

PALANZA

XXXVII National Congress on Magnetic Resonance

Under the auspices of



Villa Castagnola, Verbania Pallanza 12 – 15 September, 2007

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Gruppo Italiano Discussione Risonanze Magnetiche

XXXVII National Congress on Magnetic Resonance

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Under the auspices of





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SCIENTIFIC PROGRAM

Wednesday, September 12, 2007

- 14,00 16,40 Registration of the participants
- 16,40 17,00 Welcome Addresses
- 17,00 18,00 J. Keeler, "Broadband proton-decoupled proton spectra"
- 18,30 21,00 Welcome Reception with folk music and dance

Thursday, September 13, 2007

- 09,00 09,45 B. Hills, "Developments in Multidimensional NMR relaxometry"
- 09,45 10,30 **C. Geraldes**, "Multinuclear NMR studies of Li⁺ effects on ion transport and metabolism in neuronal cell models and rat brain: a step towards the understanding of the therapeutic action of this drug"
- 10,30 11,00 A. Minoja, "New developments in food NMR"
- 11,00 11,30 Coffee Break

Methodology

- 11,30 **G. Pileio**, "Residual dipolar couplings in solid state measured by off-MAS NMR"
- 12,00 F. Rastrelli, "Measuring homonuclear scalar coupling constants by selective J-resolved spectra"
- 12,20 E. Vacchelli, "Optimization of NMR sequences through simulation of real samples and instrumental artifacts: Application to the LAPSR sequence"
- 12,40 **M. Concistrè**, "Double-quantum NMR of the first photointermediate in vision: Bathorhodopsin"

Metabolites and Metabolomics

- 11,30 **R. Consonni**, "NMR spectroscopy and chemometrics for quality assessment of traditional balsamic vinegar"
- 12,00 **G. Balacco**, "Automatic phase correction of NMR spectra: a breakthrough in the practice of metabolomics"
- 12,20 S. Tiziani, "Effect of AKR1C3 inhibitors in acute myeloid leukemia cell lines studied using NMR-based metabolomics"
- 12,40 **A. Sobolev**, "Strong increase of foliar inulin occurs in transgenic lettuce plants (Lactuca sativa L.) overexpressing the asparagine synthetase A from E. coli"

- 13,00 14,30 Lunch
- 14,30 15,15 **S. Aime**, "MR Molecular Imaging studies for innovative diagnostic applications"

Relaxation/Imaging

- 15,15 **M. Gussoni**, "Mobility, microstructure and MRI. DTI without a DTI black box: the art of making do"
- 15,45 **G. Fanali**, "Dynamic and allosteric properties of human serum albumin investigated by nuclear magnetic relaxation dispersion"
- 16,05 S. Avedano, "Relaxometric studies of Gd(III) complexes with novel EGTA derivatives and of their protein binding"
- 16,25 **M. Gombia**, "Water imbibition in porous media by quantitative magnetic resonance imaging"

Biological Macromolecules

- 15,15 **M. Assfalg**, "Monitoring the interactions of bile acid binding proteins and selected mutants with native and synthetic ligands"
- 15,45 F. Cesare Marincola, "Competitive binding of monovalent cations to the dimeric quadruplex [d(G₄T₄G₄)]₂: a
 ²³Na and ¹H NMR investigation"
- 16,05 L. Varani, "Rapid detection and characterization of T Cell Receptor-pMHC interactions"
- 16,25 J. Fredriksson, "Automated protein NMR assignments derived from multi-way decomposition of projected spectra"
- 16,45 17,15 Coffee Break
- 17,30 20,00 GIDRM and GIRM members meetings

Thursday, September 13, 2007

- 09,00 09,45 **M**. **Paci**, "Toxins from sea Contryphan Vn and the Pseudomonads lipodepsipeptide syringomycin; NMR spectroscopy, MD and beyond by genomics"
- 09,45 10,30 **R. Harris**, "NMR crystallography, polymorphism and shielding computations"
- 10,30 11,00 D. Rice, "Solid-state NMR with fast and ultra-fast MAS"
- 11,00 11,30 Coffee Break

Biological Macromolecules

- 11,30 **G**. **Musco**, "The Autoimmune Regulator PHD finger binds nonmethylated Histone H3K4 to activate gene expression"
- 12,00 **L. Calzolai**, "Structural and functional evolution of prion proteins"
- 12,20 **S. Tomaselli**, "Deep inside the binding mechanism of bile acid binding proteins through the analysis of binding properties of selected mutants"

Materials/Solid State

- 11,30 E. Locci, "Probing polymer colloids by ¹²⁹Xe NMR"
- 12,00 **S. Borsacchi**, "Multinuclear Solid-State NMR study of PE-PEG/silica hybrid materials prepared by sol-gel processes"
- 12,20 **A. Boccia**, "Propene-norbornene copolymers: analysis of polymer microstructure"
- 12,40 **R. Anedda**, "Thermodynamics and molecular details of sorption in nanoporous dipeptides: a ¹²⁹Xe NMR study"

- 13,00 14,30 Lunch
- 14,30 17,00 Poster Session, followed by coffee break
- 17,00 18,00 GIDRM-GIRM 2007 Gold Medal Award Ceremony
 R. Gobetto, "25 Years of playing with NMR parameters"
- 19,00 on Social excursion and dinner

Saturday, September 15, 2007

- 09,00 09,45 **P. Turano**, "Combined Solid State/solution NMR for large protein aggregates: ferritin as a case example"
- 09,45 10,30 T. Carlomagno, "How much slow can it tumble?"
- 10,30 11,00 Coffee Break

Small molecules

- 11,00 **D. Donghi**, "Multinuclear NMR study of the interaction between bis(pentafluorophenyl)borinic acid and nitrogen bases"
- 11,30 A. Matteucci, "Structural elucidation of new tricyclic systems by NMR spectroscopy"
- 11,50 **D**. Caronni, "Fragment-Based Approach applied to the development of potential inhibitors of Eukaryotic Translation Initiation Factor 4E (eIF4E) using ¹⁹F-NMR Screening"
- 12,10 13,10 Best Poster Award
- 13,10 14,30 Farewell Lunch

Solid State NMR of proteins

- 11,00 **G. Mollica**, "Nuclear relaxation mechanisms in silkworm cocoons"
- 11,20 M. Lelli, "Analysis of Solid-State NMR paramagnetic restraints for protein structural investigation"

PLENARY LECTURES

BROADBAND PROTON-DECOUPLED PROTON SPECTRA

James Keeler,[‡] Andrew J Pell,[‡] Richard A E Edden[†] and Benjamin K Simmons[‡]

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It has been known from the earliest days of two-dimensional NMR that in principle a complete separation of proton shifts and proton-proton couplings can be achieved by recording a two-dimensional J-spectrum and then tilting this by 45°. However, the difficulty with such spectra is that the lines are phase-twists. Due to the dispersive component of this lineshape the resolution of the spectra are severely compromised. In addition, the 45° projection of the spectrum, which gives a proton-decoupled proton spectrum, is zero.

Over the years, much effort has been put into trying to get around this problem, principally by eliminating the phase-twist lineshape; none of these methods seem to have gained wide acceptance. Recently we have developed two new experimental methods which, in effect, result in two-dimensional J-spectra with absorption mode lineshapes and which also preserve the *natural intensities*. The first [1] is based on an anti Z-COSY experiment, while the second [2] utilizes a novel pulse sequence element proposed by Zangger and Sterk [3]. These two methods will be described and possible applications outlined.

- [1] A.J.Pell, R.A.E.Edden and J.Keeler, Broadband proton decoupled proton spectra, Magn. Reson. Chem. 45 (2007) 296-316.
- [2] A. J. Pell and J.Keeler, Two-dimensional J-spectra with absorption mode lineshapes, submitted to J. Magn. Reson.
- [3] K. Zangger and H. Sterk, Homonuclear broadband decoupled NMR spectra, J. Magn. Reson.124 (1997) 486-489.

DEVELOPMENTS IN MULTIDIMENSIONAL NMR RELAXOMETRY

Brian Hills

Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, UK

Recent developments in 2-dimensional NMR cross-correlation relaxometry will be reviewed, including applications of pure relaxation pulse sequences such as T_1 - T_2 , T_1 - T_2 * and T_2 -store- T_2 as well as diffusion based sequences used in T_2 -D and D-D cross-correlation methods. Examples will be presented from work on biopolymers, cellular tissue and porous media. This review will be followed by brief descriptions of "work-in-progress" such as extensions to a third dimension based on field-cycling, chemical shift and q-weighting as well as the development of ultrafast versions of the pulse sequences. The last part of the presentation will be more speculative in nature and discuss the possible development of multidimensional Halbach NMR relaxometry, RheoNMR relaxometry, extreme NMR and NMR multisensors.

MULTINUCLEAR NMR STUDIES OF Li⁺ EFFECTS ON ION TRANSPORT AND METABOLISM IN NEURONAL CELL MODELS AND RAT BRAIN: A STEP TOWARDS THE UNDERSTANDING OF THE THERAPEUTIC ACTION OF THIS DRUG

C.F.G.C. Geraldes^{#,&,%} and M.M.C.A. Castro^{#,&,%}

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Li⁺ has been the first-line drug for acute and prophylactic treatment of bipolar disorder. Despite the therapeutic effectiveness, its mechanism of action remains uncertain. This work aimed at contributing to a better understanding of Li⁺ pharmacological action at the molecular and cellular levels [1]. Multinuclear NMR studies were performed in chromaffin and SH-SY5Y neuroblastoma cells, cortical and hippocampal neurons and cortical astrocytes, as well as in rat brain extracts. The membrane transport pathways involved in Li⁺ uptake were studied [2]. Intracellular cAMP levels regulate Li⁺ uptake and also Li⁺ plays an important role in the homeostasis of cAMP in neuronal cells [3]. Li⁺ was also found to displace Mg²⁺ from its intracellular binding sites [2]. These studies involved ³¹P and ⁷Li NMR and fluorescence techniques.

We also studied the ²³Na DQF and TQF NMR [4] behaviour of intra- and extracellular Na⁺ in several cellular systems: human erythrocytes in suspension, and immobilized bovine chromaffin cells, human neuroblastoma SH-SY5Y cells, rat astrocytes and neurons. Both anisotropic and isotropic contributions were observed for the extracellular Na⁺, in all types of cells. Intracellular isotropic Na⁺ motions were detected in all types of cells studied, while anisotropic Na⁺ motions were only detected in erythrocytes. The effect of Li⁺ on the ²³Na DQF and TQF signals for these cell systems provided evidence for the Li⁺/Na⁺ competition for Na⁺ cell binding sites, in most cases quenching the isotropic and anisotropic signals from the extra- and intracellular environments.

Li⁺ was also able to interfere with cell energy metabolism, as shown by ¹³C NMR upon addition of ¹³C-labelled substrates. An inhibitory effect of Li⁺ on glycolytic and tricarboxylic acid cycle fluxes was observed in SH-SY5Y cells [5]. Li⁺ decreased ¹³C-labelling of γ -aminobutyric acid (GABA) from glutamate in cortical neurons, without affecting ¹³C-labelling of glutamine from glutamate in astrocytes, suggesting that Li⁺ has a more evident effect on GABAergic neurotransmission.

- [1] D.M. Freitas, M.M.C.A. Castro, C.F.G.C. Geraldes Acc. Chem. Res. 39, 283-291 (2006)
- [2] C.P. Fonseca, et.al Biochim. Biophys Acta 1691, 79-90 (2004)
- [3] L.P. Montezinho, et. al J. Neurochem., 90, 920-930 (2004)
- [4] C. Srinivasan, et. al J. Magn. Reson. 140, 206-217 (1999)
- [5] C.P. Fonseca, et.al Neurochemistry International, 47, 385-393 (2005)

MR MOLECULAR IMAGING STUDIES FOR INNOVATIVE DIAGNOSTIC APPLICATIONS

Silvio Aime

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Molecular Imaging aims at the "in vivo" quantitative visualization of molecules and molecular events that occur at cellular level. The potential towards clinical translation is huge as the same modalities used in Medical Imaging are used in Molecular Imaging investigations. Traditionally, Medical Imaging was a tool for non-invasive mapping of anatomy and for the detection of and localization of a disease process. The advent of Molecular Imaging based protocols will allow to detect the onset of diseases at an early stage well before the biochemical abnormalities result in changes in the anatomical structure. Moreover it will offer efficient methods to monitor the effect of therapeutic treatments. The Molecular Imaging agents provide the crucial link between the specificity of the target and the quantitative visualization of its "in vivo" distribution.

The possibility of carrying out Molecular Imaging protocols by means of MRI is very attractive for the superb anatomical resolution that is attainable by this technique. However, MRI suffers for an intrinsic insensitivity in respect to the competing imaging modalities that has to be overcome by designing suitable amplification procedures based on the development of reporting units endowed with an enhanced sensitivity and on the identification of efficient routes of accumulation of the imaging probes at the sites of interest.

In this lecture I'll survey the MRI probes and procedures that contribute significantly to a more effective diagnosis and follow-up of therapeutic treatments. Particular attention will be given to the use of Gd-loaded nanosized systems that allow to match the sensitivity requirements of Molecular Imaging protocols, also at the light of developing imaging –guided drus delivery procedures [1]. Furthermore, I will discuss the potential of the new classes of frequency-encoded agents represented by the CEST agents (Chemical Exchange Saturation Transfer) [2] and Hyperpolarized Probes for Metabolic Imaging.

- [1] Geninatti Crich S., et al.:"Magnetic Resonance visualization of tumor angiogenesis by targeting neural cell adhesion molecules with the highly sensitive gadolinium-loaded apoferritin probe", *Cancer Res.* 2006, 66, 9196.
- [2] Terreno E., et al.: "From spherical to osmotically shrunken paramagnetic liposomes: an improved generation of LIPOCEST MRI agents with highly shifted water protons", *Angew. Chem. Int. Ed.* 2007, 46, 966.

TOXINS FROM SEA CONTRYPHAN Vn AND THE PSEUDOMONADS LIPODEPSIPEPTIDE SYRINGOMYCIN; NMR SPECTROSCOPY, MD AND BEYOND BY GENOMICS.

Maurizio Paci

Dept. of Chemical Science and Technology. University of Rome Tor Vergata, Rome, Italy

The structural study by NMR spectroscopy of toxins from the sea world like Contryphan and from the world of Pseudomonads, mainly of agricultural interests, opens new frontiers to the possibility of design novel drugs on the basis of the effects obtained in vivo by these compounds.

The results obtained in the case of Contryphan by NMR and MD simulations are presented together with the new results in the case of syringomycin from Pseudomonads syringae about the effects on membrane of these class of compounds in a perspective view of progress in enhancing their effects in antibacterial properties by combinatorial biosynthesis Particularly those compounds, like Pseudomonads, where the genomic is in great progress the project to design a combinatorial biosynthesis appears feasible able to enhance some important properties and suppress unwanted ones.

NMR CRYSTALLOGRAPHY, POLYMORPHISM & SHIELDING COMPUTATIONS

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NMR crystallography has receiving increased prominence recently and is being much exploited. Whilst attention is often focussed on measurements of dipolar coupling constants, this lecture will concentrate on the utility of chemical shifts [1], particularly as applied to organic and pharmaceutical compounds. A key requirement in such procedures is gaining an understanding of the influence of molecular geometry and environment in crystals on observed shifts (including their anisotropies and asymmetries). The value of computing chemical shifts using the NMR-CASTEP code [2] (which operates under the translational symmetry inherent in crystals) will be emphasised, using a number of examples. The strengths and limitations of such computations will be discussed. In particular, methods of assigning resonances to specific sites in the unit cells of crystals, using a combination of NMR experiments and computations [3] will be introduced. Applications will extend to polymorphic systems and to solvates [4], illustrating the power of using NMR as a supplementary and complementary technique to diffraction experiments.

- [1] R.K. Harris, Solid State Sciences 6, 1025-1037 (2004)
- [2] C.J. Pickard & F. Mauri, *Phys Rev. B*, **63**, article no. 245101 (2001)
- [3] R.K. Harris, S.A Joyce, C.J. Pickard, S. Cadars & L. Emsley, *Phys. Chem. Chem. Phys.* 8, 137-143 (2006)
- [4] R.K. Harris, *The Analyst* **131**, 351-373 (2006)

COMBINED SOLID STATE/SOLUTION NMR FOR LARGE PROTEIN AGGREGATES: FERRITIN AS A CASE EXAMPLE

P. Turano

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A combined solution and solid state NMR approach is here presented for the assignment of very high molecular weight proteins. As a case example the results obtained for the NMR characterization of bullfrog M ferritin, a 24-mer nanocage protein of 480 kDa mass, are reported. Information in solution has been obtained using ¹³C–¹³C NOESY experiments [1-2]. By exploiting ¹³C-resonance-specific chemical shifts and spin diffusion effects, we identified 75% of the amino acids, with intraresidue C–C connectivities between nuclei separated by 1–4 bonds. Still, long range NOEs could be detected only for a couple of Met C ϵ . The observation of sequential C'/C α dipolar connectivities was hampered by severe signal line broadening. Solid state NMR is presently used to obtain sequential assignment of the identified spin systems.

