

MAIN LECTURES

NMR spectroscopy of biologically and chemically interesting molecules

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In the presentation, applications on the determination of the structure and dynamics of biomacromolecules and complexes will be presented. Dipolar couplings and cross correlated relaxation rates (1) are parameters that will be most prominently used for the analyses.

The usage of dipolar couplings for the determination of structure and dynamics of proteins and complexes will be discussed with examples (2,3). Multiple alignments will be used in order to derive local dynamics of ubiquitin. It will be shown that one can obtain detailed modes of motion.

The use of transferred cross correlated relaxation will be exemplified on the structure of the epothilon/tubulin complex (4) and for the elucidation of an enzymatically catalyzed reaction (5). Finally, high resolution NMR magic angle spinning experiments will be shown to be useful to determine that the number of ubiquinone molecules binding to cytochrome bc_1 complex III of the respiratory chain membrane proteins, is three. The stoichiometry of the binding allows to distinguish between two different mechanisms that explain the bifurcated electron flow to high-potential "Rieske" iron sulfur cluster and low-potential heme b_L that is crucial for respiratory energy conservation by the cytochrome bc_1 complex (6). Solid state measurements in order to determine the conformation of the ubiquinones in the 500 kD membrane protein will be used to derive a structural model.

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Novel Solid State NMR Strategies for the Structural Characterization of Disordered Materials.

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Because of their considerable compositional flexibility, disordered materials, including plastic crystals, glasses, and ceramics play an important role in tailoring materials to specific applications. The structural and dynamic interplay of individual atomic or molecular constituents and the order/disorder phenomena present on various scales of length and time are of considerable interest both from the fundamental science and the materials' application point of view. Solid State NMR techniques are uniquely suitable for addressing such issues. In particular, dipole-dipole interactions often lend themselves to quantifiable structural information. As an example, rotational echo double resonance (REDOR) NMR spectroscopy, pioneered by Schaefer and Gullion and applied by them with great success to distance measurements in structural biology, also turns out to be a very useful tool for the structural analysis of inorganic solids. In such materials, the situation is often complicated by the multi-spin nature of the hetero- and homonuclear dipole-dipole couplings and by the presence of nuclear electric quadrupolar interactions. Furthermore, in glasses, distributions of local environments are commonplace and the order of the spin system may be ill-defined. We have developed some simple REDOR strategies to deal with such problems and obtain some quantitative connectivity information in rather complex systems. Our approach will be illustrated with new applications to various inorganic network glasses, solid electrolyte materials and dental implant ceramics.

CONFORMATIONAL STUDIES BY LOW TEMPERATURE NMR IN SOLUTION AND IN SOLIDS.

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NMR spectra in solution at temperatures lower than $-100\text{ }^{\circ}\text{C}$ allows one to freeze a large number of internal molecular motions such as bond rotation, ring reversal, nitrogen inversion. Such experiments can be also carried out by CP MAS technique in the solid state where, usually, these motions can be frozen at temperatures higher than in solution. Thus comparison can be often made between barriers to conformational processes occurring in solution and in the solid state for the same molecule.

In addition, processes that are invisible in solution, having a too low free energy of activation, become detectable in the solid state.

A variety of examples will be presented to illustrate this type of experiments.

PARAMAGNETIC LANTHANIDE(III) COMPLEXES IN MRI: FROM
EXTRACELLULAR CONTRAST AGENTS TO PROBES IN MOLECULAR IMAGING

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In this lecture the principal issues which are at the basis of the successful use of Lanthanide(III) chelates as Contrast Agents (CA) in Magnetic Resonance Imaging (MRI) applications will be discussed. For Gd(III) chelates high relaxivity is the primary goal particularly when the paramagnetic complex has to signal changes occurring at sub-cellular level (Molecular Imaging applications). The design of Gd(III)-based probes endowed with high relaxivity may be pursued keeping in mind the relationships between structure, dynamics and the relevant parameters involved in paramagnetic relaxation processes. Moreover, the limited number of target-molecules on cellular membranes and the relatively low sensitivity of the NMR-based technique makes necessary to define strategies aimed at delivering many Gd(III)-containing units to the sites of interest. Examples dealing with Gd(III) complexes with very high relaxivities and the design of new routes for pursuing their accumulation at the targeting sites will be reported.[1] An efficient cellular uptake of Gd(III)-containing probes is the key-step for attaining the visualisation of targeted cells by MRI and selected examples regarding metal chelates bearing different recognition synthons will be presented. The problem of the assessment of the minimum amount of Gd(III) complexes necessary for the cell visualisation by MRI has been addressed by evaluating the extent of the cell-internalization of the metal probe.[2] Ln(III) complexes also possess a high potential as responsive systems. Examples dealing with Ln(III)-based probes sensitive to parameters of great diagnostic relevance like pH, temperature, concentration of metabolite and enzymatic activity will be briefly discussed. In this context, the attention will be mainly focused on a novel class of paramagnetic Ln(III) complexes (with Ln different from Gd) whose mechanism of action is based on the Chemical Exchange Saturation Transfer (CEST).[3]

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FIELD CYCLING NMR RELAXOMETRY. A VERSATILE TOOL FOR PROBING ANOMALOUS MOLECULAR DYNAMICS

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Spin-lattice relaxation of deuterons is always and that of protons is in many cases dominated by intramolecular interactions. That is, the most important spin-lattice relaxation mechanism is the reorientation of the spin-containing chemical group. As a consequence, most spin-lattice relaxation studies refer to the standard BPP formula which was derived for isotropic rotational diffusion of dipolar coupled spin pairs with a fixed internuclear distance.

However, this scenario is extremely rare in nature. Complex systems rather tend to make reorientations anisotropic, restricted or correlated with translational displacements. The dominating dipolar interactions may still be of an intramolecular nature, while mutual obstruction of molecules or chemical groups restrict the reorientation process. Another frequent source of anomalies is confinement and adsorption at liquid/solid interfaces. In all these cases the autocorrelation function describing reorientational fluctuations tends to become non-exponential in contrast to that anticipated in the standard BPP theory.

Anomalous autocorrelation functions and the corresponding spectral densities can most favorably be studied by field-cycling NMR relaxometry (see e.g. Refs 1 and 2). Combined with conventional high-field methods, a total frequency range between 10^3 and 10^9 Hz can be probed by proton spin-lattice relaxation, and between 10^2 and 10^8 Hz by deuteron spin-lattice relaxation. A unique advantage of this technique is that there is a straightforward and unambiguous connection between the spin-lattice relaxation dispersions expected in experiments and predicted by theories of molecular dynamics.

In this lecture we will focus on applications to anomalous features of molecular dynamics in polymer melts and in liquids confined in porous media. It will be shown that long, linear polymers confined in cylindrical pores strictly follow reptation dynamics whereas the famous Rouse dynamics can be verified in melts of polymers below the critical molecular mass.

Nanoporous materials and powdery agglomerates of nanoparticles or globular macromolecules such as proteins have a surface-to-volume ratio large enough to influence the reorientational dynamics of adsorbate molecules in a dramatic way. In this case, the field-cycling technique permits one to study even monomolecular surface layers. It will be demonstrated that the surface topology influences the reorientational dynamics of adsorbate molecules. A recent model based on Lévy walks along surfaces will be discussed.

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FIRST PRINCIPLES CALCULATIONS OF NMR CHEMICAL SHIFTS IN CONDENSED PHASES

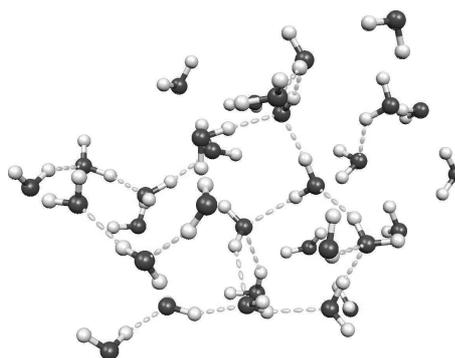
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We present a recently developed method [1] for the calculation of nuclear magnetic resonance (NMR) chemical shifts in condensed phases from first principles. The method is based on density functional perturbation theory and implemented in CPMD [2], a pseudopotential/plane-wave electronic structure code. It is suited for crystalline and amorphous insulators and liquids, as well as for isolated molecules and clusters.

The method is applied to the ¹H NMR chemical shifts of liquid water under standard and supercritical conditions [3], as well as to strongly hydrogen bonded molecular crystals [4] serving as model systems for organic water-free proton conductors. The results are in excellent agreement with experiment, confirming the validity of the description of microscopic structure in the underlying Car-Parrinello molecular dynamics simulations.



We exploit that the proton chemical shift is extremely sensitive to the detailed structure of the chemical environment, in particular the hydrogen bonding network and packing effects. This enables us to probe the characteristics of microscopic structure in disordered or only locally ordered systems, and it allows to uniquely assign experimental NMR resonance lines in solid state magic-angle-spinning (MAS) spectra to individual atoms.

