO1. Based on these chemical shift changes in combination with residual dipolar couplings, we performed docking simulations using HADDOCK. The docked protein complex has a backbone RMSD to interaction surface of 1.3 Å and 2.2 Å for SUMO1 and ZZ_CBP, respectively. These docking simulations show that the Zn-binding loop of ZZ_CBP binds to a grove on SUMO1, adjacent to the flexible N-terminal domain of SUMO1.

In order to further understand the molecular basis for the binding mechanism of SUMO1, we performed CPMG relaxation dispersion experiments on SUMO1 in the apo-state as well as for SIM- and ZZ-bound states. In the absence of any ligand, SUMO1 is exhibiting fast intrinsic exchange located predominantly to the SIM-binding site. Upon addition of a SIM-peptide, the exchange is to a large extent quenched. Comparing chemical shift changes obtained from global fits of CPMG relaxation dispersions of the bound state show a good correlation with chemical shift changes between SIM-bound and apo-SUMO1. In contrast, ZZ_CBP-bound SUMO1 exhibits a similar set of exchanging residues as apo-SUMO1 as well as additional exchange in the flexible N-terminal part.

These results show that ZZ-domains bind SUMO1 by utilizing a novel binding epitope, distinctly different from other established interactions with this protein. In addition, SUMO1 experiences intrinsic exchange, indicative of a conformational selection process for binding SIMs, and separated for the binding to ZZ-domains.

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Design principles for large dynamic multiprotein machines: the bacterial replisome

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The bacterial replisome, is the multiprotein complex that simultaneously copies both DNA strands at replication forks. Because synthesis of the lagging strand occurs discontinuously, the replisome is very dynamic. Components need to be recycled from the end of each Okazaki fragment to the beginning of the next. We study replisome dynamics as a model system to uncover rules that govern the structures and functions of nature's large, dynamic multiprotein machines. We are integrating information from many different kinds of experiments (including NMR) to develop high-resolution structural models to explain aspects of replisome function. In general terms, we conclude that:

1. Replisome dynamics are determined by a hierarchy of more than 25 different protein–protein and protein–nucleic acid interactions. These range from being very weak and transient to being strong and essentially permanent.
2. These interactions are coupled to a few irreversible chemical steps that involve nucleotide hydrolysis or incorporation. These steps act as ratchets to trap appropriate intermediates on the thermodynamic pathways.
3. Some proteins, like the sliding clamp processivity factor, act as interaction hubs where multiple proteins interact sequentially at a single binding site to simplify and direct these pathways.
4. Many protein–protein interactions occur via intrinsically disordered regions that become structured on interaction, but there are also regions of proteins that need to remain unstructured to enable large conformational changes even in fully assembled replisomes.

There are two DNA Pol III replicase complexes (five proteins and primer–template DNA) in the replisome. I will use the replicase as an example of our approaches [1–3], focussing especially on the information derived by solution-state (with Gottfried Otting, Australian National University, ANU) and solid-state NMR (with Guido Pintacuda and Anne Lesage, RALF-NMR), often coupled with cell-free protein synthesis for specific isotopic labelling and incorporation of unnatural amino acids (with Kiyoshi Ozawa, ANU). Other techniques used to infer structures and/or dynamics include X-ray crystallography (with Aaron Oakley, University of Wollongong, UOW), high mass range ESI-MS (Jenny Beck, UOW), synchrotron SEC-SAXS (Moeava Tehei, UOW), chemical crosslinking, proteomics, bioinformatics, MD and modelling (Thomas Huber, ANU), as well as bulk phase and single-molecule DNA replication assays (Slobodan Jergic, UOW, Samir Hamdan, KAUST and Antoine van Oijen, University of Groningen).

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Mechanism of spider silk formation studied by NMR

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Spider silk proteins (spidroins) consist of three structural units: a central highly repetitive region composed of polyalanine and glycine-rich co-blocks and two non-repetitive globular regions: a highly conserved N-terminal domain (NT) and a less conserved C-terminal domain (CT). While the central repetitive part accounts for spider silk's exceptional mechanical properties, the N- and C-terminal domains are implicated in the silk formation process [1, 2]. During passage from the silk gland to the spinning duct spidroins experience changes in pH, ion composition and shear forces, that have been proposed to be of importance for the silk fiber formation [3]. The CT has been suggested to undergo structural changes in response to altered ion composition and shear forces, but not to pH [4]. NT, on the other hand, has been proposed to work as a pH-regulated relay by changing its quaternary structure upon lowering of the pH from about 7 (as in the gland) to about 6 (as in the duct) [5].

As a step towards understanding the molecular mechanism of spider silk formation, we determined the NMR structures of *Euprosthenops australis* major ampullate spidroin (MaSp) NT in solution at pH 7.2 in presence of 300 mM NaCl [6], at pH 5.5 in presence of 20 mM NaCl [7], and of a monomeric NT mutant (A72R) [6]. We show that MaSp NT exists as a monomer at pH 7.2 and as a stable dimer at pH 5.5, and their interconversion is mediated by protonation of three glutamate side chains (E79, E119 and E84) in concert with relocation of a wedged tryptophan side chain (W10).

NMR studies of the *Araneus ventricosus* minor ampullate spidroin (MiSp) CT, which forms a domain-swapped dimer in solution at pH 6.8, revealed that it gets gradually destabilized and unfolds upon lowering of pH to 5.0. Furthermore, studies with CO_2 analogue CS_2 suggest that it might help unfold CT by interacting with partly buried regions in the protein [8]. Together with Trp fluorescence, CD, HDX-MS, ThT fluorescence and transmission EM data our results suggest that NT and CT work in completely different manners and that their opposite structural changes are central to the regulation of the silk formation process.

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Poster Presentations

MORe information from Paramagnetic NMR for the determination of interdomain motions

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The presence of metal ions in proteins has been considered by the X-ray spectroscopists as a blessing from above while, NMR spectroscopists were fighting against the paramagnetic effects often associated with metal ions: paramagnetism strongly affects the appearance of NMR spectra and sometimes even prevents signal detection. At the same time, however, paramagnetism-induced relaxation, contact and pseudocontact shifts (PCS) and self-orientation residual dipolar couplings (RDC) provide structural restraints that can compensate for the loss of diamagnetic restraints. One of the most promising exploitations of paramagnetism in metalloproteins is based on the combination of the various pieces of information derived from the anisotropic magnetic susceptibility tensor (PCS and RDC) to learn about the relative freedom of protein domains with respect to one another.

While calculating the NMR observables from a given conformation is a trivial task, finding the relative population of different conformers that compose the natural ensemble, based on ensemble-averaged experimental observables is an ill-posed and ill-defined inverse problem that admits an infinite number of solutions. For this reason we have introduced the concept of Maximum Occurrence (MO) [1, 2] as the only way to achieve quantitative information from averaged experimental data. Maximum Occurrence is defined as the largest amount of time that the system can spend in a given conformation and still be compatible with the experimental observables. We addressed by this method the interdomain mobility in calmodulin1 and its complexes, as well as steps preceding collagenolysis by matrix metalloproteinase-1 [3].

We now demonstrate that maximum occurrence correctly indicates the part of the conformational space that can really be sampled by the protein when limited mobility is expected (like in the case of a two domain protein with the interdomain motion drastically reduced by binding a target). We show how one can identify compact regions of space that can justify completely the experimental data, through an approach that is dubbed Maximum Occurrence of Regions (MORe). By tests on synthetic data we show that with the concept of MORe it is also possible to identify more subtle types of domain mobility, like an exchange between two centers. The method also allows to identify regions with a non-zero Minimum Occurrence (μORe), i.e. regions comprising conformations without which it is not possible to fully reproduce the experimental data.

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Backbone assignment of $^{2}H,^{13}C,^{15}N$-protochlorophyllide oxidoreductase (POR), a light dependent enzyme

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Protochlorophyllide oxidoreductase (POR) is a key regulatory enzyme in the chlorophyll biosynthesis pathway. POR catalyses the reduction of the C17-C18 double bond in protochlorophyllide (Pchlide) to produce chlorophyllide (Chlide) (Figure 1). This is a uniquely light-dependent reaction and can only proceed once an excited state of Pchlide is formed after absorption of a photon. Although there has been much interest and many studies involving POR, little is known about the protein structure and the exact role of light in the reaction.

Previous attempts to study the protein atomic structure have been unsuccessful due to failure of extensive crystal trials. POR is a relatively large (37 kDa, 322 residues) and hydrophobic protein, thus previous analysis by NMR has been problematic. The production of a highly pure, triple-labelled ($^{2}H,^{13}C,^{15}N$) sample and the use of a high field magnet through the Bio-NMR infrastructure have now produced high quality spectra for analysis. Relaxation experiments have also been carried out on the deuterated protein, in order to analyse the mobility of the protein. It looks promising that this could lead to a near full NMR assignment of POR, enabling the first structural and mobility data to be obtained for this unique enzyme.

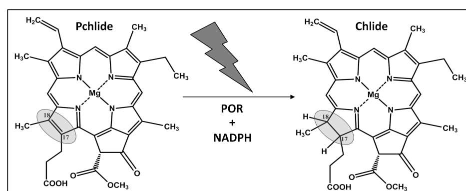

Figure 1: The reaction catalysed by POR

Practical aspects of non-uniform sampling at the sensitivity limit

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Non-uniform sampling (NUS) is an emerging technique to reduce the long experiment time typically associated with acquisition of multidimensional NMR experiments. Many techniques exist to reconstruct the NMR spectrum from sparse time-domain data sets: MaxEnt [1], MDD [2] and SIFT [3] to name a few. In general, such techniques are most reliable for high signal-to-noise ratios (SNRs), but tend to disfavor weak peaks. This makes such techniques more problematic for samples with low sensitivity. Recent algorithms like forward maximum (FM) entropy [4, 5] and related techniques like IST [6], FFM [7], and SSA [8] provide more linear reconstruction of the intensities, but are still challenged by sensitivity-limited samples. To use NUS for such samples, we have exploited the different techniques, and in particular their ability to detect weak peaks.

In this poster we present some of the characteristics of different sampling and reconstruction schemes and their effects on the reconstructed spectra [9]. Specifically, we discuss topics like NUS line broadening, linearity of peak intensities, and SNR limited peak detection. Examples are given for different types of spectra for liquid- and solid-state NMR spectra of proteins and phospholipid mixtures.

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Cold and heat unfolding and ^{15}N-CEST NMR reveal the hidden states of the antifungal disulfide protein PAF

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Antimicrobial peptides populate the border between the worlds of globular and disordered proteins and this is one of the reasons to study their unfolding propensities. We have studied cold and heat induced unfolding of PAF [1] in a broad temperature range using ^{15}N and ^{13}C NMR spy nuclei. The population of folded conformers is reflected in NMR cross-peak volumes that reach maximum at intermediate temperatures, at the "maximum stability" temperature.

In case of PAF, nine residues in the non-conserved loop regions could adequately be fitted with the accepted two-state Becktel-Schellman thermodynamic model. However, three-state models – either with or without intermediate states – are more appropriate to fit thermal unfolding. Interestingly, all models perform best if ca. 30% NMR-invisible conformers are assumed at maximum stability temperature. Supercooling of PAF in capillaries down to -15 °Cproduced neither new peaks nor a collapse of the ^{15}N-HSQC spectrum that would be expected for complete unfolding. According to conventional and cross-correlated ^{15}N relaxation, PAF is a rock-hard protein without exchange at the ps/ns timescale. Using the ^{15}N-CEST technique [2] at 298 K, we disclosed the chemical shifts of some hidden exchange conformers close to the C and N-termini. The drift of the ^{1}H/^{15}N chemical shifts as a function of temperature were also fitted using the two-and three-state models. The new, disulfide constrained PAF structure ("abcabc" pattern [3]) was used as an input for the calculation of structural ensembles reflecting internal dynamics of the observed state. In addition, key structural features of the observable state at the two temperature extremes as well as the CEST-detected hidden structure were estimated using an ensemble selection approach from accelerated molecular dynamics-derived conformer sets.

As a general conclusion, we suggest that the possibly high amount of hidden conformers of globular proteins may influence our views on molecular recognition mechanisms. In practice, it also may affect our interpretation of apparent NMR parameters and the accuracy of protein concentration measurements by NMR.

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G-quadruplex folding kinetics monitored by real-time NMR

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G-rich DNA sequences, in the presence of monovalent cations such as K^+, can form unusual base pairing and fold into four-stranded DNA structures called G-quadruplexes [1]. The folding pathway of human telomeric G-quadruplex is still unclear and the investigation of quadruplex kinetic landscapes is essential to shed light on the functions of telomeres and to develop anticancer drugs. Our aim is to characterize the species involved in the folding process at atomic resolution by NMR spectroscopy, using a rapid-mixing device [2].

We report our results on the K^+-induced folding kinetic of the human telomeric sequence Tel24 (TTGGG[TTAGGG]$_3$A) [3] in comparison with other human telomeric derived sequences [4, 5]. After injection of K^+, two distinct folded conformations are detected: a major conformation (hybrid-1) [3] and a minor conformation (Figure 1a). The folding process of Tel24 is biphasic and we propose that the minor conformer is again a quadruplex structure. NMR characterization of the topology of the minor conformer is in progress. NMR data suggest kinetic partitioning during refolding as displayed in Figure 1b.