[1] M. Matzapetakis, P. Turano, E. C. Theil, and I. Bertini, J.Biomol.NMR 38, 237-242 (2007)

^[2] I. Bertini, W. Bermel, I.C. Felli, M. Matzapetakis, R. Pierattelli, E. C. Theil, and P. Turano, *J.Magn.Reson.* 2007, in Press

HOW MUCH SLOW CAN IT TUMBLE?

P. Li,[‡] J. Orts,[‡] M. Reese,[‡] J. Tuma,[‡] C. Griesinger,[‡] M. Wahl,[†] R. Lührmann,[†] T. Carlomagno[‡]

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In the first part, we will show NMR structural studies on the 80 kD spliceosomal complex formed by the U4-RNA and the 15.5K and hPrp31 proteins. After the formation of A complex, U6/U4.U5 tri-snRNPs are incorporated into the major spliceosome. As snRNA structures are stabilized by proteins bound to them, understanding the functions of specific proteins of U4 snRNA is of great importance to visualize the dramatic structural changes taking place in U6 and U4 snRNAs. 15.5kD is one of the U4 specific proteins. Its binding to the 5' stem-loop region of U4 snRNA nucleates the binding of other U4/U6 specific proteins, namely hPrp31 (61kD) and the 20kD/60kD/90kD protein complex. The 15.5kD protein is an intriguing molecule, which is also found in the nucleoli as one of the core proteins of Box C/D snoRNAs. Although all RNAs that bind 15.5 kD seem to fold in a K-turn motif, their sequence codes for the specificity of the later binders in the various complexes. Here, we show an NMR study of the U4-RNA/15.5kD/hPrp31 complex aimed to characterize the interaction of the U4-RNA/15.5kD dimeric complex with the hPrp31 protein and to understand the mechanisms for the selectivity of the structurally similar 15.5kD/RNA complexes towards the different secondary binding proteins. The ternary RNP complex is 80 kD large and is present in solution at a concentration < 0.2mM, which represents a challenge for NMR studies. By a combined NMR and computational approach we derive a reliable model for the trimeric complex and define the Nop domain of the hPrp31 protein as a genuine RNP recognition domain [1]. In the second part, we present a new NMR methodology to characterize the interactions of small ligands with macromolecular receptors. In structural based drug design knowledge of the orientation of the ligand in the receptor-binding pocket plays a central role in the elaboration of a high affinity drug. Recently, we have developed a novel approach, called INPHARMA (Interligand Noes for PHARmacophore MApping), which allows the determination of the relative orientation of two competitive ligands in the receptor-binding pocket [2]. The method is based on the observation of interligand, spin diffusion mediated, transferred-NOE data, between two ligands A and B, binding competitively and weakly, to a macromolecular receptor T. Here, we compare the binding mode of two ligands derived from the INPHARMA method to the available crystal structures and prove the reliability and efficiency of the methodology. Furthermore, we show the application of the INPHARMA methodology to the challenging study of the complex of tubulin with the microtubule stabilizing agent epothilone. Epothilone is a potent anticancer drug, which exerts a cytotoxic activity on the cells by altering the tubulin polymerization equilibrium. We derive a structure for the Tubulin/epothilone complex that provide an excellent understanding of the structural basis of the high cytotoxic activity of epothilone and of its SAR data [3].

^[1] S. Liu et al. Science 316, 115-120 (2007)

^[2] V.M. Sanchez-Pedregal et al, Angew. Chemie 44, 4172-4175 (2005)

^[3] M. Reese et al., Angew. Chemie 46, 1864-1868 (2007)

ORAL COMMUNICATIONS

NEW DEVELOPMENTS IN FOOD NMR

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NMR is a well known analytical technique used for quality and origin control of food and beverages. For example SNIF-NMR[®] (Natural Isotope Fractionation studied by Nuclear Magnetic Resonance) was developed in the early 1980's and it is an official method recognised by EU since 1993 to detect adulteration of food and to check compliance with listed ingredients, labelling and customs declarations.

In the last decade it has been assisted to an impressive increase of the number and of types of food products investigated by means of different NMR techniques. This is mainly due to the growing request of an analytical proof of origin, properties, composition, and reliability of food, starting from the raw materials up to the final product. Moreover such a wide rage of applications has been made possible by the most recent technological developments: the better stability of the spectrometers guarantees the long term measurements and reproducibility; the increasing sensitivity gain with CryoprobeTM and micro-probes with sample tubes diameters of 1 and 1.7 mm allows one to detect lower and lower amount of sample. Consequently NMR is nowadays regarded an highly robust screening technique.

The talk will focus on different NMR applications on food. Both research investigation and quality control studies will be presented. It must taken into account that usually quality control protocols are based on screening and consequently require high throughput systems, whereas the aim of most of research studies is the structural characterization of food materials by elucidating and exploiting the role of NMR and consequently require high field spectrometers equipped with specific probes. Furthermore it will be shown how NMR is a fundamental analytical tool in metabolomic studies in order to relate consumer health and food ingredients.

For each application it will be shown which kind of info can be provided by NMR investigation and it will be discussed which is the most appropriate NMR approach and the required configuration including high Resolution NMR, hyphenated techniques, low resolution NMR and μ -imaging NMR.

RESIDUAL DIPOLAR COUPLINGS IN SOLID STATE MEASURED BY OFF-MAS NMR

<u>Giuseppe Pileio</u>,¹ Ying Guo,² Tran N. Pham,^{2,3} John M. Griffin,² Claudiu Filip,⁴ Steven P. Brown² and Malcolm H. Levitt¹

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Spin-echo magic angle spinning technique was shown to be a powerful tool to measure inter-nuclear spin-spin couplings in rotating solids [1,2] even when the coupling itself cannot be observed. Here the technique was modified to measure residual dipolar couplings in solids as a solid-state NMR analogue of the solution-state residual dipolar coupling effect (RDC) [3,4].

A small misset of the spinning angle from the magic value $(\tan^{-1}\sqrt{2})$ of the order of 0.5 degree or less is enough to modify the modulation frequency of spin echoes as a result of the reintroduction of a part of the dipolar coupling like a slightly anisotropic environment reintroduces small dipolar components in the liquid state RDC experiments. The small deviance from the MAS condition allow the measurement of RDCs which are large enough to allow the estimation of inter-nuclear distances, without significantly degrading the spectral resolution and without introducing strongly-coupled spin dynamics.

The availability of the analytical equations for the rotor-synchronized echo modulation curve at a give spinning angle misset makes the whole procedure even easier to handle. The technique is here demonstrated for a sample of ¹³C-labelled glycine.

- [1] L. Duma, W. C. Lai, M. Carravetta, L. Emsley, S. P. Brown, and M. H. Levitt, *ChemPhysChem* 5, 815 (2004).
- [2] W. C. Lai, N. McLean, A. Gansmüller, M. A. Verhoeven, G. C. Antonioli, M. Carravetta, L. Duma, P. H. M. Bovee-Geurts, O. G. Johannessen, H. J. M. de Groot, J. Lugtenburg, L. Emsley, S. P. Brown, R. C. D. Brown, W. J. DeGrip, and M. H. Levitt, *J. Am. Chem. Soc.* **128**, 3878 (2006).
- [3] Tjandra, N.; Bax, A. Science 1997, 278, 1111.
- [4] Sanders, C. R.; Prestegard, J. H. J. Am. Chem. Soc. 1991, 113, 1987.

MEASURING HOMONUCLEAR SCALAR COUPLING CONSTANTS BY SELECTIVE J-RESOLVED SPECTRA

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The ever growing refinement of modern quantum-chemistry methods has brought an impressive level of accuracy in the prediction of NMR parameters such as chemical shifts and scalar coupling constants [1], all of which need ultimate validation against reliable experimental data. In particular, the accurate measurement of homo- or hetero-nuclear coupling constants is a quite demanding task that can be accomplished by means of different approaches. In this communication we propose a simple method to obtain a selective version of homonuclear *J*-resolved spectra [2], which relies on the refocusing properties of double pulsed field gradient spin-echoes [3, 4] and provides unambiguous assignment of the measured coupling constants. The method is of general applicability, easy implementation and requires no more than a simple optimization strategy to produce clean spectra.



Fig. 1. *J*-resolved spectra of strychnine obtained from selective refocusing of protons H20a and H14 + (partially) H11a. A coupling constant spanning across four bonds is revealed.

As examples of possible applications, we have determined some long-range coupling constants in strychnine (Fig. 1) and *trans*-retinal, as well as H^{α} couplings in a tripeptide [5].

- [1] A. Bagno, F. Rastrelli, G. Saielli, Chem. Eur. J. 12 (2006) 5514.
- [2] M.J. Thrippleton, R.A.E. Edden, J. Keeler, J. Magn. Reson. 174 (2005) 97.
- [3] T.-L. Hwang, A.J. Shaka, J. Magn. Reson. A 112 (1995) 275.
- [4] T. Parella, F. Sanchez-Ferrando, A. Virgili, J. Magn. Reson. 135 (1998) 50.
- [5] F. Rastrelli, A. Bagno, J. Magn. Reson. 182, (2006) 29.

OPTIMIZATION OF NMR SEQUENCES THROUGH SIMULATION OF REAL SAMPLES AND INSTRUMENTAL ARTIFACTS: APPLICATION TO THE LAPSR SEQUENCE

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We have developed a mathematical model to simulate the effects of pulse sequences on composite samples and coded it in Matlab (it is now being converted into C++). The object-oriented code accommodates pulse sequences and samples of any complexity, including samples with any distribution of relaxation rates and offsets. Of equal importance is the fact that the most common instrumental artifacts (B₀ and B₁ inhomogeneity and pulse imperfections) can be easily taken into account.

Everything within the simulation package is an object which, in its own turn, can be a part of a more complex object. The list of objects includes: single nuclear *magnetization component, sample* (an array of *components*), *RF pulse, free-evolution delay, pulse sequence*, and *receiver*, each complete with its basic parameters and typical artifacts. As usual, the objects are complemented by methods which confer them specific functionality. The final goal is the simulation of the magnetization of a composite sample during the whole pulse sequence. This is done by simulating (using Bloch equations) the evolution of the magnetization of each sample component.

We have applied the package to the old NMR problem of a fast and efficient *presaturation* of a sample magnetization by a suitable pulse sequence. Simulating large heterogeneous samples with a wide spread of offsets, relaxation rates, and imperfections $(B_0 \text{ and } B_1 \text{ inhomogeneities})$, we have first of all gained novel insights into the severe limitations of traditional saturation sequences, such as a single 90° pulse (SR) or a train of 90° pulses with a linearly decreasing delay (APSR). We have seen that such sequences primarily randomize the direction vectors of the magnetizations of individual components, while having little impact on their individual magnitudes. The result is an apparent saturation of the overall sample signal, a situation which leaves an open door to the formation of unpredictable echoes in those sequences which employ such saturation pre-ambles to measure R_1 distributions. We have then optimized the presaturation sequence by minimizing the sum of the absolute magnitudes of all sample components (an experimentally inaccessible quantity). This corresponds to a true saturation, void of any risk of contamination by echoes. The result is the Logarithmically-distributed A-periodic Saturation Recovery (LAPSR) sequence of 180° pulses which allows to reduce the duration of R₁ measurements by a factor of about 2.5 with respect to the standard IR (Inversion Recovery) and, above all, suppresses the absolute magnetization of all components several times better than SR and APSR.

Even so, the performance of LAPSR is not perfect. We believe, however, that (i) LAPSR is very close to the best pre-saturation one can *ever* achieve by means of pulse sequences and (ii) that it is important to understand the physical limits of such techniques. Such an understanding is something that only a realistic simulation package can provide since experimental measurements can hardly ever cover a sufficiently wide range of sample-and-artifacts combinations.

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DOUBLE-QUANTUM NMR OF THE FIRST PHOTOINTERMEDIATE IN VISION: BATHORHODOPSIN.

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Rhodopsin is the photoreceptor that is responsible for dim light vision in vertebrates. After photo-excitation, the chromophore of rhodopsin, 11-cis-retinal, isomerizes in an extremely fast reaction to form the all-trans isomer. After this time the protein undergoes a series of changes in conformation that lead to a number of photointermediates. The first stable photo-product is known as bathorhodopsin and can be trapped at temperatures below 125 K [1]. In the present work a very precise procedure has been used in order to properly set the experimental conditions, sample temperature and light wavelength selection to cite the most important ones, that need to be satisfied in order to trap the photo-intermediate. The procedure is based on a home-built NMR probe that allows, through the use of optical fibres, the *in-situ* illumination of rhodopsin and the low temperature (~120 K) trapping of its intermediates. A double quantum filtered dipolar recoupling solid state NMR experiment has been used on a sample of $9,10^{-13}C_2$ -retinilydene-rhodopsin to evidence the production of the related bathorhodopsin intermediate. The chemical shifts of C9 and C10 of bathorhodospin appear clearly in the NMR spectra although their positions are very different from previous solid state NMR studies [2] and quantum mechanical calculations [3]. However, the well-controlled procedure used here make us confident of its nature.

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NMR SPECTROSCOPY AND CHEMOMETRICS FOR QUALITY ASSESSMENT OF TRADITIONAL BALSAMIC VINEGAR

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In the last years, a consistent widespread growth in the use of NMR spectroscopy in food science and authentication has been observed [1-5]. Several foods were investigated by NMR with the simple aim of component characterization [6-10] or quality assessment [11-15].

Inasmuch as multivariate statistical analysis has been largely applied to analyze the huge amount of information derived from NMR spectra, food quality assessment has achieved great improvements and different chemometric protocols (PCA, PLS-DA, O-PLS etc) were applied to NMR data to group and to discriminate samples. In this presentation, the powerful of NMR spectroscopy in combination with statistical methods is presented with the aim of quality assessment of Traditional Balsamic and Balsamic Vinegar of Modena.

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AUTOMATIC PHASE CORRECTION OF NMR SPECTRA: A BREAKTHROUGH IN THE PRACTICE OF METABOLOMICS

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In the past 30 years too many algorithms have been proposed to perform automatic phase correction of NMR spectra.[1] Every author claimed that his own new method was highly effective and the next author systematically stated that no previous methods was satisfactory. The algorithm that I describe has already been validated by practice: several independent experimentalists have already successfully applied it on thousands of ¹H-NMR spectra.

Some assumptions are made on the spectra to be processed: (a) All peaks can be brought in positive absorption mode by a first order phase correction. (b) The baseline is flat. (c) The outer regions do not contain peaks. The above requirements are normally satisfied in metabolomic analysis, for example in ¹H-NMR spectra of urine samples acquired on modern digital spectrometers. My method was indeed created to process hundreds of such spectra with minimal human interaction. It was already possible to automatize all other tasks and phase correction was the missing link. In the design of the algorithm, I allowed it to be tolerant with spectra that don't correspond to the requirements (b) and (c). In the rare cases in which the method failed, it was demonstrated that the above requirements were not respected, but the method often yields very good results even when the baseline has a parabolic shape and when the whole spectral width is disseminated with peaks.

Mine is an extension of the recipe proposed by Siegel.[2] I have corrected the merit function by removing the baseline offset and I have added two new terms to satisfy the requirement (b) above. All the terms reach their minimum value at zero. This means that the relative weight of each term is not critical for success and that the simplex algorithm, once a term has been minimized, only has to optimize the remaining ones.

My metabolomic method for phase correction has been included into the commercial program iNMR since March 2007.[3] The latter can be freely downloaded and tested. All the reports I have received confirm the accuracy and the reliability of the algorithm.



Fig. 1. A ¹H spectrum of urine after automatic phase correction.

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EFFECT OF AKR1C3 INHIBITORS IN ACUTE MYELOID LEUKAEMIA CELL LINES STUDIED USING NMR-BASED METABOLOMICS

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Aldo-keto reductase AKR1C3 has been identified as a novel target in the treatment of haemato-lymphoid malignancies [1, 2]. At present there are no AKR1C3 selective inhibitors available for clinical use but several commonly used drugs inhibit the enzyme at doses that can be achieved in vivo. These include the hypolipidemic drug bezafibrate and the synthetic steroid contraceptive medroxyprogesterone acetate (MPA). However, given the lack of specificity of bezafibrate and MPA for AKR1C3, it is difficult to directly ascribe the anti-neoplastic activity to AKR1C3 inhibition or indeed any common target. One approach to investigating this problem is to utilise NMR-based metabolomics to identify what are the common and disparate consequences of treating cells with bezafibrate and MPA and whether the changes are consistent with inhibition of AKR1C3 or other potential anti-cancer targets.

To assess the impact of AKR1C3 inhibitors on the cell metabolome exponentially proliferating KG1a, HL60 and K562 cells were treated with MPA alone, bezafibrate alone, the combination of MPA and bezafibrate. For metabolomics analysis a protocol was established to extract the polar fraction for 1D ¹H and 2D ¹H *J*-resolved NMR analysis. Two dimensional COSY and ¹H-¹³C-HSQC spectra were collected for selected samples to assign some metabolites.