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Mobile NMR: New MOUSES and Applications

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In mobile NMR the spectrometer is carried to the sample. Portable spectrometers, magnets, and probes are employed. For analysis of large objects, the polarizing and radio-frequency magnetic fields are applied from one side. This type of NMR is completely noninvasive, but the magnetic fields are highly inhomogeneous showing nonlinear field profiles within the object. Consequently each rf pulse generates a distribution of flip angles, and unless the sample is smaller than the sensitive volume of the NMR sensor, each pulse is a selective pulse independent of its shape. Only the phase of the rf pulses is uniform across the sensitive volume so that NMR in grossly inhomogeneous B_0 and B_1 fields requires new measurement strategies. For detection, signal dephasing from B_0 inhomogeneities needs to be refocused by Hahn- or CPMG-type pulse sequences. The initial magnetization can be manipulated in terms of different filters like multi-quantum filters, $T_{1\rho}$ filters, and pulsed gradient-field filters for space and displacement encoding, and even filters for chemical shift encoding (Pines et al).

Different sensors (NMR-MOUSE : Mobile Universal Surface Explorer) have been constructed for unilateral NMR, in particular the classical horseshoe geometry for the B_0 field with solenoidal, figure-8, and meander rf coils, and the dipolar geometry of a bar magnet for B_0 with the butterfly rf coil for B_1 and variants thereof. The dipolar geometry is most simple but shows poor results with simple solenoidal rf coils, which are needed when depth selectivity is required by variation of the Larmor frequency.

The construction of different NMR-MOUSE sensors is outlined and two types of application are reported: 1) Quality control of rubber products: The dipolar MOUSE has been used to test rubber samples from the rapid process analyzer for correlation of NMR results and established rubber testing procedures in the elastomer industry. The classical horseshoe-MOUSE with a solenoidal rf coil has been used for depth selective measurements of electrical cable insulation from PDMS under different conditions. 2) The same geometry has been used for CPMG measurements of water in porous bricks and in a wet Roman fresco with subsequent analysis of the transverse signal decays in terms of pore size distributions. Furthermore, the quality and degradation of paper has been studied with the figure-8 coil. The results of these measurements are reported. A general conclusion of these studies is, that the NMR parameters measured for rubber products and building materials can be reproduced within a 1 to 2% error margin when the measurements are performed at the same spot. Measurements at different spots have to be conducted for calculation of the mean and the variance. The mean relaxation time characterizes the average modulus and the variance characterizes the homogeneity of rubber products. For rapid characterization of product quality, parallel multi-sensor operation is recommended.

PARAMAGNETISM BASED STRUCTURAL CONSTRAINTS FOR THE STRUCTURE
DETERMINATION OF METALLOPROTEINS

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Several metalloproteins are paramagnetic. The unpaired electrons cause linebroadening, a contribution to the chemical shifts called hyperfine shifts, often self orient in high magnetic fields because of magnetic anisotropy, experience crosscorrelation between Curie relaxation and dipole-dipole relaxation. All of these phenomena provide structural constraints. Examples of solution structures of paramagnetic metalloproteins will be presented.

NMR SIGNALS AS INDICATORS OF TUMOUR PROGRESSION AND APOPTOSIS

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By allowing non-invasive detection of intracellular metabolites and their fluxes in either ubiquitous or tissue-specific biochemical pathways, NMR spectroscopy (MRS) offers new means to identify metabolic alterations associated with the malignant phenotype of cancer cells, as possible novel indicators of *in vivo* tumour progression and response to therapy. Moreover, some functional NMR imaging (MRI) methods allow today tissue characterisation of cancer lesions in terms of physio-pathological parameters, whose *in vivo* measurement enhances the diagnostic content of conventional anatomo-morphological maps. In particular, dynamic contrast-enhanced (CE) MRI developed to become the most sensitive modality today available for breast cancer (BC) diagnosis, allowing discrimination of malignant from benign lesions, on the basis of tissue parameters like microvascular permeability and extracellular volume fraction, related to neo-angiogenesis and tumour progression.

Among the new areas of *in vivo* biochemical research opened by MRS, over twenty years of investigations demonstrated the interest of clarifying a) the alterations of metabolic pathways and fluxes responsible for the enhancement in the levels of phospholipid (PL) derivatives detected by multinuclear MRS in cancer cells *in vitro* and *in vivo* [1]; b) biogenesis and structure of mobile lipids (ML) detected by high resolution ^1H MRS in some tumours [2], in relation to cell transformation [3] and to drug-induced programmed cell death.

Regarding the significance of PL metabolites in relation to cell proliferation and transformation, particular attention is devoted in our laboratory to i) characterise the NMR spectral alterations in the levels of phosphorylcholine (PCho) and glycerophosphorylcholine (GPC) in solid tumours, especially those of epithelial or neuroepithelial origin [4,5]; ii) investigate the contribution of mammalian phosphatidylcholine-specific phospholipases [6,7] to PCho signal intensity alterations, under different conditions of mitogenic cell stimulation or *in vivo* tumour cell invasiveness.

Nature and subcellular localization of ^1H NMR-detectable ML are currently investigated by our group in leukemia cells induced to apoptosis by either incubation with anti-tumour drugs or by exposure to anti-CD95 antibodies. An overview will be presented of the results so far obtained by NMR, Nile Red fluorescence and TLC, on the relationships between ML signals, *de novo* synthesis of neutral lipids and formation of cytoplasmic lipid bodies [8,9].

Dynamic contrast-enhanced MRI is currently utilised in a multi-centre, non-randomised, prospective study, coordinated at the national level by the Istituto Superiore di Sanità, with the aim of evaluating the effectiveness of integrating MRI with conventional X-ray mammography (XM) and ultrasound (US), in a surveillance programme specifically devoted to subjects at high genetic risk of BC. The preliminary results of this study (105 subjects recruited between June 2000 and March 2002 (mean age 46.0 years, age range 25-77); eight cases of BC detected in the trial, of whom eight identified by MRI, and only one correctly classified by XM and US) confirmed that MRI is a very useful tool to screen subjects at high genetic risk of BC (*Supported by Ricerca Finalizzata 1%, Ministero della Salute, Project N. 98/JT/T*).

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NMR OF SUPRAMOLECULAR ORGANIC AND BIOCHEMICAL SYSTEMS

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Symmetry, or the lack of it, is a key parameter in the interpretation of NMR spectra of supramolecular systems. In the first part of the lecture I shall discuss the assignment strategy for different organic molecules and supramolecular complexes formed by combination of identical units.

Dynamics is a second key aspect of supramolecular systems. Dynamical aspects include the equilibration between different species as well as the global tumbling of each of them. These processes take place in very different time scales. The range from nanoseconds to micro-milliseconds can be studied using relaxation rate measurements.

In the second part of the lecture, I shall discuss several examples of the use of relaxation measurements to study the interaction of different proteins with calcium, with purple membrane, or with itself. The information extracted in different examples includes dissociation rates, dissociation energies, detection of anisotropic motion or details about the structure of supramolecular protein-protein complexes.

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NMR, AN EFFICIENT AND RELIABLE TOOL FOR LEAD IDENTIFICATION AND OPTIMIZATION IN DRUG DISCOVERY

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Over the last few years NMR has emerged as a powerful tool for lead generation in drug discovery programs. Several ligand-based NMR techniques have been proposed for screening mixtures of compounds against the target of interest. However, the methods suffer from some important drawbacks. The Achille's heel of these methodologies is represented by the failure of detecting high affinity molecules, ligands that bind covalently to the receptor, ligands that have a slow on-rate kinetics and ligands that have low solubility in an aqueous solution. In addition, the methodologies cannot distinguish between specific and non-specific binding events.

Four novel approaches have been developed for performing High-Throughput Screening with NMR spectroscopy that overcome all these limitations. Protein concentration as low as few hundreds nanomolar can be used. Mathematical expressions are derived for the proper set-up of the NMR experiments and for extracting from a single point measurement an approximate value of the binding constant for the identified ligand. The new approaches permit screening of thousands of compounds against protein or DNA and RNA fragments in a short period of time. In addition, they can be efficiently applied in the identification of high affinity ligands present in plant and fungi extracts. Examples of these NMR based approaches for lead generation in drug discovery projects will be presented and discussed.

ABSTRUSE PULSES, QUANTITATIVE NOE BUILD-UPS AND IMPROVED FILTER DIAGONALIZATION

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This talk will focus on (i) improved adjustable, broadband selective excitation with uniform phase using a new family of frequency-modulated pulses that we have found; (ii) achieving better quantitation and improved sensitivity in small molecule NOE buildups, especially in cases where coupled spins are involved and multiplet distortions can confuse otherwise nice results; and (iii) implementation of the Filter Diagonalization Method for families of multidimensional constant-time experiments such as those often used for protein backbone assignment.

