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40 Years of NMR with Ivano... "outside the box"

We at CERM are sure that the best way to honor Ivano Bertini is to talk about his Science, that he loved so much. For this reason we are grateful to the organizers of the 2012 edition of the GIDRM for dedicating it to Ivano, for asking me to open it on Monday September 17, 2012 with a plenary in his honor, and for dedicating the whole Tuesday morning, called "The scientific legacy of Ivano Bertini" to presentations from CERMians.

Ivano had two great scientific loves: Bioinorganic Chemistry and NMR. And several of his most original scientific achievements indeed originated from conjugating NMR and metal ions. Therefore, I decided to organize my lecture as an overview of some of these achievements over the time I have been working with him - slightly over 40 years counting from when I was recruited by his mentor, Luigi Sacconi, in February 1972; only 36 years counting from the publication of my first paper with him. My overview is limited in several ways: it is restricted to NMR, while Ivano has published outstanding pieces of research "even" without NMR; it starts when I started working with him, i.e. it excludes his first 10 years of research; and finally it deals only with the research that we have done together, i.e. it does not cover Ivano's outstanding achievements with other colleagues - but this will be amended by the CERMians' presentations on Tuesday. In my overview, I will start from our first experiments in the seventies on cobalt substituted proteins. I will continue with the discovery in the eighties of the line-narrowing effects of magnetic coupling between - or among - two - or more - metal ions in the same protein frame, and the exploitation of the theory of magnetic coupling to perform the redox-specific assignment of iron ions in iron-sulfur proteins. Our achievements in the nineties will be exemplified by the discovery and theoretical explanation of the paramagnetic RACT effect, the first 3D structure of a paramagnetic metalloprotein, the exploitation of PCS and RDC as structural restraints, their incorporation into major softwares for structural determination, to finish with the detection of “invisible” paramagnetic states. For the 2000 decade I have selected the proposal of a new method to address conformational freedom in multidomain proteins, the discovery of individual metabolic fingerprints and the first solid state structure of a paramagnetic protein. I will conclude with the 2011 discovery that proteins can sediment from their solutions thanks to the artificial gravity in a MAS rotor, and can be studied by solid-state techniques with what we have called SED-NMR. Sedimented proteins can be also successfully subjected to DNP enhancements (2012, under revision).

Claudio Luchinat
CERM – University of Florence
GIDRM-GIRM 2012 Gold Medal Award
NMR SPECTROSCOPY OF MOLECULES, POLYMERS, PEPTIDES, PROTEINS

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The main steps of the experience in the spectroscopic field from the beginning of the diffusion of NMR spectroscopy in Italy from the original group of beginners. The field of NMR research and their development upon time. Some themes of the career that are more relevant for the research. From synthetic polymers to solid state NMR to biological NMR will be presented.
2011 Annalaura Segre Fellowships
ANOMALOUS ENHANCED WATER DIFFUSION IN POLYSACCHARIDE HYDROGELS STUDIED BY PGSE NMR

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Polymeric hydrogels are three-dimensional systems capable of imbibing large amounts of water or biological fluids. They are based on hydrophilic polymer networks, in which polymer chains are chemically or physically crosslinked. In hydrogels formation, polysaccharides are extremely advantageous because of their natural abundance, low cost and biocompatibility in comparison with synthetic polymers. Numerous hydrogel-forming polysaccharides have been investigated for applications in several biomedical fields and, in particular, as drug delivery systems (DDS) [1]. To predict the drug release kinetics and consequently to tailor the hydrogel features, the knowledge of diffusion phenomena inside hydrogel networks is of fundamental importance.

Diffusion of water and of model molecules inside several polysaccharide-based hydrogels was studied by the pulsed gradient spin-echo (PGSE) NMR technique and an anomalous and anisotropic enhanced diffusion behaviour [2] was observed in some systems, evidenced by the power-law dependence of the mean square displacement (MSD) with diffusion time (Fig. 1).

![Fig. 1. MSD of water in the Scleroglucan/borax sample vs. the diffusion time. Dashed line represents the fit of normal diffusing molecules data (circles). Solid line represents the fit of the first six points of the fast diffusion component (triangles) while the dotted line is the fit of the last five points.](image)

This enhanced water diffusion in polysaccharide hydrogels is very peculiar and the reported findings may lead to a deeper interpretation of drug release kinetics from these matrices used for pharmaceutical applications.

References
The possibility to trace the botanical and geographical origin of products such as honey has become more important because of market globalization. As a consequence, numerous analytical methods have been proposed for and applied to the determination of honey authenticity. In our previous works, multivariate statistical analysis of $^1$H NMR data revealed clear differences among six different unifloral honeys and polyfloral honeys and allowed us to identify important resonances belonging to specific markers [1, 2]. In this study, 23 compounds were chromatographically purified from honey samples of different floral origin and characterized using NMR and MS. These metabolites, belonging to several classes, i.e., terpenes, organic acids, flavonoids and others, are responsible for botanical discrimination. For the first time, a diacylglycerol ether and few other compounds present in each type of analyzed honey were identified and characterized.

Fig. 1. Structures of compounds 1-23

References
The Scientific Legacy of Ivano Bertini
NMR IN MECHANISTIC SYSTEMS BIOLOGY: FROM STRUCTURES TO FUNCTION

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NMR spectroscopy, with its multiple approaches and applications, has a unique role in describing functional biological processes in atomic details and in a cellular context. NMR is indeed suitable not only to characterize the structure and dynamics of biomolecules but, even more importantly, to describe functional events still maintaining atomic resolution. The comprehension of functional processes requires the knowledge of the conformational and folding properties of the proteins and biomolecules involved, and their dynamic network of interactions, within the proper cellular compartment. Along a functional process, most interactions are transient in nature. Specifically, NMR spectroscopy is particularly suitable for the atomic level characterization of the weak, transient interactions. Furthermore, structural and dynamical characterization of these systems requires the development of suitable approaches capable of addressing multiple, specific, and sometimes non conventional aspects and should be amenable to characterize functional processes, not only in vitro but, more significantly, in living cells.

Among the processes involving transient interactions are the metal transfer processes, in which metal transfer, from metal transporters to the final recipient proteins, occurs through a series of protein-protein interactions so that the metal ion is transferred from one protein to the next [1,2]. These transfer processes are determined by metal affinity gradients among the various proteins, with kinetic factors contributing to the selectivity of the processes [3]. They often also involve proteins whose folding and maturation are tightly linked to redox reactions [4]. The power of NMR approaches to describe cellular pathways at atomic resolution will be presented for a few pathways responsible for copper trafficking in the cell and for the folding of the involved proteins, with a particular focus on mitochondria. The results will be presented and discussed within an integrated approach where, from single structures to protein complexes, the processes are described in their cellular context within a molecular perspective.

References

LATEST NEWS ON SOD1

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One of the cellular defence systems for oxidative stress is Cu,Zn superoxide dismutase (SOD1), a structurally well characterized 32 kDa dimeric enzyme containing one zinc ion and one copper ion and one disulfide bond in each of its subunit. These posttranslational modifications are crucial for the structural stability and enzymatic activity of the protein. SOD1 reaches its mature active form through the interaction with the copper chaperone for SOD1, CCS [1]. A model for human CCS-dependent SOD1 maturation based on the NMR study of the interaction of SOD1 with CCS and the role of each of the three domains of CCS will be described. Understanding the SOD1 maturation mechanism is of critical importance as immature forms of SOD1 are believed to be linked to the neurodegenerative disease amyotrophic lateral sclerosis (ALS). The mechanism through which SOD1 causes this fatal neurodegenerative disease is not clear, but a prominent hypothesis involves instability, misfolding and aggregation of demetalleted SOD1 [2,3]. Although the exact mechanism of the cytotoxicity is still debated, these aggregates contribute to the pathogenesis and their removal is thought to be a promising target for new therapeutic approaches. A promising approach for the treatment of SOD1-linked ALS will be also presented.

References

MONITORING PROTEIN MATURATION STEPS IN LIVING CELLS
WITH ATOMIC RESOLUTION BY NMR

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High resolution NMR on living cells (in-cell NMR) has the unique ability to acquire structural and conformational information of biomolecules in their native cellular environment, with atomic resolution [1]. In-cell NMR was applied to describe the maturation process of human superoxide dismutase 1 (SOD1) in the cytoplasm. In E. coli cells, the first steps of SOD1 maturation were characterized: zinc binding and homodimer formation [2]. Increased binding selectivity in the cytoplasm allowed selective and stoichiometric binding to the zinc site only. The complete sequence of events in the SOD1 post-translational modification was then followed in the cytoplasm of human cells, including copper uptake and oxidation of the cysteines with formation of the intramolecular disulfide bond. Simultaneous overexpression of SOD1 with the copper chaperone for SOD1 (CCS) revealed its essential role in catalyzing the formation of SOD1 intramolecular disulfide bond through both copper-dependent and independent mechanisms.

References
MONITORING THE CONFORMATIONAL HETEROGENEITY OF MULTIDOMAIN PROTEINS

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Many multidomain proteins can easily sample a collection of conformations through the presence of flexible linker(s) connecting rigid domains. Information on the relative position of the domains can be obtained through the paramagnetism-based restraints, pseudocontact shifts (PCS), residual dipolar couplings (RDC) and paramagnetic relaxation enhancements (PRE). These restraints, in fact, act as reporters of structural information because they depend on the position of the observed nuclei (PCS and PRE) or on the orientation of the vector connecting dipole-dipole coupled nuclei (RDC) with respect to the paramagnetic susceptibility anisotropy tensor and/or the position of the metal ion. A paramagnetic lanthanide ion bound to one protein domain provides PCS/RDC/PRE restraints throughout the protein. The values measured on the nuclei of another domain depend on the relative position of the latter with respect to the metal-containing domain. In the presence of interdomain mobility, they depend on the average resulting from the protein conformations within the sampled ensemble. The analysis of the experimental data can thus provide information on the conformations actually experienced by the system. It is in fact possible to calculate the maximum occurrence (MO) of any protein conformation in agreement with the experimental average data. The approach can be conveniently extended by including into the list of the averaged experimental restraints other observables, like SAXS data. In this way, complementary information can be added to better restrict the set of conformations with the largest MO values. The above approach was applied to study the conformational heterogeneity of the two-domain proteins calmodulin and MMP-1.

References
NMR EXPERIMENTS FOR PARAMAGNETIC MOLECULES

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Since we resolved the first structure of a paramagnetic protein [1], our interest has been focused on collection and analysis of NMR data either arising from the hyperfine interaction or affected by the presence of a paramagnetic center, with the aim to convert them into unique structural and/or dynamics information [2].

Relaxation and cross correlation rates, residual dipolar couplings and NOEs, chemical shifts, are often elusive to identification via NMR experiments conceived and optimized for diamagnetic molecules; new methodological routes are often required to observe them [3-9].

Examples will be given for different metalloproteins spanning from well folded and stable proteins to highly unstable and largely unstructured proteins involved in biosynthesis of FeS clusters.

References

DIRECT $^{13}$C DETECTION NMR TO CHARACTERIZE INTRINSIC DISORDER IN PROTEINS

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The development of experimental approaches to overcome the limits of $^{13}$C detection in solution in parallel to an increase in instrumental sensitivity has brought $^{13}$C direct detection in the suitable range for any biomolecular applications. A set of exclusively heteronuclear NMR experiments has recently been proposed and can be used to quickly achieve complete sequence specific assignment of a protein in solution and to determine NMR observables useful for its characterization. These NMR experiments provide complementary information to that available through $^1$H detected NMR experiments. Initially developed to study paramagnetic metalloproteins, these experiments are generally applicable for the structural and dynamical characterization of proteins in solution and are crucial for the investigation of intrinsically disordered proteins or proteins fragments, opening the way for their characterization directly in-cell.

References
IRON PROTEINS: NEW SITES AND FUNCTIONS

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The combined use of traditional and novel NMR approaches has permitted to gain insight into novel coordination environments of iron and heme centers in proteins involved in iron uptake and storage, revealing the role of adaptable protein frames in modulating binding and release processes [1-6]. Adaptable protein surfaces, instead, are the basis for the dual function of cytochrome c: electron carrier and apoptotic mediator [7].

References
Plenary Invited Lectures
FOODOMICS AND NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY, AN INSEPARABLE COMBINATION FOR THE DYNAMIC STUDY OF FOOD QUALITY

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The magnetic resonance spectroscopy is the tool of excellence to obtain the holistic view of the food. In fact, the NMR spectrum can be considered, in all respects, a numerical representation of the molecular profile of a complex biological mixture. The latter is better studied through omics techniques (such as genomics, proteomics and metabolomics) that, in the case of foods, are included in the so-called Foodomics. In particular, the NMR spectroscopy is used to describe the metabolome of a food and, for this purpose, each sample can be localized in an n-dimensional space, where each coordinate represents the content of each detectable individual metabolite. Then, using multivariate analysis (e.g. PCA), the representation of this complexity can be condensed, and each sample is projected in a simplified space where each dimension corresponds to principal components, along which the main variations observed among all samples occur.

The significance of these principal components depends on the model described, in such a way that the difference between samples belonging to different categories, or the variation associated with a progressive time-evolution of the chemical composition of the same sample, is represented in the multivariate space.

In the latter case, the path followed in the multivariate space by a sample, during storage or processing, is an important tool for the dynamic characterization of the food quality. We used this innovative approach to define specific dynamic aspects of food quality, such as freshness and digestibility and some case studies will be presented with regard to fish, cheese and some processed meat products.
Singlet nuclear spin states are quantum states of a nuclear spin-1/2 pair that are antisymmetric with respect to spin exchange, and which have total spin zero. They are therefore non-magnetic and protected against many important relaxation mechanisms. These states often exhibit long relaxation times which may exceed the normal relaxation time T1 by an order of magnitude or even more. In the special case of the $^{15}\text{N}$ spin pair in $^{15}\text{N}$-labelled nitrous oxide, the nuclear singlet lifetime can be as long as 25 minutes.

I will discuss the phenomenon of singlet nuclear spin order in a variety of contexts - including gas-phase parahydrogen, small molecules in solution, and also some examples of nuclear singlet states in solids. I will discuss how nuclear singlet spin order may be generated from magnetization, how it is maintained, and how it is converted back into observable magnetization.

Our latest work in the field includes the generation of hyperpolarized nuclear singlet order using dynamic nuclear polarization. We have been particularly interested in the properties of nearly equivalent spin pairs, where the hyperpolarized singlet order is long-lived in high magnetic field, even without any external intervention. We have designed and demonstrated molecular systems that exhibit $^{13}\text{C}$ singlet lifetimes of more than 10 minutes.

Together with many collaborators, we have also studied the nuclear singlet states of parawater molecules trapped inside fullerene cavities, using neutron scattering and infrared spectroscopy, as well as NMR.
KALEIDOSCOPIC PROTEIN SELF-ASSEMBLY: THE AXH DOMAIN OF ATAXIN-1 UNDERGOES A COMPLEX MULTIPLE EQUILIBRIUM OF SPECIES IN SOLUTION

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Ataxin-1 is a human protein responsible for spinocerebellar ataxia type 1, a hereditary disease associated to protein aggregation and misfolding. Essential for ataxin-1 aggregation is the anomalous expansion of a polyglutamine tract near the protein N-terminus, but the sequence-wise distant AXH domain modulates and contributes to the process. The domain is also involved in the non-pathological function of the protein and in non-aberrant protein-protein interactions. Previous data have shown that the domain is dimeric in solution and forms a dimer of dimers in the crystal. Here, we have solved two new structures of the domain crystallized under different conditions and analysed the populations in solution. We prove that the domain undergoes in solution a complex multiple equilibrium between monomeric, dimeric and dimer of dimer species. This behaviour, together with the inherent tendency of the AXH fold to be trapped in local conformations and the multiplicity of protomer interfaces, make the AXH domain an unusual example of a chameleon protein whose properties bear potential relevance for the aggregation properties of ataxin-1 and thus for disease.
Small-angle X-ray scattering (SAXS) experiences a renaissance in the studies of macromolecular solutions allowing one to study the structure of native particles and complexes and to rapidly analyze structural changes in response to variations in external conditions. Novel data analysis methods significantly enhanced resolution and reliability of structural models provided by the technique [1]. Emerging automation of the experiment, data processing and interpretation make solution SAXS a streamline tool for large scale structural studies in molecular biology. The method provides low resolution macromolecular shapes ab initio and is readily combined with other structural and biochemical techniques in multidisciplinary studies. Rapid validation of predicted or experimentally obtained high resolution models in solution, identification of biologically active oligomers and addition of missing fragments to high resolution models are possible. For macromolecular complexes, quaternary structure is analyzed by rigid body movements/rotations of individual subunits. Of special interest is the joint use of SAXS with solution NMR, given that both methods yield highly complementary structural information. Thus, while the chemical shift data characterizing the intersubunit interfaces and RDC data yielding relative orientations of subunits can effectively restrain the SAXS-based rigid body modeling. A combination of SAXS and NMR is an extremely effective tool for quantitative analyses of flexible macromolecules [2]. Examples of recent applications will be presented and the perspectives of the synergistic use of SAXS and NMR in structural biology will be reviewed.

References


Acknowledgements: EU FP7 e-Infrastructures grant WeNMR (Contract number: 261572) and EU FP7 Marie Curie grant IDPbyNMR (Contract number: 264257).
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Research activity in Magnetic Resonance started in Pisa during sixties in the former Istituto di Chimica Fisica of the University and is still active in Pisa nowadays. I will try to go through research subjects, people and stories (some of them funny, others much less) that happened in this relatively long period, reviewing research from Pure Quadrupole Resonance of the early days to Electron Spin Resonance and, lastly, Nuclear Magnetic Resonance.