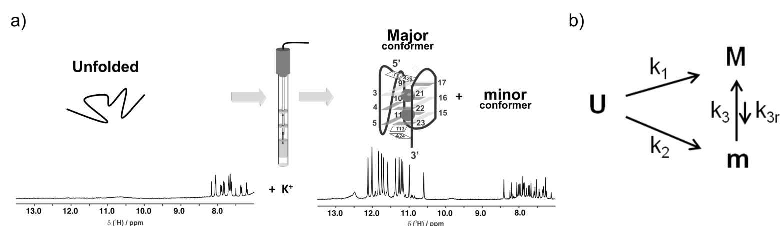

Figure 1: a) Experimental set-up for real-time NMR spectroscopy. b) Proposed folding pathway of the human telomeric quadruplex formed by Tel24.

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Novel interactions of a RING ubiquitin ligase with ubiquitin

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The posttranslational modification of proteins by ubiquitination plays an important role in many, if not all, cellular processes. Ubiquitination proceeds through the sequential activity of an E1 activating enzyme, an E2 conjugating enzyme and an E3 ubiquitin ligase. The ubiquitin ligases contain a signatory domain (RING, HECT or U-box) through which they bind to ubiquitin-charged E2 proteins, as well as domains for the recruitment of substrates, thereby providing specificity to the ubiquitination reaction. Ubiquitin ligases comprise a very large and heterogeous group, and many of the details of their regulation are not yet understood.

We are studying the activity of RNF125, or TRAC-1, a small RING ubiquitin ligase with roles in the immune system. The protein was identified as a regulator of T cell activation [1, 2], but has also been shown to downmodulate the intracellular viral RNA sensors RIG-I and MDA5 [3], thus controlling responses to viral infection. Our previous worked revealed that RNF125 contains, in addition to the RING domain, a C-terminal ubiquitin interacting motif (UIM), as well as three zinc fingers (one C2HC and two C2H2-type) of unknown function [4]. We further demonstrated that the protein associates with membranes as a consequence of myristoylation and has an extremely short half life (<30 min) as a result of extensive auto-ubiquitination and proteasomal degradation [4].

To obtain insights into the regulation of RNF125 activity and the role of its individual domains, we recently used mutational analysis combined with *in vitro* and cellular assays. Surprisingly, this revealed that, in addition to the RING domain, the C2HC zinc finger is crucial for ubiquitin ligase activity: mutations in this domain inhibit *in vitro* activity as well as auto-ubiquitination in cells, and in agreement with this, drastically increase the half-life of the protein. Molecular modelling showed that the RNF125 C2HC zinc finger shows structural similarity to ubiquitin-binding UBZ domains, leading us to hypothesize that this domain may be involved in interacting with ubiquitin and thereby enhancing the interaction with the ubiquitin-charged E2. Consistent with this, NMR analysis demonstrated spectrum perturbations of ^{15}N-ubiquitin in the presence of a construct composed of the RING and C2HC domain of RNF125. Moreover, the observed shifts correspond to amino acid positions located in regions of ubiquitin known to interact with ubiquitin-binding domains.

Thus, these initial NMR data support a novel model for regulation of RING ubiquitin ligases, whereby a C2HC domain contributes to productive E2$\sim$ub interactions, and future experiments will further map these interactions.

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Structure-function relationship of mutated forms of Arkadia E3 ubiquitin ligase RING domain

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The ubiquitin- proteasome pathway is a major pathway for the targeted degradation of proteins. In general, protein ubiquitination is catalyzed by a cascade of enzymes, including an ubiquitin- activating enzyme E1, an ubiquitin conjugating enzyme E2 and an ubiquitin ligase E3. E3 ubiquitin ligases are crucial in the selective recognition of target proteins and in the subsequent protein degradation by the 26S proteasomes [1]. Arkadia is an E3 ubiquitin ligase with a characteristic RING domain in its C-terminus. Arkadia is the first example of an E3 ligase that positively regulates TGF−β family [2].

The Arkadia RINGs, were cloned and expressed in their Zn-loaded form and studied through NMR Spectroscopy [3]. The 3D NMR solution structure of Arkadia RING was determined and deposited in PDB (2KIZ). Additionally, NMR-driven titration studies were also performed to probe the interaction interface of Arkadia RING and the partner E2 (UbcH5B) enzyme and the RING-E2 complex was constructed through an NMR-driven docking [4].

Additionally, this study resulted to the identification of Arkadia's RING functionally important residues, such as the conserved Trp972. Trp972 is considered as one of the key residues for E2 recognition and binding [5]. According to recent experimental evidence, the mutation of the Trp972 to Arg abolishes the ability of Arkadia to amplify TGF-b-Smad2/3 signaling responses in tissue culture transcription assays [6]. Various Arkadia Trp mutants are now being studied through NMR in order to obtain an atomic-level insight about the structural base of Arkadia capability to selectively interact with the appropriate E2.

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Structural and dynamical characterization of the asymmetric Ca^{2+} loaded S100A4d13-myosin complex

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S100 proteins belong to the family of low molecular weight Ca^{2+} binding proteins that generally exist as homo- or heterodimers within the cells. S100A4 gained increasing attention over the last years as its overproduction promotes cancer metastasis, chronic inflammatory and allergic diseases, therefore it is considered as a potential therapeutic target. Structure-function characterization including structural dynamic studies is a prerequisite for future drug development by inhibiting protein-protein interactions (PPIs), and requires simultaneous approach by various methods [1].

Tumor-metastasis associated cellular motility is coupled to S100A4 and myosin IIA (MPT) interaction [2]. The highly mobile C-terminus of Ca^{2+} loaded S100A4 causes aggregation and it is not involved in complex formation with the MPT fragment, thus, a truncated 91 residues long S100A4d13 form was used instead. A comprehensive multinuclear NMR investigation of both binding partners in free form and in complex permitted a detailed residue-by-residue characterization of the system. The assigned chemical shift values and measured diffusion coefficients confirmed S100A4d13 to be a homodimer, possessing the same secondary structure as the full-length S100A: 4 α-helices, 2 EF hands, and a flexible hinge region.

The binding affinity of both the full-length and the truncated S100A4 towards the MPT domain is in nanomolar range. The 45 residues long MPT tailpiece is intrinsically unstructured with an inherent helical region – proved by secondary chemical shift values and temperature - dependent reduced spectral density mapping of relaxation parameters. Upon binding to S100A, a disorder-to-order transition is detected, and the MIIA tailpiece adopts regular helical structure [3, 4]. As monitored by chemical shift mapping, the homodimer symmetry is broken in the S100A4-MIIA interaction, and the two monomer chains are no longer equivalent, but the secondary structure remains unperturbed. Relaxation measurements highlight differences in the dynamic behavior of the homodimer and the two monomer chains of the complex.

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Conserved amphipathic helix is required for stabilization of membrane curvature by a DP1 protein

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The endoplasmic reticulum (ER) is the largest and most striking organelle in the cell. It is composed of membrane sheets, both rough (ribosome studded) and smooth and an extensive, dynamic network of tubules. A class of integral membrane proteins comprising the reticulon (Rtn) and receptor expression enhancing protein (REEP) families is responsible for generating the high levels of membrane curvature required for ER tubule formation [1, 2]. Reticulon/REEP proteins contain unusual trans-membrane (TM) domains (∼30 amino acids) that are proposed to form oligomeric hairpin structures, which generate membrane curvature through a combination of hydrophobic wedging and scaffolding. In yeast *S. cervisiae*, there are three proteins involved in tubular ER membrane curvature generation, Yop1 (REEP homolog), Rtn1 and Rtn2. Currently there is no high-resolution structural information pertaining to the TM regions of these proteins, leaving unclear the exact mechanism by which membrane curvature is generated.

Using the single protein production (SPP) system in *E. coli*, we are able to purify large amounts of Yop1 (enriched with NMR observable isotopes), which can be reconstituted into detergent and bicelle systems, enabling the acquisition of high quality NMR spectra. The reconstituted protein is also able to form membrane tubules of ∼17 nm diameter and displays a polydisperse distribution of oligomers in solution. Here, we present the near complete NMR assignments and secondary structure of monomeric Yop1. We also identify a conserved amphipathic helix which is of crucial importance to the function of RHD proteins.

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Joint refinement of biomolecular structures by paramagnetic NMR and X-ray data

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We recently developed a computational tool for the joint refinement of X-ray crystallographic and paramagnetic NMR data in the framework of REFMAC5 [1]. Paramagnetic data, such as pseudo-contact shifts (PCSs) and self-orientation residual dipolar coupling (RDCs), provide useful long-range restraints that help in solving protein structures and in reconstructing the relative arrangements of complexes and multidomain proteins. For this reason, the addition of NMR data to one of the most widely used structural refinement software permits to complement the information provided by X-ray data, pointing out the differences between structures in the crystal and in solution and, in case of consistency of the data, to obtain more reliable refined structures.

The program was tested on several proteins, in some cases producing a single model consistent with both sets of observations [2, 3] and in other cases indicating the presence of non-negligible differences between conformations in solution and solid state [4].

The results obtained from the simultaneous use of solution NMR and X-ray data can also be framed in the overall evaluation of ensemble averaging approach, which relies on the retrieval of an ensemble of conformations to explain the experimental data whenever structural mobility is postulated. In this sense, the joint refinement is a necessary first step towards the discrimination between structures that require the use of an ensemble approach and structures that do not. Nevertheless, even if in some cases conformational variability should not be necessarily invoked to reproduce the experimental data, this does not rule out the possibility for the real biological system to experience mobility.

This work was supported by Ente Cassa di Risparmio di Firenze, the EC contracts Bio-NMR no. 261863 and BioMedBridges no. 284209, the COST Action TD1103 and EU ESFRI Instruct.

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Light dynamics of rhodopsin and its deactivation by arrestin

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Light absorption by rhodopsin is the initiation point for photoactivated signaling cascade in retinal rods [1]. Photoactivated rhodopsin attends meta II conformation, which interacts and activates the G-protein transducin. G-protein activation leads to a signaling cascade that finally hyperpolarizes the rod cells. Deactivation of rhodopsin is necessary to timely recover the photo response which is critical for maintaining sensitivity of the receptor in steady light. Arrestin acts as the major facilitator that downregulates the signaling pathways of GPCRs [2]. Here, we report the large scale recombinant expression of rhodopsin in mammalian cells, and the combination of NMR spectroscopy and laser techniques, as well as flash photolysis experiments, used to characterize the kinetics of the light activation process of rhodopsin and its deactivation process mediated by arrestin [3]. Our findings will provide insight into understand the signal transduction processes taking place in GPCRs.

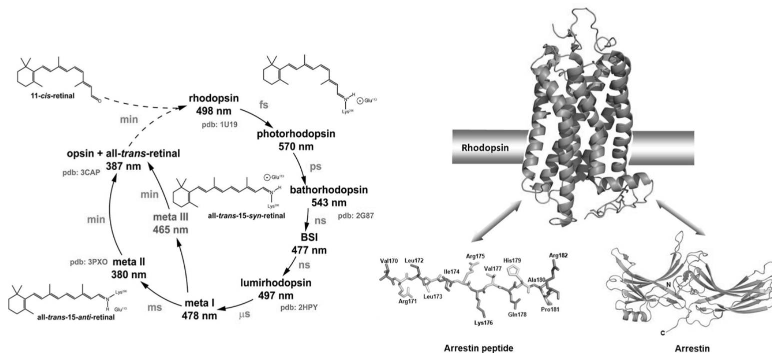

Figure 1: a) Photo cycle of bovine rhodopsin. b) Schematic representation of the interaction of rhodopsin with visual arrestin peptide and protein.

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M1 and M2 interactions and influenza virion budding

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The protein M2 from influenza is a tetrameric membrane protein with several roles in the viral life-cycle. The transmembrane helix (TMH) of M2 has proton channel activity that is required for unpacking the viral genome. Additionally a C-terminal juxtamembrane region includes an amphipathic helix (APH) important for virus budding and scission and that has been previously shown to be important for stability of the M2 tetramer [1]. The C-terminal region of M2 also contains motifs that interact with other viral proteins and the host protein LC3 [2]. One of the proteins that have been suggested to interact with M2 is matrix protein 1 (M1) [3]. M1 is essential for the structural integrity of the viral capsid and is also involved in interactions with several viral proteins. Neuraminidase (NA) and hemagglutinin (HA) are viral raft associated proteins, which initiate the formation of the viral budzone [4]. Both HA and NA have been shown to interact with M1 through their cytoplasmic tails. M1 then binds to the viral nucleoprotein and thus connects the nucleoprotein core and membrane of the virus.

Although it is known that the presence of M2 is essential for the successful exit of the nascent virus from the cell membrane, little is known about how M2 is recruited to the 'budzone' where membrane scission takes place. Here we demonstrate via SPR that M1 and M2 interact *in vitro*. We also show by NMR that beta strand residual structure is present in the otherwise unfolded C-terminal tail of M2. It is likely that the interaction with M1 provides a mechanism for the recruitment of M2 to the viral budzone, thus enabling the release of the virus from the cell.

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Regulation of TIA-1 binding to RNA by pH conditions

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TIA-1 is an RNA binding protein that regulates critical events in cell physiology by the regulation of pre-mRNA splicing and mRNA translation[1, 2]. It is constituted by three RNA recognition motifs (RRMs) and a prion-related domain. The protein binds to single stranded RNA sequences mainly through its C-terminal RRM domains, so-called RRM2 and RRM3.