For anyone who thought the techniques lakes were already fished out, I hope these developments will be a refreshing catch.

ORAL COMMUNICATIONS

Abstract JEOL

**FROM POROUS MEDIA TO BONE TISSUE:
COMMON FEATURES STUDIED BY NMR RELAXATION**

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The interest in Magnetic Resonance studies of bone is increasing as new noninvasive MR Imaging methods are being developed to study bone microstructure and the effects of diseases such as osteoporosis.

Some of the same features are found in NMR relaxation in bone as found for fluids in other porous media. Although mechanisms may differ, internal surfaces contribute to both transverse (T_2) and longitudinal (T_1) relaxation of pore fluids, and in both cases the effects depend on local surface-to-volume ratio (S/V). In both cases variations in local S/V over distances greater than diffusion distances in local relaxation times can lead to distributions of relaxation times, sometimes over decades.

Measurements were made on cow and pig femur samples under various conditions, including different degrees of dehydration, de-fatting and saturation with water, and further dehydration. Distributions of relaxation times were computed from Inversion-Recovery (IR) data for T_1 and from both CPMG and Single Echoes (SE) for T_2 .

In fresh trabecular bone, overlapping T_1 components are found near 100 ms from both fat and water, with slight tailing toward long times and an extended low tail at short times. Drying of the sample helps identify the water. In trabecular bone it is easy to see differences in dimensions of intertrabecular spaces in samples that have been de-fatted and saturated with water, with longer T_1 and T_2 for larger pores. The distributions for these water-saturated samples are usually bimodal, separating or partly separating inter- and intratrabecular water, and there is usually a small tail going down to about a ms in the T_1 distribution, indicating some very small pores, with diameters less than 2000 – 3000Å, which have been confirmed by MR cryoporometry measurements. Free induction decays show a component, probably collagen, of approximately Gaussian form ($\exp[-1/2(t/T_q)^2]$, with $T_q \approx 10 \mu\text{s}$), and another component, probably water in contact with the collagen, with approximately simple-exponential decay. Averaging and smoothing procedures are adopted to get T_1 distributions for both the Gaussian and simple-exponential components. Measurements on cortical bone show the same collagen-related effects but do not have the long T_1 and T_2 components. Because of the possibility of distinguishing between signals from inter-trabecular spaces and signals from intra-trabecular space, it is possible to evaluate the ratios of inter-trabecular and intra-trabecular pore volumes to total pore volume. From these data Bone Volume Fraction, defined as the ratio of solid bone volume (including any intra-trabecular pore space) to total bone volume (including all pore space), values can be computed consistent with data from other sources. In addition, the internal porosity of the trabeculae, defined as the ratio of intra-trabecular pore volume to the total volume occupied by the trabeculae can easily be computed. We do not know of any other method to determine this parameter in a simple and non-destructive way.

NMR STUDIES ON AGI-BASED FAST IONIC CONDUCTING GLASSES

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AgI-based conducting glasses are considered a good model system to investigate the complex relationships among local and medium range order and transport properties in highly disordered systems. In particular, they have been widely studied by means of Nuclear Magnetic Resonance experiments performed both on the glass-forming nuclei, such as, ¹¹B and ³¹P, and on the mobile cations (¹⁰⁹Ag).

In this paper we will review our recent studies on borate, phosphate and molybdate glasses, chiefly with the aim to elucidate how the Ag⁺ transport mechanisms are related to the local coordination of silver, and to the existence or not of AgI nanoscale clusters which, for a long time, have been considered to be the responsible of the high ionic conductivity of these systems. We will show that the silver cations do experience a mixed I-O coordination in the glasses, and that percolation thresholds exist in several cases for addition of about 30 mol% of AgI. These findings point towards the formation of low-impedance nanochannels in agreement with the Modified Continuous Random Network model.

STUDY OF NANOPARTICLE/MATRIX INTERFACE INTERACTIONS IN Y_2O_3 - SiO_2 SAMPLES

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Nanocrystalline Y_2O_3 has been widely investigated, due to its interesting applications in the field of the phosphors for lighting and for cathode ray tubes, when doped with rare earth metals. However, free-standing nanoparticles are unsuitable for technical applications and exhibit a severe degree of aggregation which determines important changes in the properties. Moreover, the presence of surface defects is a major factor affecting the efficiency of nanocrystalline luminescent materials. The surface of nanoparticles can be changed either by chemical reactions or by dispersing the particles in a polymer or a glass matrix. From this point of view, amorphous silica represents the ideal candidate for optical materials both because of its transparency and for the stabilising effect over the nanoparticles, protecting them from aggregation. Therefore it is used for the synthesis of yttria-silica nanocomposites. For these systems it is of fundamental importance to develop an understanding of the interactions at the interface among the nanoparticles and the dispersing phase.

We here report an ^{29}Si MAS and CPMAS NMR study of Y_2O_3 - SiO_2 nanocomposites obtained by sol-gel and impregnation methods. The interest is focused on the structural investigation of the nanoparticle-matrix interface. In the samples obtained by sol-gel, spectroscopic observations point to a description where the silica strongly interact at the interface with yttria nanoparticles through hydrogen bonds, sometime mediated by bridging water molecules, but also via direct Si-O-Y bonds. These interactions at the interface are almost constant in the nanocomposite during the thermal treatment as shown by the constancy of the amount of Q_1 sites. In such a situation, amorphous yttria nanoparticles with average size of 2 nm form, as observed by TEM, and are kept linked to the host matrix which makes difficult their coalescence and growth. In order to make the particle growing, the impregnation method with different process parameters has been used, which in the most favourable case resulted effective in the achievement of crystalline nanoparticles with average size of 8 nm. However, all the samples exhibit important interactions at the interface, which are strongly dependent on the yttrium oxide concentration, as revealed by ^{29}Si MAS NMR.

SPECTROSCOPIC MONITORING OF SOL-GEL SYNTHESSES OF FUNCTIONAL MATERIALS

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Synthetic procedures for the preparation of new materials have gone a long way from "simple" precipitation reactions. The use of more complex "wet" chemistry processes has highly improved the quality of samples allowing to achieve good size control, narrow size distribution and good crystallinity.[1-3] Among them the most successful are sol-gel routes both hydrolytic, involving the hydrolysis and condensation of metal alkoxides in controlled pH conditions, and non hydrolytic,[4] involving condensation reactions between different functionalities bound to different metal centers. The gelation of properly doped polymeric precursors are also attractive methods for the preparation of functional materials.

Once the synthetic strategy is designed, it becomes very important to develop methods to control the preparative conditions by the identification of reaction intermediates and products.

Several spectroscopic techniques that are routinely used in almost all fields of chemistry have been seldom applied to the chemistry of materials. Among them nuclear magnetic resonance (NMR) is probably the most powerful tool accessible to obtain information on the structure and physico-chemical properties of molecules. The observation of conventional nuclei (such ^1H , ^{13}C , ^{31}P) gives information on the organic residues present in the precursors solution. Furthermore many nuclei have at least one magnetically active isotope (^7Li , ^{23}Na , ^{27}Al , ^{29}Si , $^{47,49}\text{Ti}$, just to mention a few) that can be detected by NMR so that information on the inorganic residues can also be achieved with this technique. Mass spectrometry can also be of aid even when the molecular peak cannot be observed, since analysis of the fragmentation pattern of the high molecular weight inorganic or organometallic polymers formed in the syntheses can give information on the structure examined.[5,6]

The application of such spectroscopic techniques to the preparation of new materials will be discussed.

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POLYMER AND SURFACTANT MOLECULES IN NANOPOROUS SILICA MATERIALS STUDIED BY SOLID STATE NMR

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Solid State NMR has been widely applied for the characterization of silica and silica composites. ^{13}C MAS NMR provides carbon chemical shift (CS) and carbon relaxation times that can describe the conformation and motional behavior of the confined polymer or surfactant (1). In ^{29}Si MAS NMR spectra the intensity ratio between the peaks and their line width can describe accurately the silica particles. ^1H - ^{29}Si Cross Polarization (CP) dynamics are a valuable tool for studying the proximity of protonated systems, like polymers, to silica surfaces. We have shown this effect for a polyisoprene/silica system (2) where the characterization of the cross polarization dynamics allowed us to demonstrate the proximity between the polymer and the silica surface at the molecular level.

Polymer/silica interfaces in standard composites are very diluted and poorly defined. MCM-41 silica systems with extended and structured interphases, therefore, can provide a detailed model for polymer-silica composites and for the organization of surfactants on silica surface. Here we present the description of the surfactant organization inside the nanopores at low temperature. Furthermore, once removed the templating agent, the mesopores are accessible to organic molecules. Some monomers (methyl methacrylate, caprolactame) have been absorbed in MCM-41 and polymerized inside the pores; also poly(ethylene oxide) has been included in the nanochannels: conformations and mobility of the confined polymers and silica-polymer interactions will be discussed.