Principal component analysis was able to completely separate the three different drug treatments from each other and from control groups. Several discriminating compounds were identified from loadings plots of different principal components. Amongst these several amino acids were found to vary significantly in concentration after the different treatments, usually leading to increased concentrations in samples that had received drug treatment. A striking depletion of glutathione was observed when AKR1C3 inhibitors were present: MPA and bezafibrate each induced glutathione depletion when administered separately, with bezafibrate causing the most abundant change. However, the combination treatment exhibited the largest effect on glutathione concentration suggesting a synergistic effect of the considered drugs. Depletion of glutathione is related to oxidative stress in AML cells which in turn is consistent with the known activities of AKR1C3 and also the anti-neoplastic actions of the drugs.

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STRONG INCREASE OF FOLIAR INULIN OCCURS IN TRANSGENIC LETTUCE PLANTS (*LACTUCA SATIVA L.*) OVER-EXPRESSING THE ASPARAGINE SYNTHETASE A FROM *E. COLI*.

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NMR and an appropriate statistical treatment of NMR data allow the detection of complex changes in the metabolic phenotype of genetically modified organisms [1]. Like fingerprinting, an NMR spectrum gives a complete picture of the complex metabolic mixtures without necessity of time consuming separation and/or derivatization of components.

Comparing previously assigned ¹H NMR spectra of water soluble metabolites [2] we can perform an evaluation of the "substantial equivalence" of lettuce (*Lactuca sativa L.*) cv Cortina overexpressing *E. coli asnA* under the hybrid constitutive *pMAC* promoter with the commercial wild variety; that means that all metabolites present in the wild variety are also present in the GM variety. However in the transgenic variety a dramatic increase of inulin content is clear, not directly related to the target transgene activity. Inulin and oligofructose (G-F_n type, where G and F are glucose and fructose residues) are natural food ingredients, commonly found in varying percentage in dietary foods and considered to be beneficial for human health. DOSY experiment showed the presence of two fractions of inulin in lettuce: inulin I (1-kestose: G-F₂ type) and inulin II (G-F₄ type). The result demonstrates: a) unexpected effects on metabolism following genetic transformation; b) the production of a new lettuce genotype characterized by high foliar concentration of inulin useful as a reactor for further breeding programs for the improvement of this character.

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MOBILITY, MICROSTRUCTURE AND MRI. DTI WITHOUT A DTI BLACK BOX: THE ART OF MAKING DO.

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Because of its non invasive nature, NMR is a unique tool for studying the microstrusture of biological and material samples. This is usually done by monitoring the diffusion coefficients and relaxation times, whose behavior is related to the translational and rotational random molecular motion of the system components. These parameters report on motion on very different time scales, thus providing structural information at a microscopic scale well beyond the usual image resolution. However, while relaxation-weighted images are relatively straightforward to record and analyze, the study of diffusion through MRI calls for an higher level of complexity from both a theoretical and instrumental point of view and it is nowadays well established in the form of diffusion tensor imaging (DTI), a technique introduced by Basser et al. in 1994 [1]. Although software modules exist that alleviate the tedious task of setting all the parameters for the DTI sequence in newer instruments or medical scanners, our old spectrometer - a 4.7 Tesla Bruker AM WB equipped with a PFG drive unit for microimaging - did not offer the "turnkey" DT-MRI Bruker acquisition interface. We got round this by implementing a DTI acquisition module and analyzing the raw data with an open source DTI reconstruction software [2]. Our experience led us to elaborate some of the theoretical and methodological basic concepts of DTI, summarized in the following steps: i) Theoretical basis and assumptions of the equations that lead to the true and apparent calculation of the diffusion coefficient(s) [3]; ii) Acquisition scheme: a diffusion-weighted preparation module based on stimulated echo (STEAM) to provide long diffusion times with minimal T_2 relaxation [4] prior to and independent of the imaging scheme; iii) DTI scheme: the amount of diffusion weighting and at least six not collinear PFG orientations (icosahedral geometry) to estimate the six independent elements of the symmetric diffusion tensor; iv) the corrections for cross-terms interactions [5]; v) reduction of eddy currents that generate image artifacts; vi) diffusion tensor reconstruction from DTI raw data, providing information on sample microstructure and architecture for each voxel: mean diffusivity; anisotropy indices and fiber orientation mapping; vii) Phantoms to test and validate the whole procedure: isotropic (water, BSA) and anisotropic (fibers and celery) at different anisotropy degree.

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DYNAMIC AND ALLOSTERIC PROPERTIES OF HUMAN SERUM ALBUMIN INVESTIGATED BY NUCLEAR MAGNETIC RELAXATION DISPERSION

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Human serum albumin (HSA) is the most prominent protein in plasma, but it is also found in tissues and secretions throughout the body. The three-domain design of HSA provides a variety of binding sites for many ligands, including heme, fatty acids and drugs. It acts as a volume expander, allowing endogenous and exogenous compounds to be available in quantities well beyond their solubility in plasma and determining the pharmacokinetic behaviour of many drugs. HSA can hold some ligands in a strained orientation, allowing their metabolic modification and renders potential toxins harmless, transporting them to disposal site [1]. The heme-HSA complex is obtained by binding of Mn(III)heme or Fe(III)heme to HSA. The metal-heme binding to HSA endows the protein with peculiar spectroscopic properties and it is used as spectroscopic probe to follow a number of events involving the conformational of the protein. Variation of relaxivity values of metal-heme-HSA at different experimental conditions was measured.

Results reported here show that NMRD is a useful technique to investigate how interdomain interactions affect the global folding/unfolding process. We report on the chemical denaturation of heme-HSA involving three different conformational states (F, N, and B) and on the effect of prototypic drugs ibuprofen and warfarin on thermodynamics of the unfolding process [2]. Moreover, by combining NMRD and optical methods we analyzed allosteric properties of HSA and the effect of anti-HIV drugs and myristate ions as effectors [3].

As a general remark, the paramagnetic contribution to the NMR rate of solvent water protons is useful for a better characterization of the molecular environment of the metal complex binding site and of its dynamics.

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RELAXOMETRIC STUDIES OF Gd(III) COMPLEXES WITH NOVEL EGTA DERIVATIVES AND OF THEIR PROTEIN BINDING

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As it was shown some 10 years ago [1], the water exchange rate for $[GdEGTA(H_2O)]^{-1}$ complex is 3.1×10^7 s⁻¹ (298 K) and this value is one order of magnitude higher than for the clinically used MRI probes $[GdDOTA(H_2O)]^{-1}$ and $[GdDTPA(H_2O)]^{2-1}$. In spite of this favourable properties, explained by the steric compression at the water binding site exercised by the oxoethylenic bridge present in the structure, no further studies have been reported so far relative to the preparation of macromolecular derivatives of high relaxivity. We have synthesised new derivatives of EGTA where the basic structure was modified in the central ethylenic moiety, rigidified by fusion with an aromatic ring (Fig.1) in order to maintain or even improve the structural and relaxometric properties of the corresponding Gd complexes. The aromatic moiety and the hydrophobic substituents allow the complexes to interact non covalently to HSA and through competition studies we showed how the derivatives select different binding sites. Combined modelling studies and ${}^{1}H 1/T_{1}$ NMRD profiles outlined the importance of the rotational dynamics which depends on the different arrangement of the complex in the hydrophobic pockets of the protein. The limited internal mobility, associated with the high rate of water exchange led to a remarkable increase of relaxivity of the adduct of GdL2 with HSA (Fig 2) up to a value of 80 mM⁻¹s⁻¹, quite close to that predicted by theory and unprecedented to our knowledge for such systems [3].



Fig. 1 A ligand structure examined in this Fig. 2 NMRD profiles of GdL1 (bottom) and study. GdL1-HSA (top)

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WATER IMBIBITION IN POROUS MEDIA BY QUANTITATIVE MAGNETIC RESONANCE IMAGING

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Fluid imbibition in a porous medium is the process by which a non-wetting fluid, such as oil or air, is displaced by the spontaneous entry of a wetting fluid, such as water or brine, by means of capillary action. These processes affect almost every activity that directly or indirectly involves porous media, including ground water, oil reservoir rocks, building materials, in place and in the laboratory. As well known [1], the study of spontaneous water imbibition is essential to predict the production performance in a lot of oil and gas reservoirs developed by water injection or associated with active aquifers. Another example is the re-injection of produced water into geothermal reservoirs to solve the problems of reservoir pressure decline and environment pollution. In the field of Cultural Heritage, restoration and conservation treatment of stone objects, including statues and buildings, is usually done by applying polymeric compounds having hydrophobic and often cohesive properties. The efficacy and durability of the conservation treatments depend on the compounds used and also on the penetration and homogenous distribution of the polymeric product in the porous material. A high probability of pore blockage occurs when there is low penetration depth and nonhomogenous distribution of the polymer in the stone, and significant reduction of pore space in the superficial layers of the rock can cause further deterioration upon environmental stress. It is therefore essential to know the capillary properties of the untreated and treated stones [2].

Magnetic Resonance for fluids in porous media has been useful for a long time [3]. Here Magnetic Resonance Imaging (MRI) is used to follow the imbibition of water by samples of a porous rock having one side put in contact with water at a particular time, giving quantitative measurements of the positions of the advancing fluid front. In order to study wettability effects, imbibition of water was followed in time for the natural rock and for the rock with each of two different protective polymeric treatments.

The method allows measurement, in each internal section of the sample, of the height reached by water at any time during the course of the imbibition, and also to give some indication of the changes of the fluid saturation behind the front.

Measurements of the height of the imbibition front as a function of time were compared with theoretical models. The results show that the method can be successfully applied to study in a quantitative way the capillary rise of liquids in internal regions of opaque samples. The characteristic of the method is its non-destructivity, which allows us to perform quantitative measurements on the same sample after saturation with different fluids and at different times in internal regions of the samples.

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MONITORING THE INTERACTIONS OF BILE ACID BINDING PROTEINS AND SELECTED MUTANTS WITH NATIVE AND SYNTHETIC LIGANDS

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Bile acids are physiological detergents facilitating absorption, transport and distribution of lipid-soluble vitamins and dietary fats. These molecules are also involved in activating nuclear receptors and regulating cholesterol metabolism. After biosynthesis in the liver they are secreted as bile in the intestine, then absorbed by enterocytes and recycled to the liver through a mechanism termed enterohepatic circulation. The intracellular transport within hepatocytes and enterocytes is mediated by small soluble bile acid binding proteins (BABP). Our group is interested in elucidating the determinants of ligand binding using mainly NMR spectroscopy.

Earlier studies by some of us [1] and recent research [2,3] established the structural and dynamic features of the unbound chicken liver BABP as well as those of the protein bound to two bile salts. A strong conformational rearrangement of the protein and cooperativity of binding are observed. Specifically tailored diffusion measurements also indicate different mobility of the two ligand molecules. Selected mutants were engineered in order to study the role of the residues in determining the affinity and selectivity of binding towards a variety of bile salts. HSQC titration studies were carried out on samples containing labelled protein or ligand. Further mutants were designed in order to assess the relevance of amino acid residues in determining the allosteric mechanism at the basis of cooperativity. In particular, the protonation equilibria of histidine residues proposed to be responsible of triggering protein conformational rearrangements [1] were investigated through long-range ¹H, ¹⁵N-HSQC experiments.

Through the identification of a BABP in chicken ileum using bioinformatics methods [4] we were able to validate the proposed parallel model of bile acid transport in hepatocytes and ileocytes. After having established the bile acid binding capability of this protein in vitro, we are currently undertaking more detailed binding studies and starting the structural investigation of this protein which shows 42% sequence identity to the liver paralogue.

Finally, a rational drug design study was initiated for the development of hepatospecific contrast agents for MRI. Bile-acid-derived paramagnetic complexes were screened with a relaxometric approach and the binding of the most promising molecule to liver BABP was investigated by a variety of high resolution NMR experiments using both the paramagnetic ligand as well as a diamagnetic analogue [5].

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COMPETITIVE BINDING OF MONOVALENT CATIONS TO THE DIMERIC QUADRUPLEX [d(G₄T₄G₄)]₂: A²³Na AND ¹H NMR INVESTIGATION

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Guanine-rich oligonucleotides can fold into unusual secondary structures called G-quadruplex with cyclic Hoogsteen base pairs of four guanine bases (G-quartets). These DNA G-quadruplexes are of biological interest since telomeres, which are present on the ends of eukaryotic and are composed of tandem repeats of G-rich sequences, have been recognized to form *in vitro* G-DNA type structures with potential physiological roles. It is not entirely clear whether G-quartet structures exist in vivo, but if G-quartet structures are important for some aspect of telomere biology it would explain, in part, why G-rich repeats in telomere DNA are conserved throughout evolution.

The structures and stabilities of G-quadruplexes are known to be dependent on the presence and concentrations of monovalent cations because the cations can coordinate the carbonyl oxygen of the guanine and promote stacking interactions of the G-quartets. A number of structural and thermodynamic investigations demonstrated that in general the effect of ions on the stability of G-quadruplexes follows the order: $K^+>Na^+>Rb^+>NH_4^+>Cs^+>Li^+$.

To our knowledge, it appears that no systematic evaluation has been performed to study the affinity for group Ia cations for a G-quadruplex structures. In order to improve the knowledge of this kind of molecules, and in particular to provide further insight into the role of monovalent cations in the G-quadruplex folding, we have investigated the competitive binding of Na^+ with other movalent cations (K⁺, Rb⁺, and Cs⁺) to the dimeric quadruplex formed by the Oxytricha nova telomeric repeat oligonucleotide $d(G_4T_4G_4)$. Particularly, ²³Na and ¹H NMR titration experiments were performed in solution of the quadruplex $[d(G_4T_4G_4)]_2$ containing 50mM Na⁺ and by adding increasing amount of KCl, RbCl, or CsCl. For the sake of comparison, we have also investigated the influence of NaCl. The titration profiles of the measured ²³Na relaxation rate (R_{obs}) with these cations turned to be completely different. At any stage in the titration, K⁺ and Rb⁺ decrease R_{obs} more efficiently than Na⁺. This effect is much stronger for K⁺ than Rb⁺. On the contrary, adding Cs⁺ induces a very gradual reduction of Robs. Furthermore, changes in the chemical shift of the ¹H NMR spectra of $[d(G_4T_4G_4)]_2$ observed in the presence of RbCl indicate a slow exchange on the NMR time scale among the different cation forms of quadruplex. On the contrary, the addition of CsCl does not induce significant ¹H NMR modification, suggesting the absence of specific interactions with the quadruplex. Data are discussed in terms of different binding affinity of these monovalent cation to $[d(G_4T_4G_4)]_2$.

RAPID DETECTION AND CHARACTERIZATION OF T CELL RECEPTOR-PMHC INTERACTIONS

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Cells digest foreign proteins into small peptides and load them onto proteins of the Major Histocompatibility Complex (MHC). These MHC-bound peptides are then scanned by T-Cells with high sensitivity and specificity using T-Cell receptors (TCRs), which discriminate between self-antigens and foreign pathogens and stimulate a response of the immune system if appropriate.

T cell receptor recognition of peptide-MHC is central to the cellular immune response. A large database of TCR/pMHC structures will reveal general structural principles, such as whether the repertoire of TCR/MHC docking modes is dictated by a "recognition code" between conserved elements of the TCR and MHC genes.

Although approximately 15 co-crystal structures of unique TCR/pMHC complexes have been determined, co-crystallization of soluble TCR and pMHC remains a major technical obstacle in the field. If we want to analyze a vast set of structures, therefore, a faster method for structural characterization of TCR-pMHC interfaces is required.

NMR assignment of a pMHC molecule allows us to identify which residues interact with different TCRs through fast and simple chemical shift mapping experiments, and to compare the binding interfaces of different complexes. As a proof of concept, we present the comparison between two mutants of the so called 2C TCR in complex with the same pMCH. A comparison of our complex to the available structure of the same TCR bound to a different pMHC is also presented.



AUTOMATED PROTEIN NMR ASSIGNMENTS DERIVED FROM MULTI-WAY DECOMPOSITION OF PROJECTED SPECTRA

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Resonance assignments represent a critical step in most NMR studies on proteins. Often, major challenges result from extensive spectral overlap, for example for proteins with large natively disordered segments or from the need to acquire all necessary spectra in limited time, for example when working with unstable protein samples. This results in contradicting requirements for high-dimensionality spectra to resolve spectral overlap and short experimental time. We demonstrate automated assignment of the complete backbone and $C\beta H_n$ from a set of 30 spectra with coupled evolution times ("projections"), which were all recorded in less than 30 hours on a conventional 600 MHz instrument.



Fig 1. Spectrum with the linear combination $\omega_N - \omega_{C'}$ (i-1)+ $\omega_{H\alpha\beta}$ (i-1) along the vertical axis and ω_{HN} along the horizontal axis, recorded for the small protein *ubiquitin* (76 residues).

All projections are two-dimensional and have the chemical shift of HN along the directly detected dimension. The other dimension corresponds to a linear combination of several chemical shifts, including at least one shift of N or C' combined with a varying selection of shifts from the α and β carbons and hydrogens of both residues connected by the C'–N bond (see Fig. 1). Thus, for any HN resonance, signals from all spectra are connected by at least two nuclei of the central C'–N–HN fragment, and the *joint* multi-way decomposition of all spectra results in descriptions of spin systems, which each consist of all nuclei in a C β H_n–C α H–C'–NH–C α H–C β H_n fragment that stretches over two adjacent residues. These unambiguous spin systems yield each an entry in an 11-dimensional peak list results. With this list as input, the automated program GARANT provides complete and correct resonance assignments.