Here we report that the RNA binding mediated by RRM3 domain is modulated by protonation/deprotonation events of its three histidine residues, whose pKa values are around the physiological pH [3]. Although the pKa of the only RRM2-histidine (His96) is significantly below this range, it becomes biologically relevant in the complex with RNA. This work follows by Nucleic Magnetic Resonance (NMR) the behaviour of the amide groups and the ionizable centers from imidazole rings in histidine residues induced by pH changes.

The pKa differences observed between the isolated RRMs and their complexes with RNA might be relevant to explain the functionality of TIA-1 protein, given that it shuttles between two cellular compartments (nucleus and cytoplasm) with slightly different pH values. Thus, it is of particular interest to explore the effects of pH changes on the protein binding to RNA.

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Pathological mutation Y65C affects the folding of WW domain in polyglutamine binding protein. Possible insight into Golabi-Ito-Hall syndrome

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WW domains are small domains (about 40 amino acids long) present in many human proteins with a wide array of functions. They were first found in YAP (Yes kinase associated protein), a protein able to bind SH3 domain of Src and Yes kinase. The WW domain recognizes proline rich sequences, just as SH3 but has different roles. The one belonging to polyglutamine tract binding protein 1 (PQBP1) is of particular interest. This protein is highly expressed in the brain and a single point mutation in its WW domain (Y65C) correlates with several X chromosome linked intellectual disabilities, including Golabi-Ito-Hall (GIH) syndrome. Mutation prevents binding to its ligand WBP11, which regulates mRNA processing. GIH patients are severely retarded, lean, short and with a small head.

In our laboratories we have developed a protocol for expression and purification of the WW domain with the aim of gaining insight into the molecular causes of such misregulation. Preliminary results based on ^{1}H,^{15}N-HSQC NMR spectrum and calculations of the folding landscape show that mutation Y65C largely destabilizes the structure. The effect is probably due to the formation of an intramolecular disulphide bridge involving the cysteine introduced by the mutation, which is found to rapidly oxidize *in vitro*.

Study of 7-helix transmembrane proteins using ultra-fast MAS NMR

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There is great interest in obtaining high-resolution structural data on 7-helix transmembrane (7TM) proteins due to their importance as drug targets [1]. However, crystallisation of these proteins is extremely challenging, and relies on the use of conditions which differ greatly from those found in native membranes. Solution NMR presents similar difficulties since non-native detergent environments are used in order to solubilise the molecules.

Solid-state NMR has the potential to provide high-resolution structural information within a native or native-like membrane environments, preserving the native structure and function of the molecules in question. Initial studies on 7TM proteins show that spectral assignment and structural characterisation of such systems is possible [2, 3]. A key problem with all methods for protein structure determination is the large amount of sample required, which contrasts with the typically very low expression levels exhibited by membrane proteins. Recent solid-state magic-angle spinning (MAS) solid-state NMR studies of fibrillar and microcrystalline proteins have shown that high-quality spectra can be obtained using ultra-fast spinning of samples which contain smaller amounts of protein [4]. In addition to allowing the use of low-power carbon-detected pulse sequences, the ultra-fast MAS regime also enables the use of more sensitive proton-detected solid-state NMR methods [5].

Here we use MAS frequencies of 60 kHz in combination with high magnetic fields to acquire low-power ^{13}C- and ^{1}H-detected experiments of the well-characterised bacteriorhodopsin 7TM protein in its native purple membranes. We investigate if these experiments yield spectra which are both sensitive and sufficiently resolved as to enable further studies on systems where the modest sample requirements of these methods will be advantageous, opening the door to the study of more challenging and previously inaccessible membrane protein systems.

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Two-dimensional Movie-NMR with temperature sweep

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Temperature dependent multidimensional NMR is a useful technique to study phase transitions of various kinds. However, the conventional approach which involves sampling of the full Nyquist grid in each of the temperatures of interest is time consuming. Also, discrete sampling of the "temperature space" can lead to loss of information of important processes. Our approach gives a novel solution to this problem [1, 2]. In this experiment sampling is done in parallel with the linear temperature sweep, thus allowing continuous and faster insight into the phenomenon. The processing with compressed sensing algorithms allows to create a "Movie-HSQC" spectrum which shows changes in a continuous way.

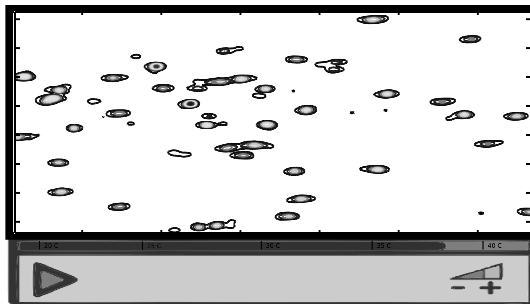

Figure 1: Movie-HSQC spectrum

We have demonstrated the technique on the unfolding process of two small proteins: SH3 domain of alpha spectrin from chicken brain (62 residues) and human ubiquitin (76 residues) using ^{15}N-edited HSQC. The results show that the technique can be used to monitor any chemical reaction or phase transition on-the-fly.

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BAZ2B bromodomain binding hotspots revealed by solution NMR and HADDOCK

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Bromodomains are epigenetic reader domains, which recognise acetylated lysines in their binding partners. These domains have recently generated much interest in the scientific community, mainly due to their involvement in disease Small molecules targeting the BAZ2B bromodomain have been reported [1], however details of the interaction with its natural binder, Histone H3 K14ac, are unknown.

We have assigned the backbone of the BAZ2B bromodomain and determined its dynamics. We then used ^{1}H-^{15}N HSQC NMR titrations to map bound histone peptides to the binding site and uncover new hotspots for interaction beyond the conserved Kac interacting residues. CLEANEX NMR experiments [2] (2) were used to monitor differences in water binding upon complexation. Models of the complex consistent with the experimental data were generated using HADDOCK's protein-peptide protocol [3, 4]. Site directed mutagenesis and peptide binding measurements obtained by isothermal titration calorimetry (ITC) were used to confirm the docking results. Overall, this study highlights hotspots on the bromodomain, providing a rationale for future inhibitor design.

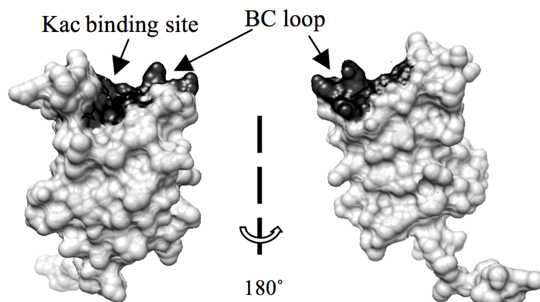

Figure 1: BAZ2B binding hotspots for the interaction with H3 acetylated lysines (Kac)

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Interaction of the mitochondrial ISC proteins Yah1 and Isu1 studied by NMR spectroscopy

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Mitochondria contain the complex Iron-Sulfur-Cluster Assembly (ISC) machinery which is crucial for the maturation of both mitochondrial and cytosolic iron-sulfur (Fe/S) proteins in eukaryotes.

Here, we determined the 3D structure of the mitochondrial [2Fe-2S] ferredoxin Yah1 in both redox states through NMR spectroscopy performing classical and non-conventional NMR experiments. In the latter approach, paramagnetic tailored NMR experiments were used to overcome fast relaxation of nuclei close to the Fe/S cluster which makes their detection impossible with standard approaches. Thereby, backbone resonance assignments for 92 and 88 out of 115 amino acid residues were obtained for oxidized and reduced Yah1, respectively. The undetected residues (stretches 96-108 and 143-145 in oxidized Yah1; 95-109 and 142-146 in reduced Yah1) were all located close to the [2Fe-2S] cluster. The NH signals in the C-terminal region (160-169), i.e. far from the cluster, were also extensively broadened, suggesting an unstructured polypeptide chain.

For better insight into the biochemical nature of the protein interaction, we used NMR spectroscopy combined with several biophysical and biochemical methods to structurally determine the contact interface between Yah1 and Isu1.

To explore the regions of interaction between Yah1 and Isu1 we performed NMR titrations through ^{1}H-^{15}N HSQC spectra under anaerobic conditions. In the presence of Isu1, spectral changes were observed only for reduced but not for oxidized Yah1, consistent with the affinity measurements by the equilibrium interaction method thermophoresis. Residues of Yah1 with largest variation were Ala133, Tyr134, Gly135, Gln88, Glu95, and Ile110. These residues are located in a region surrounding the [2Fe-2S] cluster on the α3 helix side, and likely represent the Yah1-Isu1 interaction region. Conspicuously, a group of acidic residues (Gln88, Glu95, Asp128, and Asp131) in helix α3 is present in the interaction region, suggesting a key role of these electrostatic residues in electron transfer to Isu1.

The final goal of this study is the understanding of the role of Yah1 as an electron donor in Fe-S protein biosynthesis.

How does the major birch pollen allergen Bet v 1 capture ligands?

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The major birch pollen allergen Bet v 1 represents one of the best-characterized model allergens in immunology and it is one of the main causes of Type I allergic reactions with an estimated 100 million people affected [1]. Previous studies have suggested that Bet v 1 probably functions as steroid storage protein or as transport protein that can bind different types of ligands. However, the exact physiological function of Bet v 1 remains elusive [2, 3].

The goal of our study is to gain more insight into the mechanism of Bet v 1 ligand binding and therefore we examine how binding partners are captured by this allergen and what kind of structural changes occur. Any structural rearrangements of Bet v 1 probably influence its activity [1]. We use a combination of NMR spectroscopic methods (i.e. TOCSY, HSQC titrations, PFG experiments, relaxation (CPMG dispersion, R_1, $R_{1\rho}$) and triple resonance experiments) and other techniques such as FT-ICR MS analysis, ITC measurements and computational studies to provide a comprehensive structural picture of the ligand capturing mechanism of Bet v 1.

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Molecular interactions of GIP incretin hormone with its N-terminal domain of GIP receptor

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Diabetes is a major threat to the global community. In this regard, incretin hormones play an important role for secreting insulin for the β-cell. One of the hormones, glucose-dependent insulinotropic polypeptide (GIP), is a gastrointestinal hormone that stimulates insulin secretion by interacting with a G-protein coupled receptor located in pancreatic β-cells. Due to its glucose lowering and insulinotropic properties, GIP is considered as a potential target for treating type 2 diabetes.

In our laboratory, we identified the solution structures of GIP in various solution conditions including membrane mimicking (micellular and bicellular) media using NMR spectroscopy and computational modelling techniques. In order to exploit the potential of GIP for diabetes therapy, our research focuses on understanding the GIP hormone-receptor interactions. In this work, using a NMR-based docking approach, we have determined the likely docking position of the hormone with its receptor binding region and revealed a likely interaction of GIP amino acid side chains with specific residues on the extracellular domain of the GIP receptor. These results provide a basic understanding of the interaction mechanism of GIP with its receptor that can be useful for studying the development of peptide or non-peptide drugs for treating type 2 diabetes and other related disorders.

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Probing the dynamics of a potassium channel in different functional states using solid-state NMR

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Potassium channels are tetrameric proteins that control the selective passage of potassium ions across the plasma membrane. Conductivity of ions through these channels is controlled by sensing changes in physicochemical parameters such as pH, ligand concentration, and membrane voltage. Two coupled gates direct this gating cycle: the selectivity filter (or inactivation gate) and the activation gate (Figure 1).

As a consequence of activation (C → O), potassium channels undergo a time-dependent slow inactivation process (I) that plays a key role in modulating cellular excitability. Interactions of the selectivity filter with specific residues from the pore helix and the gating hinge [1–4], specific lipid interactions [5, 6], as well as water occupancy behind the filter [7, 8], play an important role in this inactivation process.

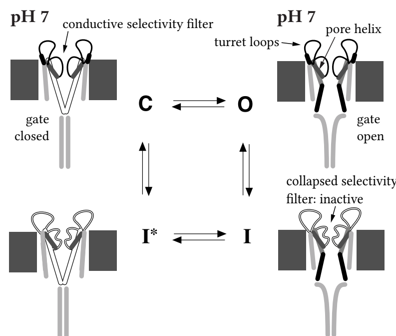

Figure 1: Schematic view of the gating cycle of KcsA, representing the closed conductive (C), open conductive (O), open inactivated (I) and closed inactivated (I*) states.

This study focuses on further understanding the role of local dynamics of the gates and their surrounding residues on gating and inactivation of potassium channels. We therefore aim at determining dipolar order-parameters and relaxation rates from MAS ssNMR experiments on KcsA in a lipid bilayer at different pH and potassium concentrations.

Initial results on the resting (closed-conductive) state of KcsA reveal that the HN dipolar order-parameters, which are sensitive to μs-ms motions, indicate a rather rigid selective filter (S^2_{dip} 0.9–1.0), while the turret region is slightly more flexible (S^2_{dip} 0.75–0.9). From the longitudinal ^{15}N relaxation rates we can conclude that more pronounced dynamics are observed on a faster ps-ns timescale in the turret region. In the selectivity filter only G79 that is located at the edge of the filter shows increased fast dynamics. These last results are in good agreement with observed backbone root-mean-square fluctuations from a 20 ns molecular dynamics trajectory of the closed-conductive state of KcsA.