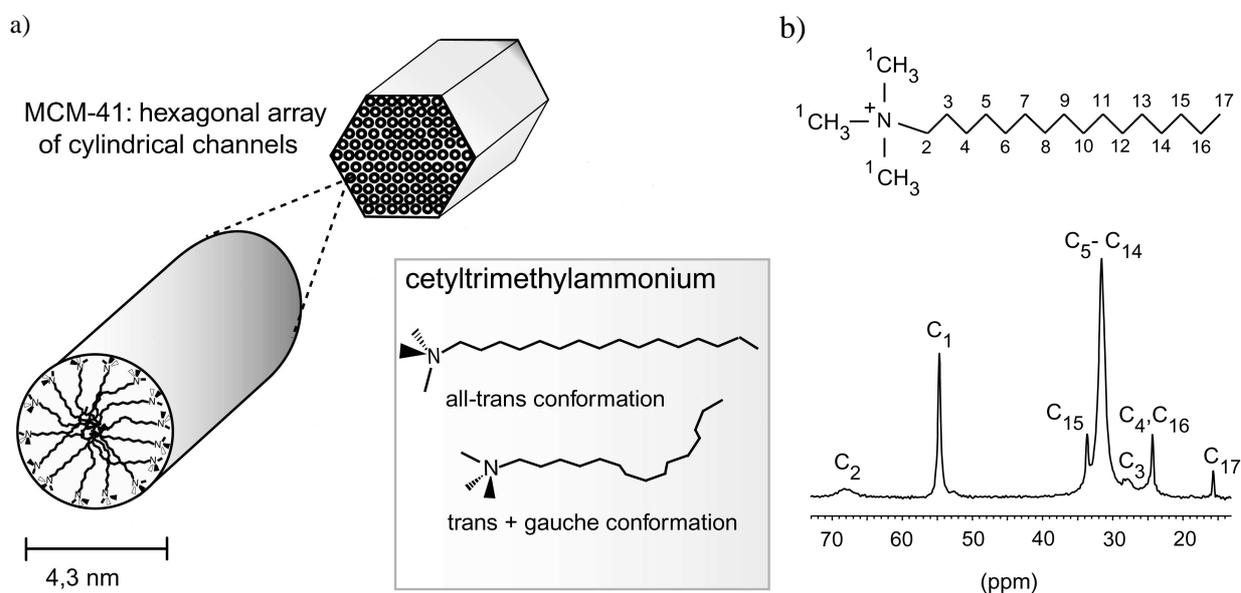


Figure 1 a) Sketch of MCM-41 nanoporous silica. b) 75.5.MHz ^{13}C SPE MAS spectrum recorded at room temperature of MCM-41, with Si/Al ratio of 10, containing the surfactant molecules.

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Novel metallothionein from a poikilotherm organism: solution structure of the metallothionein MT_{nc} from the antarctic fish *Notothenia coriiceps*

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The three-dimensional structure of [¹¹³Cd₇]-metallothionein (MT-nc) of the Antarctic fish *Notothenia coriiceps*, the first three-dimensional structure of a fish MT, was determined by homonuclear ¹H NMR experiments and heteronuclear [¹H, ¹¹³Cd]-correlation spectroscopy. Like mammalian MTs, MT-nc is composed of two globular domains, the N-terminal β-domain constituted of nine cysteines and three metal ions, and the carboxyl-terminal α-domain with eleven cysteines and four metal ions. The position of the ninth Cys of the α-domain of MT_{nc}, as in other piscine MTs, is different from the corresponding Cys of mammalian MTs. As a result of such a modification, the last CXCC motif in the mammalian MT sequence becomes CXXXCC in fish MT. In the light of the present structure determination, it is now possible to discuss this peculiarity in structural terms and to link it to the kinetics of ion exchange. In addition, NMR spectroscopy shows that the difference in dynamic behaviour between the two domains is larger than in mammalian MTs. The difference can be correlated with the structural change of the α-domain and with the different charge distribution of both domains with respect to that observed in mammalian MTs. NMR observations are paralleled by both circular dichroism and dynamic fluorescence spectra of fish MT that are more influenced by temperature than mouse MT. The differential effect of temperature on fish and mouse metallothioneins may reflect a different stability of metal-thiolate clusters of the two proteins. Such a conclusion is also corroborated by data showing differences in metal mobility between fish and mouse Zn-thionein.

NEW NMR METHODS FOR THE CHARACTERIZATION OF PROTEIN-WATER, PROTEIN-LIPID AND PROTEIN-PROTEIN INTERACTIONS

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The detailed investigation by NMR of interactions between proteins and their cellular partners invariably requires the discrimination of the ligand coherences from those belonging to the protein. This distinction can be efficiently achieved by a general approach based on a quadrature-free constant-time (QF-CT) scheme. The QF-CT methodology is very versatile and finds applications for both frequency-based and J-based coherence selection strategies. Specific examples will be presented in the context of protein hydration structure and dynamics, peptide-bicelles interactions and peptide-protein complexes.

NMR structure of the turtle prion protein

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Prion proteins are present in several high eucaryotes, including mammals, birds, reptiles and amphibians. Prion proteins are associated with prion diseases like BSE in cattle, scrapie in sheep and Creutzfeldt-Jacob Disease in humans. Up to now prion diseases have been found only in mammals.

To investigate how the structural motifs of prion proteins have evolved we determined the three-dimensional structure of a reptilian prion protein. We have solved, by NMR, the structure of the globular domain of the turtle prion protein and analyzed the dynamics of the full-length turtle prion protein.

Detemination of C-C internuclear distances via solid-state NMR in specifically labelled proteins

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Ole G. Johannessen^b, Henrik Luthman^a,
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Angelika Sebald^e, Malcolm H. Levitt^{a,b}

Abstract

We describe a method for bond-length measurements in doubly labelled ¹³C spin systems using magic-angle-spinning NMR. The method is first demonstrated on some model compounds with bond-lengths ranging from single to double bond, and then used on a specifically labelled membrane protein, rhodopsin. The aim of the investigation is to get insight about the electronic structure of the retinilidene chromophore in the dark state, with the support of experimental data.

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RIBOSOMAL PROTEIN S1 ROLE IN THE STIMULATION OF RIBONUCLEASE REGB ACTIVITY

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RegB is an endoribonuclease involved in the regulation of the T4 phage life-cycle. It, indeed, inactivates the phage early messengers by specifically cleaving a GGAG sequence in their Shine-Dalgarno regions. In addition, RegB efficiency is modulated by the ribosomal S1 protein that, most probably, acts by promoting the formation of correct RNA conformation recognized by the enzyme. In this context, we have undertaken to analyze of the molecular basis of S1 role by combining biochemical and NMR spectroscopy studies.

S1 is a modular protein composed of six repetitions of a conserved domain, called S1 motif and found in many RNA-binding protein. We first wondered if the six modules were necessary to activate RegB activity. Two RNA molecules were used as test substrates. The first possesses a well-defined secondary structure (as probed by NMR spectroscopy) whereas the second is unstructured. Interestingly, the cleavage of the first molecule only requires the presence of two contiguous fragments of S1, while the cleavage of the second is only activated in the presence of the four C-terminal modules. This suggests the RNA-S1 interaction is dependent on the nature of the substrates.

Concurrently, we have undertaken to identify, by NMR spectroscopy, the S1 residues involved in the interaction with either substrate. We also have achieved the structure determination of the last S1 domain, as a first step toward the reconstruction of the whole C-terminal region. Using this, we hope we will be able to propose a model of the S1-RNA interactions.

LOW FIELD NMR RELAXOMETRY IN LIQUID CRISTAL MATERIALS: LOCAL FIELDS AND MOLECULAR ORDER

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The goal of the field-cycling method applied to T_1 -relaxometry can be found in the possibility to extend experiments to very low Larmor frequencies, while simultaneously allowing the scan of the relaxation dispersion over a broad Larmor frequency interval (typically ranging from a few kHz to several MHz). The technique was applied for more than 30 years for the study of molecular dynamics in liquid crystal compounds, especially in looking for experimental evidence and physical properties of collective processes strongly dependent on the molecular order. A plethora of examples can be found in the literature from the 70's years to the date. A common feature of the observed dispersions, especially in calamitic systems, was the low-frequency plateau systematically dominating the lower frequency range. The phenomenon was usually interpreted in terms of a "low frequency cut-off", associated with limited coherence lengths of the order director fluctuations (ODF). A typical square root frequency dependence always dominated the proton T_1 dispersion of nematic phases between several kHz and about 100kHz. On the contrary, strong dispersions were observed in smectic A phases (as in other lamellar organized molecular settlements) starting at 20-30kHz down, ending in a low-frequency plateau at about 1kHz. Such strong dispersions were usually attributed to smectic order fluctuations (with a predicted linear frequency dependence). In a recent work, similar and even higher dispersions were found in nematic compounds under confinement, where the evolution of the magnetization during a " T_1 experiment" was clearly non-exponential. A similar result was found for smectic phases, where it was also verified that the slope of the dispersions depends on certain parameters of the experimental set-up. All these observations correspond to a Larmor frequency range where local dipolar fields are relevant and cannot be neglected. This *ultra low frequency* (ULF) band therefore defines a Larmor frequency range where a break-down of the pure Zeeman relaxation takes place. The presence of local fields demand special conditions for the magnetic field switching, resulting in any case to a false dispersion respect the Zeeman Larmor frequency. The existence of plateaus and strong dispersions within the ULF band can be explained in terms of physical effects attributed to the local fields, which in turn are closely related to the molecular order. Ultrasonically induced motional narrowing and ODF suppression comes to support this interpretation.