SOLID-STATE NMR WITH FAST AND ULTRA-FAST MAS

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New probes for magic angle spinning (MAS) at "fast" and "ultrafast" (> 30 kHz) speeds have benefited a broad group of solid-state NMR experiments, including those for organic structure determination, materials NMR and biosolids. This talk will address some new, pulsed methods for pulsed cross-polarization and decoupling at these high spinning speeds and describe the attributes of probes for new experiments in the areas of biosolids and materials NMR.
THE AUTOIMMUNE REGULATOR PHD FINGER BINDS NON-METHYLATED HISTONE H3K4 TO ACTIVATE GENE EXPRESSION

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Methylation of lysine residues on histone H3 tails regulates transcription. The plant homeodomain (PHD) fingers of ING2 [1] and BPTF [2] were reported to bind specifically trimethylated lysine 4 on histone H3 (H3K4me3), thus introducing PHD fingers as methylation-dependent chromatin-binding modules. Previously, we determined the structure of the first PHD finger of Autoimmune Regulator (AIRE-PHD1) [3], the protein defective in autoimmune polyendocrinopathycandidiasisectodermal dystrophy (APECED). AIRE is expressed in thymic medullary epithelial cells where it promotes the expression of tissue-restricted antigens. By the combined use of biochemical and biophysical methods we show that AIRE selectively interacts with histone H3. AIRE-PHD1 discriminates between different degrees of H3K4 methylation and preferentially binds nonmethylated H3K4 (KD ~4 µM). Accordingly, in vivo AIRE binds and activates promoters containing non-methylated H3K4 in HEK293 cells. We propose that AIRE-PHD1 is a prototype of a new class of PHD fingers that specifically recognize non-methylated H3K4, thus providing a new link between the status of histone modifications and the regulation of tissue-restricted antigen expression in thymus.

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STRUCTURAL AND FUNCTIONAL EVOLUTION OF PRION PROTEINS

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Prion proteins (PrP) cause fatal neurodegenerative diseases such as mad cow disease in cattle, and Creutzfeldt-Jacob disease in humans. Despite intense research there are still key open questions regarding the function of prion proteins and the mechanisms of the structural conversion that takes place from the "normal" form of the protein to the "diseased" form. I will describe the NMR structures of the first non-mammalian PrP (turtle and chicken) and how the two-domains structure (a long flexible tail and a C-terminal end globular domain) has evolved. The availability of prion protein structures for mammals, birds, and reptiles has allowed the definition of the minimal "PrP fold" onto which is now possible to map specific structural-signatures for different classes of organisms. Finally the analysis of strictly conserved amino acid residues between the three classes of organisms and the requirements for obtaining the minimal "PrP fold" has resulted in the identification of a region of the protein with a new potential functional role for PrP in specific protein-protein interactions [1].

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DEEP INSIDE THE BINDING MECHANISM OF BILE ACID BINDING PROTEINS THROUGH THE ANALYSIS OF BINDING PROPERTIES OF SELECTED MUTANTS.

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Chicken liver bile acid binding protein (cL-BABP) is involved in bile acid transport in the liver cytosol. A detailed study of the mechanism of binding and selectivity of bile acids binding proteins towards the physiological pool of bile salts is a key issue for the complete understanding of the role of these proteins and their involvement in cholesterol homeostasis. In the present study, we investigate the binding features of wt cL-BABP and the effects of selected mutations on binding mechanism.

NMR interaction and competition studies of wt cL-BABP with glycochenodeoxycholic (GCDA) and glycocholic (GCA) acids indicated a higher affinity for chenodeoxycholic acid with an upper limit for K_d of 20 μ M [1]. The binding is highly cooperative and no site selectivity was detected for the different bile salts, thus indicating that site selectivity and cooperativity are not correlated. The determinants of cL-BABP binding properties have been further investigated on selected mutants, namely L21R, T91C, H98Y, R120Q. Among the produced mutants, L21R displayed the most severe changes in the binding behaviour and its structural and binding properties have been further ligands and solution conditions. The results obtained for L21R shed light on the role of the protein open end in the binding mechanism.

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PROBING POLYMER COLLOIDS BY ¹²⁹XE NMR

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The design and control of the morphology of polymer colloids are of particular interest in the development of new aqueous latexes for coating applications. The characterization of particle properties often requires a combination of advanced techniques. This communication reports on the use of ¹²⁹Xe NMR.

Model aqueous homopolymer colloids - polystyrene, poly(methyl methacrylate), poly(n-butyl acrylate) - and a statistical copolymer - poly(n-butyl acrylate-*co*-methyl methacrylate) 40/60 – were studied. The ¹²⁹Xe NMR spectra, recorded as a function of xenon gas pressure and polymer concentration, exhibit qualitative and quantitative differences: number of peaks, line width and chemical shift values. The results are discussed in terms of particle morphology.

MULTINUCLEAR SOLID-STATE NMR STUDY OF PE-PEG/SILICA HYBRID MATERIALS PREPARED BY SOL-GEL PROCESSES

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Organic-inorganic hybrid materials have recently received a great attention because of their peculiar and attractive properties, which derive from the combination, at the nanosize level, of organic and inorganic components [1]. One of the most common and successful ways to obtain organic-inorganic hybrid materials is by means of sol-gel processes, which involve liquid inorganic precursors, such as metal alkoxides, and organic compounds such as monomers, oligomers or polymers, preferably bearing groups able to react with the inorganic phase. The properties of the final materials, from a molecular to a macroscopic level, are strongly affected by many different variables, such as nature and amounts of the reactants and preparation conditions (type of catalyst employed, curing conditions, etc..). In order to find correlations among structural and dynamic properties at a molecular level, materials preparation procedures and macroscopic behaviour, solid-state NMR can play a significant role. In this work several organic-inorganic PE-PEG/silica hybrid materials, prepared by sol-gel processes, differing for preparation conditions and final molecular architectures, were characterized by means of ²⁹Si, ¹³C and ¹H high resolution solid-state NMR techniques. The multinuclear approach [2, 3] allowed a quite extensive characterization of the materials to be obtained: information concerning the cross-linking degree of the inorganic phases and the silicon atom connectivities were obtained by ²⁹Si CP- and SPE-MAS spectra; ¹³C CP- and SPE-MAS spectra provided useful information about the conformational properties of the organic components; finally, from ¹H-MAS spectra it was possible to investigate the presence and distribution of hydrogen-bond interactions involving the silanol groups present in the final hybrid materials. The study of a quite large set of different samples allowed interesting correlations between the above cited molecular properties and preparation conditions and materials morphologies to be highlighted [4].

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PROPENE-NORBORNENE COPOLYMERS: ANALYSIS OF POLYMER MICROSTRUCTURE.

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Olefin-cycloolefin copolymers (COC) synthesized with different metallocene-based catalysts [1], represent a new class of materials showing interesting properties as excellent transparency, good heat resistance and high glass transition temperature. Specifically, concerning Propene-Norborene copolymers (P-N), an improvement of the polymer properties was expected by varying and controlling their microstructure and composition, therefore to understand the relationship between the microstructure and material properties, a detailed study of the P-N copolymers microstructure by different NMR techniques is necessary.

In general, ¹³C NMR spectra of P-N copolymers are very complicated owing the complexity of stereo- and regioregularity of the propene units as well as the stereoregularity of the bicyclic units but combining ¹³C NMR spectra and ab initio ¹³C NMR theoretical chemical shift computations, averaged over RIS conformer populations, it became possible assign a large number of resonances even for complex copolymers spectra [2].

The microstructure of P-N copolymers having different norbornene content and obtained with rac-Et(indenyl)₂ZrCl₂ and Me₂C(2Me-Indenyl)₂ZrCl₂ as pre-catalysts, were analyzed by using 2D NMR techniques, allowing the possibility to extend and confirm the assignment of the previous resonances.

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THERMODYNAMICS AND MOLECULAR DETAILS OF SORPTION IN NANOPOROUS DIPEPTIDES: A ¹²⁹Xe NMR STUDY

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¹²⁹Xe NMR has been by now successfully applied to the characterization of systems having very different nature. Nevertheless, many questions about the influence of xenon atoms on the system studied can be raised and have still to be answered. Testing the effectiveness of a probe for the characterization of a host system requires, in fact, a deep understanding of the probe-host interactions as well as the characterization of possible probe-induced modifications of the host structure.

In the solid state, although the high sensitivity of ¹²⁹Xe NMR parameters (chemical shifts, relaxation times) to the local environment (site symmetry and shape of pores, channels, cages, voids), is very useful in providing unique information on the host structure and on the host-guest interactions, their interpretation is often complicated by the concurrent influence of different effects, such as the presence of counterions in zeolites or the Xe-Xe interactions in pores that can accommodate more than one xenon atom. Different models have been previously suggested and used for the interpretation of ¹²⁹Xe chemical shifts and to account for the anisotropic line shapes observed in confined spaces, but so far a single theory does not exist which is able to provide a good explanation of experimental results. In the spirit of studying simple model compounds in order to extract general rules about the experimental outputs of ¹²⁹Xe NMR, spectra for eight different dipeptides were analyzed (VA (Val-Ala), AV (Ala-Val), AI (Ala-Ile), IA (Ile-Ala), VI (Val-Ile), IV (Ile-Val), VV (Val-Val), LS (Leu-Ser)). Each dipeptide forms cylindrical helical nanotubes, with diameters ranging between 5.3 and 3.0 Å. Also, for the narrowest channels, hyperpolarized ¹²⁹Xe NMR spectra show an intense signal with pronounced axially symmetric chemical shift anisotropy (CSA) due to the absorption of xenon inside the nanochannels. The analysis of the line shape, beyond confirming the cylindrical cross-section of the pores, provides information on their helicity and on Xe-wall and Xe-Xe interactions of the xenon absorbed inside the channels. Moreover, from variable-temperature continuous-flow hyperpolarized ¹²⁹Xe NMR experiments, information on the thermodynamics of adsorption of Xe in the nanochannels can be obtained by fitting the experimental isotropic chemical shifts to a thermodynamic model based on Langmuir theory. Analysis of signal intensities as a function of experimental temperature confirms the values extracted for enthalpy and entropy of adsorption from the variation of the chemical shifts with the temperature. A correlation between channel shape and diameter and thermodynamics of adsorption was found for the absorption of xenon in dipeptides channels.

MULTINUCLEAR NMR STUDY OF THE INTERACTION BETWEEN BIS(PENTAFLUOROPHENYL)BORINIC ACID AND NITROGEN BASES

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In the presence of nucleophiles, bis(pentafluorophenyl)borinic acid $((Ar_F)_2BOH, Ar_F = C_6F_5, 1)$ [1] shows a chameleonic behaviour forming several different, sometimes unexpected, species according to the nature of the nucleophile, the stoichiometric ratio and the temperature. The exhaustive characterization of all the species that play a role in the relevant equilibria is therefore possible mainly, and sometimes only, through NMR spectroscopy. The number of NMR active nuclei (¹H, ¹¹B, ¹⁵N and ¹⁹F), present in the studied compounds allows a fruitful multinuclear NMR approach. We have previously



shown that, in dichloromethane solution, the thermodynamic and the kinetic of the monomer-trimer interconversion $(1_m \text{ and } 1_t, \text{ see Chart } 1)$ is strongly affected by the presence of water [2], THF [3] and methanol [4].

We have studied now the reaction of **1** in the presence of nitrogen bases, namely 1,8-bis(dimethylamino)naphthalene (DMAN), which can act as Brønsted base only, and pyridine, which is a good Lewis base but weak Brønsted base. Stepwise titrations were performed at low temperature and the temperature stability of the titration products was checked.

The behaviour of the two bases was compared according to:

- a) their effect on the kinetics of the monomer-trimer interconversion;
- b) the nature of the species formed at different titration steps;
- c) the character of the interaction between **1** and the bases;
- d) the tight or loose nature of ion pairs formed.

Moreover, in the presence of DMAN, **1** undergoes progressive dearylation, which, on the contrary, is irrelevant in the case of pyridine.

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STRUCTURAL ELUCIDATION OF NEW TRICYCLIC SYSTEMS BY NMR SPECTROSCOPY

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Regioselective synthesis of heterocycles *via* cyclization reactions is an important tool in organic and pharmaceutical chemistry.

Following our research in this field [1,2], we report here the results of the reaction of the 3-acylquinolinones **1a,b** with two 1,4-bisnucleophiles carried out with the aim to obtain new biologically active seven-membered compounds. Whereas the reaction of **1a,b** with ethane-1,2-diamine gave compounds **2a,b** whose structure were determined by NMR experiments (gHSQC, gHMBC, NOESY1D) and confirmed by an X-ray analysis on **2a**, reaction of **1a** with 2-aminoethanol did not lead to the expected compound **3a** through a substitution-elimination mechanism, instead compound **4a** was isolated in good yields; structure elucidation of this compound by multinuclear NMR experiments will be discussed together with the proposed reaction mechanism.



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FRAGMENT-BASED APPROACH APPLIED TO THE DEVELOPMENT OF POTENTIAL INHIBITORS OF EUKARYOTIC TRANSLATION INITIATION FACTOR 4E (EIF4E) USING ¹⁹F-NMR SCREENING

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In the last few years Fragment-Based Drug Design (FBDD) has become an important and effective tool for drug discovery. This approach is based on the screening of small libraries of low molecular weight compounds against a given biological target with the aim of identifying weak binders (in the high micromolar to millimolar range) with a Binding Efficiency Index (BEI) comparable to potent drug molecules. A molecular fragment represents a good starting point for the development and optimization of the potency, selectivity, pharmacokinetic and pharmaceutical properties of a new drug candidate. Nuclear Magnetic Resonance (NMR)-based screening is suitable for a fragment-based approach. A major advantage is that, despite of its low absolute sensitivity compared to other techniques used for screening, the NMR technology has an intrinsic sensitivity to binding events and so the ability of detecting even weak intermolecular interactions. Utilizing the favourable properties of Fluorine NMR spectroscopy, in our laboratory was developed the FAXS (Fluorine chemical shift Anisotropy and eXchange for Screening). The FAXS is a competition-binding assay for the identification of the ligands to the target of interest and for the measurement of their dissociation binding constant. This method has been applied to the biological target eIF4E (Eukaryotic translation Initiation Factor 4E). Eukaryotic initiation factor 4E plays a central role in initiation of the translation process by recognition of the 5' terminal mRNA cap structure, 7-methylGpppN (N=any nucleoside). Formation of the eIF4E-cap mRNA complex is considered to be rate limiting for translation and its overexpression is associated with cell growth, tumorgenicity and inhibition of apoptosis. Using a small fragment library we have identified a low molecular weight compound that binds eiF4E. The crystal structure of the complex between this hit fragment and 4EHP (eIF4E Homologous Protein) was solved. The structure shows that the compound does not occupy the site of the m7GTP, but it makes a covalent interaction with a cysteine (also present in the eIF4E protein) at the back of the pocket, inducing conformational change in the binding site.

NUCLEAR RELAXATION MECHANISMS IN SILKWORM COCOONS

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Understanding the molecular structural and dynamic properties of silk and their link with its macroscopic properties is a very important task because of the extensive applications of this material in fields ranging from textile industry to biotechnologies. Many solid-state NMR studies have been performed on silks [1]. Most of them consist of structural studies of model peptides or isotopically enriched silks produced by different biological systems (mainly silkworms or spiders). On the contrary, only a few studies have been reported on the dynamic properties of silk derived from the analysis of relaxation times in nonisotopically enriched samples [2,3,4,5]. The domesticated silkworm (Bombyx mori) silk is usually constituted of two proteins, fibroin (the main component) and sericin (which coats the silk fibroin, acting as an adhesive). Notwithstanding its interesting properties, which have recently been recognized for several biotechnological applications, in the past, sericin was essentially regarded as waste, being usually removed through a degumming treatment of cocoon silk and discarded, following the procedure required by textile industrial applications. Consequently, most of solid-state NMR studies of silkworm silk have been performed on the sole fibroin component.

The work presented consists of a ¹H and ¹³C solid-state NMR study of several untreated and degummed silkworm cocoons, with a variable hydration degree. The relaxation mechanisms of proton spin-spin (T₂) and spin-lattice relaxation in both laboratory (T₁) and rotating (T_{1r}) frames have been investigated, trying to localize the relaxation sinks in the different cocoon components (sericin, fibroin, physisorbed water). Proton relaxation times, measured by low-resolution techniques, have been interpreted in terms of dynamic processes occurring at a molecular level thanks to a synergic analysis of suitable ¹H low-resolution, and ¹H and ¹³C high-resolution experiments.