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uNMR-NL: the Dutch ultrahigh-field NMR facility

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The development of molecular sciences for materials, food and health critically depends on comprehensive and non-invasive specimen characterization. Nuclear magnetic resonance (NMR) spectroscopy is one of the most widely applied analytical methods and has found abundant application in biology, medicine and materials research. The versatility of NMR to characterize equally well all kinds of materials, biomolecules, processes and living organisms makes NMR spectroscopy an indispensable tool in the search for solutions for major problems that our society faces.

To address these challenges, the five major Dutch centres for magnetic resonance research in structural biology, materials and metabolic mapping as well as imaging techniques, together with the public private partnership for analytical chemistry TI-COAST, formed in 2011 a national consortium. This concerted effort resulted in the implementation of a national ultra-high field nuclear magnetic resonance facility (uNMR-NL) that aims at providing open access to a new generation of NMR instruments operating at ultra-high field strength across scientific disciplines and industrial research. As a first step in this direction, the uNMR-NL consortium received funding to place in 2014 a 950 MHz standard bore equipped with several solution-, solid-state an micro-imaging probes that will in the near future be followed up by the first ultra-high field instrument, a 1.2 GHz standard bore NMR, in the Netherlands.

NMR insights into structural differences of cellular prion protein caused by mutations in human genome

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The development of transmissible spongiphorm encephalopathies (TSE) is associated with the conversion of the cellular prion protein (PrPC) into the misfolded, pathogenic isoform (PrPSc). In human genetic forms of these diseases, mutations in the globular C-terminal domain of PrPC are hypothesized to favor spontaneous generation of PrPSc in specific brain regions, leading to neuronal cell degeneration and death. Approximately 10–15% of TSEs are associated with the mutations. Our recent NMR studies were focused on structural characterization of different truncated recombinant human (Hu) PrPs carrying the pathological Q212P (90-231, M129) [1], V210I (90-231, M129) [2] mutations and protective E219K (90-231, M129) polymorphism [3–5]. While Q212P mutation is linked to GSS, the V210I mutation is linked to genetic CJD. The naturally occurring E219K polymorphism in the HuPrP is considered to protect against sCJD.

We have demonstrated that the determined structures of variants consist of unstructured N-terminal part (residues 90-124) and well-defined C-terminal domain (residues 125-228). Analysis and comparison with the structure of the WT Hu-PrP revealed that although structures share a similar global fold, mutations introduce some local structural differences. The determined NMR structures offer new clues on the earliest events of the pathogenic conversion process and could be used for the development of antiprion drugs.

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Apo-structure of the *M. tuberculosis* protein tyrosine phosphatase A

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At present, tuberculosis (TB) is still one of the most infectious diseases in the world and the development of new and improved drugs is crucial. The low molecular weight protein tyrosine phosphatase A (MptpA, 17 · 5 kDa, 163 amino acids) has been identified to play a major role in the interaction with host macrophages and is thus a potential target for developing small molecule inhibitors.

We elucidated the NMR solution structure of the free form MptpA and compared it to existing ligand-bound structures [1]. Our data uncovers pronounced conformational dynamics in well conserved regions that are critical for the catalytic activity of the phosphatase. The important loops flanking the active site, switch from an 'open' to a 'closed' conformation upon ligand binding. Furthermore, we characterized the interaction between MptpA and the complementary kinase PtkA (which phosphorylates two tyrosine residues in MptpA) by means of NMR titration experiments to gain more insight into the regulatory mechanism of MptpA.

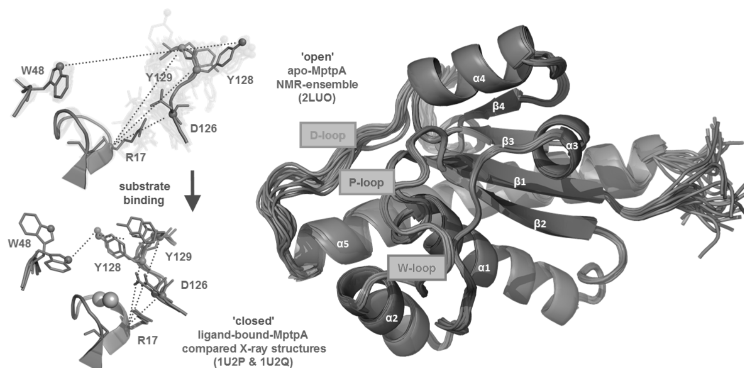

Figure 1

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Insights into complex formation between secreted phospholipases A2 and calmodulin

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Secreted phospholipases A2 (sPLA2s) are phospholipid-hydrolyzing enzymes and their enzymatic activity is involved in numerous pathophysiological conditions, such as inflammatory diseases, cell proliferation, cancer, acute respiratory distress syndrome, neurogenesis, embryogenesis and Alzheimer's disease. Tight regulation of their enzymatic activity is therefore of crucial importance. Ammodytoxin (Atx), a neurotoxic group IIA sPLA2s from the *Vipera a. ammodytes* venom, forms a high-affinity complex with cytosolic regulatory protein, calmodulin (CaM), which leads to substantial increase of its enzymatic activity (Figure 1). Until now, only the model of Atx-CaM complex exists.

In order to extend our knowledge of the intra-cytosolic CaM-associated regulation of the enzymatic activity of sPLA2s, we are studying the complex formation between Atx or the human group X sPLA2 (hGX sPLA2) and CaM using high-resolution NMR spectroscopy. To this end, we prepared fully functional recombinant unlabeled and $^{13}C/^{15}N$-labeled samples of CaM, Atx and the hGX sPLA2. ^{15}N-HSQC spectra of CaM alone and in the complex with unlabeled Atx or hGX sPLA2 were of high resolution, while the 15N-HSQC spectra of both sPLA2 alone and in the complex with unlabeled CaM were not. Comparison of the spectra of free CaM and CaM in the complex with either of sPLA2s revealed several significant changes of CaM chemical shifts. This can be interpreted with a conformational change of CaM upon its binding to sPLA2. Interestingly, the binding site on sPLA2s for CaM seems to be different from any of the canonical CaM-binding sites reported so far for other CaM-binding proteins. sPLA2s thus bind to CaM with unique binding motive.

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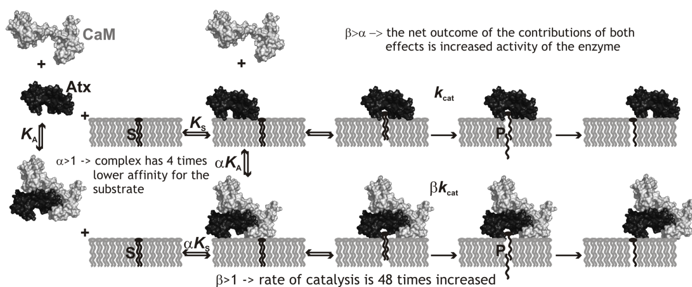

Figure 1: Schematic representation of the catalytic turnover of Atx in the presence or absence of CaM

Pga7: Fungal Heme Binding Protein

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Iron is an essential element for growth and proliferation of the majority of living organisms. However, iron bioavailability is limited, and as such commensal and pathogenic microorganisms have evolved sophisticated strategies to scavenge it. Heme is a good source of iron, as it is the most accessible iron source in the gastrointestinal (GI) tract, and serves as a cofactor in the most abundent hemoprotein, hemoglobin. Hemoglobin is the largest circulating iron pool in the human body.

Candida albicans, a commensal organism of the human GI tract, may cause severe systemic infections by disseminating into the blood in immunocompromised patients. *C. albicans* possess few iron uptake pathways. One of them is heme-iron uptake. The heme-iron uptake pathway depends on a family of cell envelope proteins characterized by the unique fungal CFEM domain. This domain is defined by eight cysteine residues with conserved spacing.

Pga7, a CFEM domain protein, is a crucial player in heme-iron uptake. This protein binds heme directly, and has a high affinity of about 65 nM to heme. The stoicheiometry of the interaction is one molecule of protein per one molecule of heme, with a specific Soret band appearing at 406 nm. The binding of heme monitored by ^{1}H NMR spectra results in a specific heme methyl resonance and small changes in the protein spectrum. Temperature dependence of heme binding follows anti-Curie behaviour, indicating the presence of a single spin-state. The effective magnetic moment calculated by Evans measurements stands at 5.5 uB and it is invariant over the investigated temperatures (298 K, 292 K, 286 K, 280 K). This is consistent with an iron(III) ($S = 5/2$) species. No shift in the heme methyl resonance peaks is observed upon pH changes (pH = 6.5, 7.4, 8.3, 9.2). This data supports penta-coordination of iron within the bound heme to Pga7.

Hemoglobin can serve as a good source of heme-iron. As such, we tested whether Pga7 interacts with hemoglobin. Our data from ^{1}H NMR spectra suggest that the heme is efficiently transfered from hemoglobin to Pga7 and results in saturation of 1 molecule of hemoglobin to 4 molecules of Pga7. This supports a model of heme extraction from hemoglobin and utilization of heme as the source of iron.

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Structural characterization of the G-triplex truncated-TBA

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Nucleic acids can adopt a variety of biologically relevant structures different from the standard Watson-Crick double helix, that can be constituted by one up to four strands. Base stacking and hydrogen-bond interactions, such as the Hoogsteen pairings, are at the basis of the stabilization of these non-canonical tertiary structures. Among these higher-order structures, G-quadruplexes have certainly played a major role in recent years because they are involved in a large number of biological processes, such as the maintenance of genome stability, and have also been exploited as attractive therapeutic targets for the development of anticancer drugs.

Recent metadynamics studies on the folding/unfolding process of the thrombin binding aptamer (15-TBA, 5'-GGTTGGTGTGGTTGG-3') suggested the formation of an energetically stable novel structural motif, the G-triplex, obtained from the G-quadruplex by the detachment of the extremity at the 3'-end [1]. A truncated form of 15-TBA, involving the last strand (5'-GGTTGGTGTGG-3') has been synthetized and analysed by NMR. The structural features of this new G-triplex folding have been investigated at atomic resolution by using external orienting media. Collectively, these new structural data show that t-TBA assumes a G-triplex DNA conformation as its stable form, reinforcing the idea that G-triplex folding intermediates may occur *in vivo* in human guanine-rich sequences.

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Individual human metabolic phenotypes

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In our group we collected the experimental evidences of the existence and uniqueness of an individual-specific metabolic phenotype (metabotype). Thorough the statistical analysis performed on NMR spectra of human urine samples an invariant part characteristic of each person was revealed. This individual fingerprint can be extracted from the analysis of multiple samples of each single subject [1]. Moreover analysis of individual phenotypes over the time scale of few years showed that the metabolic phenotypes are invariant and the constant part was significantly determined by genetic factors [2]. A new round of sample collection is ongoing, to study the evolution of the metabolic fingerprint over a longer time scale (6-9 years).

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NMR and MD studies of the new H7N9 hemagglutinin with human and avian glycosidic receptors

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Influenza A viruses are one of the most rapidly evolving pathogens with potential for new strains to adapt to human hosts and result in a pandemic outbreak, with significant economic and public health impact. The interaction between the influenza A virus surface protein haemagglutinin (HA) and the corresponding cell surface glycan receptors allows the virus to recognize the host cell to infect. Human (or human adapted) viruses recognize α2-6 Neu5Ac-Gal terminated glycan receptors, while avian viruses prefer glycans terminated with α2-3 Neu5Ac-Gal, these receptors are located on the upper respiratory epithelia of human and avian species respectively [1].

The advent in 2013 of the new H7N9 virus should be considered for its capability to infect humans, and also because human population have not the immunity on H7 virus subtype [2]. While pandemic H1N1 viruses showed greater affinity for human compared with avian receptor, the new H7N9 HA shows the ability to bind both α2-6 human and α2-3 avian glycosidic receptors with a comparable affinity [3]. X-ray crystallography of the H7/α2-6 and H7/α2-3 complexes reveals, as a static 'picture,' two different binding epitopes and different positions of the glycans in H7 receptor binding site (RBS).

In this work, STD-NMR (Saturation Transfer Difference) experiments and MD (Molecular Dynamics) simulation are applied to analyze the dynamic behavior of complexes between H7 and α2-6 and α2-3 sialylated pentasaccharides. The STD-NMR and MD simulation describe the Neu5Ac of both human and avian receptors as the main interacting residue with RBS of H7, even if the two glycans occupy different positions, according to X-ray crystallography data. On the contrary, the human glycan receptor interacts with H1 of the 'South Carolina 1918' pandemic virus, involving at least three residues of the pentasaccharide. Early structure consideration suggests how the introduction of two bulky hydrophobic groups Gln226Leu and Gly186Val [3] in H7 compared to H1 sequence reduce the affinity between the protein and the ligand. This study is a part of a project focused in undersatnding the molecular basis of the interaction between human and/or avian glycan cell surface receptors and this less known flu viral H7 HA, reinforcing knowledge elements for the pandemic event prediction and also helping in design drugs that modify the virus ability to infect cells.