* In occasion of his 70th birthday.
Oral Communications
Glutaredoxins (Grxs) are ubiquitous enzymes, conserved throughout all the kingdoms of life. They are involved in the cellular redox and/or iron sulfur cluster metabolism and usually their functions are closely linked to the glutathione system [1]. Today, they are recognized as versatile regulatory proteins with multiple functions in health and disease. In addition to the classical dithiolic Grxs with a CXXC active site, a new class of monothiol glutaredoxins (1-C-Grxs) with a CXXS active site was discovered more recently [2]. Although their functional role is still unclear, the extent of conservation of these proteins amongst prokaryotes and eukaryotes, and the poor viability of some knock-outs, suggest a decisive importance in central processes within the cells, and a role which is not redundant with dithiolic Grxs. Two subclasses of 1-C-Grxs exist, those with a single glutaredoxin domain and those with a thioredoxin (Trx)-like region followed by one or more glutaredoxin domains [2]. The role of the N-terminal Trx domain has been shown, at least in yeast, to be essential for the function of multidomain monothiol glutaredoxins whose physiological role cannot be replaced by single-domain 1-C-Grxs [3]. Trypanosomatids, the causative agents of several tropical diseases, have a unique thiol metabolism based on the dithiol trypanothione [bis(glutathionyl)spermidine] [4]. Enzymes of this parasite-specific redox metabolism are therefore attractive antiparasitic drug targets. The genome of *Trypanosoma brucei* encodes three genes for monothiol Grxs. 1C-Grx1 and 1-C-Grx2 are single domain mitochondrial proteins, while monothiol *T. brucei* 1-C-Grx3 contains an additional N-terminal Trx-like domain [5]. 1-C-Grx1 is indispensable for *T. brucei* and is not functionally redundant with the other 1-C-Grxs [6]; therefore, it represents a promising drug target. We obtained its high resolution structure, the first 3D structure for a 1-C-Grx from pathogenic protozoa with a peculiar redox system. These results will be presented here with major emphasis on the differences with other glutaredoxins. So far, no structural information is available for multidomain monothiol glutaredoxins. Therefore, we focused our attention also on 1-C-Grx3 and studied the relative orientation of the two domains. These results establish a platform for further detailed studies of structure and function of multidomain 1-C-Grxs.

References

A large variety of molecular dynamic processes can occur in solid systems, strongly dependent on the chemical nature of the systems as well as on their solid state properties. Getting insights into these processes is a very important goal, not only for improving the basic knowledge of the solid state but also in the perspective of establishing possible correlations with "macroscopic" properties of the solid systems themselves. Solid-state NMR offers an almost unparalleled variety of tools to shed light on molecular dynamic processes occurring in solids over a very wide range of characteristic frequencies, approximately from Hz to GHz [1]. The experimental approach to be chosen as well as the dynamic processes that can be effectively investigated vary from case to case. In this contribution highlights from three very different recent studies will be presented. The first study will concern the detailed characterization over an unconventional range of temperatures of all the interconformational motions occurring in Sodium Ibuprofen [2]. Such result could be obtained through the analysis of $^1$H and $^{13}$C relaxation times and spectral features measured over a very wide range of temperatures, ranging from 350 K down to 40 K, also thanks to the use of the unique custom-built NMR cryo-equipment present at University of Southampton, which allows MAS experiments to be carried out down to 13 K [3,4]. A second case study will show the investigation of the scarcely known effects of fast vibrational motions on $^{13}$C chemical shielding tensors, carried out through a novel combined approach based on solid-state NMR (again also exploiting experiments at cryogenic temperatures) and DFT calculations [5]. At last, through the presentation of the results of a low-resolution low-field proton T$_2$ relaxation times study carried out on an extended set of polyisoprene rubber samples reinforced with silica [6], it will be shown the usefulness of this approach for investigating the dynamic properties of very complex composite materials, with a particular focus on the analysis of the organic-inorganic interfacial regions.

References
Fast Field Cycling Relaxometry (FFCR) is a NMR technique used to determine the longitudinal relaxation time ($T_1$) over a range of $B_0$-fields spanning about six decades, from about $10^{-6}$ T up to ~ 1 T without varying the frequency of the spectrometer [1,2]. The main information expected from the relaxation dispersion curves, $T_1$ or $R_1 = 1/T_1$ versus the Larmor frequency $\omega$ ($\omega = \gamma B_0$, $\gamma$ is the gyromagnetic ratio) concerns molecular motions characterized by temperature-activated frequencies and described by means of spectral density $J(\omega)$. The data obtained may, therefore, be correlated directly to physical/chemical proprieties of complex materials. The use of radio frequency allows the easy penetration of most materials, thus permitting, the exploration of slow dynamics which are often difficult to study by other spectroscopic method in heterogeneous materials, that may include both liquids and solids. Furthermore, the benefit of exploring the range of low Larmor frequencies is to detect typical relaxation features associated with molecular processes characterized by very long correlation times, such as molecular surface dynamics and collective effects.

In this presentation we show developments in the FFCR method and discuss some contributions of NMRF towards a fundamental understanding of classes of materials and of phenomena predicted by theoretical models. We show in practice how relaxation experiments on protons, as well as carbons or other nuclei can be applied for qualitative structural diagnostics in solutions, quantitative structural determinations, recognitions of weak intermolecular interactions and studies of molecular mobility. The findings that have been established more recently are noteworthy for their potential use in quality assessment and off-line process monitoring.

References

Organosilanes have been successfully applied in the anticorrosion field to increase the durability of polymeric clearcoats [1]. Due to their efficient properties as coupling agents, organosilane molecules represent an interesting and environmentally friendly alternative in the field of surface conversion treatment. Hybrid sol-gel films can be applied onto galvanized steel to increase the adhesion between the metallic substrate and the organic coating but also for corrosion protection, acting as physical barrier against water and aggressive ion diffusion to the substrate [2].

In the present work, sol-gel derived layers containing clay nanoparticles (SM) were prepared from sols of tetraethoxysilane (TEOS), glycidoxypropyltrimethoxysilane (GPTMS) and methyltriethoxysilane (MTES) containing 2 wt% of sodium montmorillonite (Na-MON), which were sonicated for different times before the dip-coating process on previously etched metal substrates. The coatings were finally cured at 150°C for 30 min. It was previously reported [3] that the sonication time of the organosilane-nanoclays sols influences significantly the rheological properties of the films, with beneficial effects on the corrosion protection ability. In this work we aim to investigate, by multinuclear solid state NMR, the sonication role on the disaggregation/exfoliation of Na-MON platelets in the silica-based sols and characterize the silica matrix/Na-MON interactions in order to get insights in the structure-properties relations, which drive the materials development.

In order to highlight the structural differences between the samples, the NMR characterization was performed on pristine Na-MON, matrix xerogel and on powders obtained by gelling the solutions employed for coating metal substrates. According to $^{29}$Si NMR data, the nanoclay addition produces a decrease in degree of condensation (DOC) in the siloxane network, corresponding to a clear rearrangement of T and Q units distribution with respect to the pure silane-derived xerogel. These results suggest the formation of chemical linkages between matrix and fillers, probably mainly involving the trifunctional Si units. This interaction is confirmed by $^{27}$Al spectra, which point out an increase of tetrahedral Al sites and the possible formation of fivefold-coordinated Al atoms. Si and Al spectra reveal also some differences in SM samples as a function of sols sonication time. The NMR study suggests that the barrier properties are not only determined by the exfoliation process, which increases the electrolyte diffusion path length, but could also be ascribed to network structural changes due to the formation of linkages between silica and clay platelets.

References
EXPLORING THE INTERACTION OF COPPER WITH MEMBRANE-BOUND α-SYNUCLEIN

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α-Synuclein (αS) is a small cytosolic protein localized near synaptic vesicles and mitochondrial membranes in vivo [1,2]. αS misfolding and dysfunction are involved in the pathogenesis of Parkinson's disease and neurodegenerative dementias.

In aqueous solution, αS adopts a dynamically disordered backbone conformation, but its N-terminal 100-residue segment adopts an ordered α-helical conformation upon binding to the surface of small unilamellar vesicles (SUVs) or detergent micelles [3-6]. Protein-vesicle interactions are involved in the physiological function of αS, and it has been suggested that αS-membrane association promotes aggregation, and that the membrane surface serves as an initiation site for amyloid fibril formation [7,8].

Oligomerization rate of αS in water solution is enhanced by the interaction of the protein with metal ions such as copper and iron [9]. It has been recently shown that Cu(II) binding to the N-terminus of αS affects its helical propensity [10]. Since αS-membrane interactions are relevant to the protein’s native function, it is possible that alterations in cellular copper levels could influence protein conformation leading to increased toxicity. The interaction of Cu(II) with αS in aqueous solution has been widely characterized [11,12], while the binding features of the metal to membrane-associated αS remain poor elucidated.

In this work we have investigated the structural details of Cu(II) binding to αS in the presence of SDS micelles at pH 7.4, using NMR, EPR and CD spectroscopy. Cu(I) binding to αS in the presence of SDS micelles was also investigated, using Ag(I) as a probe. The obtained results were compared with those obtained for the interaction of both metals with the dynamically disordered αS in aqueous solution.

References

NUCLEAR MAGNETIC RESONANCE IN THE STUDY OF MATERIALS

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Examples of application of Nuclear Magnetic Resonance methodologies in the characterization of different types of materials will be shown. In particular the study of the firing process of clays, the characterization of different types of tuff materials, the characterization of materials constituting detached and re-supported mural paintings, and the characterization of new stationary phases for the separation of a variety of polar compounds.

Fig. 1. $^{27}$Al MAS NMR spectrum (left) and $^{27}$Al 3Q MAS NMR map (right) of a sample of tuff.


**1H DQ CRAMPS SOLID-STATE NMR EXPERIMENTS FOR PROBING HYDROGEN-BOND NETWORKS IN Ru(II) BUILDING BLOCKS**

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1H CRAMPS, 2D double quantum (DQ) CRAMPS techniques have been used for probing the hydrogen bond network of four (pseudo-)polymorphs (1α, 1·H₂O, 1β and 1γ) of the Ru(II) complex [(p-cymene)Ru(kN-INA)Cl₂] (INA = isonicotinic acid) [1]. The resolution improvement provided by the PMLG pulse sequence with respect to fast MAS experiments has been highlighted (Fig 1). The presence of DQ signals in the 1H DQ CRAMPS spectra allows detecting proton-proton proximities up to 3 Å (Fig. 1). The solid-state structures of 1β and 1γ have been solved from powdered samples by combining XRPD and solid-state NMR data. 13C CPMAS and 1H-13C off-resonance FSLG-Hetcor solid-state NMR data agree with the structure solved from XRPD. None of the four polymorphs shows the expected cyclic dimerization of the carboxylic functions of INA, owing to the presence of chlorine atoms as strong hydrogen bond acceptors. The hydration-dehydration process of the complex 1α, from which all forms arise has been discussed in terms of structure and hydrogen bond rearrangements.

Fig. 1. Left: comparison between 1H MAS (dotted lines) and 1H CRAMPS (solid lines) spectra of 1α (a), 1·H₂O (b), 1β (c), 1γ (d) and 2 (e). Right: 1H DQ CRAMPS spectrum of 1α together with skyline projections.

References

ELECTRONIC AND NUCLEAR SPIN DYNAMICS IN THE THERMAL MIXING MODEL OF DYNAMIC NUCLEAR POLARIZATION

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A novel mathematical treatment is proposed for computing the time evolution of dynamic nuclear polarization processes in the low temperature thermal mixing regime. Without assuming any a priori analytical form for the electron polarization, our approach provides a quantitative picture of the steady state that recovers the well known Borghini prediction [1,2] based on thermodynamics arguments, as long as the electrons-nuclei transition rates ($T_{\text{ISS}}$) are fast compared to the other relevant time scales. Substantially different final polarization levels are achieved instead when the latter assumption is relaxed in the presence of a nuclear leakage term ($T_{1n}$), even though very weak, suggesting a possible explanation for the deviation between the measured steady state polarizations and the Borghini prediction. The proposed methodology also allows to calculate nuclear polarization and relaxation times, once specified the electrons/nuclei concentration ratio ($N_e/N_n$) and the typical rates of the microscopic processes involving the two spin species. Numerical results are shown to account for the manifold dynamical behaviors of typical DNP samples.

Fig. 1. Polarization build-up curves at 3.35 T and 1.2 K of a DNP system with $N_n = N_e = 1000$, electronic relaxation time $T_{1e} = 1$ s for different values of contact time $T_{\text{ISS}}$ (0.001 s (thick lines) and 0.1 s (thin lines)) and nuclear intrinsic relaxation times $T_{1n}$ ($\rightarrow\infty$ (solid lines), 10000 s (dashed lines) and 1000 s (dotdashed lines)).

References
ADVENTURES WITH qESTIMATE

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Who does not know about qNMR these days? And who would not like to have systems that provide analytes concentrations with good accuracy and precision – possibly –with a few clicks of the mouse? Finally, what about the possibility of generating automatic reports of qNMR results?

In this communication some real life results obtained with qEstimate (Agilent’s qNMR system) will be presented and discussed. A few recipes will be also proposed for keeping user’s interactions with the spectrometer at minimum when carrying out bulk, lengthy (and potentially boring) quantification analyses.
FORENSIC DIFFERENTIATION OF LATEX GLOVES AND POLYURETHANE FOAMS BY TD-NMR

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Several polymer materials, including latex from gloves and polyurethane pieces from cushioning, are sometimes found on the scene of crime. If properly analyzed, these items may provide useful information to investigators. Polymer materials contain several kinds of additives and inorganic fillers to regulate mechanical properties and make up bulk inexpensively. Thus they display a compositional variability that can depend on the type, producer and batch, allowing to perform meaningful differentiation from the forensic point of view.

In this work we demonstrate that by measuring appropriate parameters with time domain NMR we can provide high discriminating power between families of similar products. Glove samples, in particular, were indistinguishable in respect to microscopic analysis and IR spectroscopy.

Fig. 1. PU foam (left) and latex glove rubber (right) samples represented as points in the H\%-%Rigid fraction and H\%-T\textsubscript{2} planes respectively. Error bars for gloves include variance in the same batch.

A generally applicable experiment is the “proton counting”, a method that provides the weight \% content of proton nuclei in a given sample. It is based on the linear dependence between number of protons and NMR signal intensity. The initial FID intensity is measured and compared to a calibration curve of compounds with known proton content. To increase discriminating power, samples of the same family were investigated also with specific techniques. Gloves, being rubbery materials were analyzed using Hahn Echo technique that provides information on chain mobility [1]. The hard polyurethane material was instead analyzed with a magic sandwich echo (MSE) sequence that allows quantitation of rigid fraction [2].

References
NOVEL Gd(III)-BASED MRI PROBES FOR THE MOLECULAR IMAGING OF MMPs

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Novel Gd-based contrast agents (CAs) for the molecular imaging of matrix metalloproteinases (MMPs) by MRI were synthesized and characterized in vitro and in vivo. These probes are based either on the PLG*LWAR peptide sequence, known to be hydrolyzed between gly and leu by a panel of MMPs, or on the NIPVS*LYA sequence, which is more specific to MMP-2. A GdDOTA chelate was conjugated to the N-terminal position through an amide bond, either directly to proline or through a hydrophilic spacer. Both CAs were made strongly amphiphilic by conjugating an alkyl chain at the C-terminus of the peptide sequence. Upon MMP-dependent cleavage, the CAs lose the C-terminal tetrapeptide and the alkyl chain, thus undergoing an amphiphilic-to-hydrophilic transformation that alters tissue pharmacokinetics. These probes were systemically administered to mice bearing a subcutaneous B16.F10 melanoma, either pre-treated or not with the broad spectrum MMP inhibitor GM6001 (Ilomastat). The washout of Gd-contrast enhancement in DCE-MR images was significantly faster for untreated subjects (displaying MMP activity) as compared to the treated ones (MMP activity inhibited). Ultimately, the washout kinetics of Gd-contrast enhancement from the tumor microenvironment gave a qualitative picture of tissue MMP activity. To get a more quantitative description of MMP-activity, the measurement of the molar ratio of intact versus cleaved probe within the ROI is needed, in a manner which is independent from the absolute concentration of gadolinium within the tissue. To this purpose, the Gd-probes were inserted into stealth liposomes to obtain systems amenable to the evaluation of the uncleaved vs cleaved probe through the ratiometric approach, which consists in the measurement of the $R_{2p}/R_{1p}$ ratio [1]. The factors affecting the sensitivity of the ratiometric approach are discussed.

References

EXPLORING THE LIGAND BINDING CAPABILITY OF HUMAN LIVER FATTY ACID BINDING PROTEIN

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Human Liver Fatty Acid Binding Protein (hL-FABP) is a 14 KDa cytosolic protein whose function is the intracellular transport of long chain fatty acids. In the liver it is highly abundant, constituting about 2-5% of the cytoplasmic proteins. In addition to fatty acids, it has the ability of binding a variety of other endogenous ligands in its capacious binding cavity (about 600 Å³). Given these characteristics it has been proposed that hL-FABP could act as a bile salt carrier in the liver [1].