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ANALYSIS OF SOLID-STATE NMR PARAMAGNETIC RESTRAINTS FOR PROTEIN STRUCTURAL INVESTIGATION

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Solid-State NMR (SS NMR) of biological systems has recently seen a strong increase of interest, mainly when it was demonstrated as valuable technique for structural determination of protein in solid-state and of membrane protein [1,2]. Recently this technique has also been applied to the investigation of paramagnetic metallo-proteins in solid-phase [3-5]. In solid-state metallo-proteins carrying a metal with sizable anisotropy of the magnetic susceptibility it is possible to observe pseudocontact shifts (PCS) of nuclei sufficiently close to metal.

We describe the study performed on the catalytic domain of the matrix metalloproteinase MMP12 (159 AA, 17.6 kDa) in the microcrystalline state. This protein binds one Zn(II) ion (diamagnetic) in the catalytic active site, which can be replaced by a paramagnetic Co(II) ion. ¹³C SS NMR PCS's were measured by subtracting the observed shifts in the native diamagnetic protein to the Co(II)-replaced paramagnetic protein. The analysis of these data demonstrates that PCS's are valuable restraints for the SS NMR as well as in solution NMR [5].

For a limited number of peaks, it is also possible to observe contributions to PCS arising from the metal of the neighboring proteins in the crystal lattice. These *inter*-molecular contributions can be separated from the *intra*-molecular terms (due to bound Co ion) by using *paramagnetic diluted* samples. Co-crystallizing the ¹³C-¹⁵N labeled paramagnetic cobalt-MMP12 protein with an excess of unlabelled diamagnetic zinc-MMP12 the effects of the *inter*-molecular PCS are strongly quenched allowing one to separate them from the *intra*-molecular terms.

These terms are rich of structural information: the *inter*-molecular PCS's provides unique information for the protein crystal lattice investigation through NMR, while the *intra*-molecular terms provide restraints for the protein structural determination. Starting from the protein structure, the detailed analysis of the *inter*-molecular PCS allowed us to fix the position of two neighboring protein in the crystal lattice, in good agreement with the arrangement determined by X-ray crystallography [6].

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POSTERS

IT TAKES A LOT OF INGREDIENTS TO MAKE AN INTERACTIVE DNMR

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The problem of simulating an NMR spectrum subject to exchange phenomena was already solved 40 years ago with the program DNMR.[1] Apparently this opened the way to the straightforward and correct quantification of exchange rates by NMR spectroscopy. Unfortunately, many of the practiced protocols to fit an experimental spectrum (trial and error; systematic search; least squares fitting) have proved to be inappropriate. The nature of the problem lends itself very well, instead, to real time, interactive fitting by a human operator, because the spectrum at the lowest temperature is, usually, easily interpreted and each of the remaining spectra presents small variations from the preceding one. In recent years the programs WinDNMR [2] and SwaN-MR [3] paved the way to such manual fitting, but in practice they can only solve the simplest cases. After years of practice, we came to the conclusion that all the following ingredients are necessary for an interactive fitting routine to be generally applicable:

A fast executable code to provide real-time visual feedback.

Visualization of the experimental spectrum as an overlay over the simulation. The freedom for the user to define any kind of relations, symmetries and

equivalences that reduce the degrees of freedom of the problem to the bare minimum.

Incorporation of the simulation module into a full-featured processing software, to improve the quality of the experimental spectrum.

Generic formulation of the problem in the Liouville space, without the assumptions and simplifications that limit the validity to a few special cases.

The above ingredients, together with clear anti-aliased graphics, were therefore incorporated into the program iNMR.[4] With it we have easily measured the exchange rates of a complex of naphthyridine mono-coordinated to the platinum. The same spectra had resisted, until today, to all attempts of simulation up to the point of suggesting the substitution of di-methyl-phenanthroline for the naphthyridine. [5]



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Gd(III) COMPLEXES OF NEW TRIAZINE-BASED AMINOCARBOXYLIC LIGANDS: A ¹H AND ¹⁷O NMR RELAXOMETRIC STUDY

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s-Triazine is a well-known and tough structural motif for the construction of a large variety of intriguing molecules, showing an outstanding ability in intermolecular interactions [1]. In alternative to several pyridine-containing ligands already reported [2], we were prompted to explore the possibility to use the triazine ring as a coordinating unit and relying on its structural versatility to implant auxiliary coordinating unit and tailor-made recognition moieties. Hydrazino-N,N-diacetic groups complete the overall heptadenticity of the ligand in order to obtain a stable coordinating environment for lanthanide ions (Fig.1).

We have investigated the relaxometric properties of the Gd(III) complexes of two derivatives in aqueous solutions. Chemical exchange of the coordinated water molecule/s was determined through the measurement of the ¹H and ¹⁷O VT longitudinal and transverse relaxation rates, while the rotational dynamics was evaluated by recording and analyzing the $1/T_1$ Nuclear Magnetic Resonance Dispersion (NMRD) profiles. The lipophilic complex GdL2 aggregates and forms a micellar systems at low concentration, as assessed by measuring the R_1 data versus the concentration at 20 MHz and 25°C. The NMRD profile of the aggregated system was also recorded and analysed in detail using the Lipari-Szabo model free approach of the rotational dynamics that takes into account both the internal rotation superimposed to a global motion.



Fig. 1. Chemical structure of the ligands.

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PROBING THE SURFACE ACCESSIBILITY OF THE ARCHAEAL PROTEIN SSO7D

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Understanding how proteins approach surrounding molecules is fundamental, since biological processes mostly depend on the specific bindings occurring at the protein surface. An enhanced surface accessibility of protein binding sites has been already observed in some cases, but the molecular basis of this finding are not established. Here protein surface accessibility of the archaeal protein Sso7d has been investigated, by using water and paramagnetic molecules as probes to correlate hydration, probe accessibility and activity of the protein. A combined analysis of the hydration data derived from molecular dynamics simulations and NMR ePHOGSY [1] spectroscopy is here proposed to get a reliable picture of solvent dynamics around Sso7d [2-3], a small and multifunctional protein from *Solfolobus solfataricus*. The Sso7d DNA binding domain appears to be, at the same time, scarcely hydrated and very accessible to Gd(III)(DTPA-BMA). Conversely, the protein ATP binding site appears to be strongly hydrated and scarcely accessible both to Gd(III)(DTPA-BMA) and TEMPOL. This finding suggests that protein Sso7d hydration may controls its intermolecular approach.



Fig. 1. ATP-bound structure of hydrated Sso7d as derived from molecular docking.

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COBALT (II) ALLYL COMPLEXES: SYNTHESIS, NMR CHARACTERISATION AND POLYMERISATION BEHAVIOUR.

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A series of cobalt (II) allyl complexes of general formula $L_1L_2Co(PPh_2R)$ (Figure 1) [L_1 = diene monomer (e.g. butadiene, isoprene); L_2 = butenyl- or substituted butenyl-group; R = alkyl or cycloalkyl group] were obtained, following an already reported experimental procedure [1], by reacting CoCl₂ with a diene monomer (e.g. butadiene, isoprene), in the presence of metallic Zn.

All the complexes were characterized by NMR spectroscopy and comparisons have been made with the cobalt complex $(PPh_3)Co(C_4H_6)(C_4H_{10})$ previously reported [1].

The behaviour of these novel complexes in the polymerization of butadienes and allenes has been investigated.

The interest on these complexes lies also in the fact that their structure is likely analogous to that of the active site in the polymerization of butadiene with $CoCl_2(PRPh_2)_2/MAO$ systems [2], giving indeed useful information on some aspects of the diene polymerization mechanism [3].



Figure 1: But-2-enyl-buta-1,3-diene (Methyl-diphenylphosphine) Cobalt (II) complex

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A MULTINUCLEAR SOLID-STATE NMR CHARACTERIZATION OF TWO POLYORGANOPHOSPHAZENES EMPLOYED AS SUPPORTS FOR CATALYTICALLY ACTIVE PALLADIUM NANOPARTICLES

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Polyorganophosphazenes, $-[N=PR_2]_n$, are polymers constituted by an inorganic backbone formed by alternating nitrogen and phosphorus atoms, with two organic side groups bonded to each phosphorus atom. They are known to exhibit interesting physical and chemical properties, as stability against strong acids, bases and aggressive chemicals, high thermal stability, flame resistance, unusual flexibility, biocompatibility and photosensitivity [1]. Recently, the basic properties of the backbone nitrogen atoms, which make them able to coordinate metal atoms and cations, have been exploited in the synthesis of polyorganophosphazene supported metal nanoparticle catalysts [2], found to be active in the hydrogenation of a wide range of organic compounds. Quite few solid-state NMR studies of polyorganophosphazenes have been reported, mostly concerning the polymers structural and dynamic properties [3-7]. Here we present a multinuclear solid-state NMR study of two different polyorganophosphazenes (polydimethylphosphazene and poly[bis(phenoxy)]phosphazene), both employed as supports for palladium nanoparticle catalysts [8]. By means of ³¹P, ¹³C and ¹⁵N highresolution and ¹H low-resolution techniques the different coexistent polymer phases have been detected and characterized and interesting effects have been observed as a consequence of the presence of Pd nanoparticles. The employment of a multinuclear and comparative solid-state NMR approach and the analysis of two different polymers have turned out to be very useful in investigating the interaction between the catalytically active metal nanoparticles and the polymer supports.

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LANTHANUM SULFATE HYDRATES: A SOLID-STATE ¹³⁹LA NMR STUDY

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The dehydration and subsequent decomposition process of the rare earth metal sulfate hydrates has been quite extensively studied in the past and it is still object of interest, not only from a pure cognitive point of view, but also for several applicative implications ranging from metallurgical industrial processes to potential employments in the storage of solar energy and nuclear heat, and in fuel gas cleanup process [1-3]. Most of the literature studies have been performed using thermal analysis techniques, while, especially as far as the dehydration process is concerned, only few structural data of the possible sulfate intermediate hydrated forms have been reported, thus making a clear and unambiguous picture of the dehydration process still difficult to be obtained.

In this study we tried to get some insights into the dehydration process of the lanthanum (III) sulfate by means of ¹³⁹La solid-state NMR spectroscopy. ¹³⁹La is a quadrupolar nucleus (I = 7/2), with natural isotopic abundance of 99.9% and a moderate nuclear quadrupole moment ($Q = +20 \text{ fm}^2$). Despite only few studies performed in the solid state have been reported, ¹³⁹La solid-state NMR experiments resulted to be feasible and potentially very powerful in the molecular investigation of both simpler inorganic and coordination compounds [4, 5]. In this case we performed a ¹³⁹La solid-state NMR study on various lanthanum sulfate hydrates, prepared by heating commercial samples of La₂(SO₄)₃•xH2O at different temperatures. The water content of the different samples was determined by means of thermogravimetric analysis (TGA) and the effects of hydration on the molecular structure were observed via acquisition and analysis of solid-state ¹³⁹La NMR spectra. Simulations of the NMR spectra enabled the ¹³⁹La electric field gradient and chemical shift tensor parameters to be obtained for several La sites in lanthanum sulfate nona-hydrate, different intermediate hydrates, and anhydrous lanthanum sulfate, as well as to obtain important information concerning the occurrence of the dehydration process.

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GEOGRAPHICAL CHARACTERIZATION OF Crocus sativus : ¹H NMR AND MULTIVARIATE STATISTICAL ANALYSIS

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In the last years, PDO certification from E.U. became a valorization tool for foods. The products showing this trade mark posses typical, qualitative and sensorial characteristics strictly related to the geographical origin and to the production procedures, expressed into the set rules. In Italy, actually, more than 100 products got the PDO certification. The main aim of several research groups is the development of new analytical techniques for geographical characterization of foods and in particular search for qualitative and territorial markers. In this work ¹H NMR studies in combination with multivariate statistical analysis are presented. PDO saffron samples from Aquila and from S. Gimignano (RE 205/2005) and Sardinian saffrons were analyzed in comparison with samples from different geographical origins (Italian and extra European). PCA, PLS-DA, and LDA have been applied to NMR data with the aim of sample discrimination.

STUDY OF THE OXIDATION PROCESS OF SOME CULINARY OILS BY ¹H NMR SPECTROSCOPY

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The importance of oils in the processing of several foods as well as in human nutrition is well established. They are constituents of many foods and play an important role in their organoleptic and nutritional properties. The thermal stress to which oils can be exposed during standard culinary practice causes their oxidative deterioration. Oils oxidative stability is strictly related to their composition. The main reaction involved in the oxidative deterioration is the thermally-induced, radical-mediated autoxidation of polyunsaturated fatty acids (PUFAs) that primarily leads to conjugated hydroperoxydiene species. Such species further evolve to a wide variety of secondary peroxidation products, including a range of aldehydes (mostly *n*-alkanals, *trans*-2-alkenals, 4-hydroxy-*trans*-2-alkenals and 4-hydroperoxy-*trans*-2-alkenals) [1, 2].

Some of these aldehydes are cyto- and geno-toxic and are related to several diseases in humans. For these reasons characterization of edible vegetable oils, detection and quantification of specific products arising from the oxidation of culinary oils are essential for evaluating their suitability for different uses in the food industry.

The oxidation process of corn, peanut, soybean and sunflower oils at 70° C in air has been investigated by means of ¹H-NMR (Fig. 1).

The evolution of the oxidative process, the rate of degradation of the acyl groups, the formation and the degradation of primary oxidation products, as well as the formation of secondary oxidation products as been studied for the four oils tested. The data obtained from ¹H-NMR spectra have been compared with the iodine value parameter measured according to AOAC (Association of Analytical Communities) method at each step of the degradation process. The data reported represent a preliminary phase of our study that will be extended to other vegetable oils. Work is in progress to study the thermal stability of the lipidic part of hazelnuts of different cultivars [3].

87 86 85 84 83 82 81 80 79 78 7.7 7.6 7.5 7.4 7.3 7.2 7.1 7.0 69 68 67 66 65 64 63 62 6.1

Fig. 1. Region between 5.5 and 10.0 ppm of the¹H-NMR spectra of soybean oil after 10d of treatment

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SOLID-STATE NMR AND MECHANICALLY-INDUCED TAUTOMERISM OF BARBITURATES

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Solid-state grinding, carried out manually with a mortar and pestle or mechanically in a mill, is generally used in solvent-free reactions. This simple procedure can induce also a variety of solid state transformations, including crystalline to amorphous, amorphous to crystalline, as well as polymorph conversion [1].

Here we report the mechanical conversion of the barbituric acid and of the 5methylbarbituric acid into one of their tautomers: the trihydroxyl and the hydroxyl tautomer, respectively (Figure 1). Both acids are known to exist in the solid state only in the trioxo structure as shown by X-ray diffraction study. However, they may give rise to many (11) possible tautomers supposed to be the intermediate forms in many reactions involving barbiturates [2].

For both compounds the obtained tautomer has been identified by mean of NMR experiments. In the former case the tautomer has been isolated in the solid state since it immediately converts to barbituric acid in solution in many solvent (protic, aprotic and amphiprotic). By combining 1D ¹³C and ¹⁵N (CPMAS, NQS) and 2D (PASS, ¹H-¹³C FSLG-HETCOR) solid-state NMR data it has been possible to ascertain the formation of the trihydroxyl form. In the latter case the tautomer shows in the ¹³C CPMAS spectrum very broad peaks due to the presence of static disorder. However it is stable in solution and it has been characterized by means of 1D ¹³C and 2D (HMQC, HMBC) solution-state NMR.



Fig. 1. Mechanically induced tautomerism in barbituric acid and in 5-methylbarbituric acid.

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EFFECTS OF THE INTRODUCTION OF A DISULPHIDE BRIDGE ON DYNAMICS AND INTERACTION PROPERTIES OF CHICKEN LIVER BILE ACID BINDING PROTEIN

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In the liver, two paralogous groups of FABPs have been described: liver-FABP14, extensively characterized in mammals, and liver basic-FABP, that have not yet been found in mammalian liver but have been described in several non mammalian vertebrates [1]. We have previously reported on the role of chicken liver basic Fatty Acid Binding Proteins (FABPs) in the binding and transport within cytosol of insoluble bile acids and this role is emerging as the specific function of the liver basic-FABPs, hence called liver bile acid binding proteins (BABP) [2]. BABPs are small molecular mass proteins (14-15 kDa) exhibiting the typical fold of the family in which 10 strands of antiparallel β -sheet surround the hydrophobic ligand binding cavity and two short α helices are located between the first and second strands. We have previously described the solution structure of apo (cl-BABP) [3] and holo chicken liver BABP (cl-BABP) [4] binding bile salts with a 1:2 stoichiometry.NMR ¹⁵N relaxation studies on apo and holo proteins revealed a substantial conformational flexibility, on the microsecond to millisecond time scales, mainly localised in the C-terminal face (EFGHJ) of the betabarrel of apo protein that is quenched in the presence of the bile salts. The observed dynamics has been primarily related to the protonation/deprotonation equilibrium of H98 located on the flexible face. A network of polar buried side-chains (T72, C80, S93, H98, E109, R120), defining a spine going from E to J strand, is likely to provide the long range connectivity needed to communicate motions from H98 to EF loop at the open end of the protein.