MATRIX EPR INVESTIGATION OF THE CHEMICAL BASES OF RADIATION BIOLOGY

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The chemical bases of radiobiology are related to the damage induced by radiations in key biomolecules, particularly DNA and proteins. Within the time scale of the radiolytic events the earliest analysis are made by pulsed techniques at microseconds and picoseconds resolution. The EPR spectroscopy coupled with low temperature matrix isolation of the reactive intermediates allows even more detailed information to be obtained about the reaction mechanisms. The radiolysis is quenched at the primary stages by performing the irradiation at 77 K and later the process is allowed to evolve outside the radiation field under controlled increasing temperature. The primary and secondary species and their reactions can thus be followed by continuous recording of the EPR spectra. In this work, the results of an investigation regarding the solid state radiolysis of DNA (DNA, DNA bound to nucleoproteins, DNA/Pt(II) complexes) and of sulphur rich proteins (lysozyme and ovalbumine). The experiments performed with DNA/Pt(II) complexes were aimed to selectively modify the ionisation potential of the guanine residues which according to a mechanistic model act as sinks of the positive holes stemming from the ionisation events. The resulting guanine cation-radicals are thought to be the precursors of strand breaks in the DNA structure which are the lethal events for the cell. The results are consistent with the model of long range migration of the holes which is not significantly influenced by a severe modifications of the base stacking structure .

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Electron Spin Echo and ENDOR studies on metalloenzymes
ESEEM Study on F₁-ATP-ase from Bovine Heart Mn(II)-substituted

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ATP-synthase is the enzyme synthesizing ATP from ADP + P_i for any eucariotic cell. The synthesis is taking place in three catalytic sites, which are in the water soluble part of the enzyme, called F₁. This part can hydrolyse ATP and it is called ATP-ase. ATP-ase has a hexagonal structure with six sites, three catalytic and three structural containing nucleotides and Mg (MgADP, MgATP). Mg (II) can be substituted by Mn (II) leaving intact the biological activity. Mn (II) is paramagnetic and allows the use of EPR spectroscopies. ESEEM (Electron Spin Echo Envelope Modulation) and pulsed ENDOR (Electron Nuclear Double Resonance) allow to measure the hyperfine coupling constants with the nuclei surrounding the metal cation.

Till now only plants and bacteria ATP-ase have been studied with ESEEM.

In this work we have studied by ESEEM and pulsed ENDOR the ATP-ase obtained from bovine heart.

F₁ was isolated and treated in such a way to empty of nucleotides and Mg a structural site and two catalytic sites. Then the sample has been added in turn with Mn(II), Mn(II)ADP and Mn(II)AMP-PNP (an analogue non hydrolysable of ATP).

The hyperfine coupling due to ³¹P, ¹⁴N, ¹H have been detected in the samples, both by ENDOR and ESEEM. The information obtained by the two types of techniques are compared and discussed.

USING NMR CROSS-CORRELATION EFFECTS TO DETERMINE STRUCTURAL AND DYNAMICAL INFORMATION ABOUT MACROMOLECULES

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In the last few years, there has been much interest in cross-correlation between the fluctuations of two interactions such as dipole-dipole (DD) couplings and chemical shift anisotropies (CSA). These rates have been exploited to obtain a measure of dihedral χ - and ϕ -angles in proteins (1-3). In general, over-determined measurements will not be compatible with a unique set of dihedral angles χ , ϕ and ω . Degeneracy inherent to these kind of parameters can be removed by measurements of additional rates such as those involving DD/DD and CSA/CSA interactions in successive residues. The effects of conformational exchange on these rates have been investigated (4).

Fast internal motions in the backbone of the protein are usually probed by determining relaxation parameters of the amide nitrogen nuclei. However, these parameters are insensitive to rotation about the NH_N vectors and often cannot distinguish between different models of motions. These problems can be alleviated by measuring cross-correlation rates like $\text{C}'/\text{C}'\text{H}_N$ (CSA/DD) (5).

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Solution ^1H NMR Study of the Magnetic Properties of High-Spin Ferrous Deoxy Myoglobin

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Magnetic Properties of deoxy *sperm whale* myoglobin, in solution, were investigated through ^1H and ^{15}N NMR spectroscopy. The chemical shifts were compared with those of the diamagnetic CO-derivative. The differences provide the pseudocontact shifts, which are related to magnetic susceptibility tensor and to the coordinates of the resonating nucleus. The ^1H pseudocontact shifts were used to obtain the magnetic susceptibility tensor parameters, by fitting the data on the X-ray structures available. $\Delta\chi_{ax}$ was found to be $2 \times 10^{-32} \text{ m}^3$ and $\Delta\chi_{rh} = 1 \times 10^{-32} \text{ m}^3$, the z axis being in the heme plane orthogonal to the proximal His plane and the x axis tilted 35° from the perpendicular to the heme in the proximal His plane. A principal direction orthogonal to the proximal histidine plane appears to be determined by the π bond of the histidine and accounts for the hyperfine shift patterns of the heme methyls in similar systems. The tilt of the other axis from the normal to the heme plane can be the result of low symmetry. This surprising alignment is consistent with the analysis of the residual dipolar couplings, which are due to partial auto-orientation of the molecule at high magnetic fields. In order to rule out the possibility that the present data are the result of some structural reorganization in solution, we checked the consistency of the interproton distances derived from X-ray with pseudocontact shifts and residual dipolar couplings through an algorithm for solution structure determination. These detailed info may be related to the biological function.

PERFLUOROARYLBORON ADDUCTS WITH LEWIS BASES: STRUCTURE AND DYNAMICS IN SOLUTION

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Metal-catalyzed olefin polymerization requires, as a first step, the activation of the catalyst precursor. The activation is due to a species, the cocatalyst, able to transform the catalyst precursor into an active cationic metal site, which is then stabilized through an ion pair interaction. For the economical relevance of the polymerization processes, it is not surprising that many efforts have been made in the recent years to establish the requirements for a good cocatalyst, the nature and the extent of the ion pair interactions, and more generally the structure-activity relationship for catalyst precursor/cocatalyst couples to direct and improve the polymerization process (1, 2).

Many 13th group organometallic compounds are commonly employed as cocatalyst in the activation of group 4 metallocene alkyls. With boron or aluminum based Lewis acids, (A), catalyst activation occurs through the abstraction from the metallocene of an alkyl group, as R^- , giving rise to a coordinatively unsaturated cationic metal site stabilized by the AR^- anion formed accordingly. When trityl or trialkylammonium borate (or aluminate) salts are used, activation occurs by abstractive cleavage or protonolysis of the M-R bond, and the resulting cationic metal site is stabilized by the borate anion.

Perfluoroaryl boranes, like $B(C_6F_5)_3$ (3) and similar derivatives, are very active and effective cocatalysts. However, the water content of the solution must be carefully controlled, for the adducts with water are Brønsted acids strong enough to protonate the alkyl groups bound to the metal center leading therefore to different ion pairs and eventually to cocatalyst decomposition.

We have studied through multinuclear NMR spectroscopy, mainly ^{19}F , the reactions of $B(C_6F_5)_3$ in toluene solutions with different Lewis bases. The nature, the structure and the dynamics of its adducts with water, with nitrogen bases and with some new dimethyl zirconocenes (4) will be presented and discussed.