Recently, X-ray and NMR structures of the hL-FABP in its apo form and in complex with fatty acids have been reported [2, 3]. For the first time we used mass spectrometry to confirm the unique ability of hL-FABP to accommodate two molecules of fatty acids. Moreover, a variety of NMR experiments have been used to shed light on the binding mechanisms toward different amphiphilic molecules. We performed NMR titration experiments using unlabeled and labeled (¹⁵N and ¹³C ¹⁵N) glycocholic acid (GCA). The chemical shift induced upon binding and the presence of NOEs with side chains of aromatic residues let us conclude that GCA is indeed binding in the interior of the cavity.

Finally, we used hydrogen/deuterium exchange experiments and backbone ¹⁵N relaxation NMR techniques on hL-FABP in its apo form and in complex with both GCA and oleate to obtain residue-specific information about dynamics on various time scales.

References
INSIGHT INTO THE DYNAMICS OF CARBOXYLATE GROUPS
AS REVEALED BY $^{17}$O DYNAMIC NMR

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$^{17}$O NMR is not routinely used for structure characterization, moreover, kinetic studies are seldom undertaken because the combination of poor sensitivity, fast quadrupole relaxation and baseline distortions is frequently regarded as an intractable issue [1]. The observation of coalescence may indeed be a challenge and the $^{17}$O natural linewidth, which typically exhibits a strong temperature dependence, cannot be neglected in the data analysis. The present communication shows how, nowadays, quantitative $^{17}$O dynamic NMR studies on small organic molecules are feasible, extending the use of dynamic NMR to the characterization of processes that are not amenable to study by $^1$H or $^{13}$C NMR. Here we report on various acetoxy derivatives of iodine ($\lambda^3$ and $\lambda^5$ periodinanes) and silicon (Fig. 1). By combining $^{17}$O NMR and DFT calculations, these compounds are shown to be fluxional as a consequence of a [1,3] sigmatropic shift of the iodine/silicon atom between the two oxygen atoms of a carboxylate group [2-4]. The methods used (i) for recording natural abundance $^{17}$O NMR spectra showing minimal baseline distortion and suitable for quantitative integral or linewidth measurements, and (ii) for proper determination of the kinetic activation parameters without the observation of coalescence being needed, will be presented. In addition, recent results reporting on the dynamics of the carboxylate arms of diamagnetic and paramagnetic $^{17}$O-enriched lanthanide-DTPA complexes will be shown [5]. The $^{17}$O NMR spectra of Pr-DTPA and Eu-DTPA reveal that these compounds are fluxional, and estimations of the activation free energy characterizing the carboxylate dynamics are provided for the first time.

Fig. 1. Variable temperature $^{17}$O NMR spectra of a silicon tetraacetate and schematic representation of the [1,3] sigmatropic shift.

References

SOLUTION AND SOLID STATE NMR STUDY ON METAL-COORDINATED WHITE PHOSPHORUS


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The dynamic behaviour in solution of eight mono-hapto tetraphosphorus transition metal complexes (see Fig. 1) was studied by variable temperature NMR and $^{31}$P{$^\{1\}$H} EXSY experiments. For most of the species, it was ascertained that the metal-coordinated P$_4$ molecule experiences a dynamic process consisting in a tumbling movement of the P$_4$ cage while remaining chemically coordinated to the central metal. For complexes [2]BF$_4$ and [3]PF$_6$, MAS 31P NMR experiments revealed that the dynamic processes observed in solution (i.e. rotation and tumbling) take place also in the solid state.

NMR data were exploited to calculate activation parameters for the dynamic processes of complexes 1', 2', 3', 4', 6', 8' in solution.[1,2]

Fig. 1. mono-hapto tetraphosphorus transition metal complexes.

References

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Metabolomics represent the last technique in the ‘omics’ era. Along with genomics, transcriptomics and proteomics define the core flow of biological information from the genes and receives the information from the environment integrating the effects of the other three levels with it. NMR can be easily used to obtain metabolic fingerprints (endometabolome) or footprints (exometabolome) of cell lines, extracellular medium, tissues and biofluids [1].

We have used liquid NMR metabolomics to study the in vitro differentiation of B cells into antibody secreting plasma cells using both the endometabolome and the exometabolome in order to understand the biochemical changes and requirements of a B cell to become a plasma cell, an antibody producing cell factory.

Multiple Myeloma (MM) is a lymphoid malignancy, responsible for 13% of haematological neoplasias and one of the commonest causes of bone cancer [2]. In MM, neoplastic plasma cells grow at multiple foci in the bone marrow, their physiological niche, secrete monoclonal immunoglobulins, and induce end-organ damage [3].

HR-MAS based NMR allows to perform metabolomics studies on intact tissue, thereby preserving the specimens for further analysis and allowing for simpler sample preparation that is easily transferrable to clinical practice [4]. In order to specifically address intralesional heterogeneity, as well as to set up a method for human bone cancer which, to our knowledge, had only been applied to non-cancer mouse models, we set out to characterize by HR-MAS the local heterogeneity in a myeloma osteolytic lesion causing pathological fracture.

References
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 and localization of a disease process. The advent of Molecular Imaging based protocols will allow the detection of the onset of diseases at an early stage well before the biochemical abnormalities result in change in the anatomical structures. 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 [1].

Moreover, MRI probes may be designed to become responsive to a specific physical or bio-chemical parameter of the micro-environment in which they distribute. The *in vivo* monitoring of such parameters may significantly improve early-diagnosis and therapy follow-up of many pathologies. An interesting application of this peculiarity is the possibility to follow “cell decision” *in vitro* and most of all *in vivo*. MRI cell labeling probes have been designed to report on the chemical event(s) which stay behind a given cell decision. Examples will be reported on the possibility to visualize gene expression and cell differentiation [2,3].

References

INVESTIGATION OF FERRITIN BY SOLID-STATE NMR

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Ferritins are members of the broad superfamily of iron storage and detoxification proteins found ubiquitously in nature. They have a supramolecular nanostructure designed to sequester thousands of iron atoms, in a mineralized and biologically available form, and release them in a controlled manner [1]. The first step of the iron uptake process occurs at the ferritin ferroxidase site, where two iron(II) ions react with dioxygen to form diferric species. Once released, such products migrate into the cavity where the biomineral is formed and stored. Solution $^{13}$C-$^{13}$C NOESY NMR experiments allowed the identification of about 75% of amino acid side chains [2]. The paramagnetic broadening induced by iron(III) species on the resonances of nearby residues permitted the recognition of the residues signals along this path [3]. However, the iron path definition requires a sequence specific assignment of the spectra that could not be achieved in solution. Indeed, the slow molecular tumbling of the protein (480 kDa) induces rapid transverse relaxation and hampers solution NMR experiments based on coherence transfer. Immobilization of the protein in solid state overcomes this problem: magic angle spinning (MAS) Solid-State NMR $^{13}$C detection experiments provided the sequence specific assignment of 59 residues out of a total of 176 [3]. We are now using the ultrafast MAS Solid-State NMR $^1$H detection experiments to extend the sequence specific assignment.

References
HOW THE STRUCTURAL METAL ION INFLUENCES THE MECHANISM OF FOLDING OF THE PROKARYOTIC ZINC FINGER DOMAIN

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Metal ions play an important role not only as local structural stabilizing elements in the native state contributing to the maintenance of a given specific structural fold, but also as potential key nucleation points during folding [1-3]. Their binding to the unfolded protein imposes a conformational restriction which lowers the entropy of the unfolded state and favours a specific folding pathway speeding up radically the folding reaction [1]. Furthermore in some cases protein folding is totally metal-dependent and involves the coordination of one or more metal ions to drive the folding into the fully functional native conformation while in other cases metal ions are also capable to bind proteins in a later stage of the folding reaction. These considerations suggest for metallo-proteins different folding pathways which can imply the presence of intermediate states. In the present work we analyze, by means of CD, DSC and NMR methodologies, the differences in the folding mechanism of two highly homologous proteins, Ros87 protein from A. tumefaciens and Ml4 from Mesorizhobium loti [4, 5] that share the same three-dimensional structure although one of them lacks the structural metal ion. Our data show how in these two proteins the same fold is achieved using two complete different mechanisms of folding. Notably, the structural Zn(II) in Ros87 implies a switch from the fully cooperative folding of Ml4 to a partly downhill mechanism.

References

NANOPARTICLES AS CELL LABELING TOOLS IN MAGNETIC RESONANCE IMAGING

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Recently, nanoparticles have been proposed as diagnostic tools in MRI, both for preclinical and clinical studies. Several ions have been used for providing nanoparticles with magnetic properties, but, at the present, most widely used nanoparticles contain iron. These nanoparticles are constituted by an iron oxide core and an organic coating which makes them biocompatible and allows for functionalization. From a diagnostic point of view, the interesting property of such nanoparticles is their high transversal relaxivity which make them visible in MRI with high sensitivity.

Functionalization may include antibodies (or other molecules providing specificity for a given receptor), drugs or probes detectable with different imaging modalities including Ultrasounds, PET, Optical Techniques.

Interestingly, iron-based nanoparticles have the capability of being internalized by cells, e.g. stem cells, without relevant cyto-toxic effects. After labeling, cells are detectable in MR images of intact organisms, non invasively and with high sensitivity. Labeling can occurs in vitro (cells are labeled and then injected in the organisms) or in vivo (nanoparticles are injected in the organisms and then internalized by cells). This property has been widely used in investigating homing of cells after transplantation, both in preclinical and clinical studies [1-3].

In this paper we will describe several biomedical applications of iron based nanoparticles in labeling different kind of cells and studying their fate in vivo. Strengths and potential pitfalls of the methodology will be discussed.

References

TIME DOMAIN NMR FOR NANOPARTICLE SIZE MEASUREMENT AND SURFACE CHARACTERIZATION

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Control of particle size is an essential element in tuning the properties of nanoparticles, and its measurement is an open and appealing challenge to the nanotechnologist [1]. Following the pioneering work of Cosgrove on silica particles at high field [2], we propose a low field time domain NMR method especially suited for direct investigation of nanoparticles in colloidal dispersion. The physical principle is the enhanced relaxation of protons in contact with a surface. Rapid exchange with the solvent then produces an average relaxivity $R_2$ that depends upon total area and chemical nature of the available surface. Here we focus our attention on crystalline titania nanoparticles. A variety of possible synthesis (sol-gel hydrolytic or non hydrolytic, hydrothermal, with surfactants) result in TiO$_2$ particles with different crystal structure and chemical surface.

![Graph](image)

Fig. 1. (left side) Effect of increasing quantity of different titania particles on relaxivity. In the case of Anatase _aqu, a bimodal decay was detected and both values are plotted. For the well defined rutile and anatase _org particles, relaxivity is also expressed as function of total surface in 1 g solution (right side).

$R_2$ measurements, as function of weight content in water, clearly differentiate rutile from anatase nanoparticles. Furthermore TD-NMR could differentiate between anatase prepared in organic (Anatase _org) [3] or in aqueous environment (Anatase _aqu). By independent measurement of the available surface, performed by BET, it is possible to separate the specific effect of surface, attributed to different chemical nature. Finally, in the case of unknown particle concentration, as in the case of partial sedimentation, it is possible by a simple measurement of $R_2$ determine the concentration of the nanoparticles in the colloid.

References

NMR STUDIES OF HP1-HISTONE3 INTERACTION IN THE HIGH MOLECULAR WEIGHT NUCLEOSOME SYSTEM


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A large number of diverse post-translational modifications on histones direct chromatin structural states and modulate chromatin dynamics. Methylation of lysine 9 within the H3 N-terminus governs the packaging of DNA into heterochromatin, a highly condensed and transcriptionally inactive form of chromatin. The recognition of trimethylated Lys9 in histone3 (H3K9me3) by heterochromatin proteins 1 (HP1) constitutes a key step in the establishment and maintenance of heterochromatin. Previous works on component systems using isolated histone peptides and/or individual domains of the protein [1, 2], as well as using HP1 proteins of different species or isoforms [3, 4], have determined various domains and interaction interfaces of HP1 binding to chromatin. The large variability of findings precludes from deducing a coherent picture of HP1/nucleosome binding. In particular structural and biophysical insights have been missing in this picture so far.

Here, we have investigated the molecular basis of hHP1β binding to nucleosomes by using a combination of NMR and biophysical experiments on a fully assembled recombinant system. NMR techniques for large proteins [5, 6, 7] revealed single-residue details of alternative binding interfaces in dependence of H3K9me3 and the highly dynamic feature of the complex.

References
DETAILED STRUCTURE OF MICROEMULSIONS: A PULSED GRADIENT SPIN-ECHO NMR APPROACH

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Under suitable conditions (T, P, composition) water, apolar compounds, and surfactants give rise to microemulsions. A peculiar feature of microemulsions is that the water and oil domains can arrange itself in a variety of shapes (e.g. lamellae, cylinders, spheres and so on) depending on the intensive variables of the systems. Under favorable conditions, the fine structure in microemulsion can be profitably investigated by means of PGSE-NMR technique; examples of this approach will be presented for either discoid micelles in water [1] and wormlike reverse micelles [2,3]. Finally, the combination of PGSE-NMR data with results coming from different techniques (23Na and 2H quadrupolar splitting [4], rheometry [1,5], rheo-SANS [6]) allows insight on the response of the system to mechanical deformations.

References
THE MECHANISM OF 3D DOMAIN SWAPPING IN BS-RNASE:
WHEN TWO IS BETTER THAN ONE

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3D-domain swapping (DS) is a protein aggregation mechanism consisting in the mutual exchange of structural elements between two or more identical polypeptide chains. It is often associated with new biological functions with respect to the parent, monomeric proteins and it has been also suggested as one of the possible routes leading to amyloid deposits. Despite the increasing interest in this phenomenon and the high number of domain swapped structures reported on PDB, the details of DS process are still poorly understood. Current views are based on two distinct, opposite mechanisms: after the historical hypothesis of Eisenberg (1995) [1], consisting in a partial opening of the protein subunits followed by their assembly, two recent NMR studies [2, 3] indicated that the protein subunits need to be fully denatured in order to swap. In all the cases investigated so far the DS is associated with a monomer to dimer conversion. In contrast, the protein we have studied here, bovine seminal ribonuclease (BS-RNase), is a homodimeric protein whose subunits are linked through two disulfide bridges. The native form is isolated as a mixture of two isoforms, indicated as MxM and M=M, with and without swapping of N-terminal ends respectively [1]. A careful structural and dynamical investigation of the monomeric derivative of BS-RNase [4], led us to conclude that the protein subunit has an extremely compact, globular structure, with no tendency to global or local unfolding. We have then undertaken a comparison of the dynamical properties of the two dimeric protein isoforms by solution NMR. The swapping process, monitored by chemical shift analysis, suggested a cooperative process whose energetic balance is in agreement with a partial opening of the polypeptide chain. Moreover, comparative chemical shift analysis, H/D exchange, heteronuclear NOEs and urea denaturation studies, besides evidencing minor differences between the two isoforms, revealed an evident increase in the protein plasticity going from the monomeric derivative to both dimers. This finding suggests an alternative hypothesis for 3D-domain swapping mechanism in BS-RNase, assigning a primary role to the interaction between the two subunits. Further studies are needed to understand if this mechanism can be extended also to other proteins, which in general convert from closed monomers to swapped dimers, or indeed is linked to the special case of BS-RNase, which is anyway a dimer also in the unswapped state.

References

Acknowledgements: Utrecht Large Scale Facility for the transnational access is acknowledged.
NMR APPLIED IN MEASUREMENT OF PHYSICAL PROPERTIES OF DRUGS: THE CRITICAL MICELLE CONCENTRATION

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Physical properties of drugs can be studied through a variety of techniques. Among them, NMR plays an important role in understanding the behaviour of drugs in solution. The Critical Micelle Concentration (CMC) is one of the parameters that can be determined by NMR, and/or by other techniques, which measures the tendency of a drug to give self-association in solution. Self-association phenomena are responsible for surface activity, and drugs which exhibits such behaviour are known to cause morphological changes of the blood cells in the process of drug-induced hemolysis, their safety and tolerability upon injection after intravenous administration being related to the CMC [1].

NMR spectroscopy was used in combination with surface tension measurement (SFT) for determining the micellar properties of casopitant mesylate, a new NK1 antagonist drug. Casopitant’s CMC was measured by both NMR and SFT leading to comparable values in glycine buffer. Afterwards, the two techniques allowed completing the study with the determination of thermodynamic of the micellization (NMR) and of effect of electrolyte (SFT). The use of orthogonal and complementary techniques allowed us to get a sufficiently clear physical understanding of the self-association phenomena for this compound [2].

Fig. 1. A consequence of the self-association is a remarkable shielding trend for the aromatic protons of casopitant mesylate in the spectra collected at increasing concentrations.

References

DISCOVERING FUNCTION FROM PROTEIN STRUCTURE: THE BA42 PROTEIN FROM THE ANTARCTIC BACTERIA B. ARGENTINENSIS IS A NOVEL PHOSPHATASE


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The bacteria Bizionia argentinensis sp. Nov (BA) was isolated from surface seawater in Antarctica and the phylogenetic analysis showed that it belongs to a novel species of the genus Bizionia [1]. The BA genome was recently decoded [2] and constitutes a relevant source for the discovery of new proteins showing biological activity in extreme conditions of low temperatures. In particular, we are interested in those proteins for which function cannot be inferred solely on sequence, and for which structural homologues are not available. To study this type of proteins, a network of NMR Laboratories was created, giving birth to the Extremophiles Structural Genomics consortium (ESG). ESG includes the NMR Laboratories of Fundación Instituto Leloir, Buenos Aires, Argentina, Verona University and Parma University, Italy.