In order to understand the role of each spine residue site-direct mutagenesis approach was followed. Residue 91 is a Thr or a Cys in the different isoforms reported for cL-BABP. Most of BABPs belonging to the liver non-mammalian species show a cysteine residue in position 91 involved in a disulphide bridge with the conserved spine residue C80. We report here the chemical shift assignment and ¹⁵N relaxation data analysis of apo and holo T91C mutant. Changes in backbone dynamics of cL-BABP, induced by the presence of a disulphide bridge, are observed with respect to WT protein. NMR interaction studies of T91C with 15N-glycochenodeoxycholic acid suggest a conserved stoichiometry, accompanied by changes in binding affinity for the superficial site.

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STRUCTURAL CHARACTERIZATION OF THE HELIX STABILTY OF A DE NOVO ENGINEERED VEGF MIMICKING PEPTIDE

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Modulating angiogenesis is an attractive goal because many pathological conditions depend on the growth of the new vessels. Angiogenesis is mainly regulated by the VEGF(Vascular Endhotelial Growth Factor), a mitogen specific for endothelial cells. In the last years, many efforts have been pursued to modulate the angiogenic response targeting VEGF and its receptors¹. Based on X-ray structure of VEGF bound to the receptor², we have designed and synthesized a peptide, QK, reproducing a region of VEGF binding interface: the helix region 17-25 of VEGF³. CD and NMR experiments demonstrated that the peptide assume a monomeric helical conformation. Biological assays in vitro suggested that QK binds to the VEGF receptors and competes with VEGF. Furthermore, QK induced endothelial cells proliferation and increased the VEGF biological response. The high biological activity of QK appears to be strictly related with its helical propensity; here, we therefore examine the structural bases of QK helix stability through CD, NMR and MD analysis. CD and NMR experiments have clearly shown that QK helical conformation is chemically and thermally very stable.

MD and mutagenesis studies suggested that residues located at the N-terminal or central region of QK sequence should play a relevant role in stabilizing the helical fold. Consequently, the single point QK mutants have been synthesized to be structurally analyzed to verify the role of those residues in QK helical conformation.

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THE UNFOLDING MECHANISM OF THE CHICKEN LIVER BILE ACID BINDING PROTEIN

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The chicken liver bile acid binding protein (cl-BABP) is a member of the intracellular lipid binding protein family, a group of small, approximately 15 kDa proteins, that are thought to facilitate the cellular trafficking and metabolic regulation of fatty acids, retinoids, cholesterol, and bile salts. In particular cl-BABP plays a role in the enterohepatic circulation of bile salts [1].

In the present study we characterize the unfolding mechanism of the cl-BABP by means of NMR spectroscopy and of Differential Scanning Calorimetry (DSC).

The chemical denaturation has been followed in a residue specific manner, collecting a series of ¹H-¹⁵N HSQC spectra and comparing the intensities of the peaks at increasing concentrations of Urea. To obtain an estimate of the conformational stability we used the linear extrapolation method [2] adapted to a three-state mechanism. In this way it is possible to obtain the ΔG_D for each residue and for each step of the denaturation path.

DSC technique provides direct measurement of the thermal-induced conformational transition energy and allows thermodynamic characterization of the thermal denaturation process.

The comparison of the information obtained using these two techniques with the dynamic data [3] will be presented.

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LOCAL AND GLOBAL CONFORMATIONAL PLASTICITY OF THE HPV-16 **E2 DNA BINDING DIMERIC B-BARREL DOMAIN BY NMR**

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Regulation of gene transcription, genome replication and viral episome maintenance in Papillomavirus requires E2 protein and its capacity to bind specific DNA sequences through its dimeric C-terminal domain. A disfunction of this protein leads to cervical carcinoma. We analyzed the structural dynamics and solvent exchange of HPV16 E2 DNA binding domain. Despite being a highly stable and cooperatively folded dimer, amide exchange measurements of the free domain show that over 90% of the protons are exchanged after 12 hrs. ¹⁵N relaxation studies indicate that this protein is characterized in the DNA-free state by a global and local flexibility functional to its DNA binding activity. High mobility is present at the DNA recognition helices, especially at their N-cap portions. Extensive and slow conformational motions are observed at the dimer interface. This global plasticity is regulated by protonation of the ring of a key histidine (H318), which displays also the inversion of the tautomeric form as revealed by long-range ¹H-¹⁵N HSQC spectra performed at different pHs. Titrations of backbone NHs fit to pK_a values of the five histidine residues present in this protein. The overall dimer stability is compromised at low pH, when protonation of H288 disrupts crucial hydrophobic interactions and causes dimer unfolding.

Upon binding of DNA, most of the protein becomes more rigid, in particular the recognition helices, which increases their helical characteristics. Interestingly, only the loop connecting B2 and B3 remains flexible in the complex despite the fact that it faces the DNA target, strongly suggesting transient but effective interactions, and that the loop evolved to retain flexibility even in the DNA bound state. Magnetization exchange with solvent and ¹⁵N relaxation data indicate that the main differences between the free and DNA bound domain lie at the N-cap of the recognition helix, at the region around K349, recently reported to play an essential role in the complex stabilization, and at the intermonomer contact area.

α-SYNUCLEIN AND NEGATIVELY CHARGED BICELLES: CHARACTERIZATION AND STABILITY OF THE MEMBRANE-MIMETIC SYSTEM AND PRELIMINARY STUDIES ON PROTEIN-BICELLE INTERACTION

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Bicelles, described as bilayered micelles, are a very useful membrane-mimetic system for NMR structural studies in solution: they have a planar region made up of long-chain phospholipids surrounded by a rim composed of short-chain phospholipids [1]. They are usually characterised by two parameters: q (the molar ratio between the long-chain phospholipids and the short-chain detergent), that is related to the size of the bicelles, and c_L (the total lipid concentration in weight percent). The interest in such a system is due to the ability of bicelles having 2<q<5 to form a well-oriented discotic nematic phase when placed in a strong magnetic field [2]. This particular property allows the detection of residual dipolar couplings as additional structural restraints to be used in molecular dynamics calculations of proteins and peptides. Furthermore, if their size is sufficiently small (0.5 < q < 1), they can be used to study the conformation of membraneassociated biomolecules even when they are non aligned [3]. Up to now, many authors have described the preparation of bicelles composed of DMPC (1,2-dimyristoyl-snglycero-3-phosphocholine) in the planar region and DHPC (1,2-dihexanoyl-sn-glycero-3-phosphocholine) in the rim; this system has been characterised in depth by several techniques, such as ²H-NMR, ³¹P-NMR and electron microscopy at different q and c_L values and at different temperatures, but very little is known about similar structures composed of acidic long-chain phospholipids. In this work, we prepared bicelles with DMPG (1,2-dimyristoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]) in the bilayer and DHPC (1,2-dihexanoyl-sn-glycero-3-phosphocholine) in the rim in 2:1, 1:1 and 3:4 molar ratios at different pH, ionic strength and concentration, and we studied their stability in solution by dynamic light scattering, TEM and DOSY. This kind of bicelles can be prepared as already described in literature [4], but their stability strongly depends on temperature, concentration and probably on ionic strength.

 α -Synuclein is a small protein involved in Parkinson's disease and with an unknown function, but it can interact with negatively charged membranes. We tested the conditions to have the best bicelle-protein interaction and we performed preliminary NMR experiments of the protein bound to our new membrane-mimetic system to elucidate its structure in a bilayer.

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NMR DATA ACCUMULATION IN POLAR COORDINATES NUMERICAL METHODS TO MINIMIZE SYSTEMATIC ERRORS

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In this paper, we discuss a hardware implementation of numerical methods to minimize Rician noise data contamination effects, during NMR signals magnitude accumulation.

The accumulation of signal magnitudes of NMR data in low signal-to-noise ratio (SNR) regimes produces signal-dependent noise bias that reduces the accuracy of the measurements. This is particularly important for T1 and T2 measurements when the relaxation curve are fitted by a multi-exponential acquisition from polar coordinates. In fact the white noise, with a Gaussian distribution, that affect the NMR signal, become a Rician noise, characterized from the Rayleight distribution, on the accumulated signal magnitudes resulting in a systematic error.

To apply the proposed numerical method we need to: acquire noise parameters such as mean and noise variance, estimate the signal-to-noise ratio and calculate the correction factor with dependences from signal amplitude.

We have developed the correction scheme on a reconfigurable digital hardware architecture, to apply the factor directly over the data-flow on our acquisition channel. In order to estimate noise parameters only a few phantom measures are needed and the technique is applied without any additional or external operation resulting in better than 1% signal correction on our tests.

Further developments are focused about the improved hardware implementation of numerical methods used to calculate the correction factor. We have also proposed a different technique in order to better evaluate SNR dependencies and extract the real signal intensity from noisy magnitude MNR signals. This alternative technique presumes the possibility of measuring the power of the noisy signals.

Several numerical approaches are been proposed in literature to de-noising NMR signals including a non-linear and wavelet transform. This methods are suitable to use in a post-processing data evaluation, used in conjunction with our hardware correction technique, becomes a complete tool to compensate and/or remove noise from digitalized NMR signals.

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MEASUREMENT OF THE SPIN-SPIN RELAXATION TIME T₂ AT VERY LOW MAGNETIC FIELD BY MEANS OF THE FAST FIELD CYCLING NMR METHOD

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Field Cycling NMR is a reliable and powerful method used so far for measuring the spin-lattice relaxation time T_1 at low fields as well as its dependence on the magnetic field strength. Moreover, the Field Cycling NMR is the only technique which allows to extend these measurements down to very low fields, typically close to hearth field or lower.

As far as the spin-spin relaxation time T_2 is concerned, it was almost impossible till now to exploit the same advantages offered by the technique because of instrumental limits and the intrinsic problems you get with the acquisition and the accumulation of an echo (compared with the acquisition of an FID) in a fast field cycling experiment.

In this work we present new instrumental concepts and new NMR methods developed exactly on the objective to overcome these limits to allow the acquisition of a Hahn echo decay in the same magnetic field range used in the measurement of a T_1 NMRD profile.

In case of experiment with lower S/N systematic errors are introduced. These errors can be removed using suitable acquisition procedures as well as proper weighting functions of the signal and noise during the accumulation process. Several T_2 (spin-echo decay) profiles acquired on different samples by means of a Field Cycling Spin-echo experiment are presented and discussed.

TRACEABILITY OF SYNTHETIC DRUGS BY POSITION SPECIFIC ²H **ISOTOPE RATIO ANALYSIS**

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It is well established that natural abundance isotope ratios provide powerful analytical criteria for the authentication of the origin of products and several applications in the field of the food science have been developed. Stable isotope analysis can also be used to trace back the reaction pathway of synthetic compounds such as drugs. In the field of illicit drugs, such as methamphetamine and ecstasy, we have shown that useful information can be obtained from quantitative natural abundance ²H NMR spectra for the identification of the precursors and of the methods employed for the synthesis [1]. The same methodology may be applied to the analysis of active pharmaceutical ingredients to discover counterfeiting and patent infringement. FDA repeatedly published warnings on the possible dangers of buying medicine online [2], in particular from various Canadian websites [3]. Tests made on these products revealed that the formulations often contain dangerous species or do not have the proper amount of active ingredient. Thus it is socially relevant to develop analytical methods for the traceability of synthetic drugs in order to establish their synthetic history and relate them to a specific manufacturer.

We have investigated three drugs of large use: fluoxetine 1, a antidepressant marketed as Prozac, ibuprofen 2 and naproxen 3, two nonsteroidal anti-inflammatory agents.



We performed a ²H NMR study on several samples of these drugs either isolated from commercial samples or prepared according to the main known synthetic routes. As expected the deuterium content at the various sites of the molecule bears memory of the synthetic procedure and provides a chemical fingerprint useful to trace back the history of the material.

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PLANT METABOLIC ANALYSIS THROUGH NMR TECNIQUES

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Metabolomic is considered as an emerging "omic" tool for gene function establishment, in according with trascriptome and proteome, and for metabolic pattern analysis. It's involved with the study of chemical processes occurring in living organism, thus constituting the group of small molecules, called "metabolites", necessary for growth, maintenance and plant defence.

One of plant metabolomic applications, is the investigation of metabolic profile change induced by both abiotic (UV, temperature, moisture) and biotic stresses (pathogen attacks). These factors lead to qualitative and quantitative modification of primary and secondary metabolites [1,2]. In particularly, the plant biochemical changes induced by biotic and abiotic stresses, involved the change of secondary metabolites (i.e. phenilproanoids), essentially compounds for plant defence. Detection and identification of metabolites changes, are the first steps to understand plant adaptation to stress conditions. Another application of plant metabolomic, is identification of economic interest compounds in pharmaceutical, cosmetic and nutritional field (i.e. alkaloids, terpenes and carotenoids)[3,4,5].

Different techniques can be successfully applied to plant metabolic analysis: classical approaches like TLC, FT-IR, HPLC-UV or GC-MS are strongly biased by limited separation and/or detection of particular class of chemical compounds. In the recent years, NMR techniques have acquired great importance due to its features of rapidity, specificity and non selectivity.

Here, we present a preliminary study of metabolic content of rice and tobacco leaves in normal growth condition through multidimensional NMR spectroscopy and selective tecniques. Primary metabolites like amminoacids and sugars have been identified in the two species, known to be essential for growth and maintenance of plant. Besides, in rice we have identified also secondary metabolites, like nucleosides and phenilpropanoids precursors. These identifications will be used to understand the metabolic changes induced by abiotic (salt) and biotic (infection by *M.grisae*) stresses. In tobacco, we have identified secondary metabolites belonging to alkaloid and phenilpropanoid (i.e. chlorogenic acid) classes, which could have applications in pharmaceutical field.

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SULFUR HEXAFLUORIDE: A PROMISING SPIN-SPY MOLECULE FOR PROBING SYSTEMS IN SOLUTION

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The chemical shift of ¹²⁹Xe is exquisitely sensitive to the xenon environment and, therefore, ¹²⁹Xe is used as a monatomic spin-spy for characterizing systems in gas, liquid or solid states. The major drawbacks of ¹²⁹Xe are its extremely long longitudinal relaxation time in diamagnetic environments and its rather poor NMR sensitivity. This usually prevents quantitative interpretation of the integrals and severely limits the scope of feasible NMR experiments.

This communication reports on the interest of sulfur hexafluoride (SF₆) in the framework of the spin-spy methodology. Solvent effects are explored, relaxation mechanisms are investigated and the inclusion of SF₆ within the cavity of α -cyclodextrin is studied.

Sulfur hexafluoride comes forward as a promising spin-spy molecule for probing systems in solution notably because (i) its detection limit by ¹⁹F NMR reaches the μ M range with standard equipments, (ii) integral measurements allow to quantify the amount of SF₆ in solution, (iii) chemical shift, diffusion, longitudinal relaxation time measurements as well as intermolecular Overhauser effect can be used for characterization.



[SF_]

500

400

0.0

100

200

300

NMR evidence of the inclusion of SF₆ by α -cyclodextrin in aqueous solutions: ¹⁹F integral (*a*) and chemical shift (*b*) variations, region of the ¹H (*c*) and ¹⁹F-¹H 1D HOESY (*d*) spectra.

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ORIENTATIONAL ORDER OF FLUORINATED LIQUID CRYSTALS BY MEANS OF A COMBINED ANALYSIS OF ¹³C NMR, OPTICAL AND DIELECTRIC MEASUREMENTS

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In the past few years, liquid crystals displays (LCDs) have become a nearly inseparable part of our daily life. In this field fluorinated liquid crystals (FLC) represent an important class of materials with possible applications, especially thanks to their broad nematic mesophase ranges, low rotational viscosity and high dielectric anisotropy [1].

Structural and orientational order properties of **3Cy2CyBF2** [Cr-34.8°C-N-103.2°C-I] and of **5CyCy2BF2** [Cr-25°C-SmB-73.3°C-N-121.3°C-I] have been investigated by means of ¹³C NMR, optical and dielectric spectroscopy methods.

For both compounds ¹³C-{¹H} NMR static spectra have been recorded by means of the Linear-Ramped Cross-Polarization technique, under SPINAL-64 decoupling [2]. The assignment of the ¹³C resonances have been carried out thanks to the comparison with solution state spectra and with DFT calculations using Gaussian'03 [3].

The order parameters have been calculated simultaneously analyzing all the ¹⁹F-¹³C dipolar couplings (D_{C-F}) according to the procedure described in ref. 4, as well as by fitting of all the experimental ¹³C chemical shift anisotropies. Dielectric measurements were performed in a broad frequency range for two orientations of the nematic director with respect to the measuring field. At low frequencies (static case) a positive dielectric anisotropy was determined, which enabled the calculation of the order parameter according to the procedure described in ref. 4. At high frequencies the dielectric anisotropy changes its sign which can be useful in designing a dual addressing display. The optical birefringence $\Delta n = n_e - n_o$ was measured as a function of temperature which yielded the order parameter [4]. The nematic order parameter determined from optical birefringence data, dielectric anisotropy and NMR have been compared. The results so obtained have been discussed, also with reference to those previously reported for two different fluorinated nematogens [4].