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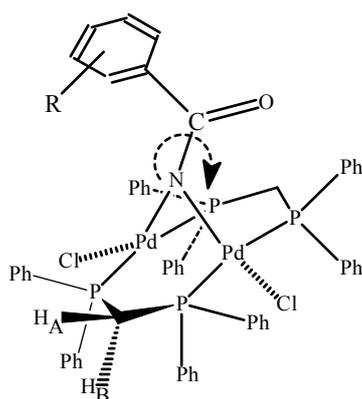
SOLUTION AND SOLID-STATE NMR CHARACTERISATION OF DIMERIC PALLADIUM A-FRAME COMPLEXES

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Several acylimido derivatives have been investigated by multinuclear approach both in solution and solid state. These molecules are of interest because they are likely intermediates of certain catalytic transformations. In solution, apart from the benzamide, they possess C_2



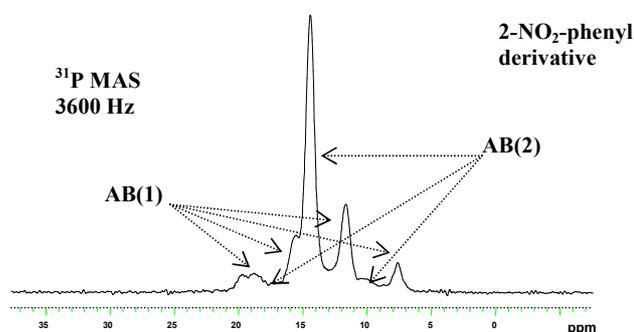
symmetry, four pairs of P-phenyl groups can clearly be distinguished in the proton and carbon spectra. Methylene protons of the P-CH_AH_B-P moiety (dppm) exhibit the AB part of an ABXX' spin system, likewise *ortho* proton pairs display virtual triplets due to the presence of second-order P-P interactions.

Similar AXX' type spin systems are observed in the ¹³C spectrum for the *ipso*, *ortho* and *meta* carbon atoms of the P-phenyl rings and, also for the methylene carbon.

Based on room temperature NOESY and COSY spectra protons of each phenyl group can be assigned unambiguously, furthermore distances of the *ortho* protons from H_A and H_B could be estimated from TOE and

transient NOE experiments. While the overall structure is rigid, all phenyl rings rotate freely at ambient temperatures. The C_2 symmetry axis is perpendicular to the A-frame and goes through the N atom.

The ³¹P spectra are always of second order, sometimes slightly asymmetric, approaching an AA'XX' system. Simulations of the observed patterns carried out so far failed to give satisfactory solution for the coupling and chemical shift values. Hindered rotation of the amide bond is suspected. The amide bond is presumably coplanar with the benzene ring, therefore P atoms of the same chelate ring are not equivalent. Position of the R substituent on the benzene ring has an impact on rotation and therefore on chemical shift difference of the P atoms and also on the observed spin system.



In solid phase, however the C_2 symmetry is lost. We always observed two pairs of AB patterns which represent, as judged from the magnitude of the ²J(P...P) coupling values (they are somewhere between 450 and 490 Hz), mutually *trans* P atoms. ²J(P,P)_{cis} couplings are too small to be observed. Line widths of the phosphorus signals were about 180-200 Hz, what can be attributed to some residual dipolar coupling

between the phosphorus and the quadrupolar Pd atoms. Between 3000 and 10000 Hz the spectra did not show spinning-rate-dependent MAS line shape.

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PARAMAGNETIC Ln-Na COMPLEXES WITH MACROCYCLIC SCHIFF BASES AS SHIFT REAGENTS OF IMPROVED EFFICENCY

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A series of stable Na(I)-Ln(III) complexes with a macrocyclic ligand containing two adjacent cavities, a N₃O₂ Schiff base site and a O₂O₃ crown-ether like site was prepared. The preference of Na⁺ for the polyoxa cavity is the clue for the synthesis of well-defined, stable hetero-dinuclear Ln-Na complexes, where Na⁺ resides in the O₃O₂ chamber and the Ln(III) cation in the Schiff base site. A detailed ¹H and ¹³C NMR study was carried out for both diamagnetic and paramagnetic derivatives. The complexes show a high degree of isostructurality both in methanolic solution and in the solid state. Furthermore, unlike the mononuclear complexes, the [LnNa(L)(Cl)₂(CH₃OH)] complexes are characterized in solution by a marked stereochemical rigidity, as a consequence of the presence of two metal ions into the two adjacent coordination sites. The ²³Na NMR resonance of the bound cation is markedly shifted from that of the free ion by the paramagnetic Ln(III) center. This shift depends upon the geometrical position of the sodium ion relative to the magnetic symmetry axis of the complex and upon the Ln-Na distance and is proportional to a term of the type $(3\cos^2\theta - 1)/r^3$, where θ is the angle between the Ln-Na vector and the magnetic axis of the complex and r is the Ln-Na distance. In the etherodinuclear Ln-Na complexes the distance r is only 3.55 Å, much shorter than that estimated for the corresponding polyaminopolycarboxylic (about 3.9 Å) and polyoxa tetraaza macrocyclic (5.3 Å) complexes. At temperatures above ca. 40 °C a fast exchange occurs between the “free” and “bound” sodium cations and an average signal is observed. All these properties make the etherodinuclear Ln-Na complexes very promising candidates for the development of more effective shift reagents for metal cations of biological importance.

PROTON AND HETERONUCLEI POLARISATION BY PARA-HYDROGEN ADDITION
TO SUBSTRATESSilvio Aime^a, Daniel Canet^b, Roberto Gobetto^a, Francesca Reineri^a, Luca Salassa^a^a *Dipartimento di Chimica I.F.M., Università degli Studi di Torino, Torino, Italy*^b *Laboratoire de Méthodologie RMN, Université H. Poincaré, Nancy, France*

The use of hydrogen enriched in the para spin state (para-H₂) has been recently introduced in the investigation of hydrogenation mechanisms by NMR spectroscopy.

Moreover para-H₂ proved to be a powerful tool in the hyperpolarisation of low sensitive heteronuclei like ¹³C. In fact the pairwise addition of H₂ enriched in the para spin state gives rise to strongly enhanced absorption and emission signals in the reaction products[1]. This phenomenon derives from the non equilibrium population of the spin levels in the product molecule and can be observed if the two transferred protons are magnetically distinct.

Thanks to the enhancement in the product molecules, para-H₂ allows, in principle, the detection of very low abundant species like hydrogenation intermediates [2], however the direct observation of them is not always possible. Another way of using para-H₂ in the investigation of reaction pathways is based on the experimental observation that different patterns are obtained from the same substrates depending on the catalyst[3].

In this contribution we show that on the base of the different hyperpolarised patterns it is possible to find the differences in the hydrogenation pathways of a Rhodium catalyst and the same complex supported on SiO₂. In our case the differences in the polarised patterns can be attributed to the formation of a di-hydride intermediate in which the two para-H₂ nuclei are coordinated to the metal. This species, containing two magnetically distinct protons, reduce the characteristic para-H₂ product operator ($\frac{1}{4}I - I_1I_2$) to the longitudinal two spin order ($\frac{1}{4}I - I_{1z}I_{2z}$). The di-hydride intermediate is formed only with the supported catalyst, while in the homogeneous catalytic pathway the symmetry of the H₂ molecule is not broken before the addition to the substrate. Para-H₂ is the only mean that allows to obtain such information.

As mentioned before another important feature of para-hydrogenation of substrates is the possibility of polarisation transfer to heteronuclei. The enhancement that we have obtained on ¹³C of para-hydrogenated molecules can be very high: we have obtained a 20000 times increased signal for some symmetric alkenes. Taking into account the conditions upon which this increment is possible, we used the deuteration of alkynes for obtaining polarisation both on ¹³C and on ²H.

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New avenues for NMR using cryo-cooled rf-coils and preamplifiers.

Available Probes, Practical Aspects for Installation & Examples of Applications

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NMR spectroscopy is a powerful analytical tool for a large range of applications. So far, the most important limiting factor for NMR was its inherent “low” sensitivity compared to other analytical techniques. In addition, more and more recent NMR applications (such as complex triple resonance experiments or industrial applications like drug screening) require instruments with a high sensitivity and/or allowing a high throughput. Recent Bruker probehead developments show a way out of this dilemma: cryoprobesTM are able to reduce the electronic thermal noise giving roughly an increase in Signal-to-Noise of a factor of 4 compared to conventional probeheads. The actually available probeheads will be presented and some practical aspects for cryoprobe installation and operation will be discussed.

Among the presented applications of cryoprobesTM experimental results for direct H-bond detection for a 16 kDa monomeric enzyme will be analyzed. They have been obtained with conventional spectrometers operating at 600 MHz and 800 MHz, and with a 600 MHz spectrometer using a cryoprobeTM. Conventional long-range HNC0 and TROSY-like approaches will be compared.

NMR METHODS IN THE STUDY OF POLYSACCHARIDES

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Polysaccharides constitute an important class of compounds with interesting properties mostly related to their behavior with water. The NMR methods necessary for their characterization are usually non standard and rather peculiar. The best method to assess their purity is DOSY. Structural determination in solution usually requires sequences using selective pulses or *ad hoc* sequences such as 2D (NOESY-HMQC). Full spectral simulation is also necessary.