In this work we present the first structure of a BA protein of unknown function, the BA42 protein, codified by the ORF 00042 contig 3 [3]. BA42 presents significant sequence identity with a PfamA family, DUF477 (Domain of Unknown Function). Proteins of this family are found both in eukarya and eubacteria. The three-dimensional structure consists in a three-layer sandwich with three α-helices in the upper layer, four β-sheets in the middle layer, and one α-helix in the lower layer. Using the DALI server [4], we found three remote structural homologues of BA42. One of these homologues was recently characterized as an acid phosphatase of the thylakoid lumen in plants [5].

We will present the first biochemical evidences showing that BA42 presents phosphatase activity as well, pointing to the discovery of a novel type of phosphatases associated to the DUF477 domain.

References
LEAF METABOLOMICS AND WATER STATUS AS SEEN BY NMR

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Leaves are organs specialized for photosynthesis vitally important for plants. Thus, leaf composition, physiological state and metabolism are outstandingly interesting for biological studies. Moreover, in some cases, leaves themselves constitute the nutritional plant item. Therefore, it is of the utmost interest to determine their metabolic composition as completely as possible. As a high-throughput metabolite profiling technique high resolution NMR spectroscopy is a suitable tool for the study of leaves metabolome. The applications of high resolution NMR to various plant species (lettuce [1-3], endive, escarole, soybean [4], kiwi) has given plenty of information relative to leaves metabolic composition and its variation associated with natural plant variability, plant development, seasonal changes, genotype (differences between cultivars, the expression of exogenous genes in transgenic plants), and water stress. Other interesting information on the leaf water status can be obtained nondestructively using a portable unilateral NMR as a non invasive technique. In particular, low-field proton NMR relaxometry measurements have been carried out using a portable unilateral instrument in situ to study the water status of leaves of herbaceous crops and trees giving important information on the physiological state of leaves under a developing water stress [5].

References
SOLID STATE NMR AS A USEFUL APPROACH TOWARD THE COMPREHENSION OF HOW MORPHOLOGY INFLUENCES BHJ SOLAR CELLS PERFORMANCE

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In the last two decades a lot of efforts have been made in order to develop polymeric solar cells that, in comparison with the traditional silicon solar cells, should lead to lower production costs and more mechanical flexibility.

In polymeric solar cells, two different organic materials are necessary, respectively with donor and acceptor properties. In the first works the two materials were disposed in a bilayer structure, but it was then understood that an increase in efficiency could be achieved with a bulk heterojunction structure (BHJ), where the two materials strongly interpenetrate one into the other: in this way a greatly increased interfacial area could be obtained which helps the charge separation and transport to the electrodes.

But, although encouraging progress has been made up to now, the power conversion efficiencies of the BHJ solar cells are still too low. It has already been well understood that an improvement of the performances of these devices can be obtained with an accurate control of their morphology.

Besides, many studies have demonstrated improved power-conversion efficiency after a thermal annealing treatment of the active layer at elevated temperatures (100-150 °C) and a lot of effort has been made in order to understand what really happens in the film during the annealing process but not everything has been completely clarified.

TEM, SEM and/or AFM analysis, and the comparison with the corresponding solar cell parameters were, up to now, the most popular approaches for the comprehension of the relationship between morphology and performance. Solid State NMR can represent a useful complementary technique to characterize the organization and morphology of polymeric blends in the bulk.

The object of this study are BHJ blends, composed by poly(3-hexylthiophene) (P3HT) as electron donor and [6,6]-phenyl-C61 butyric acid methyl ester (PCBM) as electron acceptor.

HETCOR 2D experiments proved the close proximity of the two phases through their dipolar couplings. In addition, a morphological study was conducted by means of T1(1H) and T1(13C) relaxation times measurements. T1(1H), thanks to the process of spin diffusion, provide information on the level of heterogeneity on a nanometer scale and on the domain dimensions that can be related to the percolation pathways for charge transport to the electrodes, while T1(13C), not influenced by spin diffusion, are a probe of the local molecular dynamics.

The annealing process seems not to have a great influence on the domain dimensions with these blend components at the actual working ratio (1:0.8).
Table grapes are food products of considerable commercial value for several countries (USA, Brazil, Italy, South Africa, China, Chile, India and Australia are the most important producers). In Europe, Italy ranks first place for table grape production with more than eight million tons per year (ISTAT, 2011).

We have studied the variability of the metabolic profile of table grapes by multivariate statistical analysis applied to $^1$H NMR data (See Fig. 1). Our investigations revealed that the inter-vineyard variability of the metabolic profile has a greater discriminating effect over the intra-vineyard one. [1] The effect of the application of plant growth regulators during production step were also considered with particular attention to the abscisic acid [2]. Moreover, suitable expert classification systems (based on three different algorithms including an Artificial Neural Network) were used to discriminate grapes on the bases of some common features (variety, vintage, use of plant growth regulators, trunk girdling, vineyard location) [3].

In this presentation, metabolic profiling of table grapes during pre- and post-harvest periods along with the performances of the expert classification systems in the classification of the grapes will be shown.

References

NEW DEVELOPMENTS IN SOLID STATE NMR

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Solid state NMR is a fundamental technique in many different areas ranging from polymers to material science to biological macromolecules. The technical requirements for performing state-of-the-art experiments are demanding: high power, short timings, extreme MAS speed, high field etc. Moreover systems working at extreme temperatures and Dynamic Nuclear Polarisation (DNP) have opened up new area of applications.

In the recent years Bruker has dedicated much attention in developing hardware and software solutions for uncompromised performances. In this talk we will present some of our latest achievements.
HUMAN Sp140 PHD FINGER HAS AN UNUSUAL FOLD RECOGNIZED BY PIN1

C. Zucchelli, G. Quilici, M. Saare, P. Pärts, G. Musco

Sp140 is an IFNγ-inducible leukocyte-specific protein, member of the Sp100 protein family and a component of PML-nuclear bodies. It is expressed in mature B, plasma and some T cells and in vitro behaves as transcription co-activator for a reporter gene [1]. Despite the involvement in B-cell Chronic Lymphocytic Leukaemia [2] and HIV-1 replication [3], Sp140 protein is unknown and unexplored at both structural and functional levels. Its amino acid sequence indicates a modular structure with a N-terminal “Sp100-like” domain, a SAND domain, a PHD finger and a C-terminal bromodomain. We solved by NMR and ARIA 2.3.1 software the solution structure of Sp140 PHD finger. PHD fingers are protein domains (~60 residues) characterized by a short antiparallel β-sheet and a conserved C4HC3 motif for Zn2+-coordination in a cross-brace arrangement. They are found in ~150 human protein and commonly act as readers of epigenetic marks on histone H3 tail [4]. Despite the high sequence identity with respect to AIRE and TRIM24 PHD fingers (respectively, 52% and 46%) Sp140 PHD structure is peculiar for both secondary structure and Zn-coordination mode. Moreover it shows the first case of both cis-trans peptidyl-prolyl isomerization and S-S bridge in the PHD finger family. In line with its peculiar structure, we found by in vitro binding assays (fluorescence and NMR titrations, binding assays with histone peptide arrays) that Sp140 PHD finger does not bind to modified or unmodified histone tails (H3, H4, H2A and H2B). On the other hand by NMR we showed a direct interaction between human PIN1 and Sp140 PHD finger, via the peptidyl-prolyl bond. PIN1 is a highly conserved peptidyl-prolyl isomerase (PPIase) which isomerizes specific phosphorylated Ser/Thr-Pro bonds in a wide range of proteins, thereby inducing conformational changes. In this way PIN1 is a key regulator of transcription, cell cycle progression, apoptosis, neuronal differentiation, stress and immune responses [5]. In conclusion, our data reveal a completely unexpected peculiarity of Sp140 PHD in the PHD finger family. Moreover they add Sp140 protein to the expanding list of PIN1 targets, suggesting a structural regulation of Sp140 PHD function.

References
Posters
Saturation Transfer Difference (STD)-NMR spectroscopy has been extensively exploited to study receptor-ligand interactions [1]. This technique has also been used to study molecular recognition events involving membrane receptors and their ligands, by working on samples which contained platelets or whole cells, and exploiting liquid state NMR [2]. This strategy allowed, at least in some particular cases, to overcome the inherent problems associated with the extreme difficulty of isolating and maintaining certain membrane receptors in solution with the correct folding and the proper functionality. Nevertheless, the authors employed platelets and cells, that live in suspension, particularly in the bloodstream, and that present a low tendency to aggregate and precipitate. In contrast, cells derived from solid tissues show a strong tendency to aggregate and precipitate which, in most cases, prevents the application of STD-NMR experiments. In this context, we aimed to develop a robust NMR methodology to study the interaction of ligands with membrane proteins, employing samples which contain whole and vital cells, without restrictions related to the nature of the tissue of origin. In particular, the use of high resolution magic angle spinning (HR-MAS) NMR techniques has been explored to exploit the rotation at a relative high speed as a tool to maintain cells into the sample “active window”.

To verify the feasibility of this approach, we selected a model system composed by the hSGLT1 cotransporter interacting with two of its known ligands. hSGLT1 is a Na+/glucose co-transporter membrane protein that uses the energy from a downhill sodium gradient to transport glucose across the apical membrane. The glycoside phlorizin and naphthyl-β-D-galactoside are both competitive inhibitors of hSGLT1, but presenting affinities for the receptor that differ for more than three orders of magnitude. The phlorizin/hSGLT1 and naphthyl-β-D-galactoside/hSGLT1 interactions have been verified and characterized by STD experiments acquired on samples containing the ligands and cells expressing h-SGLT1 [3].

References

Acknowledgements: The research leading to these results has received funding from the CARIPLO Foundation (CARIPLO 2008-3175 “Development of NMR techniques for tissue engineering studies”) and NEDD (Regione Lombardia, FONDO PER LA PROMOZIONE DI ACCORDI ISTITUZIONALI Progetto n. 4779 ”Network Enabled Drug Design”), which we gratefully thank.
NMR CHARACTERIZATION OF THE BINDING OF GENISTA TENERA ETHYL ACETATE EXTRACT AND ITS MAJOR COMPONENT TO Aβ1-42 PEPTIDE

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Genista tenera is a native plant from the island of Madeira (Portugal), used as an antidiabetic agent by the local population, whose major component is 8-β-D-glucosylgenistein (8GEN) [1]. This compound has shown an extensive therapeutical impact on an animal model of beta-cell failure and diabetes. It completely normalized the fasting hyperglycaemia and radically ameliorated the excessive glucose excursions to values similar to those observed in normal rats. It was also observed that 8GEN is able to increase insulin sensitivity and shows a tendency to increase glucose-induced insulin secretion [2].

Type 2 diabetes, along with other age-related degenerative diseases, including Alzheimer’s disease, is related with the accumulation of amyloid fibrils [3]. Saturation Transfer Difference (STD) and tr-NOESY NMR experiments demonstrated that 8GEN binds to Aβ oligomers and with higher affinity than the corresponding 7-O-glucoside indicating that this compound may have a potential anti-amyloid aggregation effect, useful in the therapeutics of diabetes and/or Alzheimer’s disease [2,4].

NMR binding study results will be presented in this communication.

References

Acknowledgements: This work was supported by the European Community's FP7 programme under contract CP-IP 212043-2 NAD and NEDD (Regione Lombardia, FONDO PER LA PROMOZIONE DI ACCORDI ISTITUZIONALI Progetto n. 4779 "Network Enabled Drug Design"), which we gratefully thank.
NEW Gd-CONTAINING NON LAMELLAR LIPID-SUPERSTRUCTURES: RELAXOMETRIC, STRUCTURAL CHARACTERIZATIONS AND BIMODAL IMAGING OF HUMAN OVARIAN CARCINOMA CELLS

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During the course of the last decades an increasing interest has been devoted to the study of nanoparticles able to vehicle imaging probes as well as for drug delivery. A newsworthy alternative to the more classical lamellar phase materhials such as micelles and liposomes is represented by non lamellar nanoparticles, formed by self-assembling of lipid mesophases. Their peculiar internal structure, in which intercrossing water channels are partitioned by lipid bilayers, featuring both hydrophilic and hydrophobic domains, allows for the encapsulation of high payload of imaging and therapeutic molecules [1]. Very few systems loaded with chelated metal ions included in the internal phase framework have been reported [2]. In particular, to our knowledge, this is the first Gd-containing “cubosome like” system, with potential applications as MRI probe, in which the Gd(III) metal ion is included in a thermodynamically highly stable complex. Here we report the synthesis, the structural and the relaxometric characterization of liquid-crystalline lipid nanoparticles (NPs) constituted by glycerol mono-oleate (MO) or di-oleate (DO) lipids, a Gd-DTPA like complex functionalized with two C18 lipophilic chains (1÷20% wt), and the fluorescent Rhodamine-PEG2000-PE (0.1%). NPs were stabilized by the nonionic block copolymer Pluronic F127 and, for cellular uptake experiments, functionalized with an amphiphilic folic acid containing molecule (3%wt). Systems were characterized using relaxometric measurements, DLS and Cryo-TEM analysis for particle size and morphology. The relaxivity values of MO- and DO-based NPs containing different amounts (1÷20% wt) of (C18)2 DTPA(Gd) were measured at 20 MHz and 298K. The paramagnetic systems were also characterized through the registration of NMRD profiles. MO- and DONPs relaxivities are quite similar for the different loading values, with a slight increase when the percentage of Gd-complex is brought to 20%. These particles are characterized by diameters ranging from 110nm to 200 nm and a negative surface charge. To determine the specificity and the efficacy of the delivery, the GdNPs were evaluated in IGROV-1 and Hela, FR-positive cells lines and in FR-negative B16F10and K562 cells. A preferential uptake efficiency of DONPs-FA in IGROV-1 cells with respect to DONPs without folic acid, expesically at low concentrations of nanoparticles or at short incubation times, was observed. By comparing the uptake efficacy of DONPs-FA and DONPs in other cell types with lower or absent expression of folate receptors (B16F10, K562 and HeLa) we observed a drastic drop in Gd(III) and Rhod-PE content.

References
IMPROVED CHARACTERIZATION TECHNIQUE FOR IDENTIFYING AND QUANTITATIVELY DETERMINING END GROUPS IN FLUORINATED MATERIALS

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Chain end groups are known to greatly influence chemical, thermal and mechanical properties of fluorinated polymers [1]. For this reason there’s a continuous need for improving and optimizing procedures for the identification and quantitative determination of end groups, in particular for VDF/HFP copolymers [2]. The now optimized analytical procedure performed on Agilent DirectDrive System 500 instrument enables us to identify all types of end groups, even at very low concentration. An efficient solution for improving the sensitivity has been developed based on an appropriate SPIN ECHO technique aimed at reducing the intensity of the signals of polymer backbone in comparison with those from end groups. By careful tuning and setting of the sequence, a very clean spectrum is obtained enabling very precise integration of the peaks, so as to reach very low sensitivity, down to levels of about 0.1 mmols of end groups per kg of rubber.

Fig. 1. End group determination: CPMG and s2pul comparison.

References
Fluorinated and perfluorinated materials are extensively employed in fields of use that require extreme chemical and thermal resistance. A new technological platform has recently been developed in Solvay Specialty Polymers for the synthesis of new PFPE block copolymers, and in particular PFPE-TFE copolymers [1]. Depending on the length of PTFE block, these new classes of materials could be provided in a liquid, wax or solid form and, consequently, be applied either as lubricant oils, or as processing aids and additives in polymers formulations. A complete determination of structural parameters has been performed by fluorine high resolution NMR, either on neat samples (for samples available in liquid form) or on solutions in perfluorinated solvents (for wax samples). A similar approach has been developed based on SS-NMR enabling us, not only to detect and assign PFPE and PTFE signals, but also to determine the length of PTFE blocks. This approach, tested by comparison with solution-state NMR results, has been found extremely reliable, and has made possible the structural assessment of insoluble solid copolymers, endowed with long PTFE blocks.

Fig. 1. $^{19}$F-NMR spectrum of PFPE-TFE wax in hexafluorobenzene

References

THE COMPLEX PROCESS OF CELLULAR VITAMIN A UPTAKE: FIRST INSIGHTS BY NMR AND OTHER BIOPHYSICAL TECHNIQUES

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Vitamin A is essential for diverse aspects of life, ranging from embryogenesis to the proper functioning of most adult organs. It circulates in blood bound to serum retinol binding protein (RBP) and is transported into cells by a membrane receptor termed Stimulated by Retinoic Acid 6 (STRA6) [1]. The mechanism of the STRA6-mediated translocation of retinol from holo-RBP into target cells appears to be unique [2]. There are also evidences that a specific binding site for the intracellular carriers (CRBPs) might exist on the cytoplasmic side of the membrane [3] and that CRBP-I plays a key role other than simply to sustain retinol transport [4]. However, until now it is not clear whether the apo-CRBPs may bind STRA6 or they interact with the membrane for retinol uptake.

To gain first insights into this complex process, we have investigated the interactions of CRBP-I and CRBP-II with biomembrane mimetic systems. NMR experiments were performed at different protein:vesicles molar ratios and by varying the composition of the phospholipid liposomes and the ionic strength. Chemical shifts perturbations and line shape analysis provided insights into the interacting residues and proteins conformational dynamics. As the signals were broadened beyond detection at latest steps of the titration, the NMR data have been complemented by other biophysical measurements.