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STRUCTURE, ORIENTATIONAL ORDER AND DYNAMICS OF A LIQUID CRYSTAL BY ²H AND ¹³C NMR SPECTROSCOPY AND DFT CALCULATIONS

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Structural, orientational order properties and dynamics of 4,4'-heptylazoxybenzene (**HAB**) were critically re-investigated in its nematic and smectic A phases combining ²H and ¹³C NMR techniques, as well as theoretical DFT calculations. To this aim, two isotopomers, **HAB-d₄** and **HAB-d₈**, partially and fully deuterated on the phenyl rings, respectively, were also studied.

¹³C-{¹H} NMR static spectra were recorded on **HAB** by means of the Linear-Ramped Cross-Polarization technique, under SPINAL-64 decoupling [1] in the whole mesomorphic range. ¹³C resonances were assigned with the help of solution state spectra and DFT calculations performed using Gaussian'03 [2]. Chemical shift anisotropy (CSA) trends with temperature were obtained for all of the chemically distinguishable carbons. Moreover, deuterium quadrupolar and dipolar (deuterium-proton) splittings were determined from ²H NMR quadrupolar echo spectra, recorded on **HAB-d**₄ at different temperatures within the mesophases. ¹³C and ²H data were used to determine the complete order matrix and the angle between the *para* axes of the **HAB** phenyl rings through a non-linear least-squares global-fitting procedure, in which geometrical parameters and ¹³C shielding tensors calculated at the DFT level of theory (B3LYP/6-31G(d) and MPW1PW91/6-311+G(d,p) combination of hybrid functional and basis set; Gauge-Including Atomic Orbitals, GIAO method [3]) were employed. The results were discussed in comparison with those previously obtained from deuterium data only [4].

 13 C spin-lattice relaxation times (T₁) were determined for all three compounds throughout the liquid-crystalline phases. The combined analysis of these data with the already available deuterium relaxation times [5] allowed detailed information to be obtained on **HAB** reorientational dynamics in the mesophases.

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PRELIMINARY NMR STUDIES CONCERNING E. COLI GRX4 DIMERIZATION

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The so called monothiolic Glutaredoxins belong to a recently discovered class of putative Glutaredoxins and are characterized by a Cys-X₁-Phe/Tyr-X₂ site which corresponds, by alignment, to the active site of the well known dithiolic Glutaredoxins. Up till now, neither the physiological reductant nor the substrate of these proteins are definitely proved, and this implies that their functional role is actually elusive. Nevertheless, the extent of conservation of these proteins amongst prokaryotes and eukaryotes, and the poor viability of some knock-outs, suggest a decisive importance in a very central process within the cells, and a role which is not redundant with dithiolic Glutaredoxins. Defective mutants seem to show an accentuated sensibility to oxidative stress.

The Glutaredoxin we are interested in is the *E. coli* Grx4 (grxD gene), a 115 residue protein with a thioredoxin-like fold. An NMR structure obtained on ¹³C and ¹⁵N isotope labelled samples is available [1]. It is remarkable that Grx4 has a well defined patch of spatially co-localized residues on the contact surface with the solvent which is perfectly conserved in other monothiolic Glutaredoxins of different pathogens. This portion of surface is contiguous to the monothiolic site and is very close to a hydrophobic, exposed region. The amino acids involved in the interaction between glutathione and dithiolic Glutaredoxins are partially conserved in Grx4, both for charge distribution and for steric outline of the lodging groove. It is not clear if the structural gap which renders Grx4 inactive in traditional dithiolic Glutaredoxins functional assays could be overcome by an interacting partner.

While performing other NMR studies over Grx4, we noticed a marked tendency to selfaggregation, a propriety already underlined for other proteins of the thioredoxin superfamily and proposed also for Glutaredoxins [2].

In this work, we report some preliminary data concerning Grx4 oligomerization obtained monitoring NMR variations with time and concentration. Through ¹⁵N-HSQC we were able to identify the backbone amides most affected by the process and to propose a probable superficial region involved in the interaction. Then, we tried to predict the translational diffusion coefficient of the monomer and of a hypothetic dimeric structure, with the purpose of verifying these calculations through DOSY experiments at different sample concentrations.

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NMR CHARACTERIZATION OF CHICKEN ILEAL-BILE ACID-BINDING PROTEIN

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Bile acids play an important role in efficient digestion and absorption of dietary fats and in the regulation of cholesterol homoeostasis [1]. They are synthesized from cholesterol in the liver and secreted with bile into the small bowel, where they form micelles with luminal lipids. On reaching the terminal ileum, the luminal bile acids are actively reabsorbed by enterocytes and returned to the liver via portal circulation. This recycling system for bile acids (enterohepatic circulation) is important for the maintenance of bile acid and cholesterol balance [2]. In the bile acids enterohepatic circulation, three key steps are mediated by: i) a receptor system, that binds bile salts on one surface and translocates them into the cell; ii) a cellular bile salt binding protein, that moves them across the cell and iii) an exit system, which moves bile salts out of the other side of the cell [3]. Much has been learned about the mechanism by which bile acids enter and exit liver and intestinal cells, but very little is known about how bile acids are shuttled in the cytosol [4]. It has been proposed that intracellular carrier proteins mediate active transport of the bile acids within hepatocytes and ileocytes, during the enterohepatic circulation [5-7]. In mammalian species only ileal bile acid binding proteins have been so far identified, while liver cytosolic carriers have never been found. On the contrary, in non mammalian vertebrates, only liver, and not ileal, bile acid binding proteins were reported. A bioinformatic search allowed us to identify a non mammalian putative bile acid binding protein in the chicken ileum (cI-BABP), which we recombinantly expressed and purified. The protein exhibits the capability, tested by in vitro NMR experiments, of binding bile acids [8]. Here we report the preliminary data on the interaction between cIBABP and the bile salt glycochenodeoxycholate.

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IRON REMOVAL AND NMR QUANTIFICATION OF THE IMPURITIES IN COMMERCIAL IRON FERTILIZERS BASED ON 0,0-EDDHA

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Iron chlorosis is a nutritional disorder that affects crops in alkaline soils. Fertilization with iron chelated by synthetic ethylenediamine-N,N'-bis(o-hydroxyphenylacetic) acid (o,o-EDDHA) has proven useful to relieve this problem, but an issue arises regarding the purity of the commercial preparations [1]. While at least one of the impurities present in such iron fertilizers has been claimed to be useful for improving iron uptake in calcareous soils [2], little is known about the concentration of the condensation by-products that form during the synthesis [3]. This lack of information is however not surprising, because iron chelates are usually analyzed by chromatography (HPLC) where quantification can only be obtained after calibration with pure specimens of the compounds under study, most of which are in this case unknown. NMR does not need any previous calibration and would be more useful than HPLC for by-product quantification; however, because of the severe line broadening caused by iron paramagnetism, the NMR spectra of the iron chelates only show one very broad and featureless line, thus calling for iron removal.

Iron removal from this type of fertilizers is by no means an easy task, owing to the extremely high stablity of the iron chelates. In an attempt to obtain useful NMR spectra of the organic molecules contained in the commercial EDDHA/Fe³⁺ chelates, we have developed a protocol for iron removal based on the well known reaction between Fe³⁺ and Fe[(CN)₆]⁴⁻ yielding Prussian Blue as a precipitate. In this work we show that using the above reaction iron is removed to a negligible level, thus permitting the detection of many minor compounds with good NMR resolution.

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A DIFFUSION-ORDERED SPECTROSCOPY STUDY OF SOLUBILISATION OF ARTEMISININ BY SODIUM DODECYL SULPHATE MICELLES

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Artemisinin is the active principle of the plant Artemisia annua L., a Chinese herb known for its antifebrile properties and which first reports of medicinal use dated as far back as 1596 B.C. Since 1971, several studies have demonstrated artemisinin activity against malaria, including chloroquine- and quinine-resistant cases, with no signs of serious toxicity [1]. Recently, this natural product and its synthetic derivatives also showed anticancer activity in tumorous cell lines [2]. However, artemisinin is very poorly soluble in both water and pharmaceutical oils and is not readily bioavailable in vivo. To improve artemisinin water solubility, so that to increase its potential as an effective drug for human treatments, we prepared alternative forms of solubilised artemisinin, including dispersions of sodium dodecyl sulphate (SDS), singly and in combination with ascorbyl 6-O-octanoate (ASC8; a radical scavenger which can be useful to preserve artemisinin stability [3]). By quantitative ¹H-NMR, we monitored the solubility changes of artemisinin in such dispersions and, by two-dimensional diffusionordered spectroscopy (DOSY) [4], its micellisation. We found that this natural product can be solubilised by SDS micelles and this event is accompanied by a slowing of its diffusion. Artemisinin solubility was enhanced by as much as 570-fold with SDS (40mM) and 1050-fold with SDS (80mM). In ASC8/SDS binary mixtures, the apparent diffusion coefficients of the two surfactants were very similar, indicating formation of slow diffusing, mixed micelles. However, in contrast with the remarkable solubilisation obtained with micelles of SDS (40mM) alone, artemisinin solubility increased only by 330-fold with combined SDS (40mM)/ASC8 (60 mM) dispersions. Our investigation confirms DOSY as a useful and non-invasive technique to resolve mixtures of fragile structures, such as micelles, and to provide a global view of particle sizes in mixtures.

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HIGH- AND LOW-RESOLUTION SOLID-STATE NMR CHARACTERIZATION OF CHITOSAN VESICLES

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High- and low-resolution solid-state NMR techniques have been employed for the structural and dynamic characterization of vesicles obtained combining carboxymethylchitosan (CMC) [1] and trimethyl-chitosan (TMC). On one hand, the study has concerned the investigation of the individual polyions for what concerns their morphology and dynamic properties. On the other hand, these properties have been compared with the behaviour observed for the resulting vesicles, so as to understand the mechanism of their formation and stabilization, which results extremely important in the perspective of their use in pharmaceutics as carriers of drugs in specific controlledrelease systems. In particular, the combined use of ¹H-MAS and ¹³C selective experiments allowed significant differences in both the structural order and the dynamic behaviour of the two polyions to be detected. The carbon spectra of these modified chitosans have been assigned with the support of semi-empirical calculations, which revealed very informative for the understanding of the main spectral modification observed in the vesicles ¹³C spectra. The dynamic properties of both the chitosans and the resulting vesicles have been investigated through the analysis of ¹H FIDs acquired on resonance under low-resolution conditions. The results obtained from the different experiments were able to reveal the mechanism of formation of the vesicles, demonstrating the presence of an interaction between the carboxylate group of CMC and the trimethylammonium group of TMC.

Moreover, to detect possible differences in the behaviour of the vesicles following different mechanical treatments, low-resolution ¹H FID analysis has been performed on vesicles either simply liophilized, or liophilized and compressed. Both dry and D₂O-swollen vesicles samples were investigated, so as to simulate their behaviour *in vivo*. The results did not show any significant difference in the dynamic properties between the two dry samples, demonstrating that the mechanic compression of the vesicles does not produce any remarkable change. The molecular mobility significantly increases, and in the presence of an excess of water, the vesicles swell but do not dissolve, thus supporting their possible use as carriers even in the release of little soluble drugs.

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COTTON FIBERS ENCAPSULATED WITH HOMO- AND BLOCK-COPOLYMERS BY MEANS OF ATRP GRAFTING-FROM TECHNIQUE: A SOLID-STATE NMR STUDY

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In the last few decades, great efforts have been devoted to the development of new processes and chemistry aiming at modifying the surface of cellulose [1] fibers to make them more compatible with synthetic polymers, to reduce their hydrophilicity, to increase their elasticity, resistance to abrasion and heat, sensitivity to chemical and biological agents. In particular, the main interest in this field has been recently directed towards the modification of the physical and chemical properties of native cellulose-based natural fibers, with the purpose of obtaining new functional materials or to employ them as components in high-performance composites based on renewable resources.

In the present study, among the various methods available for the surface modification of cellulose fibers used in cellulose-polymer composites synthesis, grafting from cellulose fibers was achieved by means of Atom Transfer Radical Polymerization (ATRP) [2], a controlled/living "grafting-from" process that results in both higher grafting density than the "grafting-on" processes and low-polydispersity grafts with well-defined molecular weights, also showing good performances in the growth of block copolymer grafts. In particular, natural cotton fibers were modified with poly(ethyl acrylate) (PEA) and poly(ethyl acrylate)-*b*-polystyrene (PEA-*b*-PS) grafts grown from the surface of the fibers after binding a suitable ATRP initiator.

Solid-state NMR (SSNMR) has proved to be a successful technique in the investigation of the molecular structure, conformational properties and dynamics of both synthetic and natural polymers and, among them, of several kinds of natural and modified polysaccharides as well as various synthetic block copolymers. With the purpose of detecting the structural and dynamic modifications induced in either cellulose or PEA domains after the PEA grafting and PS copolymerization, as well as to get more information about the degree of mixing among the different domains of the synthesized composite materials, both high- and low-resolution SSNMR experiments (¹³C CP-MAS, ¹H T₁ and ¹H on-resonance FID analysis) have been performed on pure components and composite samples. Passing from pure cellulose to cellulose with grafted-PEA and then to cellulose with grafted-PEA-*b*-PS, a noticeable increase of the rigid and intermediate domains of the sample has been observed, which has been ascribed to a substantial stiffening of the PEA chains.

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SOLUTION STRUCTURE OF THE BACTERIAL MONOMERIC COPPER, ZINC, SUPEROXIDE DISMUTASE FROM SALMONELLA ENTERICA

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Several Cu,ZnSODs have been characterised either with X-ray crystallography or NMR spectroscopy showing that the eukaryotic enzymes of this class display an impressive degree of structural and functional conservation. In contrast, the Cu.ZnSOD isolated from different bacterial species show much greater structural variation, not only with respect to the eukaryotic enzymes, but also between different bacterial species. To better understand this, an hybrid approach, in which both proton and carbon based experiments has been used [1] to obtain the complete assignments and the structure in solution of the Cu,ZnSOD encoded by the sodC2 gene isolated from a Salmonella enterica serovar Choleraesuis strain. This protein is one of the very few monomeric Cu,ZnSODs so far identified. In this work we applied automated methods for structure calculation based on the ATNOS/CANDID/CYANA package for NOESY peak picking, assignment and structural calculation. After restrained energy minimization with the AMBER 8.0 program, a family of 20 conformers with a backbone pairwise RMSD of 0.79 ± 0.09 Å for the backbone atoms and 1.12 ± 0.10 Å for the heavy atoms, has been obtained. [1]. The overall structure and the active site are compared to those of other bacterial monomeric SOD as well as to those of dimeric human and bovine SODs. Protein hydration have also been investigated via PHOGSY and CLEANEX experiments, while the routine 15N relaxation studies are compared with information obtained from the measurement of cross correlated relaxation rates in order to provide a more informative picture of internal dynamics.

Jimenez B., Mori M., Battistoni A., Sette M., Piccioli M. (2007) NMR assignment of reduced form of copper, zinc superoxide dismutase from *Salmonella enterica*. Biomolecular NMR Assignment (in press).

CSM/CSM CROSS CORRELATION RATES OBSERVED VIA ¹³C DIRECT DETECTION

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It has been shown recently that relaxation rates of zero and double quantum coherences involving neighboring carbonyl C' and amide N nuclei in protein backbone provide evidences of slow motion in proteins [1]. Such relaxation rates reflect dynamics on time scales in the range of micro to milliseconds and vary significantly along the protein backbone. In this work, the original ¹H detected pulse sequence, developed to study slow internal dynamics, has been modified in order to excite and observe directly the carbonyl nuclei. The resulting sequence is a modified version of the CON experiment, in which ¹⁵N is observed in the indirect dimension and homonuclear C'C^{α} couplings are removed via an IPAP scheme during C' acquisition in the direct dimension. At the expenses of a reduction in sensitivity, the sequence is efficient in circumventing signal losses due to fast transverse relaxation arising from various sources such as exchange phenomena or paramagnetism.

The new sequence was tested using a copper, zinc Superoxide Dismutase, a 154 amino acids protein. Relaxation measurements were performed with relaxation periods of 20, 40 and 80 ms. In Figure are shown two CON spectra acquired with a delay of 20. Signal intensities are modulated by autorelaxation (A) or by CSM/CSM (isotropic chemical shift modulation) between C' and N nuclei (B). More than 90 peaks could be identified and quantitatively analyzed.



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BIOCHEMICAL AND NMR STUDIES OF THE INTERACTION BETWEEN HUMAN α -SYNUCLEIN AND 14-3-3 ζ PROTEIN

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Alpha-Synuclein, the major constituent of Lewy bodies in Parkinson's disease, is a neuronal protein of undefined function that is normally localized to presynaptic terminals in mammalian brain. Several biophysical approaches have demonstrated that the protein is unfolded in its native state. However, alpha-synuclein has great structural plasticity as it can acquire a mostly helical structure in association with negatively charged membrane systems, and it adopts a beta sheet structure in pathological protofibrils and fibrils [1]. Different biochemical methods have identified several potential protein partners of alpha-synuclein, but to date, the structural evidence of these protein-protein interactions is very limited [2].