Crosslinked polysaccharides are insoluble. Their structure must be obtained using CP-MAS methods. Therefore the cross polarization dynamics must be investigated. Again full spectral simulation is demanded.

Some polysaccharides form hydrogels. Lyophilized and re-hydrated gels can be characterized using relaxometric techniques. In this case an inverse Laplace transformation performed using a "uniform penalty" method leads to a clear visualization of relaxation data. In this way gel forming networks can be clearly differentiated.

HR-MAS methods apply quite well to hydrogels. An example from a thermo-responsive hydrogel will be presented.

FROM GASES TO POLYMERS IN NANOCANNELS: A NMR POINT OF VIEW

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The interest of our group has been focused for several years on molecules (especially macromolecules) as diffused and confined to open nanochannels of 0.5-4 nm. This provides a mean of observing the properties of isolated molecules and small collections of molecules in restricted and anisotropic environments (1).

In this presentation the anisotropic diffusion of gas atoms and molecules within the nanochannels are determined by solid state NMR. For this purpose we prepared: (A) molecular and (B) covalent nanoporous materials.

A) Novel nanoporous crystals have been prepared by self-assembly of molecules (TPP) containing the inorganic cyclophosphazene unit as the central ring and presenting a three-fold symmetry D_{3h} (2). They are stable with 20% open nanochannels constituted by aromatic carbons with 0.5 nm cross-section. The molecular architecture is sustained by weak interactions. Xenon atoms, can quickly diffuse at room temperature into the porous material (3) as isolated atoms running along the channels and are subjected to *single file* diffusion phenomenon (4). The anisotropic NMR signal of xenon-129 learns the axial symmetry of the nanochannels. The anisotropy is pressure dependent because the pressure modulates xenon-xenon interactions against xenon-walls interactions. Hyperpolarization techniques allowed us to study at very high sensitivity 1-2% mixtures of xenon diluted in other gases. In the channels of TPP it is possible to confine active molecules with interesting non-linear optical and electro-optical properties like trans-stilbene, trans azobenzene, diphenylhexatriene and others. The peculiar feature of these new nanocomposites is the absence of close interaction between the active molecules, being wrapped by the walls of the nanochannels. New solid state 2D ^1H - ^{13}C HETCOR sequences, based on the Lee-Goldburg homonuclear decoupling scheme, are applied in order to elucidate the structure of the nanocomposites. The dynamics of polymer chains included in TPP will be also discussed.

B) Nanoporous materials prepared by MCM-41 silica containing polymer chains confined to restricted spaces, produce a special case of organization, mobility and reactivity. The ^{13}C and ^{29}Si characterization of the novel nanocomposites is presented. In particular, ^1H - ^{29}Si cross polarization dynamics in 1D and 2D experiments indicate the close proximity of the protonated organic chains to the silica surfaces: non-protonated Q_4 silicon atoms close to the surface receive effectively the magnetization from the hydrogens of the polymer. The dynamics and solid-solid transitions of the surfactant molecules within the nanopores of MCM-41 show at room temperature a gel-like material with a high fraction of *gauche* conformations. The surfactant molecules exhibit anisotropic motions progressively reduced from the chain ends towards the polar heads (5). The crystallization in the confined state was observed as a *gauche-trans* conformational transition of the hydrocarbon chains due to crystallization occurring at low temperature.

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Synthesis, orientational order and dynamics of deuterated liquid crystalline polymers

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Connectivity of mesogenic monomer units through a macromolecular chain may result in special mesophase structures and transitions of side-chain liquid crystalline polymers. Typically, a flexible spacer segment is used to attach either conventional or chiral (ferroelectric) mesogenic cores as pendent substituents to a polymer chain, which allows for an offset of the conformational disorder of the polymer backbone by the orientational ordering field of the side chains. However, at the microscopic level the interplay between the polymer backbone and the side chains imposes mutual restrictions on the respective conformational and orientational orders and dynamic processes. This is manifested in the occurrence of spatial-temporal heterogeneities over different length scales in polymers that can be detected by several spectroscopic methods. Specifically, ^2H -NMR spectroscopy is a powerful technique to probe the order and dynamics in deuterated liquid crystals.

In this work we use ^2H -NMR spectroscopy to highlight rather peculiar behaviors of side-chain chiral polymers as far as mesophase structure, order and phase transitions are concerned. Such effects will be presented and discussed, with emphasis on the unwinding of the helical structure of chiral mesophases by the magnetic field.

The dynamics was then investigated as a function of temperature in various states of the polymers by ^2H -NMR. We tested different diffusional models that have been developed for low molar mass liquid crystals. The interpretation of the spectral densities of our side-chain polymers by using these models is not simple, and a relatively complex approach for the internal motions had to be considered. This problem is significant and its addressing is necessary for understand the properties of these materials.

CYCLO-OLEFIN COPOLYMERS: INFERRING MICROSTRUCTURE FROM ^{13}C NMR SPECTRA WITH MOLECULAR MODELING

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Ciclo-olefin copolymers (COC) are endowed with interesting physico-chemical properties (as high T_g and transparency) which strongly depend on the microstructure (i.e., not only from the rough composition, but also on the degree of regio- and stereo-regularity), which in turn depends from the catalyst.

The correlation between microstructure and ^{13}C NMR spectra of polymers has been much investigated and some quantitative relationships established; as an example, methods of computational chemistry allowed to extend the well-known Lindeman and Adams' evaluation of C chemical shifts by accounting for conformational effects.

The quantitative interpretation of the spectra would then lead to a better knowledge of the reaction mechanism, thus helping in obtaining polymers of choice

Due to eterogeneity of the polymers (e.g.: Ethylene-Norbornene and Propylene-Norbornene), their NMR spectra are quite complex and a good deal of peaks are only tentatively assigned with standard procedures, asking for more sophisticated analysis and more physical interpretation.

Recently, *ab initio* methods have been exploited in the search for quantitative evaluation of chemical shifts: while absolute values agree only within a few ppm with experimental data, differences due to conformational effects are in nice agreement with the values predicted on the basis of empirical rules previously established (e.g., the so called γ -gauche effect).

Our approach with polymer spectra makes use of combined computational techniques. Molecular-mechanics evaluations with MM2/MM3solv force-fields give conformer energies for suitable model compounds: a RIS treatment of each chain bond yields population statistics for polymer segments. Energy of most stable conformers is further minimised with DFT (B3LYP) at 6-31G** basis set level: chemical shifts of C atoms are then computed for the optimized structures with GIAO using Barone's density functional MPW1PW91 - basis set 6-311+G(2d,p).

The differences in chemical shifts weighted according to the statistical populations, while confirming previous assignments of signals, allow new identifications.

As an example, the considerable splitting (due to the N-N interactions in "defect" sequences as ENNE) between "internal" and "external" carbons of the pairs C1/C4, C2/C3, and C6/C5, is well reproduced. Also the spectra of P/N copolymers have also been rationalised for most of the peaks.

THE RELATIONSHIP BETWEEN PROTEIN SEQUENCE, STRUCTURE AND FUNCTION: A COMPUTATIONAL BIOLOGY CHALLENGE

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In 1953 Rosalind Franklin, Maurice Wilkins, James Watson and Francis Crick published three articles describing the determination of the structure of a molecule of DNA. In 1962 Watson, Crick and Wilkins won the Nobel prize for their discovery (Rosalind Franklin had prematurely died in 1958). The most striking implication of this discovery was the elucidation of the mechanism of DNA replication. But, even if the technical details had to wait about twenty years, the structure also contained the principle that allowed a DNA filament to be sequenced. Even more importantly, the procedure was simple enough that it could be completely automated. In a relatively short time after the Nobel prize assigned to Sanger and Gilbert for the discovery of two different methods for the determination of a DNA sequence, one of the most exciting dreams of scientists could come true: the sequence of the complete genetic repertoire of humans and of many other species has been completely determined.

We have now the possibility of understanding life at a molecular level, of modelling and simulating the behavior of whole cells and of understanding the molecular basis of diseases that we might cure or diagnose at an earlier stage. We might be able to predict the propensity of an individual to a certain pathology and minimize its probability of occurrence or devise methods to delay its onset, we might catalogue individuals according to the probability that they respond to a specific pharmacological treatment, and so on.

All this should be possible using the information contained in a genome sequence and that is the linear sequence of four "letters", the four nucleotides. How can we exploit this information effectively? How can we make sense of this large amount of mono-dimensional information and translate them into the three dimensional complex interconnected picture of a living organism?

The size of the problem makes it unfeasible to tackle it only with experimental methods. I will discuss how computational biology or bioinformatics, the science devoted to the development of appropriate methods to bridge the gap between this linear information and its biological meaning, is trying to help in unraveling the complex relationship between protein sequence, structure and function.