The results revealed striking differences between CRBP-I and CRBP-II, despite they exhibit the same fold (a β-barrel with two short α-helices) and identical retinol-binding motifs. Moreover, the interactions of the two homologs with the lipid bilayers depend upon the phospholipid composition and ionic strength. These new evidences complement the lessons learned from our former studies which had suggested that the two primary cellular retinol carriers exhibit different mechanisms of ligand uptake [5, 6]. These differences may account for their distinct functional roles in the modulation of intracellular retinoid metabolism.

References

MD AND TEMPOL AIDED DESIGN OF PPI DISRUPTORS

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Interfering with protein-protein interactions, PPI, by using suitable small molecules represents an always attractive strategy to combat tumor progression and dissemination [1,2]. However, designing such PPI disruptors is not an easy task, due to the fact that molecular interfaces of PPI are large and flat regions of the protein surface and, conversely, small molecule-protein interactions occur only wherever surface pockets are available [3]. It follows that standard drug design procedures are not practicable for designing PPI disruptors [4]. It has been suggested that the time evolution of protein surface shape, monitored by Molecular Dynamics, MD, simulations can be a suitable starting point for delineating the formation of transient pockets where small molecules can bind proteins [5]. In the present report, the interaction of the chemokine CXCL12 with 3-(2-naphthoylthiocarbamoylamino)benzoate, a potential antitumoral drug, has been analyzed in TEMPOL spin-labeled solvent. Obtained NMR data have been interpreted in terms of protein surface dynamics, as derived from a 1 μs MD in explicit water on the CXCL12 crystallographic PDB structure 1QG7. Chemical shifts changes of CXCL12 backbone amides upon additions of 310454 and strong TEMPOL induced attenuations of 15N-1H HSQC signals are not observed in the very flexible amino- and carboxy-terminal moieties. Enhanced TEMPO L accessibility to the CXCL12 surface is apparent in three different regions, i.e. the protein binding sites to CXCR4 receptor and 310454, together with the A35-L36 fragment, whose relevance in the protein dimerization has already been shown [6]. 310454 interacts also with the flexible 23-24 protein region where a transient pocket is predicted by the MD simulation. The overall data suggest that a combined use of TEMPOL accessibility profiles and MD simulations can offer a suitable framework for dynamic drug design for PPI interference.

Fig. 1. Left: ZINC ID 310454; Right: RMSF calculated from a 1 μs MD simulation in explicit water.

References
PRELIMINARY NMR STUDY ON THE DIMERIZATION OF 1-C-GRX1, AN ESSENTIAL IRON-SULFUR PROTEIN FROM *TRYPANOSOMA BRUCEI*

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Glutaredoxins are small thiol proteins, ubiquitous in all the kingdoms of life, belonging to the thioredoxin-fold superfamily [1]. They were initially identified as glutathione-dependent oxidoreductases, but today their multiple regulatory functions inside the cell have been recognized. They can be distinguished into two major groups: dithiol glutaredoxins, displaying a CXXC active site, and monothiol glutaredoxins (1-C-Grx), with a CXXS active site. African trypanosomes, responsible for the African trypanosomiasis, encode three monothiol glutaredoxins [2]: 1-C-Grx1, 1-C-Grx2, and 1-C-Grx3. *Trypanosoma brucei* 1-C-Grx1 occurs exclusively in the mitochondrion and it has a CAYS active site; it forms non-covalent homodimers and like other monothiol glutaredoxins it lacks any oxidoreductase activity. Instead, experimental evidence suggests a possible role of 1-C-Grx1 in forming iron sulfur clusters, playing an essential role in the iron metabolism of trypanosomes [3]. The mechanism that leads to the dimerization of the protein is still unknown, but it has been shown that an N-terminal segment of 35 residues, exclusively found on 1-C-Grx1 from trypanosomatids, is responsible for the dimerization of this protein. The solution structure of a 1-C-Grx1 mutant lacking this sequence has been recently solved in our laboratory [to be published]: this domain shows a very similar structure to other glutaredoxins and it has been proven, by 15N relaxation experiments, to be monomeric even at the relative high concentration required for NMR, underlying the importance of the N-terminal extension in the formation of the dimer. Interestingly, many peaks in the 15N – HSQC spectrum of the wild-type dimeric protein show large differences with respect to the monomer, suggesting a significant structural change upon dimerization; furthermore, the spectra are consistent with the presence of a symmetric dimer. We have proven by NMR and circular dichroism experiments that the dimerization domain alone is unstructured, and it does not interact significantly with the monomeric mutant of the protein. Further studies are still required to understand the dimerization mechanism. Considering that most orthologues of *T. brucei* 1-C-Grx1 only form monomers and that the sequence of the dimerization domain is conserved only in the trypanosomatids, it is important to obtain new information on the dimerization process that can be eventually exploited in the future for the development of new antiparasitic drugs.

References


GdHPDO3A: SLIGHTLY ANOMALOUS RELAXOMETRIC PROPERTIES EXPLAINED BY ELUSIVE ISOMERIC STRUCTURES IN SOLUTION

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GdHPDO3A is a clinically approved $T_1$ contrast agent for MRI, known under the trade name of ProHance. Moreover, the related complex YbHPDO3A has been recently proposed as a highly efficient responsive paraCEST agent [1]. The LnHPDO3A complexes exist in solution as a mixture of diastereomeric pairs whose population changes across the series. Despite the very crowded spectra, it is possible to detect the presence of two sets of resonances associated with different isomers in solution in slow exchange in the NMR timescale. In principle, the LnHPDO3A complexes may be present in solution as eight isomeric forms (four enantiomeric pairs) differing in the layout of the acetate arms ($\Delta$ or $\Lambda$), in the conformation of the macrocyclic ring ($\delta\delta\delta\delta$ or $\lambda\lambda\lambda\lambda$) and in the configuration of the chiral center (R or S). Variable temperature $^1$H NMR spectra and 2D EXSY maps acquired at 298K and 278K (Ln = Pr, Nd, Eu) allowed to assign the spectra and to elucidate the interconversion process between the two diasteroisomers. In turn, this information enabled us to assign these diasteroisomers to the forms $\Lambda(\lambda\lambda\lambda\lambda)$-R (TSAP-1R) and $\Lambda(\delta\delta\delta\delta)$-R (SAP-2R) that interconvert through the inversion of the macrocyclic ring. Then, we assessed the relative population of the stereo-isomers across the lanthanide series by integrating the area of the corresponding NMR peaks. Analysis of the data led us to assume the presence in solution of a third isomeric species, indicated as TSAP'($q=0$), whose population becomes relevant for the heavier lanthanides (Ln = Ho-Lu). Finally, the two isomeric forms present in the aqueous solution of GdHPDO3A were considered in the analysis of the relaxometric $^1$H and $^{17}$O data as a function of magnetic field strength and temperature. A global fit of the data suggest that the TSAP form, present in lower concentration, is characterized by an extremely fast rate of water exchange that affects both the shape of the VT-$^{17}$O profile and the amplitude of the $^1$H NMRD profiles. This finding is in excellent agreement with recent ab-initio simulations [2].

Fig. 1. Chemical structure of LnHPDO3A.

References
NMR STUDIES OF HONEY

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In the last years several studies have focused their attention on food authentication [1-4]. In the case of honey, an appreciated product worldwide, being a readily available source of energy and due to its antibacterial and antioxidant activities [5,6], authentication of botanical and geographical origin as well as compositional assessments (sugars or syrups can’t be added) are relevant requirements. Nowadays botanical and geographical origin of honey is evaluated by melissopalynology that nevertheless presents some limitations [7]. NMR has already demonstrated its potentiality in food authentication [8] and in this study it is proposed as a valid alternative tool. In this respect, saccharides content of honeys of different botanical [9] and geographical origin was considered as the discriminating factor.

References
ARCHAEOMETRIC STUDY OF OPUS SIGNINUM BY SOLID STATE NMR

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In the last century, cementitious materials geopolymers were proposed as a green alternative to ordinary Portland cement [1]. The idea is however not new, since the Romans widely used the so-called Opus signinum for beaten earth floors and waterproof plasters. They can be obtained from a mixture of earthenware and slaked lime through a geopolymeric reaction of the metakaolin component followed by a slow carbonation of the unreacted lime mortar [2]. These ancient floors are considered as an index of diffusion of Roman civility. They are often the only evidence of the first built structures, revised or covered several times during the centuries, due to their resistance. For example, in Verona many sites rich in beaten earth floor, commonly used for urban domus of the period between I and II a.C. centuries, are found. At that time, these floors were made by mixing mortar with various other materials, such as small stones, grey grout and tuff. The objective of our research is the study, by means of multinuclear solid state NMR, of floor samples collected in different sites in Verona. ssNMR is a powerful tool for analysing amorphous materials and is used in this case for getting information about the structural organization of the aluminosilicate network [3] and for demonstrating that Opus signinum is one of the first and ancient human-made geopolymeric materials. The samples for NMR analyses were prepared separating matrices and earthenware and, for a deeper inspection, analysing separately grain boundaries and internal parts.

Fig. 1. Detail of a floor made of Opus signinum in the S. Cosimo site (Verona)

The $^{29}$Si NMR spectra show, for all samples, two signals attributable to $Q^4(2\text{Al})$ units and pure quartz. Moreover, in the matrices highly overlapped resonances accounting for a minor presence of silica units and $Q^4$ units bearing 1 to 3 Si-O-Al bonds can be detected. These findings suggest the occurrence of a very slow geopolymeric reaction. $^{27}$Al NMR analyses highlight the predominance of tetrahedral Al sites with respect to octahedral ones confirming the meta-kaolin nature of the material.

References

SOLID STATE $^{13}$C NMR TECHNIQUES FOR THE CHARACTERIZATION OF CHAR OBTAINED FROM BIOMASS CARBONIZATION

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With the aim of contributing to the solution of global energy and environmental sustainability problems, biomass is currently being transformed by thermochemical processes for the production of biofuels but also char, which can be considered as highly valuable “green” Carbon. Indeed, the conversion of biomass into char represents per se an efficient way of taking the CO$_2$ out of the carbon cycle; furthermore, biomass-derived char can be combusted for heat and power or used for applications in several crucial fields, such as water purification, catalysis, electronics, and biomedicine. Different technologies can be used to produce carbonized organic matter, i.e. pyrolysis, gasification, hydrothermal carbonization, and flash carbonization [1]. Depending on the method as well as on the conditions used for its production, mainly temperature, speed and duration, the char produced can have fundamentally different physical and chemical properties, which in turn determine its possible applications. For this reason, in recent years, several groups have responded to the challenge of gaining more information on the chemical and physical properties of char by using several instrumental techniques as well as chemical analysis methods, and comparing the final properties of the materials obtained using the same precursor but different carbonization methods. Among the techniques used, solid-state $^{13}$C NMR spectroscopy has revealed of value for qualitatively and quantitatively characterizing the forms of carbon present in char, allowing in particular specific functional groups and chemical structures to be identified and the aromatization degree as well as the size of the aromatic clusters to be estimated [2-6].

In the present work, the results obtained from the application of several $^{13}$C NMR techniques (Direct Excitation, Cross Polarization, Dipolar Dephasing, Long Range Dipolar Recoupling), to char samples produced from carbonization of corncob and Miscanthus using different methods, i.e. hydrothermal carbonization, flash carbonization and slow pyrolysis, are presented and discussed.

References
Poly(amidoamine)s (PAAs) are polymers containing amido and amino groups regularly arranged along their polymer chains, which can be obtained by addition of primary amines or secondary diamines to bis-acrylamides. In particular, we studied the products of reaction between N,N'-methylenebisacrilamide (MBA) and ethanolamine (EA) (Fig. 1) or aminopropyltriethoxysilane (APTES) under different experimental conditions. The main aim of this paper is to find the optimum conditions for the synthesis of the resulting polymer through NMR spectroscopy. The influence of solvent (water, ethanol, methanol or no solvent) and the way in which the two reagents were reacted (whether EA was quickly or slowly added to MBA or MBA dropped into the ethanolamine and vice versa) have been investigated.

Furthermore, the structure of hybrid syloxane-polyaminoamides obtained with different APTES/EA ratio, using water as solvent, has been also studied. $^{13}$C NMR spectra allowed us to follow the reaction through the observation of methylene, vinyl and carbonyl signals. We have observed that both the solvent and the operational mode affected the polymerization (Fig. 2). Specifically, the slow addition of reagents promoted the polymerization reaction and the chain growth. Accordingly, the spectra showed the disappearance of the signals due to the vinyl groups of the MBA. Regarding hybrid syloxane-polyaminoamides, the APTES/EA ratio influenced the formation of polymer.

Fig. 2. $^{13}$C NMR spectra of PAA with methanol as solvent a) ethanolamine added to MBA fastly, b) slowly and c) MBA added dropwise to the ethanolamine.

References

LOW TEMPERATURE NMR MEASUREMENTS FOR THE CHARACTERIZATION
OF MOLECULAR DYNAMICS IN CRISTALLINE SOLIDS:
STUDY OF SODIUM IBUPROFEN AT CRYOGENIC TEMPERATURES

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The study of molecular motions occurring in solid systems is of particular interest, not only for the intrinsic value of such a deep knowledge of a chemical system, but also because molecular dynamics can be connected with fundamental properties, such as stability of solid phases, solid–solid phase transitions, intra- and intermolecular interactions, and chemical reactivity. Sodium Ibuprofen represents an interesting case study for a thorough dynamic investigation. Molecular fragments in its dihydrated form are characterized by interconformational motions in the kHz and MHz regimes even below room temperature, as found from a previous solid-state NMR (SSNMR) study [1]. Low-temperature SSNMR may give a crucial contribution to the quantitative determination of motional parameters such as activation energies and correlation times. However, performing MAS NMR experiments at temperatures below 100 K is challenging. The experiments here reported have been carried out using the custom-built NMR cryo-equipment at University of Southampton. In particular, the experiments done at temperatures ranging from 300 to 140 K have been performed in a system where a standard Varian VT stack was modified such as that both bearing and drive gas flows are cooled by thermal contact with the exhaust gas of the cooling flow [2]. On the other hand, for temperatures below 140 K a cryogenic NMR equipment that uses supercritical helium to reach temperatures as low as 1.8 K in static conditions and 13 K under 15 kHz MAS has been used [3]. The simultaneous analysis of $^{13}$C and $^1$H relaxation time measurements, as well as $^{13}$C CP-MAS and 2D PASS spectra, performed in the temperature range between 230 and 40 K, along with previously acquired higher temperature data, led to a comprehensive and quantitative characterization of all interconformational motions occurring in crystalline Sodium Ibuprofen.

References
A NEW Gd-AAZTA DIMER BEARING AN ADAMANTYL MOIETY SUITABLE FOR THE PREPARATION OF SUPRAMOLECULAR ASSEMBLIES

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There is an ongoing interest in developing high-relaxivity Gd-based MRI contrast agents to overcome the low sensitivity issue inherent to the technique. The increasing of the hydration number $q$ and lengthening of the reorientational correlation time ($\tau_R$) are two possible ways to increase the relaxivity of Gd$^{III}$-CAs. An attractive approach to attain long $\tau_R$ values relies on the reversible formation of adducts between suitably functionalized complexes and macromolecular substrates. We synthesized a new MRI probe that combines the following features: $q = 2$; fast rate of water exchange, presence of an adamantyl moiety able to form supramolecular assemblies with a variety of substrates. Its relaxivity ($r_1 = 16.7$ mM$^{-1}$ s$^{-1}$, at 20 MHz and 298 K) is comparable and even higher than that of other Gd$^{III}$-dimers reported in the literature with two coordinated, fast exchanging, water molecules ($q = 2$) on each metal ion. Furthermore, an efficient relaxation enhancement can be achieved through the interaction of the adamantane group with slowly tumbling macromolecular substrates. The complex forms high-molecular-weight host-guest adducts with $\beta$-cyclodextrin ($\beta$-CD) and poly-$\beta$-CD, and also interacts strongly ($K_f = 1.17 \times 10^4$ M$^{-1}$) with hydrophobic binding sites on human serum albumin (HSA). We have also prepared a new macromolecular architecture consisting of eight $\beta$-CD units attached to a PAMAM-G1 dendrimer through a disulfide bond that can be cleaved under reducing conditions like those where specific enzymes or high radical concentrations are associated with a disease state (e.g. tumors or strokes). The ditopic Gd complex showed a very large affinity ($\sim 10^6$ M$^{-1}$) for the CD units of the dendrimer. The MRI contrast effect is sensibly enhanced (up to +70%) upon binding of the Gd-complexes to the polymeric structure.