Our research aim is to investigate the mechanism of molecular recognition of human alpha-synuclein and some of the potential physiological partners involved in the regulation of neurotrasmission. The protein partner presented in this structural study is protein 14-3-3 zeta, member of a highly conserved and widely expressed family of molecules that bind a wide variety of proteins and regulate several cellular processes, such as the synthesis of neurotransmitters in neurons. Several biochemical results indicate that alpha-synuclein interacts with 14-3-3 proteins both in physiological and pathological conditions [3] [4].

This work is focused on the use of different biochemical and spectroscopic methods to study this protein-protein interaction in vitro. Gel filtration and electrophoretic experiments show that recombinant alpha-synuclein and protein 14-3-3 zeta fail to form a stable complex in solution. The formation of a transient complex was investigated by NMR. Specifically, the titration of ¹⁵N-labelled alpha-synuclein with protein 14-3-3 zeta was followed by ¹H-¹⁵N HSQC experiments.

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EXPRESSION AND PURIFICATION OF HUMAN GLYCODELIN AND STRUCTURAL STUDIES ON clBABP

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The subject of the present study is the structural characterization by NMR spectroscopy of proteins belonging to the calycin superfamily.

Glycodelin (Gd) is an extracellular glycoprotein found in several mammalian organisms, showing angiogenic, immunosopressive and contraceptive properties.[1,2,3] Gd's cDNA was inserted into vectors suitable for expression in bacteria and eukaryotes. Deglycosilated Gd was expressed as inclusion bodies in *E.coli* strain BL21(DE3) and purified by affinity chromatography in denaturating conditions. Several refolding protocols were tested and each sample analyzed by ¹H 1D NMR spectroscopy. Glycosilated Gd was expressed in the soluble fraction in Pichia pastoris KM71H cells. The sample was purified by affinity and size exclusion chromatography and checked by ¹H 1D NMR. All the analyzed samples show low dispersion of signals, typical of poorly structured proteins. A soluble expression of Gd was achieved using *E. coli* strain Rosetta DE3 but the amount of purified protein was not sufficient for NMR characterization. We are now developing a fermentor protocol to increase the yield of the protein in the soluble fraction.

Another calycin protein, well characterized in our laboratory, is the chicken liver Bile Acid Binding Protein (clBABP). [4] This protein is involved in the recycling of bile acids, a regulatory mechanism of cholesterol homeostasis. In a previous study we suggested that protonation equilibria of H98 could be responsible for the opening-closure mechanism of clBABP that affects bile acids binding. [5] In order to elucidate the function of H98 we developed a mutant by substituting Histidine (H) at position 98 with a Glutamine (Q). The mutated protein was expressed in *E.coli* BL21(DE3) in ¹⁵N labeled minimal media and purified by ion exchange and size exclusion chromatography. The purified protein was used for HSQC measurements at various pH values and the results where compared with the data from wild type clBABP. Further work is in progress to elucidate the mechanism of pH induced conformational change.

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MUP AND ITS MUTANTS: IN SEARCH FOR THE KEY RESIDUES IMPLICATED IN CONFORMATIONAL STABILITY, LIGAND BINDING AND ALLERGENIC ACTIVITY.

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Mouse Urinary Protein (MUP) is an abundant pheromone-binding component of male mouse urine. During its complex physiological cycle the protein moves through completely different chemical environments where it acquires functional structures able to bind and release small molecules, and to be recognized by specific receptors. Limited information are available with respect to the mechanisms controlling the protein conformational transitions responsible for the binding and release of the ligands. Besides this, another important biological property that characterizes MUP is its activity as human allergen present in the environment as aeroallergens [1,2].

Focus of our work is to describe the molecular mechanisms that underlay the folding and unfolding of the protein, recognizing they may be a key information to understand how the protein can express its multiple biological functions.

The initial questions we addressed are:

1. In mice the concentration of urea increases 200 times on going from blood plasma to urine where it reaches 1.8 M [3]. Which are the structural determinants that maintain the MUP active fold in such high urea concentration?

2. It has been postulated that the molten globule state could be involved in physiological processes [4] such as ligand release. Does MUP acquire a molten globule state while exploiting its biological activity? Which are the environmental factors that allow the acquisition of that transient conformational state?

3. Recombinant allergens are highly performing diagnostic tools [5], and will shortly proceed to therapeutic applications [6]. Can we find a hypo-allergenic variant of MUP suitable to produce a vaccine?

To tackle these questions, beside the wild type MUP, we expressed three single point mutants (MUP-Y120F, MUP-Y120A and MUP-C138S) and characterized them structurally and for their binding ability by circular dichroism, fluorescence and nuclear magnetic resonance. Skin prick-tests were used to quantify their activity as allergens.

Briefly, the results indicate that, Tyr120 occupies a critical position in the protein architecture controlling both the overall structural flexibility and binding ability. As for free Cys138, we found it is responsible for the structural instability and aggregation of the protein at high temperature. Finally the Y120A mutant turned out to exhibit a reduced allergenic activity.

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LONG-LIVED SPIN STATES: BEYOND T₁ AND DEEPER INTO RELAXATION THEORY.

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Long-lived nuclear spin states have been observed in systems of two weak-coupled proton nuclei [1-3]. Their lifetimes were shown to be up to 37 times the longitudinal relaxation delay, T1. This has some important application in the wide NMR field. For instance, long-lived spin nuclear states have already been used for studies of spatial diffusion [4] and chemical exchange [5] in presence of very slow dynamic and are expected to be very useful for transporting hyperpolarized nuclear spin order. In view of this last target and for the sake of new applications some steps in understanding the nature of such a long-lived states should be done. Here we present some theoretical calculations to investigate the nature of long-lived states in system of two nuclei and their dependence on relevant parameters. Recently, long-lived states have been also observed in multiple-spin systems of four coupled proton nuclei [6]. These states can be easily seen as a sort of localized long-lived singlet state between couple of nuclei, hypothesis challenged by the existence of a non vanishing spin-spin J-coupling between nuclei belonging to the singlet state and not. This latter interaction should results in a quench of the singlet lifetime within a time of the order of the inverse of the highest Jcoupling. Here we present a theoretical justification of why and when such states can be observed despite their mutual J-coupling interactions [7] based on an ideal system of three coupled nuclei plus some new exciting experiments.

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KEY FEATURES OF EF-HAND SUPERFAMILY STRUCTURE USING PARAMAGNETIC PROBE.

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The calcium binding proteins of the EF-hand super-family are involved in the regulation of all aspects of cell function, through the modulation of the activity of a number of enzymes. Despite a great diversity in composition, structure, calcium binding affinities and target interaction properties [1], a common feature, common to several EF-hand proteins, is that they may undergo a major conformational change upon calcium binding from a 'closed' to an 'open' state, which allows exposure of a hydrophobic core and target recognition [2]. There are however also examples in which the protein is always in an open or close conformation, independently of the presence of calcium. Understanding the mechanism which promotes the conformational change and being able to predict the state of these proteins a priori without a detailed structure determination of both the calcium free and the calcium loaded forms is desirable, especially in view to structural genomic studies.

Here, we propose to use paramagnetic probes to be able to obtain information about the conformational state of EF-hand proteins. Paramagnetic probes have long been used to identify solvent-exposed protein surfaces or regions that are of particular interest for the detection of intermolecular contact sites in protein-ligand complexes and protein-multimers [3-4]. The advantage of this method is that the information is obtained through quick HSQC NMR spectra and does not need a complete 3D structure determination [5-6].

In the present work the difference on protein surface accessibility to the Tempol and Gd(DTPA-BMA) probe of apo and holo-Calmodulin is discussed. Calmodulin is studied as a model, since it is probably the prototype of EF-hand proteins both from a structural and functional point of view. The difference in the attenuation profile of calmodulin in the calcium free and calcium loaded forms can be used to draw general conclusions on their conformational states in solution.

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THE PROKARYOTIC CYS₂HIS₂ ZINC FINGER DOMAIN ADOPTS A NOVEL FOLD AS REVEALED BY THE SOLUTION STRUCTURE OF AGROBACTERIUM TUMEFACIENS ROS DNA BINDING DOMAIN

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The first putative prokaryotic Cys_2His_2 zinc finger domain has been identified in a transcriptional regulator, the Ros protein, from *Agrobacterium tumefaciens* [1] indicating that the Cys_2His_2 zinc finger domain, originally thought to be confined to the eukaryotic kingdom, could be widespread throughout the living kingdom from eukaryotic, both animal and plant, to prokaryotic. Here, we report the NMR solution structure and backbone dynamics of Ros DNA binding domain (Ros87), providing the first structural characterization of a prokaryotic Cys_2His_2 zinc finger domain. We show that the putative prokaryotic Cys_2His_2 zinc finger sequence is indeed part of a significantly larger zinc binding globular domain that consists of fifty-eight amino acids arranged in a $\beta\beta\beta\alpha\alpha$ topology and stabilized by an extensive 15 residues hydrophobic core. These new structural features define a novel fold not previously reported in literature and are likely to mediate functional variations between this domain and the classical zinc finger domain that lead to a different and novel way of interaction with the DNA.

NMR SPECTROSCOPY: IDENTIFICATION OF COW, SHEEP AND GOAT CHEESES AND DETECTION OF COW MILK IN SHEEP CHEESES

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Cow, sheep and goat cheeses are important constituents of the Italian diet. In Veneto cheese is considered a very high-quality product and a large number of dairy products are made with milk obtained from species other than cow.

In the present study, lipid extracts from cow, sheep and goat cheeses were investigated by using ¹³C NMR (Nuclear Magnetic Resonance) spectra.

Recent analytical authentication methods (i.e. analysis of protein fraction, electrophoretic methods, etc.) have explored the possibility of searching for parameters that could unequivocally identify the animal origin of milk. These techniques are very slow and laborious.

We have analyzed the composition and the distribution of fatty acids from cow, sheep and goat cheeses in order to evaluate whether NMR can be used to determine the animal origin of cheese.

Moreover the presence of cow milk in sheep cheeses was detected by means of ¹³C and ¹H NMR spectra since one of the problems in sheep cheese production is its adulteration with cow milk.

The positional distribution of fatty acid chains on the glycerol was identified by adding triacylglycerols pure standards. A complete assignment of fatty acid resonances in the ¹³C and ¹H spectra was obtained by TOCSY, HMQC, HMBC. These resonances were quantified too.

The results suggest that the use of ¹³C NMR spectra and multivariate statistical analysis allow the differentiation of cheeses of different species and the detection of the presence of cow milk in sheep cheeses.

Even through ¹H NMR spectra have a lower cost than ¹³C spectra and should be faster to obtain they do not allow complete resonance assignment.

The rapidity in obtaining information on a large number of compounds, the absence of chemical manipulation of the sample and the small amount of sample required for analysis could make NMR spectroscopy the most advantageous technique for the above-mentioned characterizations.

¹H NMR INVESTIGATION OF THE REACTION BETWEEN NEODYMIUM CARBOXYLATES AND ALUMINIUM ALKYLS

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Neodymium-based catalysts are among the best systems for efficient formation of highcis 1,4-polydienes. Although this great relevance, relatively little is known about the nature of the active sites. Typical systems have three components: a neodymium carboxylate, an aluminium chloride reagent and triisobutylaluminium or diisobutylaluminium hydride (DIBAH).

According to the most reliable hypothesis in a first step the neodymium carboxylate reacts with the aluminum chloride reagent to form an intermediate, probably a sort of NdCl₃, which is subsequentely alkylated by the isobutylaluminum compound to make an active Nd-C bond which initiates the catalysis. The most commonly used neodymium carboxylate is neodymium versatate, made from a mixture of isomers of carboxylic acids obtained from petrochemical plants.

In order to more deeply investigate the mechanism of the catalyst activation, we have carefully analyzed, by means of NMR spectroscopy, some model systems such as Nd(Ethylesanoate)₃ (Nd(EHA)₃) and Nd(Pivalate)₃ (Nd(piv)₃) and their reactivity toward diisobutyl-aluminium hydride (DIBAH). In this sense we have focused our attention, for simplicity reasons, on the interaction of only two components, leaving for the moment outside the clorurating agent and the diene monomer that obviously have their influence on the activation of the catalytic system.

By reacting Nd(EHA)₃ and DIBAH the first result is the transfer of ethylesanoate ligands on the aluminium site; here two different complexes come out, (AIH), having both an alkoxide and an hydride, as bridging groups between Al(Isobut)₂, units. The difference between the two complexes could be ascribed to the nuclearity: dimer and trimer, or tetramer. The two complexes afford to the complex having only alkoxides as bridging groups (*AlOCH*₂) as the DIBAH concentration approximates to zero. The presence of the alkoxide functional groups proves the reducing properties of the environment while the exchange of one EHA ligand from neodymium to Al(III) proves that "something else" has gone on the neodymium site, hydride or isobutyl group, in both cases preparing the way to the starting polymerization site.

Careful dynamic NMR experiments as function of time have proven that at lower levels of Al/Nd the reaction goes faster toward the alkoxide species because less DIBAH is at disposal for the formation of the bridging complexes.

Quite differently Nd(piv)₃ with DIBAH goes, in a first moment, toward the formation of a bridged complex Al-Al having an hydride and a carboxylate as bridging groups (*AlCOO*), thus again proving the transfer of an isobutyl group to aluminium; this evolves toward the complex with the hydride and the alcolate as bridging group (*AlH*) and from this toward the formation of the alkoxide complex (*AlOCH*₂). In this case we observe a sort of "three steps" reaction, thus leading to the conclusion that the reduction of the carboxylate is now a more difficult step than with Nd(EHA)₃. The presence of a tertiary carbon α to the reducing group instead of a secondary group as "assisting group" can perhaps have an influence.

ASSESSING THE MONO-EXPONENTIAL CHARACTER OF RELAXATION AND DIFFUSION CURVES

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This poster first reviews traditional approaches to assessing whether a relaxation curve (be it a decay or a build-up) is mono-exponential or not, keeping in mind that the question is actually ill-posed since, in the presence of noise, a multi-exponential decay may appear as mono-exponential. One should therefore ask: given a significance level, can I exclude that a given relaxation data set is mono-exponential? If I can't, it then leads to a second question: Within my application context, does this justify me to proceed assuming that the relaxation curves are mono-exponential? Far too often, these two totally independent questions are treated as a single one!

The traditional answer to the first question is based on the statistical analysis of the residuals after fitting the relaxation data with a mono-exponential curve.

This poster introduces an alternative method of assessing mono-exponentiality which is as good as the residue analysis when the analyzed curve is truly mono-exponential, but superior otherwise, since it provides a *statistically sharper distinction of border-line cases* and a wealth of useful additional information in the case of non mono-exponential situations. The superiority of the new method is illustrated using Monte Carlo analysis.

All that has been said above regards also the diffusion decay curves (such as those measured in DOSY) where the importance of a reliable discrimination between mono-exponential and poly-exponential decays goes beyond the extraction of the diffusion constant value (consider the overlap of lines belonging to different molecules).

The new algorithm, as well as the traditional ones, will be made available as an opensource, freeware utility for fitting and assessing mono-exponential decays, complete with statistically rigorous confidence interval estimates of all fitted parameters.

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TEMPOL PROBING OF RNA STRUCTURES: A NEW TOOL IN THE INVESTIGATION OF RNA DYNAMICS AND BINDING PROCESSES

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The analysis of paramagnetic perturbations generated by soluble and uncharged paramagnetic molecules on conventional NMR spectra has been extensively applied in the investigation of protein surface dynamics [1-4]. Here, TEMPOL induced paramagnetic attenuation are used to investigate the surface accessibility of RNA. By investigating RNA structures of increasing size and complexity, we show that relative paramagnetic attenuation correlates with the degree of surface exposure of the atom in most cases. Dynamic regions result in higher levels of paramagnetic attenuation than would be predicted from the corresponding static structures, as expected. Therefore, TEMPOL probing provides a facile way to independently validate structure models and dynamic measurements.

Finally, we show that TEMPOL is able to efficiently detect RNA conformational switches as well as RNA-ligand binding interfaces.



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PEAK ASSIGNMENT IN CROSS CORRELATION NMR RELAXOMETRY

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The disclosure of a fast algorithm for bidimensional Laplace inversion [1] has paved the way for the development of new multidimensional time-domain NMR relaxometry experiments [2-4]. While it is now sufficiently easy to *obtain* many bi- and multidimensional "spectra" cross-correlating the relaxation and water diffusion properties of biological and model systems, *interpretation* of the results (such as – for example - the assignment of a pool of signals to the originating protons) remains a challenging task.

In this work a number of NMR protocols have been developed for overcoming this difficulty and assigning the peaks found in ¹H cross-correlation spectra of model sucrose [5] and BSA solutions to the corresponding protons pools. Identification was accomplished by resorting to T_1 - T_2 cross-correlation plots obtained at variable CPMG pulsing rate and spectrometer frequency. Three-dimensional chemical-shift, diffusion-and field-cycled-weighted T_1 - T_2 cross correlation spectra were also explored as a further mean for peak assignment. The feasibility of the proposed protocols was also tested on inhomogenous-multicompartment systems such as BSA gels and meat samples.



Fig. 1. Representative field-cycled T_1 - T_2 spectra acquired when the t_1 dimension was recovering in the indicated relaxation fields: a) 100kHz. b) 1MHz c) 2MHz d) 4MHz.

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