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Investigation of the structure-activity relationship of E3 ubiquitin ligases: mutations of Arkadia RING-H2 domain

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Protein ubiquitination and subsequent degradation of intracellular proteins plays a crucial role in many cellular functions. Ubiquitination occurs through an enzymatic cascade which includes an ubiquitin-activating enzyme E1, an ubiquitin-conjugating enzyme E2 and an ubiquitin ligase E3. E3 ubiquitin ligases are responsible for target substrate recognition and degradation by the 26S proteasome [1]. RING type E3 ligases function through a characteristic domain in their C-terminus. Arkadia is the first example of an E3 ligase that positively regulates TGF-β family signaling through its C-terminal RING domain [2], while its homologue, ARKADIA-2, is implicated in BMP pathway.

The Arkadia RINGs were cloned and expressed in their Zn-loaded form and studied through NMR spectroscopy [3]. The 3D NMR solution structure of Arkadia RING was determined and deposited in PDB (2KIZ). Additionally, NMR-driven titration studies were also performed to probe the interaction interface of Arkadia RING and its partner E2 (UbcH5B) enzyme. The RING-E2 complex was constructed through an NMR-driven docking, too [4]. In order to investigate the role of Zn ion in the structure and function of Arkadia, two mutations were designed, H962C and H965C. Further research is currently taking place to explore their structure and interaction with E2 enzyme via NMR spectroscopy, while Arkadia-2 is being studied as well.

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Cytochrome c_1 exhibits two binding sites for cytochrome *c* in plants, as revealed by NMR in solution

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In plants, cytochrome *c* participates in channeling electrons rather than in simply shuttling them between complexes III and IV [1]. However, the mode cytochrome *c* behaves inside a supercomplex such as the respirasome, formed by complexes I, III and IV, remains veiled from a structural point of view. Here, we report NMR-driven docking computations showing two well-defined binding sites for cytochrome c at the head soluble domain of cytochrome c_1, namely a non-productive (or distal) site with a long heme-to-heme distance and a functional (or proximal) site with the two heme groups close enough as to allow electron transfer. The two binding sites exhibit equilibrium dissociation constants for the reduced species within the micromolar range, so revealing the transient nature of such a respiratory complex. Although the docking of cytochrome *c* at the distal site occurs at the proximity to the interface between cytochrome c_1 and the Rieske subunit, it is fully compatible onto the complex III structure. In our model, the extra distal site in complex III could indeed facilitate the functional channeling of electrons towards complex IV by building a “floating boat bridge” of cytochrome *c* molecules (between complexes III and IV) in plant respirasome.

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NMR spectroscopy reveals phosphorylation of Sp140 PHD finger and interaction with Pin1

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Sp140 is a IFNg-inducible leukocyte-specific nuclear transcriptional activator, member of the Sp100 family [1, 2] expressed in mature B, plasma and some T cells. Despite increasing evidences showing an involvement of Sp140 in the etiology of chronic lymphocytic leukemia (CLL) [3] the real function of Sp140 protein is still unknown. Sp140 has modular structure with a N-terminal "Sp100-like" domain for putative dimerization, a SAND domain, a PHD finger and a C-terminal bromodomain.

In a first attempt to get some molecular insights into Sp140 function, we have solved the solution structure of Sp140 PHD finger [4]. The structure presents an unexpected switch from an α/β to an all α-helical fold, characterized by the presence of an unprecedented *cis-trans* isomerization of a peptidyl-prolyl bond (Thr726-Pro727). In line with its structural peculiarity, *in vitro* binding assays indicate that Sp140 PHD finger does not bind to modified or unmodified histone tails. Prompted by the *cis-trans* isomerization of the Thr726-Pro727 bond, we asked whether Sp140 PHD finger could be a substrate for Pin1, a unique human PPIase able to catalyze the *cis-trans* isomerization of phosphorylated Ser/Thr-Pro bonds in a wide range of targets. By NMR titrations, we showed that Pin1 binds to Sp140 PHD finger and that the interaction surface is centered around the Thr726-Pro727 bond. With ^{1}H-^{1}H ROESY NMR experiments, we proved that Pin1 catalyzes the isomerization of a synthetic peptide containing the phosphorylated Sp140 PHD finger Thr-Pro bond. Co-immunoprecipitation in HEK293T cells confirmed in vivo Sp140 as a new Pin1 target.

We also exploited in-extract NMR experiments to test the propensity of the PHD finger to be phosphorylated. SOFAST ^{1}H-^{15}N HSQC experiments acquired on ^{15}N-labeled Sp140 PHD finger showed that the Thr-Pro is phosphorylated upon addition of a lysate from MEC1 cells (a CLL-derived cellular line). Importantly, after phosphorylation the *trans*-pThr-Pro bond is converted into *cis*-pThr-Pro, likely through Pin1 catalytic activity. To identify the Thr kinase specific for Sp140 PHD finger, we are performing NMR monitoring of *in vitro* phosphorylation reactions with different kinases and in-extract NMR experiments in the presence of kinase inhibitors. Although Sp140 function needs further investigations, data provided in this study include Sp140 in the list of the increasing number of Pin1 targets and suggest a Pin1-regulated modulation of the biological role of Sp140 PHD finger.

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Structural and dynamical characterization of human BolA3

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BolA-like proteins are generally grouped into three subfamilies designated BolA1-, BolA2-, BolA3-like proteins and have recently emerged as novel players in the iron-sulfur (Fe/S) protein biogenesis. In particular, it is known that BolA3 is involved in human mitochondrial assembly machinery (ISC), but its protein partners as well as its functional relevance in the Fe/S protein biogenesis are not yet defined. Genome-wide yeast two-hybrid studies have shown a physical interaction between cytosolic monothiol glutaredoxins (Grx) and BolA-like proteins in *Saccharomyces cerevisiae* and *Drosophila melanogaster* [1, 2]. Therefore, it can be postulated that BolA3 interacts with human glutaredoxin 5 (Glrx5), which is involved in the transfer of [2Fe-2S] clusters to protein partners.

NMR heteronuclear relaxation measurements were performed in order to obtain backbone mobility parameters and information about protein aggregation state. The results indicate that apo BolA3 is monomeric in solution and it is a rigid protein. Using triple resonance experiments, backbone and side chain resonance assignment of BolA3 was performed. Empirical prediction of ϕ and ψ backbone torsion angles was then obtained with the TALOS+ program. A structural model predicted with I-TASSER and a chemical shift-based protein structure prediction from CS-ROSETTA were then obtained and compared. These models show that the conserved Cys 33 is close to His 70, which was identified as Fe/S ligand in BolA2 [3], suggesting that Cys 33 is also involved in Fe/S binding.

After chemical Fe-S reconstitution in anaerobic conditions, BolA3 is not able to bind to iron-sulfur clusters. Fe-S reconstitution with the cysteine desulfurase Nfs1, the mitochondrial protein partner involved in ISC machinery, is now under investigation. Protein-protein interaction between BolA3 and Glrx5 was also investigated by NMR. Specifically, ^{1}H-^{15}N HSQC experiments were performed by titrating one ^{15}N-labeled protein with an unlabeled partner using different protein ratios. These data showed that two proteins interact, forming a stable complex. The functional role of this complex is now under investigation with the final aim of defining its role in Fe/S protein biogenesis.

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NMR studies on the *M. tuberculosis* PtkA-MptpA regulatory system

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Protein phosphorylation, regulated by phosphatases and kinases, is one of the most important mechanisms for cellular signal transduction. Characterization of the involved proteins and their interactions is of crucial importance for understanding the regulation of various disease related processes. Our studies on the *M. tuberculosis* regulatory system involve the low molecular weight protein tyrosine phosphatase A (MptpA) and the complementary kinase PtkA. To complete our previous structural studies on MptpA [1], we aim to solve the structure of PtkA (30.6 kDa, 291 amino acids) and the complex. Moreover, protein tyrosine phosphatases (PTPs) are known to be highly susceptible to oxidation of the catalytic active cysteine residue by reactive oxygen species (ROS) [2, 3]. Therefore, we investigate the oxidation of the MptpA cysteines (C11, C16 in the active site and C53) and consequently the influence on its phosphatase activity level.

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Structural studies of Zn(II) binding to minimal zinc hook peptides

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Protein-protein interactions play a crucial role in most cellular processes. Protein-protein complexes are usually stabilized by electrostatic and hydrophobic interactions, nevertheless some proteins utilize other mechanisms for this role. One such mechanism involves intermolucular coordination of metal ions that control protein assembly. Formation of the Rad50 protein complex, responsible for tethering of two DNA molecules during repair of double-stranded DNA breaks [1], relies on formation of ZnS_4, crosslink between their zinc hook domains. Despite biological importance of this protein complex and the fact that zinc binding is crucial to Rad50 complex formation, it is not clear how this complex achieves stability, given that only two ligands from each interacting molecule take part in zinc binding and that contact surface between protein subunits is relatively small.

It was recently shown that the 14 amino acid long minimal zinc hook peptide has zinc binding affinity comparable to that of native Rad50 monomer [2], and that even shorter peptides are able to bind zinc with high affinity. Our studies indicate that those peptides form stable dimers, with zinc coordinated by two peptides.

The goal of this study is to analyze the tendency towards complex formation between peptides and zinc as a function of peptide length, and to fully elucidate what kind of interactions are responsible for stable dimer formation.

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NMR characterization of DNA damage-inducible 1 protein (Ddi1) that acts as proteasomal shuttle

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The ubiquitin-proteasome system plays an essential role in the biology of eukaryotes. Through turnover of short-lived proteins, it regulates such vital processes as cell cycle progression, transcription, misfolded protein degradation, and immune response. One scenario of how ubiquitinated proteins are driven to the 26S proteasome for degradation involves UBL-UBA shuttle proteins (Rad23, Dsk2, or Ddi1), which recognize their substrate with the UBA domain and the proteasome with their UBL domain.

Ddi1 (DNA damage-inducible 1) protein has an unusual composition for a UBL-UBA protein, as it also contains a conserved retroviral protease fold domain (RVP). The detailed substrate specificity of Ddi1 as a shuttle is not known. However, it was ascertained that Ddi1 is required for the degradation of Ho endonuclease and F-box protein Ufo1, two proteins involved in cell cycle progression and regulation [1, 2]. In *Saccharomyces cerevisiae*, Ddi1 contains both UBA and UBL domains required for its shuttling function. Interestingly, over the course of evolution, Ddi1 has lost its UBA domain in mammals, which poses the question how shuttle protein can perform its function without the domain that binds Ub. Furthermore, the presence of UBL domain is also questionable, since the N-terminal gene sequence of Ddi1 in yeast shares low identity with Ub and other known UBL domains. In order to fully confirm that yeast Ddi1 is an UBL-UBA shuttle, we determined the solution structure of the nominal UBL and UBA domains. In both cases, we confirmed that they have the expected UBL and UBA fold. In addition to structural properties of both domains, we also studied their functional properties by examining the interactions with their known binding partners. To that end, we characterized Ddi1UBA interaction with Ub and Ddi1UBL interaction with UIM domains of the Ufo1 protein, since it was previously discovered that the turnover of Ufo1 depends on Ddi1 presence and specifically on the presence of its UBL domain.

UBL-UBA shuttle proteins are one of the better studied proteins in the ubiquitin proteasomal pathway. Nevertheless, it is not known in detail how they deliver target proteins to the proteasome for degradation. Our characterization of Ddi1 as an UBL-UBA protein confirmed its potential to be a shuttle protein, together with other UBL-UBA proteins like Dsk2 and Rad23.

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Sedimented, fibrillized, silica-entrapped and microcrystalline proteins

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Solid-state NMR (SS-NMR) is the method of choice for the study of insoluble objects, such as amyloid fibrils [1]. However, experimental efforts are devoted to the characterization of soluble objects, mostly as models for more complex/larger systems. This is usually accomplished by crystallization. We have demonstrated that pseudocontact shifts (PCSs) measured on microcrystalline powders of the paramagnetic cobalt(II) substituted matrix metalloproteinase 12 allow for solid state NMR crystallography [2]. Along with usual restraints from ^{13}C-^{13}C and ^{15}N-^{13}C mixing SS-NMR experiments, the protein molecular structure as well as the correct crystal packing are obtained. With this approach paramagnetic dilution is not needed for the discrimination between intermolecular and intramolecular pcs contributions [3, 4]. The first observation of strongly paramagnetically shifted signals in a microcrystalline iron-containing protein is also reported.

Crystallization is not the only way out: centrifugation confines biomolecules by gravity into a hydrated solid and, when gravity is removed, they revert to solution; these hydrated solids are known as sediments. We have shown that biomolecular sediments are extremely suited for SS-NMR [5], with their rotational motion prevented on a NMR-relevant timescale [6, 7]. Sedimentation can be achieved by magic angle spinning (MAS) of the rotor [5, 8], or by the use of an ultracentrifuge [9, 10]. Contextually, we have adapted the equation for sedimentation to the conditions of MAS [9], and created a web interface for the calculation of the experiment-relevant parameters [11].

Biosilica entrapped proteins are gaining great interest for industrial and analytical applications, yet characterization of these systems have so far relied only upon activity tests. We show that two entrapped proteins (MMP12 and hSOD) yield high quality SSNMR spectra, that can contribute to the understanding of the structural features in such systems in their artificial but functional environment [12].