References
Commercial gadolinium(III) based Magnetic Resonance Imaging (MRI) contrast agents consist of discrete Gd(III) complexes with octadentate polyalminopolycarboxylate ligands and are nowadays administered in up to 30% of clinical scans to improve the quality of the images and enhance the diagnostic accuracy. The interest in further studies on small Gd(III) complexes raises from the need to improve their properties in order to find complexes with all the parameters optimized. As it was already shown in the past [1], the water exchange rate for the [Gd(EGTA)(H$_2$O)]$^-$ (EGTA = ethyleneglycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid) complex is $3.1 \times 10^7$ s$^{-1}$ (at 298 K), one order of magnitude higher than that typically found for clinically relevant MRI contrast agents and one of the fastest reported for nine-coordinate polyaniminopolycarboxylate Gd(III) complexes [1,2]. The thermodynamic stability of the complex, however, is not that high ($\log K_{ML} = 16.97$) to allow its application in MRI studies. A novel octadentate ligand derived from the basic structure of EGTA with two piperidine-cis-dicarboxylic acids moieties spaced by a triethylenedioxy chain (L1) has been synthesised with the aim to improve the structural rigidity and thus thermodynamic and kinetic stabilities of the Ln(III) complexes. The $^1$H- and $^{13}$C NMR spectra of the diamagnetic La$^{3+}$, Y$^{3+}$ and Lu$^{3+}$ complexes show a pronounced stereochemical rigidity and a structural change along the lanthanide series. The relaxometric data showed that [Gd(L1)]$^-$ maintains the optimal relaxometric properties of [Gd(EGTA)]$^-$ even with a three times faster water exchange rate. Unexpectedly, the stability constants of [Ln(L1)]$^-$ complexes shows a weaker binding affinity for Ln$^{3+}$ ions of L1 than EGTA. Also in terms of kinetic inertness both acid catalysed and metal assisted decomplexation of [Gd(L1)]$^-$ showed faster rates of decomplexation with respect to [Gd(EGTA)]$^-$. 

Fig. 1. Structure of L1.

References
STUDIES OF THE INTERACTION BETWEEN A BILE ACID BINDING PROTEIN AND MEMBRANE MIMETIC SYSTEMS USING SOLUTION NMR

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The interaction of proteins with lipid membranes is often at the core of essential biological events. As opposed to integral membrane proteins or lipid-anchored proteins, some peripheral membrane proteins adhere only temporarily to the biological membrane [1]. In the interface, large changes in chemical composition, viscosity, water activity and electrostatic forces, can stabilize different protein conformations. The binding to the interface is then coupled to conformational changes in the protein with possible consequences on its biological activity [2].

The fatty acid binding proteins are a class of cytosolic proteins known to participate in lipid transport and homeostasis [3]. These relatively small biomolecules are responsible for the translocation of hydrophobic or amphiphilic molecules across the cell, delivering their cargo to the cell membrane or to other cellular components [4].

A number of studies have started to address the mechanisms of lipid transfer from and to the membranes, such as for Liver Fatty Acid Binding Protein (L-FABP), Intestinal Fatty Acid Binding Protein (I-FABP). However, the binding equilibria describing the partitioning of these proteins between free and membrane-bound states remain poorly characterized. Some members of the FABP family, called Bile Acid Binding Proteins (BABPs), are responsible for the translocation of bile acid in the enterocytes and in the hepatocytes [5]. This transport is achieved mainly through a protein mediated mechanism that involves BABPs as intracellular carriers. Bile acids undergo a recycling pathway between the intestine and the liver, called “enterohepatic circulation”, which assure the recovery of these molecules and their subsequent reutilization. Alterations of intracellular BA transport are linked to cholestatic diseases and BA accumulation leads to liver damage and may promote the development of liver tumors [6].

We explored the possibility to use solution NMR techniques to investigate the interaction between BABPs and membrane mimetic systems in order to understand the nature of these interactions and to determine binding affinities. The system under study prevents a direct investigation of the lipid-bound protein state by solution NMR due to the impossibility to observe the corresponding signals (“dark state”) [7]. It is in principle possible, however, to explore the protein:liposome adduct through experiments that report on the observable free protein signals perturbations arising from chemical exchange between the two states. We further explored the role of phospholipids composition, vesicle size, and ionic strength in the modulation of binding and their role in protein conformational changes.

References

Solid state NMR at cryogenic temperatures offers an increase in the signal-to-noise ratio and the opportunity to study scientifically and technologically important physical phenomena happening at such low temperatures. Examples include superconductivity, quantum tunneling and quantum molecular rotations. In the biological area, cryogenic NMR has a major potential in the study of protein assembly or membrane proteins, to cite a few examples. Cryogenic NMR, when combined with DNP, can deliver a large gain in sensitivity, resulting in a very strong tool of investigations.

We have custom-built cryogenic NMR equipment able to perform both static and magic-angle-spinning solid-state NMR experiments in a field of 14.1 Tesla. The static cryogenic probe can perform solid state NMR at temperatures that ranges from room temperature to 1.8 Kelvin. The cryogenic MAS probe spins a 2 mm zirconia rotor at up to 15 kHz and at as low as 13 K (true sample temperature). Furthermore, sample temperature and spinning frequency can be adjusted independently, since bearing, turbine and sample cooling lines are well separated and mutually insulated. The cooling is done using cold He gas produced in a custom-made Helium boiler that works by evaporating liq-He in the supercritical regime. The sample temperature is calibrated by using the longitudinal relaxation decay constant of $^{127}$I in cesium iodide (CsI).

We show results for a series of experiments performed in the cryogenic regime under both static and magic-angle-spinning conditions:

1. **Endofullerenes (H$_2$@C$_{70}$, H$_2$O@C$_{60}$):** These systems are interesting examples of molecular rotors constrained into a cage. Our equipment give access to the dynamics of molecular H$_2$ confined into the fullerene cage as well as to the direct observation of the ortho-para conversion between the two spin-isomers of H$_2$.
2. **β2-microglobulin (B2M):** A protein chosen to test the sensitivity enhancements provided by cryogenic operations and the effects of low temperature on spectral resolution, in the context of biological solid-state NMR.
3. **Ibuprofen sodium salt:** The different fragments of its ibuprofen’s dihydrate form undergo several motions on the ns timescale. The molecule is therefore an interesting probe for the study of molecular motions occurring in the solid state, which are connected with fundamental properties. We used cryogenic solid-state NMR in order to characterize these multiple internal molecular motions.
ANISOTROPIC NUCLEAR SPIN INTERACTIONS IN $\text{H}_2\text{O}@\text{C}_{60}$
DETERMINED BY SOLID-STATE NMR

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We report a solid state NMR study of the anisotropic spin interactions in $\text{H}_2\text{O}@\text{C}_{60}$ at room temperature. We found that both dipolar coupling and chemical shift anisotropy interactions affect the room temperature solid-state NMR spectrum of a spinning sample. We used a combination of solid-state NMR and numerical simulation to estimate the size of these anisotropic interactions. The source of the chemical shift anisotropy is discussed within three different models based on a. the partial alignment of water; b. the distortion of molecular orbital due to the water electric dipole moment; c. the distortion of molecular orbital due to the distortion of the cage. Our data exclude model a, but cannot discriminate between the other two.


**METAL IONS COMPLEXES OF BRI2-23: NMR AND MD INVESTIGATION**

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This study is centered on a short chain synthetic peptide called Bri2-23. Bri2-23 is a 23 amino acids peptide derived from the transmembrane British Dementia Protein-2 (Bri2) that consist of 266 amino acid residues. The biological roles of this protein in human brain are not well known so far, but there are large evidences that Bri2 is involved in neurodegenerative disease like familial British dementia [1-3]. Patients afflicted by Familial British Dementia (FBD) suffer from progressive dementia, spasticity, and cerebellar ataxia; usually the disease is around the fifth decade of life and full penetrance by the age of 60 [4].

Bri2 is expressed at very high levels in the brain and presumably cleaved by furin or furin-like proteases as its C-terminus to produce Bri2-23. Bri2-23 has shown inhibitory effects on amyloid-β deposition both in vivo and in vitro, thus suggesting its possible role in modulating amyloid-β aggregation and deposition [4-7].

In this study we have investigated Bri2-23 in water either in the presence or in the absence of metal ions like Cu (I), Ag (I) and Hg (II). The aim of this project is to analyze and to characterize the obtained metal complexes by using Nuclear Magnetic Resonance (NMR) and Molecular Dynamics (MD) techniques. NMR has provided informations about the metal binding sites and the secondary structure rearrangement induced by the metal binding.

References

NMR CHARACTERIZATION OF A POTENT ANGIOGENIC PEPTIDE FUNCTIONAL MECHANISM IN LIVING CELLS

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Angiogenesis, the process of the growing new blood vessels from an already established vasculature, is a fundamental biological mechanism, whose disregulation results in relevant pathological conditions [1]. The Vascular Endothelial Growth Factor (VEGF), is a homodimeric protein and has been characterized as a prime regulator of angiogenesis and vasculogenesis; when cells lose the ability to control the synthesis of VEGF, angiogenic disease ensues. In vitro studies show that VEGF is a potent and specific angiogenic factor involved in the development of the vascular system and in the differentiation of endothelial cells. VEGF biological function is mediated through binding to two receptor tyrosine kinases: the kinase domain receptor (KDR or VEGFR-2) and the Fms-like tyrosine kinase (Flt-1 or VEGFR-1) [2], which are localized on the cell surface of various endothelial cell types. This binding activates signal transduction and can regulate both physiological and pathological angiogenesis. In fact, VEGF and its receptors are overexpressed in pathological angiogenesis, making this system a potential target for therapeutic and diagnostic applications [3].

In the last years we have reported the structural characterization and biologic properties of novel designed VEGF mimicking peptide [4,5], reproducing a region of the VEGF binding interface: the helix region 17-25. In this work, we have carried out a NMR study, by STD and trNOESY techniques, to elucidate the structural requirements for the interaction of this bioactive peptide with intact cells in which the receptor VEGFR-2 is highly expressed.

References
PROBING THE POSITIONING OF CRBPs UPON PHOSPHOLIPID BILAYERS INTERACTION BY A SUITE OF NMR EXPERIMENTS

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Vitamin A must be adequately distributed within the mammalian body to produce visual chromophore in the eyes and all-trans-retinoic acid in other tissues. The cellular trafficking and metabolism of vitamin A are regulated primarily by specific high-affinity carriers called CRBP-I and CRBP-II. Both proteins deliver retinol to microsomal membrane-bound enzymes, either for esterification with fatty acids (LRAT) [1, 2] or for oxidation to retinaldehyde (RDH) [3]. Our current understanding of these processes remains incomplete, but there is evidence that the membrane microenvironment plays a role in the interactions of holo CRBPs with enzymes [3]. To address this hypothesis, we have performed a suite of NMR experiments with retinol-bound CRBP-I and CRBP-II in the presence of model membranes composed of either anionic or zwitterionic phospholipids, at varying protein:lipid molar ratios and ionic strength. Besides NMR, other biophysical techniques were employed to achieve a better understanding of the ongoing processes. TROSY spectra provided insights into the involved protein residues and conformational dynamics. The interaction with the liposomes differs significantly between the two homologous proteins; moreover, it depends upon the phospholipid charge and ionic strength, suggesting an electrostatic nature of the binding. The data indicate that holo CRBP-I, in contrast to CRBP-II, interacts more strongly with the anionic lipid vesicles and some conformational changes are observed in the ligand entry portal. Nevertheless, there is apparently no protein region embedded inside the bilayer, as judged by H/D exchange measurements. This study may help to understand certain aspects of the mechanisms of ligand delivery by CRBPs.

References

SOLID-STATE NMR STUDY OF IONIC CO-CRYSTALS OF NICLOSAMIDE


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In recent years, there is an increasingly interest about the potential use of co-crystals especially in the pharmaceutical field for improving the properties of active pharmaceutical ingredients [1,2]. Here we present new ionic co-crystals used for increasing the solubility of Niclosamide (Nic) an anthelmintic mainly used for the treatment of worm infestations in humans and animals. The co-crystals, of formula Nic·MNic·nH2O (n= 1-4 and M= Na, K), were obtained as powders by grinding Nic with carbonate salts (MHCO3 with M= K, Na). All samples, but Nic·KNic·1H2O whose structure was solved by X-ray diffraction (see Figure 1), were characterized by means of solid-state NMR (SS-NMR) (1H MAS, and 13C CPMAS). In particular, it allowed to establish symmetry and number of independent molecules in unit cell (Z'), and to predict the number of water molecule present in the crystal structure. In the 13C CPMAS spectra the high frequency shift (154 ppm) of the C-OH signal around at 168 ppm reveals the formation of the anion Nic-. On the same time, the 1H signal around 18.7 ppm in the 1H MAS spectra indicates the presence of a strong O···H···O hydrogen bond similar to that observed for Nic·KNic·1H2O. Thermal stability and dissolution properties of Nic in these compounds have been also studied. It has been observed that the formation of the co-crystals leads to an increase in the thermal stability of about 60°C and to an enhancement of the solubility in water.

Fig. 1. H bond distance of Nic·KNic·1H2O [grey, carbon; blue, nitrogen; white, hydrogen; red, oxygen; pink, potassium; green, chlorine].

References
Glucan Particles (GPs) are particles of 2-4 µm derived from Saccharomyces Cerevisiae through a process consisting in chemical extraction of protein, lipid, and mannan. They can be considered an innovative class of microcarrier as they show a high affinity towards immune system cells [1]. When loaded with imaging probes, they could allow the in vivo visualization of cell tracking on animal models of inflammation.

The most common approach used so far in MRI cell imaging has dealt with iron-oxide nanoparticles. However, the use of these agents generates hypointensity in the MR images, thus preventing the contrast detection in endogenous areas of low intensity. Positive contrast agents could be considered a promising alternative, but the entrapment of 10⁷-10⁸ complexes per cell is required to obtain enough contrast. In this respect, GPs may represent an interesting carrier for this kind of applications.

The inner core of GPs was loaded with amphiphilic Gd- or Rhodamine-DPPE-labeled agents using a sudden change in solvent polarity. Cell uptake experiments were carried out using J774A.1, mouse melanoma (B16-F10) and rat hepatoma (HTC) cell lines. It was estimated that each particle is loaded with ca. 1.6×10⁷ Gd³⁺ ions, yielding an unprecedented relaxivity per particle of 3.5×10⁸ s⁻¹mM⁻¹.

A model of severe hepatocellular liver failure was obtained by injecting 8-week-old male C57BL/6 mice with CCl₄, (1 mL/kg) intraperitoneally. Macrophages labeled with GPs were administered intravenously and MR images were acquired using a T1-weighted, fat suppressed, multislice multiecho protocol. Histological analyses were performed to validate MRI results. A considerable increase in liver signal was observed, indicating that the tracked macrophages successfully reached the inflamed site.

Moreover, GPs loaded with a phospholipid-based cyanine dye were also used for the optical visualization of the macrophages recruitment in a mouse model of collagen-induced rheumatoid arthritis, exploiting the natural affinity of GPs for immune system cells, particularly abundant in sites of inflammation. Furthermore, liposomes encapsulating small hydrophilic compounds, which could not be loaded in GPs, were formed into the inner core of GPs, thus opening new possible future applications for this microcarrier.

References
NUCLEAR MAGNETIC RESONANCE STUDY OF ISOMERIZATION IN IMINOETHER PLATINUM COMPLEXES WITH ANTITUMOR POTENTIAL

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The development of new platinum anticancer agents still continues in order to provide drugs which are less toxic than cisplatin, cis-[PtCl₂(NH₃)₂] (cis-DDP), and are active against different types of tumors [1]. Different types of carrier ligands have been exploited. Most of them are nitrogen donors, but also phosphorous-donor ligands have been employed [2]. In previous works we have extensively investigated platinum complexes with iminoether ligands, HN=C(R)OR'. The thermodynamic stability of the platinum-iminoether moiety, Pt(H)N=C(R)OR', is influenced by the steric interaction between cis substituents at the C=N double bond. When R and R' = Me, the E configuration is by far the preferred one; in contrast, when R = tBu the Z configuration is preferred [3]. The configuration of the iminoether ligands can modulate the antitumor potential of the platinum complexes, the species with Z configuration being generally more active than those with E configuration [4].

![NMR spectra of trans-[PtCl₂{E-HN=C(Me)OMe}₂(PPh₃)]Cl, at various time intervals after solubilization in D₂O, 300K, pH 6.5.](image)

Our investigation shows that the configuration of the iminoether ligand (E or Z), in complexes of the type trans-[PtCl₂ₓ{E-HN=C(Me)OMe}₂(L)ₓ]Clₓ, can be modulated also by the cis ligands (L= PPh₃, AsPh₃, DMSO, thiourea; x = 1, 2). Looking for a possible explanation we performed 1D and 2D multinuclear NMR experiments (Fig. 1, L= PPh₃ and x=1). Moreover, in the case of reaction with thiourea, a McLafferty rearrangement, with an aminic group of thiourea substituting a methoxy group of a cis iminoether, leads to formation of a cyclic compound. Such a rearrangement could play a role in the drug-DNA adduct formation.

References
MOLECULAR TWEEZERS FOR RECOGNITION OF POLYMER SEQUENCES: READING INFORMATION BY NMR SPECTROSCOPY

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One of the most common methods for information storage, so far, is writing digital information on the surface of flat disks using lasers or electromagnets. An alternative way, in principle, is to chemically encode information into synthetic polymers [1] in much the same way that genetic information is encoded in DNA. This approach has the advantage of exponentially increasing the information density because it is encoded at the molecular level. In the present work, the key role is played by selective non-covalent interactions between \( \pi \)-electron-rich pyrene-armed molecular tweezers and \( \pi \)-electron-poor aromatic diimide copolymers [2]. Tweezers can recognize specific sequences of the copolymer, hence they can read information that the synthetic material may contain [3].

The reading process is based on \(^1\)H-NMR spectroscopy. Some molecular tweezers are able to recognize and selectively bind to a specific diimide monomer in a copolymer. This complexation leads to a shift and splitting of the NMR resonances respectively caused by the changing of the electronic environment from the free to the complexed form of the sequences involved. The present project aim is to prepare new molecular tweezers, new electron-poor receptors and to study their complexation and sequence recognition properties.