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Functional amyloids from the opportunistic pathogen *Aspergillus fumigatus*

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Hydrophobins are small fungal proteins (< 20 kDa) characterised by their amphipatic properties and an idiosyncratic pattern of eight cysteine residues involved in four disulfide bridges. The soluble form of these proteins self-assembles at hydrophobic/hydrophilic or air/water interfaces to form an amphipatic monolayer. Hydrophobins are used by fungi to breach the air/water barrier and develop aerial hyphae, to prevent water-logging, to cover aerial hyphae and spores rendering them hydrophobic thus facilitating aerial growth and spore dispersal, to participate in the extracellular matrix or to form a protective layer during fruiting body development. Hydrophobins can also participate in host-fungi interactions [1]. The RodA hydrophobin of the threatful opportunistic pathogen Aspergillus fumigatus forms an amyloid monolayer with a rodlet morphology that covers the surface of fungal spores. This rodlet coat renders the spores, which are the infectious form of the mould, inert relative to the immune system, preventing the recognition of pathogen-associated patterns (PAMs) [2].

We aim at describing the self-association of RodA into rodlets, characterising the structure of the amyloid rodlets and shedding light on the possible relationships between structure and immunological inertness. We have shown by NMR that recombinant RodA expressed in Escherichia coli can be successfully refolded in vitro into a native protein with the same structure than the protein extracted from fungal spores and that it can auto-associate into amyloid rodlets in vitro that show the same morphology as the rodlets at the surface of the spores. As a first step in the analysis of the self-association of RodA, we have studied the structure and dynamics of RodA by solution NMR and shown that the protein displays new as well as conserved structural features relative to other hydrophobins. A mutational analysis has highlighted important residues for rodlet formation that may be involved on the one hand in the spine of the amyloid fibres and on the other hand on the lateral association of the rodlets to form a monolayer. We have also explored the relationship between structure and immunological inertness.

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Structural basis for SINEUP RNA activity

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Recently, it has been shown that a new class of long non-coding RNAs (lncRNA), named SINEUP, can regulate protein synthesis at a post-transcriptional level [1]. These antisense (AS) RNAs interact with their sense mRNA partners through a short overlaping sequence and also contain an embedded inverted SINEB2 element. Previously, an inverted SINEB2 element within the AS lncRNA of the mouse ubiquitin carboxyterminal hydrolase L1 gene (Uchl1), which is involved in brain function and neurodegenerative diseases, has been identified as key for the RNA's activity [1].

The secondary structure of the SINEB2 element (183-nt) has been determined utilizing DMS and CMCT chemical footprinting. The SINEB2 RNA folds into a structure with mostly helical secondary structure elements (Figure 1). The structure exhibits several bulges, asymmetric internal loops and hairpins. A deletion mutant in the region of the stem-loop 1 (SL1) completely abolished the ability of the AS RNA to up-regulate UCHL1 protein production, while no such effect was detected with deletions in other regions.

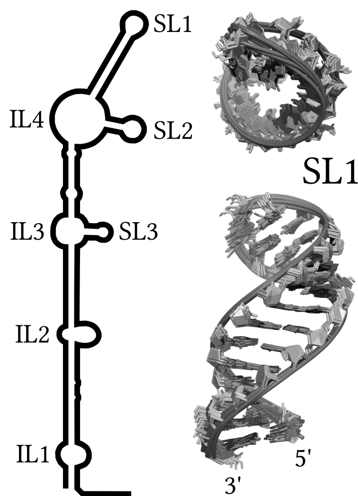

Figure 1: Structure of SINEB2

A high resolution structure of the relevant hairpin (SL1) has been derived utilizing NMR spectroscopy in solution (Figure 1). SL1 (38-nt) RNA folds into a stable hairpin structure. The stem region with observable imino protons is comprised of seven G:C, three A:U and one G:U base pairs with an additional U:U mismatch. After careful examination of the SL1 structure a similarity with the anticodon arm of tRNA molecules becomes apparent. Current data suggests that SINEUP RNAs could function in a similar matter as IRES (internal ribosomal entry site) elements, whose unique structural features enable them to initiate protein production. The tRNA like structures of these elements are constrained by the shape of the ribosomal tRNA binding sites. The tRNA anticodon arm features five base pairs terminated by seven unpaired nucleotides, three of which are the anticodon. According to molecular dynamics based on NMR data Uchl1 SL1 exhibits a triloop, however, the loop is followed by two unstable A:U base pairs, which we believe could open readily (e.g. upon ribosome binding). Since no conserved sequence was found between several active SINEUP RNAs, the possible interaction with the ribosome is most likely based purely on structural rather than sequential recognition.

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Metabolic analysis in *Trypanosoma cruzi* employing steady-state free precession (SSFP) NMR

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High resolution NMR is one of the most widely employed techniques for metabolomic/metabonomic analysis [1, 2], due to the large amount of information obtained from a single ^{1}H spectrum. Complementary analyses, such as ^{13}C and 2D-NMR, can improve the final datasets, providing ways to evaluate the composition of a complex mixture, illustrated in Figure 1 by its ^{1}H NMR spectrum.

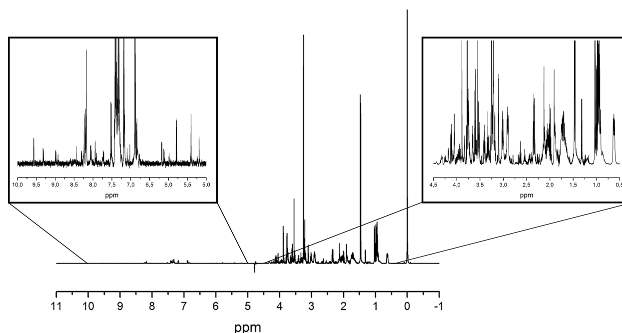

Figure 1: ^{1}H NMR spectrum of *T. cruzi* cell extract, showing the complexity of the sample

However, due to the low sensitivity of ^{13}C, the complete study of a single sample can take several hours, depending on its concentration. Steady-state free precession (SSFP) NMR has been studied as a fast method for ^{13}C acquisition, showing good results in pure samples [3, 4]. In this work, we evaluate the viability of SSFP-NMR in the analysis of a complex mixture, such as a cell extract. In this case, we used an extract from *Trypanosoma cruzi*, since Chagas' disease is one of the most concerning tropical diseases in the Southern hemisphere. Metabolomic/metabonomic analysis can provide valuable information about the parasite's metabolism and its response to external stimuli, such as medium changes, temperature changes or the administration of a drug, e.g. benznidazole [5].

SSFP-NMR technique leads to spectra with improved signal-to-noise ratio when compared to the conventional technique, allowing the analysis of more samples in a given period of time. On the other hand, it causes severe phase distortions, preventing the use of regular Fourier transform for data treatment. Thus, this work also investigates alternative methods for FID processing, including apodization functions and mathematical treatments. This technique can represent an interesting tool for fast ^{13}C acquisition and/or acquisition in very low concentrations, where a better SN ratio is desirable.

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Conformational studies of SAA protein fragment (86-104) and their mutants. Consequence of Pro → Ala point mutations for peptide structure and flexibility

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Human serum amyloid A (SAA) is a highly conserved apolipoprotein associated with high-density lipoproteins (HDL). Predominantly produced by the liver, it takes part in HDL metabolism, pathogen defense and cholesterol transport. It also plays a major role in host defense during the acute phase of inflammation. It is known that SAA has amyloidogenic properties [1, 2]. Analysis of protein sequence and circular dichroism data together with theoretical algorithms revealed typical globular character of the SAA protein. Based on the homology to the N-terminal domain of hemocyanin, it was suggested that 80% of the molecule may consist of helical conformation and only the C-terminal part of SAA is potentially disordered [3]. The C-terminal part contains three proline residues, which are probably responsible for the disordered structure.

Recently, a specific interaction between SAA and human cystatin C, the ubiquitous inhibitor of cysteine proteases, has been suggested [4]. Using a combination of selective proteolytic excision and high-resolution mass spectrometry [5–7], the binding site in the SAA sequence as SAA(86–104) was determined. In search for the conformational details of the binding SAA fragment, we performed structural and affinity studies of peptides representing different Pro → Ala variants of SAA(86-104) (Figure 1). The results of our studies point to the importance of proline residues for the structure of the binding fragment.

SAA(86-104)	GRSGK^{90}DPNHFRPAGL^{100}PEKY
SAA(86-104) P(92,97)A	GRSGK^{90}DANHFRAAGL^{100}PEKY
SAA(86-104) P(92,101)A	GRSGK^{90}DANHFRAAGL^{100}AEKY
SAA(86-104) P(97,101)A	GRSGK^{90}DPNHFRAAGL^{100}AEKY
SAA(86-104) P(92,97,101)A	GRSGK^{90}DANHFRAAGL^{100}AEKY

Figure 1: Amino acid sequence of the SAA fragment binding to hCC and its Pro → Ala variants

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Selective non-uniformly sampled 4D H4'C4'(P)C4'H4' experiment for sequential assignments in ^{13}C-labeled RNAs

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The determination of RNA structure by NMR spectroscopy is a challenging task, since it is often associated with severe spectral overlaps. The low proton density and less chemical-shift dispersion in RNAs makes the task even more difficult. The sequential resonance assignment in RNA is achieved using through-space NOE-type [1] or through-bond HCP-type experiments [2] and the efficacy of both types of experiments is severely affected by spectral overlaps, which further increases with the size of RNA.

Here we propose a novel through bond, non-uniformly sampled, H4'/C4' selective, four-dimensional HC(P)CH experiment which provides sequential connectivity via $H4'_{(i)}$-$C4'_{(i)}$-$P_{(i)}$-$C4'_{(i+1)}$-$H4'_{(i+1)}$ links. The experiment is designed with an emphasis on achieving higher resolution, reducing sensitivity losses and selective coherence transfer from ^{13}C4' to ^{31}P and from ^{31}P to ^{13}C4'. For obtaining reasonable resolution, non-uniform sampling is employed in indirect evolution of t_1 (^{1}H) and t_2/t_3 (^{13}Cs). To reduce sensitivity losses, multiple quantum coherences are preserved during evolution/transfer delays. Selective inversion pulses are used for selective coherence transfer and prevent CC coupling evolutions. An interesting aspect of the experiment is the suppression of auto (out-and-back) peaks and enhancement of the cross (out-and-stay) peaks, which further facilitates the unambiguous resonance assignments in RNAs. The performance of the experiment was tested on a fully ^{13}C,^{15}N-labeled 34-nt hairpin RNA consisting of two A-RNA form stems, one adenine bulge, an asymmetric internal loop and a GAAA terminal loop [3].

The proposed experiment complements the set of two recently reported high dimensional experiments, 5D-APSY HCNCH [4] and 4D-NUS C(aro),C(ribo)-NOESY [5] for resonance assignment in RNAs.

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Does the staggered-rotamer model do what we think it does?

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If we agree that it takes four J-coupling measurements in order to determine a torsion angle unambiguously [1], why then do people traditionally attempt to retrieve amino-acid side-chain rotamers on the basis of only two J-coupling constants? Just how trustworthy can the population parameters for those staggered states be?

We demonstrate that the popular staggered-rotamer model in its classical form describes rotamer equilibria inadequately. The population parameters obtained are counterintuitive and sytematically wrong, even if extended or complete sets of up to 6 or 9 coupling constants per torsion are available. This is a model-inherent consequence of referring, first, to only three fixed equi-spaced angles and, second, to only two focal values of J_{trans} and J_{gauche} associated with these [2]. A Karplus equation is notoriously not being applied in the process and, in fact, the bimodal curvature of the coupling-angle dependency is being missed entirely in the classic rotamer analysis. The issue will be highlighted with J-coupling data acquired for χ_1 torsions in the amino-acid side chains of the enzyme RNase T1. We also devise a numerical procedure that, albeit incapable of fixing the problem, does help ameliorate it.

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Solution structure of H-NS C-terminal domain bound to its target DNA

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H-NS, one of the most abundant proteins in *Escherichia coli*, is able to condense the DNA and to act as a transcriptional repressor of many genes [1]. The protein consists of an N-terminal protein oligomerization domain and a C-terminal DNA-binding domain separated by a linker. Oligomerization allows HN-S to bind to multiple sites on the DNA, thereby regulating the activity of many different genes.

The peculiar features of the interaction of the C-domain of H-NS with a target DNA fragment have been illustrated in a previous article [2]. Here we show the structure of the complex between the C-domain of H-NS and a target DNA fragment 20 base pairs in length and discuss the main features of this interaction. We show that H-NS binds to the minor groove of DNA through a loop previously characterized by NMR titration experiments.

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Membrane mimicking systems used in conformational analysis of short peptides: structure and dynamics of mixed micelle of dodecylphosphocholine and sodium dodecyl sulfate

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Solutions containing micellar aggregates are frequently used as membrane mimetic systems to perform conformational analysis of short peptides acting at the cell membrane interface. The most common membrane-mimicking aqueous media compatible with NMR studies in solution are detergents such as n-dodecylphosphocholine (DPC) and sodium dodecyl sulfate (SDS), at a concentration much higher than their CMC. The surfactants form spherical aggregates with polar head groups on the surface and the hydrophobic tails in the core, and can solubilize peptides. The dodecyphosphocholine (DPC) provides a zwitterionic surface on the micelles that adequately mimics biological membranes of the vertebrates, whereas the SDS micelles, with a negatively charged head group, adequately imitate the bacterial membrane [1]. However, to mimic electrostatic properties of the vertebrate plasma, a membrane characterized by a slight prevalence of the negative charge, mixed DPC and SDS micelles can be used as well [2–4]. Although the pure DPC and SDS micelles are well described in the literature, the characterization of DPC:SDS mixed micelles is still insufficient. Recent studies of these binary systems, performed by Manzo et al. [5], showed that the SDS and DPC media are synergetic and can be used jointly to prepare mixed micelles with different negative/zwitterionic surfactant molar ratios.