Pyrene-armed tweezers with different spacers and electron-poor diimides have been prepared. The spacers are substituted isophthaloyl diamides and diamines. The influence of the nature of the spacer on the binding strength has been studied by both UV and NMR techniques. The tweezers having electron-poor spacers were found to be able to self assemble, however the complexation with diimides is preferred. It was found that the complexation strength with diimide changes if the receptor is linear or a macrocycle. The nature and the size of the spacer play a significative role on the binding. The presence of hydrogen bonds can enhance the strength of the complexation and the proper length of the spacer enables the pyrene arms to lie parallel in order to reach an optimal aromatic-interaction.

References

Lactobacillus plantarum is a major component of the urogenital and intestinal microflora of most mammals, including humans. Among the probiotics it is one of the most used in processes of conservation and food processing, and one of the most studied for its involvement in the immune stimulation and balancing of the intestinal microflora.

We have identified, within the genome of *L. plantarum* WCFS1, three putative proteins, hereby named FlmA, FlmB, and FlmC. Interestingly, their deduced aminoacid sequences show a significant percentage of identity with the BrpA (biofilm regulator protein A) protein. Chatfield and coworkers have suggested that BrpA is a cell-surface protein, involved in maintaining the structure of the cell wall. BrpA, as well as FlmA, FlmB, and FlmC, contains the highly conserved LytR-cpsA-psr (LCP) domain [1].

Kawai and coworkers, in addition, have identified the LCP protein family as novel enzymes required for major anionic cell wall polymers synthesis (teichoic acids and acidic capsular polysaccharides); indeed, the LytR-CpsA-Psr family seems to play a role in bacterial cell envelope maintenance [2]. Therefore, this protein family represents a promising target to gain more insight into virulence and antibiotic resistance development [3].

The function and structure of the LytR-CpsA-Psr domain, however, is not yet well known. Here we report the preliminary structural and functional characterization of LytR-CpsA-Psr domain of the FlmC protein, in order to gain insight into the structure and the function of this interesting domain.

References

DETERMINATION OF THE ORIENTATIONAL ORDER OF A FLUORO-
AND ISOTHIOCYANATE- SUBSTITUTED LIQUID CRYSTAL:
A COMBINED NMR AND DFT STUDY

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Isothiocyanate liquid crystals, characterized by high birefringence, low viscosity and considerable chemical and photochemical stability, find a lot of applications in active matrix liquid crystal displays [1]. Fluorosubstituted isothiocyanates (F-ITCs) are interesting not only for technological but also for biomedical applications [2, 3]. In the present work we investigated the orientational order of a mesogen in which, in addition to the isothiocyanate group, a fluorine atom is present on the same molecular fragment (1F-NCS in Fig. 1). To this aim, a combined DFT-NMR study was performed. 13C NMR spectra were recorded in the melt phase and in solution (CDCl3 as solvent) through Direct Excitation (DE) techniques, while for 13C NMR spectra in the nematic phases the Single Pulse Excitation (SPE) technique has been used. Orientational order parameters at different temperatures in the nematic phase were determined through the quantitative analysis of 13C-19F dipolar couplings and 13C chemical shift anisotropy, measured in the SPE spectra. 13C chemical shift tensors and their orientation with respect to a molecular frame were calculated by DFT methods. In the calculations the effect of the anisotropic environment characteristic of the liquid crystalline phase has been taken into account with the polarizable continuum model (pcm) [4].

Fig. 1. Chemical structure of 1F-NCS.

References
Mycobacterium tuberculosis, the causative agent of tuberculosis, is able to enter into a non-replicating state and survive in an intracellular habitat for years, evading host responses and establishing a latent infection. The probability of reactivation from dormancy is strongly affected by the type of host immune response and it is significantly enhanced in immuno-compromised patients, e.g. suffering from AIDS. Understanding and controlling of entry and exit from dormancy is important for the development of new anti-tubercular therapies. In recent years, a class of proteins (Resuscitation-Promoting Factors, RPFs) that act like growth factors for dormant mycobacteria has been identified. The first protein of this class, known as Rpf, was originally identified in Micrococcus luteus. It is ~16–17 kDa and it is secreted by actively growing bacteria [1]. The protein in picomolar concentrations is able to resuscitate dormant M. luteus [1]. Rpf homologues have been also recognized in M. tuberculosis, which has five genes encoding for Rpf (rpfA-E) [2]. RpfS, containing a transglycosylase-like domain that is homologous to lysozyme, have been proposed to act as peptidoglycan hydrolases on the cell wall of dormant bacteria, thus altering the mechanical properties of the cell wall and favouring cell division and/or release of anti-dormancy factors [3]. Here we report the first step of the structural characterization via NMR of the RpfC catalytic domain from M. tuberculosis.

References

NUCLEAR MAGNETIC RESONANCE OF A HYDROGEN MOLECULE
CONFINED IN FULLERENE C\textsubscript{70}

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We report the solid state NMR of molecular hydrogen H\textsubscript{2} confined inside the cavity of the axially symmetric fullerene C\textsubscript{70} between 300 and 5 K. We show that the proton line shape is determined by the homonuclear dipolar interaction combined with the chemical shift anisotropy induced by the cage. The effective values of the dipolar constant and of the chemical shift anisotropy are determined by the quantum and thermal average over the spatial states. Above 15 K the line shape is interpreted by a fast exchange between the populated quantum states. Below 15 K the NMR line shape is interpreted in terms of hydrogen molecules slowly exchanging between definite quantum states. The proton spin-lattice relaxation has been interpreted by extending Fedders’ theory of relaxation for molecular hydrogen trapped in solid non-magnetic hosts.
REORIENTATIONAL DYNAMICS OF P3HT IN PHOTOACTIVE MATERIALS FOR SOLAR CELLS BY MEANS OF SOLID-STATE NMR

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Polymeric solar cells (PSC’s), characterized by low-cost polymeric photoactive layers, are arising a great interest in the field of solar energy, but several issues, such as the efficiency of these devices, need to be improved before their industrial breakthrough could take place [1]. The investigation of the morphology and dynamics of the polymeric photoactive layers and of the correlations between these properties and the efficiency of the final PSC’s is considered of primary importance in order to develop systems with improved and optimized performances. To this aim solid-state NMR (SSNMR) is one of the most promising techniques, as demonstrated by the increasing number of applications in the field [2-4].

Blends of poly(3-hexylthiophene) (P3HT), as electron-donor, and [6,6]-phenyl-C60-butyric acid methyl ester (PCBM), as electron-acceptor, intimately mixed on a nanometric scale (bulk-heterojunction), are one of the most performing photoactive materials developed so far, and were object of several studies in the literature [2,3]. In this work we investigated in detail the dynamic properties of P3HT in the frequency range of MHz in both the neat polymer and the photoactive blends P3HT/PCBM before and after termal treatment (annealing), with the final aim to find correlations between these properties and the efficiencies of the correspondig devices. In particular, 1H and 13C T1 vs temperature curves have been measured by means of low-resolution and high-resolution experiments, respectively, and were simultaneoulsly fitted by means of a suitable model and a home-made software. This analysis allowed the main riorientational motions of P3HT alklyic side-chains and the twisting motion of P3HT main-chain to be characterized, and the corresponding correlation times and activation energies to be quantitavely determined. Comparing the results obtained for the neat P3HT and the P3HT/PCBM blends, it was found that the dynamic behaviour of P3HT significantly changes among the different systems, and possible correlations between the mobility of P3HT side-chains in the blends before and after annealing and the corresponding increase of efficiency of the final PSC were identified.

References

APPLICATION OF NUCLEAR MAGNETIC RESONANCE TECHNIQUES TO THE IDENTIFICATION OF WINE AROMA PRECURSORS IN GOLDMUSKATELLER GRAPE

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In this work, 1D and 2D NMR techniques are used to identify aroma precursors, in particular glycosilate terpenes [1], in grape juice. Goldmuskateller grape juice was purified with a few HPLC steps and divided in fractions, each containing several aroma precursors. Using two-dimensional homo- and hetero-nuclear experiments, nine molecules were identified. Some of these molecules seem to have never been described before, such as a compound which contains the terpene 1,2-dihydroxyinalool. The focus of this work is to find an analytical way to determine these precursors in a complex matrix such as wine or juice and to improve the scarce knowledge about this particular kind of molecules.

References

NMR STUDIES OF THE FIRST SAM DOMAIN OF ODIN AND ITS ASSOCIATION WITH THE SAM DOMAIN OF EphA2

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EphA2 is a tyrosine kinase receptor that is over-expressed in many tumors and presents several pro-cancer activities [1]. The process of receptor endocytosis and the consequent degradation have been analyzed for their potential linkage to decreased tumor malignancy. A recent report shows that Sam (Sterile Alpha Motif) domains of Odin, a member of the ANKS (Ankyrin repeat and sterile alpha motif domain-containing) family of proteins, may regulate EphA2 receptor degradation [2]. Odin contains two Sam domains in tandem (Odin-Sam1 and Sam2).

In the present study, we implement a multidisciplinary approach consisting of NMR, SPR (Surface Plasmon Resonance), ITC (Isothermal Titration Calorimetry), molecular modeling and mutagenesis techniques to study the solution structure of Odin-Sam1 and its association with the Sam domain of EphA2. The two Sam domains bind in the low micromolar range by adopting a head-to-tail topology typical of several Sam-Sam complexes [3]. This observed binding mode is similar to that we have previously proposed for the interaction between the Sam domains of the lipid phosphatase Ship2 and EphA2 [4].

Our research reveals additional structural features that are crucial for Sam-Sam heterotypic associations and suggests novel possible ways to design potential therapeutic compounds able to modulate processes connected with EphA2 receptor endocytosis.

References
RECOGNITION PROCESSES IN NON-HOMOGENEOUS MEDIA BY HR-MAS NMR: ARTIFICIAL LECTIN INTERACTION WITH CARBOHYDRATES

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NMR Spectroscopy allows to obtain information necessary for a deeper understanding of ligand-receptor recognition processes and nowadays high-resolution magic-angle-spinning (HR-MAS) NMR spectroscopy [1,2] is a well established tool for the study of heterogeneous systems. Here we show the development of an “artificial” receptor used to explore the possibility to reveal its interactions with a panel of soluble ligands, by using STD HR-MAS NMR spectroscopy. In particular, we based our studies on the carbohydrate recognition process: the basic receptors for sugars in Nature are lectins, which show a high specificity for particular sugar moieties. We obtained the synthetic pseudo-receptor, that mimics lectin binding site, coupling a Tryptophan residue to a Sepharose resin and we characterize its interaction with different methyl-pyranosides. The results support the model according to which lectins bind carbohydrates exploiting the CH-π interactions that occur in their active site [3, 4].

Moreover, the experimental approach here described can be generally applied when the interacting species do not have the same solubility properties in physiological conditions.

References

DIPOLAR COUPLING MEASUREMENTS IN MULTIPLE-SPIN COUPLED NETWORKS

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The dipolar coupling is one of the most important spin interactions in NMR as it provides a means of determining intermolecular distances, and from these, the geometry and conformation of molecules. In solid-state NMR, dipolar couplings are eliminated by spinning the sample at the magic-angle and/or using suitable decoupling schemes, but they can be reintroduced during selected periods of the experiment using carefully designed pulse sequences. However, the measurement of weak homonuclear couplings remains difficult in the case where the sample contains clusters of many magnetic nuclei, for example 13C nuclei in organic solids with a high density of 13C labeling, or proton nuclei in almost any organic material. Recently, a new methodology has been introduced for estimating weak homonuclear dipolar couplings in 13C multiple-spin coupled networks [1, 2]. We demonstrate here that this methodology can be extended to 1H nuclei and present results of 1H-1H dipolar couplings measurements where dipolar couplings corresponding to distances exceeding 350 picometers have been measured [3]. The principles, advantages and drawbacks of this methodology will be further discussed.

Fig. 1. Solid-state NMR can give access to accurate 1H-1H internuclear distances.

References
TOWARDS THE RATIONALIZATION OF THE MALDI PROCESS: A COMBINED MASS SPECTROMETRY/SOLID-STATE NMR APPROACH

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Despite matrix-assisted laser desorption/ionization (MALDI) is widely used as an efficient ionization method to characterize synthetic polymers [1], mechanisms underlying this desorption/ionization process are still not fully understood. A key point in the success of a MALDI-MS analysis is sample preparation, namely, matrix and cation selection as well as solid-state organization. The matrix actually plays multiple roles in the MALDI process and should accordingly present specific properties. Some criteria are clearly defined, such as a strong absorptivity at the employed laser wavelength or good vacuum stability, while other requirements, such as a good miscibility of the matrix with the analyte in the solid state, are not easily related to physico-chemical properties of the matrix. As a result, prediction for the optimal matrix-polymer system is still not possible and sample preparation methods are usually developed from published protocols that were shown to work for a given polymeric system. In an attempt to rationalize the preparation of MALDI samples and investigate the mechanisms of MALDI process, the molecular organization of MALDI samples was studied by solid-state NMR. The model system investigated here was composed of 2,5-DHB as the matrix, a small poly(ethylene oxide) (PEG) as the analyte and cesium chloride as the cationizing agent, allowing ¹H, ¹³C and ¹³³Cs sites to be directly probed using NMR [2]. To avoid any solvent effect, the polymer samples to be mass analyzed were prepared under solvent-free conditions. To ensure reproducible data, the vortex method was chosen to grind the sample components in the solid state. Depending on the sample grinding time, the NMR spectra of the MALDI samples revealed the presence of up to three different phases. Water molecules were evidenced for the first time to be part of the MALDI sample. The variety of structural information obtained by solid-state NMR on the investigated model sample showed how this technique can be successfully employed to gain new insights in the MALDI mechanism (see Fig.1).

Fig. 1. Solid-state NMR is used to investigate the MALDI process.

References
Glutaredoxins (Grxs) are ubiquitous enzymes conserved throughout all the kingdoms of life. They belong to the so-called thioredoxin fold super-family and were first described as glutathione-dependent oxidoreductases. Grxs take part in a wide variety of biological processes, the most important being the maintenance of the redox status of cysteine-containing proteins through the reduction of both intramolecular and mixed disulphides with glutathione of protein cysteinil groups [1]. More recently, it was demonstrated that some Grxs are involved in the biogenesis of, or their activity is regulated by, Iron Sulfur clusters [2]. Depending on the amino acid composition of the active site, Grxs can be divided into two subclasses, the classical dithiol and the more recently described monothiol glutaredoxins [3].

Trypanosomatids are parasitic protozoa of the Order Kinetoplastida and they are the recognized causative agents of some diseases spread throughout the tropical areas of the South American (Chagas' disease) and African (Human sleeping sickness and Nagana cattle disease) continents. These parasites exploit a unique thiol-dependent redox system based on bis(glutathionyl) spermidine rather than glutathione. This peculiarity, together with the pivotal role of monothiol glutaredoxins in iron- and iron-sulfur metabolism, makes these proteins potential candidates as drug targets [4].

Trypanosomes encode three monothiol glutaredoxins: Grx1 and Grx2 are mitochondrial single domain proteins while Grx3 is probably cytosolic with a thioredoxin domain fused to its N-terminus [5]. Tb-Grx1 displays an atypical binding site characterized by the CAYS sequence and it lacks classical oxidoreductase activity. It is able to assemble a [2Fe2S] cluster in vitro but its biochemical roles are still unclear. Interestingly, it was demonstrated that Tb-Grx1 is fundamental for parasite survival despite the occurrence of a mitochondrial Grx2 [6].

With the aim to shed light on its biochemical properties and its potential role as drug target, we solved the solution structure of Tb-Grx1 and studied its backbone dynamics via NMR.

References


Acknowledgements: Financial support by the Access to Research Infrastructures activity in the 7th Framework Programme of the EC (Project number: 261863, Bio-NMR) is gratefully acknowledged.
1H-NMR SPECTROSCOPY AS METHOD TO ASSESS FRESHNESS QUALITY IN DIFFERENT SPECIES OF FISH

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The quality of fish can be estimated by sensory tests, microbial methods, measuring volatile compounds and lipid oxidation, determination of changes in muscle proteins, ATP breakdown products and physical changes (including electrical properties of skin) in fish [1]. Among these, Quality index, based on sensory analysis, together with K-index and N-TMA, are the most important methods used to give a measure of freshness of fish. As most of these methods are costly, time-consuming and not user friendly tools, we tried to develop a simple and easy system for the evaluation of fish freshness unresolved goal.

In this work it is demonstrated how 1H-NMR in combination with metabonomics [2-3], could replace the common methods to evaluate freshness of fish.

With this spectroscopic technique is possible to evaluate with a single analysis both fresh indexes: TMA and the nucleotides of the ATP pool used for the calculation of the K-Index; moreover is possible also to have an overview on the whole molecular profile, viewing the changes for example in amino acids concentration [1].

Other important advantages of this spectroscopic approach are: avoiding the use of toxic solvents, such as toluene and picric acid, normally used in the conventional determination of TMA by HPLC or spectrophotometric method and a huge reduce of the time of analysis.

As the results obtained by 1H-NMR spectroscopy are comparable with the classical ones (HPLS and picric acid), this new 1H-NMR method could be used as an alternative, innovative and user friendly way of measuring fish freshness.