In our studies, we decided to investigate the behavior of mixed DPC:SDS micelles at three molar ratios of 1:0; 9:1 and 5:1. The critical micellar concentration (CMC) was determined for all studied media using surface tension measurements and isothermal titration calorimetry. The intermolecular and intramolecular surfactant interactions were characterized by the spin-lattice relaxation (T1) and the spin-spin relaxation times (T2). The ^{1}H diffusion studies were carried out to understand the effect of head group interactions on the structural organization of the constituent components in the mixed micelles. For DPC and DPC:SDS 5:1 systems, CG MD simulations yield detailed insights into the process of self-organization.

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Solution structure determination of the cocaine-binding DNA aptamer

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Aptamers represent a class of nucleic acids with the ability to bind ligands with high selectivity and specificity. The structural characterization with X-ray crystallography or solution NMR spectroscopy led to important insights into the molecular mechanisms how nucleic acids can form intricate molecular architectures and accommodate a certain small molecule.

About ten years ago, a DNA sequence able to specifically recognize the alkaloid cocaine was obtained via an *in vitro* selection process. Using solution NMR spectroscopy, a secondary structure proposal for the cocaine-binding DNA aptamer was obtained. Further, the ligand binding mechanism could be elucidated by other biophysical methods. Although these significant efforts were undertaken to address the details of the binary DNA-cocaine complex, no high-resolution 3D structure was reported so far [1–3].

To obtain the solution structure of the DNA-cocaine complex, we introduced an efficient site-specific deuteration protocol to facilitate the first mandatory step of the project – the resonance assignment step. With this information, the next step will be the determination of the solution structure of the cocaine-binding aptamer. This will give interesting insights on how this DNA sequence is able to specifically bind cocaine and will further deepen our understanding of how nucleic acids can expand their functional repertoire by intricate 3-dimensional folds.

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Fragment-based approach to targeting Mycobacterium tuberculosis dephospho-coenzyme A kinase – using NMR spectroscopy to explore fragment binding sites

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Coenzyme A (CoA) is an essential enzyme cofactor in intermediary metabolism. It functions as an acyl group carrier and a carbonyl-activating group, and provides the 4'-phosphopantetheine prosthetic group incorporated by carrier proteins such as acyl-carrier protein. The enzyme-mediated pathway by which CoA is synthesised from pantothenate (vitamin B5) is an attractive target for the development of drugs against pathogenic microorganisms including *Mycobacterium tuberculosis*, the bacterium that causes tuberculosis. In this project, we targeted *M. tuberculosis* dephospho-CoA kinase (MtDPCK) – the essential enzyme [1, 2] that catalyses the final step in CoA biosynthesis – using a fragment-based approach.

A library of fragments (compounds of low molecular weight and complexity) was screened against MtDPCK using a combination of biophysical techniques (a fluorescence-based thermal shift assay and ligand-observed NMR) as well as a biochemical assay. To investigate the binding site of fragment hits, ^{15}N-labelled *Escherichia coli* DPCK (EcDPCK; a 25 kDa, single-domain DPCK used as a surrogate for the catalytic domain of the 46 kDa, two-domain MtDPCK) was titrated with fragment hits. Protein backbone resonance assignment was carried out by performing triple resonance NMR experiments using ^{2}H,^{13}C,^{15}N-EcDPCK, which enabled chemical shift perturbations observed upon titration with fragments to be mapped to the EcDPCK crystal structure.

We present here fragments found to bind MtDPCK with high ligand efficiency and discuss their binding modes as implicated from the protein-observed NMR experiments. SAR investigations and strategies for developing the fragment hits into high-affinity inhibitors of MtDPCK will also be discussed.

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NMR insights into the conformational plasticity of the extracellular domain of a prokaryotic nAChR homologue

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Pentameric ligand-gated ion channels (pLGICs) of the Cys-loop family are transmembrane glucoproteins, which are important regulators of rapid chemo-electrical transduction. However, the ion permeation and gating mechanisms of these membrane proteins remain elusive. Recently, the X-ray structures of two prokaryotic homologues of the nicotinic acetylcholine receptor (nAChR), which is the most studied member of the LGIC family, have been determined. The first is the bacterial Gloeobacter violaceus pentameric LGIC homologue (GLIC) studied at 2.9 Å resolution in an apparently open conformation [1] and the second is the bacterium *Erwinia chrysanthemi* (ELIC) pentamer, studied at 3.3 Å resolution defining a closed conformation of the channel [2]. It is interesting to note that the extracellular soluble domain of GLIC has been found to remain in monomeric state in solution, though it assumes a hexameric quaternary structure when is crystallized [3].

The 200-residue extracellular domain of GLIC was cloned and expressed in high yields in *E. coli.* The ^{1}H-^{15}N HSQC exhibits signal dispersion typical for polypeptides with mainly beta structure. $^{13}C/^{15}N$ labeled GLIC was studied using heteronuclear multidimensional NMR spectroscopy and < 40% of the backbone nuclei were originally identified. Deuterated, triple labeled $^{2}H,^{13}C,^{15}N$ samples were used for the acquisition of triple-resonance NMR spectra and 50-60% of the backbone resonances have been identified by the analysis so far. Selective ^{15}N labeling and unlabeling techniques were utilized for 12 different amino acids in prototrophic and auxotrophic *E. coli* strains aiming at the identification of approximately 80% of the backbone resonances. NMR data suggested that various GLIC segments are characterized by conformational exchange behavior. NMR data in higher temperatures, H/D exchange experiments and ^{15}N relaxation measurements were used to determine the dynamics of the protein and the determinants of the GLICECD assembly and oligomerization [4].

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High-dimensional experiments for the quantification of cross-correlated relaxation in IDPs

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NMR has proven particularly useful for investigation of intrinsically disordered proteins (IDPs) as it provides unique means for characterization of internal dynamics, conformational changes and structures they adopt upon binding. However, IDPs are also quite demanding for NMR spectroscopy due to inherent spectral crowding and exposure of HN to solvent exchange. In addition, NOE-based methods, which are routinely employed for structure elucidation of well-folded proteins, are of minor relevance.

Recently, we have extended the portfolio of NMR experiments suitable for IDPs by the introduction of a four-dimensional experiment for quantification of dipolar HNN – CSA (C') cross-correlated relaxation (CCR) [1]. We have demonstrated that CCR rates are sensitive indicators of subtle conformational transitions that may occur upon environmental changes or binding.

In this communication, we propose new pulse schemes for measurement of the following CCR rates: *intraresidual* dipolar $H^{\alpha}C^{\alpha}$ – CSA (C'), and dipolar–dipolar *intra-* and *interresidual* HNN – $H^{\alpha}C^{\alpha}$. The experiments were devised are redesigned from previosly reported lower-dimensional counterparts [2–5]. Ensuring high spectral resolution was of particular importance. This was achieved by the suppression of passive *J*-couplings, and incorporation of evolution periods (shared-time whenever possible) for nuclei featuring reasonable chemical shift dispersion, i.e. CO and ^{15}N. Also, the symmetric interconversion principle [5] is exploited to avoid line splittings, which are otherwise necessary for discrimination of differently relaxing DQ/ZQ coherences.

The issue of severe signal overlap is adressed by the use of non-uniform random sampling, high-dimensionality and appropriate data processing which ensures linear reproduction of peak intensities and preserves high sensitivity [6].

We envisage that novel cross-correlated relaxation experiments should complement information available currently from NMR methods (RDCs, chemical shifts, PREs, PCSs).

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Structure determination of the dithiol glutaredoxin Grx1 from the pathogen *Trypanosoma brucei*

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Trypanosoma brucei trypanosomatids are protozoan organisms of the order *Kinetoplastida* which cause African sleeping sickness, also known as sleeping sickness in humans and nagana in animals. The parasite thiol metabolism is based on trypanothione [bis(glutathionyl)spermidine] and the flavoenzyme trypanothione reductase [1]. The absence of the trypanothione system in mammals, the lack of a functional redundancy within the parasite thiol system, together with the sensitivity of trypanosomes against oxidative stress, render the components of this redox metabolism attractive as drug targets.

Glutaredoxins are small ubiquitous proteins that catalyze glutathione-dependent redox reactions. The genome of *Trypanosoma brucei*, the causative agent of African sleeping sickness, encodes for three monothiol glutaredoxins (1-C-Grx1, 1-C-Grx2 and 1-C-Grx3) and two dithiol glutaredoxins (2-C-Grx1 and 2-C-Grx2) [2]. Recently we have solved the structure of 1-C-Grx1, highlighting some peculiarities of this parasitic protein [3]. *T. brucei* 2-C-Grx1 is the first dithiol Grx with a classical CPYC active site shown to coordinate an iron-sulfur cluster [4]. Here, we reveal the structure of the dithiol 2-C-Grx1. In addition, we investigate the different binding properties of glutathione and trypanothione with 2-C-Grx1 [1].

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Metabolomics and its applications to disease fingerprinting

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Metabolomics is a new "omic" science with the purpose of elaborating a comprehensive analysis of the metabolome, which is the complete set of metabolites in a biofluid or cell. Nuclear magnetic resonance (NMR) is an extremely powerful technique to provide the metabolic profile of a subject through the acquisition of spectra that require relatively short acquisition times and little or no sample handling. This technique has been successfully exploited by our group in different pathological contexts providing significant information on a wide range of pathologies, such as celiac disease [1, 2], heart failure [3] and breast cancer [4]. It is expected that metabolomics will be the basis for a future new paradigm in personalized medicine and in prevention, allowing us to really switch from the classical reactive medicine to a true predictive and preventive medicine [5].

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Qualification and traceability of Tuscany milk through NMR-based metabolomics

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The value of agricultural products is associated with their origin, species and composition. Growing conditions of vegetables and fruits, and in case of dairy products, nutritional habits of animals produce a variability in the metabolic fingerprint of the resulting products. ^{1}H-NMR spectroscopy can be used to profile agricultural samples to extract information about traceability and composition. In this sense, we evaluated metabolic composition of Tuscany bovine milk and short and long supply chain fruits and vegetables.

Engineered non-fluorescent Affibody molecules facilitate studies of the amyloid-beta (Aβ) peptide in monomeric form: low pH reduces Aβ/Cu(II) binding affinity

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Aggregation of amyloid-beta (Aβ) peptides into oligomers is considered a causative factor in Alzheimer's disease (AD). Aβ binds metal ions such as Cu(II) via ligands provided by the N-terminal histidines and the N-terminus. As metal ions are over-represented in the brains of AD patients, and as Cu(II) modulates the Aβ aggregation pathways, metal binding likely plays a role in AD progression. Aβ peptide aggregation is known to be pH-dependent, and inflammatory conditions leading to physiological acidosis appear to be involved in AD. The specificity of metal binding to Aβ varies at different pH values and between different metal ions, but so far binding affinity between metal ions and Aβ has not been quantitatively investigated at sub-neutral pH levels. This may be explained by the difficulties involved in studying the monomeric peptide under conditions that promote aggregation.

We have recently shown that a modified Affibody molecule, ZAβ3(12-58), binds Aβ with sub-nanomolar affinity and locks it in a monomeric form without affecting the N-terminal region where the Aβ metal binding site is located. Here, we have designed non-fluorescent Aβ peptide-binding Affibody variants that keep Aβ in monomeric form and only slightly affect the peptide's metal binding properties. Using fluorescence spectroscopy, we show that Aβ(1-40)/Cu(II) binding is almost two orders of magnitude weaker at pH 5.0 (apparent KD = 51 μM) than at pH 7.3 (apparent KD = 0.86 μM). This is most likely caused by protonation of the histidines involved in metal ligandation. Our results indicate that engineered variants of Affibody molecules are useful for studying metal-binding and other properties of monomeric Aβ under physiological conditions, which will improve our understanding of the molecular mechanisms involved in AD.

Partial flower-like micelles studied by ^{1}H NMR relaxation spectroscopy

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^{1}H NMR relaxation spectroscopy allowed to study the self-assembly [1] of a double thermoresponsive diblock copolymer, poly(N-ethyl acrylamide)-b-poly(N-propyl acrylamide), in dilute aqueous solution into mixed star-/flower-like micelles [2]. A biphasic T_2 relaxation of the TMS-labeled end-group attached to the shell-forming poly(N-ethyl acrylamide) block above the cloud point of the poly(N-propyl acrylamide) block at about 25 °Creveals two different environments for the hydrophobic end group. A slow T_2 relaxation time reflects the TMS end groups at the periphery of a star-like micelle, whereas the fast T_2 relaxation corresponds to the TMS-end groups which folded toward the hydrophobic core, forming a flower-like micelle. The existence of both contributions in ^{1}H NMR relaxometry reveals the formation of mixed star-/flower-like micelles from a doubly TMS-labeled poly(N-ethyl acrylamide)-b-poly(N-propyl acrylamide) diblock copolymer above the aggregation temperature [3].