References

A SEALABLE RESERVOIR OF NUCLEAR SPIN HYPERPOLARIZATION


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Magnetic Resonance capabilities are often limited by low sensitivity and fast relaxation time of nuclear longitudinal magnetization. The low sensitivity is due to the low degree of spin polarization achievable at room temperature in the magnetic field range typical for conventional NMR instruments. The fast relaxation time is due to the combination of different relaxation mechanisms that discharges any non-equilibrium spin configuration.

Techniques like dissolution-DNP prepares hyperpolarized longitudinal solid-state magnetization that results in a signal-to-noise enhancement of up to five orders of magnitude when the hyperpolarized solid is dissolved in a solvent and inserted in a conventional NMR magnet. The hyperpolarized signal, however, still decays with the same time constant, T1, of thermal magnetization.

Recently, some of us set up a Singlet State NMR technique, which allows nuclear spin polarization to be stored for many tens of minutes (and >40 times longer than T1) in the form of singlet spin order.

We now present a novel technique, dubbed hyperpolarized Singlet Magnetic Resonance, which combines the enhanced lifetime provided by Singlet State NMR with the signal enhancement provided by hyperpolarization techniques such as dissolution-DNP. This technique allows the in-vitro (and in-vivo) prolonged observation of metabolites, tumour markers or other endogenous substance.

Here we highlight the potentiality of the technique by showing how hyperpolarization can be stored as singlet order, read and restored in the singlet state, repetitively, allowing more than half-an-hour long observation of the same original hyperpolarized material. We also show how hyperpolarization can be stored as singlet order in the high field of a pre-clinical MRI scanner, retrieved and imaged at will.
CAN A BILE ACID BINDING PROTEIN BE EMPLOYED FOR OPTICAL DEVICE APPLICATIONS?

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A driving force to study biomolecular optics is the idea that biological molecules are optimized by evolution and natural selection for given tasks, such as recognition and molecules sequestering, which may be of interest for hosting π-conjugated organic molecules, generally used as laser dye. Hybrid and biohybrid host-guest materials are considered as one of the main research areas in materials science and technology for developing functional and structural advanced materials [1].

The potential of an intracellular lipid binding protein for optical applications stems from its stability and its versatile binding pocket, capable of hosting a wide variety of exogenous ligands [2]. We report here interaction studies of a bile acid binding protein (BABP) with oxazine-4 (Ox4) and rhodamine-6G (R6G), two well-known cationic laser dyes also used as fluorescent tags (Fig. 1).

Fig. 1. Molecular structure of oxazine-4 (Ox4) and rhodamine-6G (R6G).

The presence of the ethoxycarbonyl-phenyl group on R 6G appeared to have an important role in BABP binding. NMR titration studies coupled to HADDOCK docking simulations allowed to derive: i) the binding stoichiometry and dissociation constant; ii) a structural model for the BABP-R6G complex, showing that R6G is hosted within the protein cavity.

Preliminary optical characterization, by means of electronic absorption and steady-state photoluminescence, supported the inclusion of R6G dye within BABP nanocontainer. The good film quality formation aptness makes this fluorescent host-guest system very interesting for optical device applications.

References

Acknowledgements: M.A., H.M., S.T., and S.Z. acknowledge FIRB 2008, Grant RBFR08R7OU, for funding.
In this study $^1$H-Nuclear Magnetic Resonance (NMR) spectroscopy was used to analyze pot honey samples [1] of different geographical and entomological origin. An NMR-based metabolomic approach, applied to 67 samples, tested and confirmed the validity of the multivariate statistical analysis in the discrimination. We developed an efficient tool to differentiate the honeys by their geographical origin; additionally, we showed that within limited geographical areas it is possible to distinguish honey samples in terms of the bee species that produced it. Furthermore, the structural identification of geographical marker compounds was achieved.

References

MOISTURE DETERMINATION IN SINGLE GREEN COFFEE BEANS BY TIME-DOMAIN NUCLEAR MAGNETIC RESONANCE

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Moisture content in green coffee beans is a crucial determinant for quality. A water content ranging from 8% to 12.5% is considered adequate to avoid possible development of off-flavours and undesired microbiological phenomena during transportation and storage. Both under-dried and over-dried green coffee beans may affect cup flavor quality. A number of different methodologies are normally used to determine moisture content in samples of green coffee beans. Depending on the method, from one to several grams of green beans are employed. The average moisture content is generally used as Quality Control parameter.

Using time-domain nuclear magnetic resonance, the moisture content of 8 different commercial lots of C. arabica was determined. Specifically, 200 beans were randomly selected from each lot and spin-echo experiments were recorded on every single bean. Moisture distributions were therefore determined. Interestingly, several lots showed different moisture distribution, even though they are characterized by the same average moisture content. In some cases, the distribution included beans for which the moisture value was out of the adequate range, suggesting that moisture distribution, along with the average value, should be monitored for quality purposes.
NATURAL COMPOUNDS AGAINST ALZHEIMER’S DISEASE: NMR INVESTIGATION ON THE INTERACTION BETWEEN ROSMARINIC ACID AND AMYLOID BETA PEPTIDES

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The amyloid peptides Aβ1-40 and Aβ1-42 represent one of the main molecular targets for the development of potential drugs for the treatment of Alzheimer's Disease (AD). Oligomeric and fibrillar aggregates generated by these peptides are among the principal components of amyloid plaques found post-mortem in patients suffering from AD [1]. Synthetic or natural compounds effective in preventing the aggregation of amyloid peptides in vitro and to delay the progression of the disease in animal models are already known [2]. However, little information is available about the molecular mechanism by which these compounds exert their effect. Among the molecules known for their anti-aggregating activity against Aβ peptide, but for which the mechanisms of action is not known, there is rosmarinic acid (fig.1) [3].

Fig. 1. Rosmarinic acid structure

To elucidate rosmarinic acid mechanism of action at molecular level, STD-NMR and trNOESY experiments were performed. They confirmed the interaction between Aβ1-42 oligomers and rosmarinic acid in physiological conditions and allowed the identification of its binding epitope. NOESY spectra revealed also some differences in rosmarinic acid conformation after the binding to Aβ peptides. Moreover, some rosmarinic acid derivatives were synthesized and tested to further clarify the role of the two aromatic rings in the interaction.

References

Acknowledgements: This work was supported by the European Community's FP7 programme under contract CP-IP 212043-2 NAD and NEDD (Regione Lombardia, FONDO PER LA PROMOZIONE DI ACCORDI ISTITUZIONALI Progetto n. 4779 “Network Enabled Drug Design”) which we gratefully thank.
NMR CHARACTERIZATION OF GLYCOFUSED TRICYCLES AS LIGANDS FOR AMYLOIDOGENIC PEPTIDES: GOING TOWARDS THE DEVELOPMENT OF NEW TOOLS FOR THE DIAGNOSIS AND THERAPY OF NEURODEGENERATIVE DISORDERS

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Alzheimer’s and prion diseases belong to neurodegenerative disorders in which takes place a conformational change of normally expressed proteins: β-amyloid in Alzheimer’s disease and prion protein (PrP) in prion disease [1]. These proteins lack any significant primary sequence homology, yet their aggregates possess very similar features, specifically, high β-sheet content, fibrillar morphology, relative insolubility, and protease resistance [2].

Many small molecules that are able to bind amyloid peptides and inhibit their aggregation are already known; most of them are natural compounds bearing aromatic moieties.

We generated a small library of glycofused aromatic tricyclic compounds [3] and we verified their ability to bind Aβ and PrP oligomers by STD-NMR and trNOESY experiments. All these compounds were able to bind amyloidogenic peptides but their affinities were modulated by the functional groups present at the aromatic ring. By molecular dynamic studies, we demonstrated that all these molecules have the same 3D-structure and conformation, so the diverse affinity may be only due to the different polarity determined by the aromatic substituents. No influence on the binding was observed for the sugar moiety, which in fact displays only a minor involvement in the interaction with amyloid peptides.

The compounds here presented, apart their binding properties, are also characterized by chemical stability and water solubility; moreover, the presence of free hydroxyl groups allows their easy functionalization with other entities able to modulate their solubility properties, for example to allow the molecules to cross the blood brain barrier.

In conclusion, these compounds represent a ductile tool that may be useful for the diagnosis and therapy of neurodegenerative disorders.

References

Acknowledgments: This work was supported by the European Community's FP7 programme under contract CP-IP 212043-2 NAD and NEDD (Regione Lombardia, FONDO PER LA PROMOZIONE DI ACCORDI ISTITUZIONALI Progetto n. 4779 “Network Enabled Drug Design”) which we gratefully thank.
AN NMR STUDY ON THE INTERACTION BETWEEN
THE ANTI-APOPTOTIC PROTEINS BCL-XL AND DJ-1

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The molecular characterization of Protein-Protein Interaction (PPI) is a fundamental step in the design of small molecules able to block the physiological and pathological effects of this protein complex interaction. Here, we present a study on the interaction between two anti-apoptotic proteins, Bcl-Xl and DJ-1, that were recently identified as partners under UVB irradiation by immunoprecipitation [1]. Bcl-Xl is an anti-apoptotic member of the Bcl-2 family; it is fundamental in the survival of several cancer cell lines and its inhibition by peptides or small molecules represents a very promising strategy in cancer treatment. DJ-1 is a multi-tasking protein related to Parkinson disease and several cancers. Its roles is still unclear even thought its ability to play as oxidative-stress sensor is widely accepted. Interestingly, DJ-1 was also identified as a zymogen, gaining a further function by a C-terminal cleavage under oxidative conditions [2-4].

In this framework, we initially performed different $^{15}$N-HSQC experiments to evaluate the binding affinity of Bcl-Xl and DJ-1 and to identify the regions involved in the interaction. No significant peak shifts were observed in the spectra, at the conditions used, suggesting no or only weak interaction between the two proteins. To simplify the protein complex and to reproduce the putative cleaved DJ-1 form, we produced a library of fragments obtained by chemical and enzymatic cleavage of wild type DJ-1. Preliminary NMR-based screening of this library revealed the presence of peptides, resulting from the digestion of DJ-1 with trypsin, with affinity for BCL-Xl.

References

IN VIVO MRI VISUALIZATION OF DRUG RELEASE INDUCED BY NON FOCUSED ULTRASOUND IN AN EXPERIMENTAL TUMOR MODEL

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Introduction: Doxorubicin is one of the clinically most used drugs to treat solid tumors, but shows a very high toxicity. To reduce collateral effect of Doxorubicin and to carry it directly in the site of the tumor, it has been loaded into liposomes. Several approaches aimed at controlling and optimizing the propter release of the drug, are studied, and among them the use of Ultrasound as external triggering factor is considered and studied on experimental tumor model mice. We have recently demonstrated that low frequency non focused ultrasound can induce a mechanical release from water-filled liposomes, both in vitro and in vivo, visualized by MRI that provide an excellent spatio-temporal control of the release process.

Subjects and methods: A stealth liposomes mimicking Doxyl-like formulation (containing DPPC, DSPC, Cholesterol and DSPE-PEG2000 methoxy) was prepared, encapsulating in it MRI contrast agent Gadoteridol and Doxorubicin. At first it has been tested in vitro in order to find the best experimental conditions and optimize the release of doxorubicin and gadoteridol. Interestingly, the release of the drug and the contrast agent from the same nanocarrier was very similar (see figure 1).

As in vivo proof-of-concept, the liposomes were injected in the tail vein of mice grafted with a syngenic breast cancer model. The subcutaneous tumor was exposed to a single shot of pulsed non-focused ultrasound (duty cycle pilot modulation) after the liposomal injection to maximize the local release of the drug and its diffusion in the tumor mass. The mice received a single dose of chemotherapeutic (5 mg/kg bw) per week for three weeks and the “US-group” was insonated after each injection. Mice were monitored by MRI over the three weeks and T1 and T2 contrast was measured in tumor, kidneys, liver, spleen and bladder. Furthermore, MRI was also used to monitor the tumor growth (see figure 2). Organs were explanted at different time-points and subjected to conventional histology.

Results: After US exposure, tumors showed a good T1 contrast due to release of the liposomes content, while control groups didn’t have any T1 contrast. Furthermore a more rapid tumor shrinkage was observed for mice exposed to US.

Conclusions: In this work, the ability of pulsed low intensity non-focused US to trigger mechanical release of the content from water- filled liposomes open opportunities to develop innovative therapeutic protocols for tumor pathologies decreasing drug doses and collateral effects.
PURE SHIFT: AN NMR TOOL FOR ANALYSIS OF COMPLEX MIXTURES

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One of the main advantages of NMR spectroscopy applied to the analysis of complex mixtures is that structural and quantitative information can be obtained on a wide range of chemical species in a single experiment. In the case of matrices of high complexity, such as the case for food or biological matrices, spectral resolution is a crucial element for NMR spectral analysis. In this respect, a valuable tool is represented by “pure shift” NMR techniques [1], in which broad multiplet structures collapse to sharp singlets, producing a simplification of the ¹H NMR spectrum.

Here, we report the preliminary results of our investigation on different complex mixtures using a novel, quantitative ¹H NMR “pure shift” experiment [2]. Our data indicate that chemometric analysis on spectra with increased resolution, allows good characterization and differentiation of samples of different origin facilitating the identification of signals important for this separation (see fig. 1). Furthermore, the “pure shift” experiment can be used in the chemical identification and quantification of constituents in complex mixtures.

Fig. 1. 1D ¹H-NMR spectra of chloroform extracts of honey of different botanical origins recorded at 500 MHz, ¹H resonance frequency. Red traces: conventional 1D spectra. Black traces: quantitative pure shift spectra. The botanical origin is indicated at the bottom.

References

METABOLOMICS BY $^1$H-NMR IN HUMAN PLEURAL EFFUSIONS:
PRELIMINARY RESULTS

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$^1$H NMR-based metabolomics has been applied, for the first time to our knowledge of published literature, to investigate lung cancer metabolic signatures in human pleural effusions (PE) collected by thoracentesis, with the aim to assess the diagnostic potential of this approach and to gain novel insights into lung cancer metabolism and related systemic effects. The innovative aspect of this preliminary work is based on the usage of pleural effusions as biofluid samples. Generally, the biofluids used for NMR metabolomic investigations are urine and plasma or serum: the pleural effusions have the advantage, when compared with the urine samples, of being much less influenced, if not at all, by the diet and lifestyle of the patients and, when compared with the plasma or serum samples, of having a very low content of proteins, that generally cover, at different extent depending on protein type and concentration, the NMR metabolite signals. Pleural effusions samples obtained from cancer patients (n 36) and from a control healthy group (n 30) were analyzed by high resolution $^1$H-NMR (300 MHz) with standard NMR pulse sequences (1D NOESY sequence, CPMG spin-echo sequence), at 310 °K and pH 7.4. The spectral profiles were subjected to multivariate statistics (AMIX software) according to Principal Component Analysis (PCA). A partial separation between patients and control group was achieved by the multivariate modeling of the pleural effusion profiles (Fig.2). The possible confounding influence of other factors (e.g., gender and age) on the PCA results have been taken into account and excluded by our results. Four spectral regions (0.80-0.90, 1.30-1.40, 2.00-2.10 and 4.10-4.20 ppm) emerged as the most interesting for the individuation of the metabolites involved in the pathology (Fig.1) The metabolites mainly contributing to this discrimination, as highlighted by multivariate analysis, seem to be lactate, triglycerides (increased in patients), some amino acids and metabolites that have yet to be identified. Such preliminary results should be confirmed by applying the investigation to a wider patient cohort, and reinforced by the identification of as much as possible of the metabolites emerged as significant in the present study.

Fig.1. Two of the four NMR spectral regions interesting to discriminate between healthy group and cancer patients
Fig.2. Score plot: PC1 vs PC2 resulting from PCA statistical analysis
HIGH RESOLUTION NUCLEAR MAGNETIC RESONANCE METHODS TO MONITOR MOLECULAR PROBES IN EUKARYOTIC CELLS

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NMR spectroscopy, in conjunction with specific isotope enrichment, is a powerful approach to investigate the conformation, interactions and dynamics of small ligands and macromolecules within their natural and highly complex cellular environments [1]. However, the application of “in-cell NMR” to eukaryotic cells is still hampered by the difficulty to deliver proteins and drugs into cells at high concentrations. In this context, the aim of this study is to provide a working protocol to investigate intracellular delivery at an atomic-level of detail using high resolution NMR.

As starting point for “in-cell NMR” experiments we optimized the conditions for detection of signals from small molecules inside human cells. For this purpose, we monitored the uptake and metabolism of reporter molecules by 13C NMR. 13C-labeled glucose or 2-deoxyglucose (2-DG) were incubated with leukemia cells for different time intervals and concentrations. In the tumor cells, the former is consumed at a high rate and released in the medium as lactate [2,3], while the latter is transported into the cells where it is phosphorylated but not further metabolized [4]. Unfortunately, extracellular signals of the phosphorylated product suggested approximately 30% of cell death induced by 2-DG [5]. In order to reduce the toxicity of the reporter molecule, 2-DG-encapsulated liposomes were prepared as drug carriers for the intracellular delivery. To verify the efficiency of the entrapped sugar in the liposomes, NMR diffusion experiments were performed to distinguish between trapped and free 2-DG. The apparent diffusion coefficient values observed for the 2-DG-encapsulated liposomes were different from that of 2-DG in solution, suggesting the localization of the reporter molecule within the liposomes.

These preliminary data provide the basis to implement an experimental platform suitable for observing and monitoring intracellular delivery of different compounds by in-cell high resolution NMR.

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