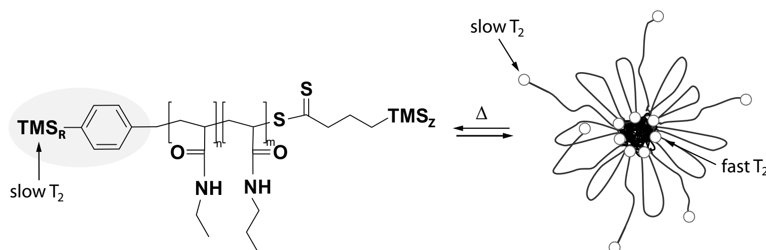

Figure 1

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Reduction of cellular toxicity of α-synuclein oligomers by epigallocatechin gallate

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Oligomeric species of various proteins are linked to pathogenesis of different neurodegenerative disorders. Consequently, there is intense focus on the discovery of novel inhibitors to block their toxicity. In Parkinson's disease, α-synuclein forms cytotoxic oligomers, consisting of $\sim$30 monomers on average [1]. Epigallocatechin gallate (EGCG) has previously been shown to redirect the aggregation of α-synuclein monomers and remodel α-synuclein amyloid fibrils into disordered aggregates [2, 3]. In this study, we investigated the effect of EGCG on the toxicity of the α-synuclein oligomers.

EGCG inhibited the ability of α-synuclein oligomers to permeabilize membranes and rescued rat neuronal cells from their toxicity. Liquid-state NMR spectroscopy showed that the N-terminus and the central hydrophobic region of α-synuclein build up the oligomer core whereas the C-terminus remains disordered in the oligomer state, and the flexibility of the C-terminus decreased upon EGCG binding. EGCG binds to the oligomers without changing either their secondary structure or size distribution. Thus inhibition of membrane permeabilization and the cellular toxicity is not due to dissociation or aggregation of the oligomers. Rather, EGCG inhibits the toxicity by reducing their binding affinity to membranes, highlighted as a viable therapeutic approach against Parkinson's disease.

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Solid-state NMR of native curli amyloid fibrils from *E. coli*

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Curli fibers are the major proteinaceous component of a complex extracellular matrix produced by many enterobacteriaceae. They are involved in adhesion to surfaces, cell aggregation, and biofilm formation. Curli are also potent inducers of the host inflammatory response. Curli are amyloid fibers 6–12 nm in diameter and several µm in length. CsgA is the major subunit of curli, while CsgB is the minor nucleator subunit. The primary structure of CsgA and CsgB can be divided into three domains, a sec signal domain, an N-terminal 22- or 23-residue domain, and a C-terminal amyloid core composed of five imperfect repeating units (R1-R5) [1].

Curli fibers are insoluble and non-crystalline, prohibiting structural studies of curli fibers by X-ray crystallography or solution NMR. However, solid-state NMR is a powerful biophysical tool that has been used to study amyloid fibers such as Aβ and HET-s [2–4]. High-resolution structure of curli fibers is a prerequisite to gain insights into its assembly and functions, and might help to understand the differences between disease-related and functional amyloids.

In the case of disease-related amyloids, material isolated from diseased tissue is typically not isotope labeled and therefore not amenable to detailed NMR studies. It is therefore necessary to rely on recombinantly produced amyloid fibrils, which may result in alternative structural states of the amyloidogenic protein. Bacterial amyloids offer therefore the unique option to compare the structural properties of recombinant and natively produces amyloid fibrils. Here, we developed the methods to express and purify uniformly ^{15}N,^{13}C-labeled native Curli from the *Escherichia coli* MC4100 strain. We have recorded ^{13}C-^{13}C solid state NMR correlation spectra and compared them to spectra of fibrils form from recombinantly produced CsgA. Our results revealed a striking similarity between the two samples, implying that recombinant CsgA fibrils adopt a highly similar tertiary structure compared to native curli.

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Novel NMR experiments for direct phosphorylation studies of intrinsically disordered proteins

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The phosphorylation of a specific amino acid residue is usually detected with NMR by observing a chemical shift change between the phosphorylated and dephosphorylated state of an amino acid [1, 2]. Unfortunately, because of the severe signal crowding on spectra of big disordered proteins, unambiguous identification of a modified residue using a 2D experiment such as 2D NHSQC, especially when several phosphorylation sites exist, can be very difficult.

The presented approach relies on a direct detection of the phosphorylation state. Thanks to the employment of phosphorus filtering within the pulse sequence, direct detection of the phosphorylated residues is possible. To provide good signal dispersion, a three dimensional experiment was used. The result is a triple resonance experiment HNCO(P) with a phosphorus filter and a quadruple resonance experiment HNCOP. 3D HNCO(P) allows an unambiguous identification of the phosphorylated and dephosphorylated amino acid residues. Moreover, 4D HNCOP allows one to obtain an additional phosphorus chemical shift for each phosphorylated residue.

The presented experiments were tested on a numerously phosphorylated human osteopontin, an intrinsically disordered protein containing 302 amino acid residues. Agilent 600 MHz spectrometer equipped with a room temperature Penta probe was used.

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What bio-NMR contributes to nano-medicine and nano-pharmacology

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Nanomedicine and nanopharmacology are rapidly developing scientific fields in which various biophysical methods (e.g. NMR, X-ray crystallography, MS, AFM, EM and SPR) are combined with molecular modelling approaches (including molecular docking and *ab initio* calculations) [1–3]. The resulting data of such approaches have to be further combined with biochemical as well as molecular and cell biological methods [4, 5] in order to address so-far unsolved clinical problems on a nano-scale level. Neuroscience, e.g. provides a number of open questions when anti-infection processes and neurological diseases have to be analysed on a sub-molecular size-scale. Two examples are chosen to emphasise this statement: In the case of anti-infection strategies one can observe that lysozymes of various animal species slightly differ in their structure from each other which may be related to the diverse bacteria which have to be attacked. Protein-carbohydrate interactions are of highest importance when humans and animals protect themselves against infections but these kind of interactions are also essential in the case of neuronal differentiation and regeneration processes [5–7] especially when sialic acids are involved [8]. Therefore, we are discussing beside lysozymes as second example also polysialic acid (polySia) receptors such as the lectin from the Chinese bird hunting spider *Selenocosmia huwena* Wang (SHL-1) used as suited role model. An improved understanding of the molecular interplay between carbohydrate chains and their receptors opens deeper insights into the relation between neurological disorders and the anti-infection strategies of nature.

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Transmembrane fragments of bilitranslocase transporter protein in lipid media

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The transmembrane protein bilitranslocase (BTL) is involved in the transport of bilirubin and several other anions from blood to liver cells. It is one of the identified human influx carriers. BTL has been identified as a potential target for cellular uptake of several drugs, so the detailed information about its 3D structure and mechanism of action is very important. Chemometric model developed on base of the counter-propagation neural network (CPNN) has been used to predict transmembrane (TM) regions in BTL. The stability of the polipeptide chains defined as four TM regions (TM1, TM2, TM3, and TM4) were studied by molecular dynamics simulation performed in two layers of dipalmitoyl phosphatidyl choline (DPPC) lipids. At present, the 3D structures of TM2 and TM3 BTL fragments in SDS micelle are evaluated [1, 2]. It has been confirmed that both segments – TM2 (^{73}Ser...Leu^{99}) and TM3 (^{220}Ser...Thr^{237}) – are folded into helix-loop-helix motifs with kinks around the central proline residues (Pro85, Pro231).

Here we present a 3D structure of a mixture of two transmembrane domains, TM2 and TM3, dissolved together in SDS lipid media evaluated with NMR spectroscopy (Figure 1). The DPFGDSTE (Pulsed Gradient Double Stimulated Echo) experiment was employed to extract the values of diffusion coefficients and calculate the hydrodynamic radius of the SDS micelle in the studied systems. The existence of both TM2 and TM3 in the same micelle was confirmed with the Förster Resonance Energy Transfer (FRET) technique. The evaluated structure demonstrates significantly different positions of TM2 and TM3 fragments in mixture than reported in our previous studies [1, 2]. Detailed analysis of the orientation of side chains in hydrophobic and hydrophilic residues suggests that the mixture of TM2 and TM3 peptides could facilitate the formation of some part of a BTL transmembrane anion channel.

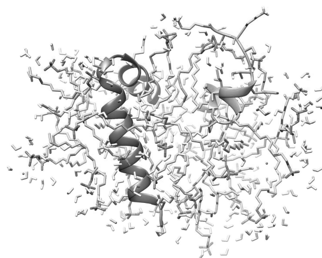

Figure 1: High-resolution 3D structure of TM2/TM3 mixture of TM fragments from BTL in SDS micelle after 50 ns molecular dynamic simulations with AMBER 11

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Head-to-tail connection of the bacteriophage virion: an NMR and EM study

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Bacteriophages, the viruses that infect and replicate within bacteria, constitute the largest known group of viruses. They occur everywhere in the biosphere where bacteria are found. The total number of phage species is estimated to reach millions, and their classification remains a challenge nowadays. However, the viral particle of most bacteriophages exhibits a head, mainly constituted by an icosahedral capsid that protects the double-stranded DNA viral genome, and a tail specialized in DNA delivery to the bacterial host cytoplasm. Further, structural studies of a few phage structural proteins suggest that the capsid, the head-to-tail connection and the tail are formed by structurally analogous proteins [1]. Thus, phage proteins with highly divergent amino acid sequences could assemble into similar virion architectures [2].

We focused on the structural analysis of the virion of the long-tailed SPP1 phage. The structure of the bacteriophage SPP1 capsid was determined at subnanometer resolution by cryo-Electron Microscopy and single-particle analysis [3]. We here report the solution structures of four head-to-tail connection proteins and the structural analysis of the major tail protein from this bacteriophage [4–6] achieved from solution-state nuclear magnetic resonance data. The SPP1 head-to-tail connection proteins are folded before assembly in the virion but also feature several unstructured regions. Docking of their structures into the EM density of virion subcomplexes provided a pseudo-atomic structure for the capsid portal vertex revealing that large unstructured loops of the head-to-tail connection proteins gp15 and gp16 acquire a defined conformation during construction of the virion [4]. The SPP1 major tail protein is poorly folded in its monomeric form, but is capable of forming tubes *in vitro* whose diameter is similar to that of virion tails. Analysis of the tube assembly process from infrared and solid-state nuclear magnetic resonance data and modeling of the assembled major tail protein structure from that of bacteriophage λ allowed the calculation of a pseudo-atomic model for the assembled virion tail. Analysis of the evolution of the analyzed structures during virion assembly and infection will be discussed.

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NMR structural studies of ubiquitin immobilized on mesoporous silica materials as a model of protein interactions with biogenic silica

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The cell wall of oceanic organisms such as the diatoms is made of diverse biosilica structures which are formed under the regulation of specialized proteins. Multidimensional MAS NMR experiments for in situ structural studies of these proteins adsorbed to silica were hampered by poor sensitivity and resolution. Here we demonstrate feasibility of such endeavors, by examining a model protein, ubiquitin (Ubq), immobilized on two similar forms of nanoporous synthetic silica materials, MCM41 and SBA15. The two silicas differ in pore diameter, density of surface OH groups and surface disorder. Ubq adsorbed onto MCM41 is shown to adopt a static conformation observed via ^{13}C CPMAS measurements and a dynamic unfolded conformation detected via ^{13}C INEPT and ^{15}N HMQC measurements. In contrast, on SBA15 it adopts only a static conformation. 2D ^{1}H-^{15}N HETCOR spectra indicate fewer transfers from backbone amide and sidechain Arg and Lys to surface OH and interfacial water in SBA15 than in MCM41 in consistency with lower enthalpy change in Ubq adsorption to SBA15 and in line with weaker interactions between the protein and the more hydrophobic surfaces of SBA15. Resolution is adequate for 2D ^{13}C DARR, 3D NCOCX and 3D NCACX on [U-^{13}C,^{15}N] Ubq on both surfaces with only sensitivity precluding complete backbone walk at the moment. DNP-enhanced 3D measurements at 180 K are shown, which partly alleviate the S/N limitation. Understanding protein-controlled material synthesis in organisms such as diatoms has implications to mineral formation in hard tissues.

Sugar profiles of Bulgarian oak honeydew honey by NMR

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In the last decade, there is a growing market in Europe for honeydew honey. In Eastern Europe, as well as in Germany, Switzerland, and Austria, the price of honeydew honeys is in general higher than the one of floral honeys, because they are regarded as more beneficial for human health [1]. A number of studies have demonstrated that honeydew honey possesses higher antibacterial and antioxidant activity compared to floral honeys [2]. For this reason, it is important to develop reliable procedures to discriminate honeydew honey from blossom honeys. Honeydew honeys contain specific microscopic elements (fungi and algae), but there are no quantitative quality criteria described so far [3]. The situation is further complicated by the fact that there are many different types of honeydew honeys [4]. Secondary metabolites and chemometric approaches, mostly PCA have also been suggested as potential solution for discrimination of honeydew honey [5]. In general, there are no internationally accepted quality criteria for the different types of honeydew honeys.

Based on the NMR carbohydrate profiles of the anomeric region of 24 Bulgarian honeys, we derived some specific features which set oak honeydew apart form floral honeys. The oak honeydew honeys are characterized by lower F/G ratio. Another important difference is the presence of kestose in all studied oak honeydew honeys and its absence in all studied floral honeys. Together with the presence of quercitol [6], detected in the course of the same measurement, NMR sugar profiling is a useful tool for discrimination of oak honeydew honey from floral honeys.

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