COMPLETE PREDICTION OF THE ^1H NMR SPECTRUM OF ORGANIC MOLECULES BY DFT METHODS

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NMR spectroscopy is probably the most powerful technique for structure elucidation in solution. However, the full understanding and assignment of the ^1H spectrum even of small organic molecules is often plagued with difficulties, since such spectra are often crowded and strongly coupled (second-order).

We present a computational protocol aimed at predicting the major features of a ^1H NMR spectrum by DFT calculations of the nuclear shieldings and spin-spin coupling constants. Such calculated values are used to simulate the ^1H NMR spectra of organic molecules with complicated spin systems, obtaining (see Figure 1) a generally very good agreement with experimental spectra with no prior knowledge of the involved parameters(1). In test cases the performance of several functionals and basis sets has been analyzed, and the various contributions to spin-spin coupling (Fermi-contact, diamagnetic and paramagnetic spin-orbit) have been evaluated. The latter two components cancel each other, so that the calculation of the contact term only is sufficient. The efficiency of is such that these calculations may become a computational aid to the elucidation of the structure of new molecules.

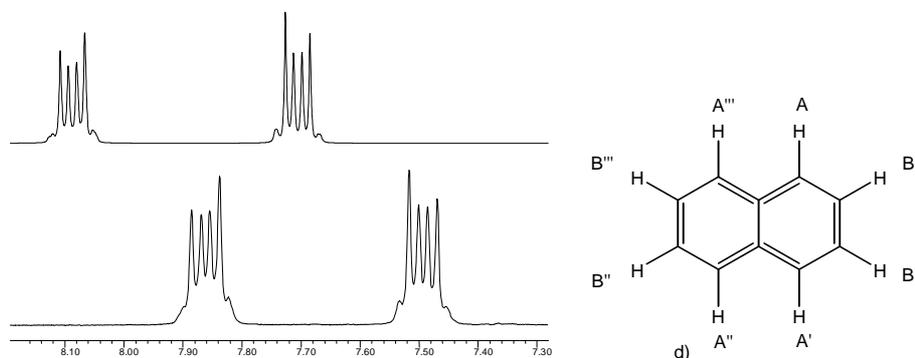


Figure 1. Calculated (top) and experimental (bottom) spectrum of naphthalene (B3LYP/cc-pVTZ//B3LYP/6-31G(d,p)).

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CALCULATION OF NMR PARAMETERS BY COMPUTER SIMULATION

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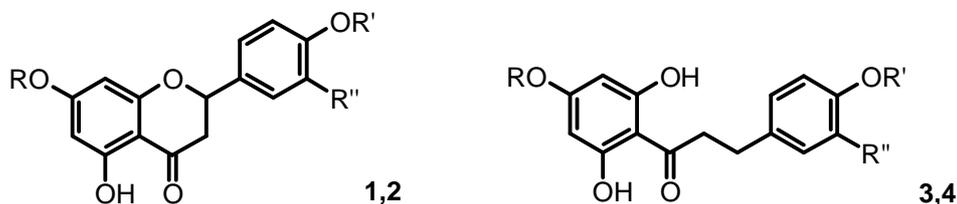
A recent ^{13}C NMR experiment (Smith *et al.*, Nature Struct. Biol. 1996, 3, 946-950) on the Asp25-Asp25 dyad in pepstatin A/HIV-1 protease measured two separate resonance lines, which were interpreted as being a singly protonated dyad. We have addressed this issue by performing *ab initio* molecular dynamics calculations on models for this site accompanied by calculations of ^{13}C NMR chemical shifts and isotopic shifts. Our calculations suggest point to a different protonation state in which both aspartic groups are protonated. Despite the symmetric protonation state, the calculated ^{13}C NMR properties are in good agreement with the experiment. The pepstatin A binding to the diprotonated form are consistent with the inverse solvent isotope effect on the onset of inhibition of pepsin by pepstatin and the kinetic iso-mechanism proposed for aspartic proteases (Cho, T.-K.; Rebholz, K.; Northrop, D.B. Biochemistry 1994, 33, 9637-9642).

STRUCTURAL INVESTIGATION OF THE β -CYCLODEXTRIN INCLUSION
COMPLEXES OF SOME FRUIT INGREDIENTS: DIFFERENT COMPLEXATION
BEHAVIOUR OF BITTER AND SWEET COMPONENTS

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Naringin **1** and neohesperidin **2** are flavonoid glycosides constituent of citrus fruits occurring principally in the peel. Their main characteristic is the **bitterness** which can be detected at very low concentrations. For this reason high quantities of such substances are undesirable in citrus juices and various methods are used to lower the level of these flavonoids in their preparation. Among them direct addition of β -cyclodextrin (β CD) to the juices or their treatment with insoluble β CD polymers are reported.



- 1 R= α -L-Rha-(1-2)- β -D-Glc; R'=H; R''=H (Naringin)
- 2 R= α -L-Rha-(1-2)- β -D-Glc; R'=CH₃; R''=OH (Neohesperidin)
- 3 R= α -L-Rha-(1-2)- β -D-Glc; R'=H; R''=H (Naringin dihydrochalcone)
- 4 R= α -L-Rha-(1-2)- β -D-Glc; R'=CH₃; R''=OH (Neohesperidin dihydrochalcone)

In contrast with the bitter **1** and **2** the corresponding dihydrochalcones (DHC) **3** and **4** are sweet. In particular neohesperidin DHC **4** is very sweet and finds many applications. The structural difference between **1-2** and their DHC derivatives resides in the link between the aromatic rings which is a dihydrobenzopyran ring in the native glycosides and an open chain in the DHC derivatives. Such structural variation produces a different behaviour with respect to the taste receptor, i. e. **from bitterness to sweetness**. We will show that compounds **1-2** and their DHC derivatives behave differently also in their mode of complexation with β CD thus suggesting that the nature of the link is crucial in determining the molecular recognition mechanism of molecules of this type.

Methods and results. The stoichiometry was determined from Job plots built up from the chemical shift variations of suitable signals. The complex geometry was obtained from bidimensional or monodimensional ROE spectra.

For all complexes the stoichiometry is 1:1. The intermolecular NOE contacts show that the inclusion involves the aromatic rings of the guests. The complexation occurs from the side of the larger rim of β CD truncated cone for naringin **1** and neohesperidin **2** while it occurs from the opposite side, i.e. from the narrower rim, for the dihydrochalcones **3** and **4**. The interactions contributing to the stabilisation of the different geometries, in particular that between the disaccharidic unit and the external surface of β CD, are discussed.

A QUANTUM CHEMICAL STUDY OF THROUGH-SPACE SPIN-SPIN COUPLING IN VAN DER WAALS DIMERS AND CH/ π INTERACTING SYSTEMSAlessandro Bagno, Giacomo Saielli and Gianfranco Scorrano

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Recently, non-negligible through-space nuclear spin-spin coupling constants have been predicted, at various levels of theory, for simple van der Waals dimers of noble gases, He \cdots He, Xe \cdots Xe and Xe \cdots H (1). Although the results for these simple systems certainly prove the existence of through-space spin-spin couplings it is desirable to extend such investigations to more realistic models and, eventually, real molecules. Therefore, we have investigated the through-space J_{HH} and J_{CH} spin-spin coupling constants of model van der Waals dimers, involving methane, ethylene and benzene, and of selected compounds showing the CH/ π interaction, by means of DFT and *ab initio* calculations. In the range of intermolecular separations for which the interaction is stabilising, weak couplings (0.1–0.3 Hz) are predicted for J_{CH} , while the corresponding J_{HH} couplings are much smaller (2). The relative contributions (Fermi-contact, spin-orbit and spin-dipole) are strongly dependent on the geometry of the dimers and on the distance; the non-negligible values of J_{CH} for π systems stem largely from an incomplete cancellation of spin-orbit terms.

The results obtained for larger molecules, by necessity only at the DFT level of theory, as the aryl ester shown in the figure, are consistent with those on the model dimers. For the aryl ester, the occurrence of a through-space mechanism for the transmission of coupling is established by examining trends in the magnitude of couplings as a function of the number of intervening covalent bonds (3) for the *extended* and *folded* conformer.

Currently we are also exploring the possibility of through-space spin-spin coupling in van der Waals dimers between Xenon and organic molecules. These results may be of importance due to the extensive use of Xenon as an NMR probe in various environments.



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IN VIVO MAGNETIC RESONANCE SPECTROSCOPY:
FROM BIOCHEMISTRY TO MOLECULAR DIAGNOSTIC

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Magnetic Resonance Spectroscopy (MRS) is a non invasive tool allowing the *in vivo* study of biochemical processes. Several nuclei have been using so far: ^{31}P , ^1H , ^{13}C , ^{19}F ; although mainly phosphorus and proton are extensively used to assess the functionality of different metabolic pathways in living tissues. Typically, ^{31}P -MRS is performed to examine the energy metabolism of brain and skeletal muscle and hence the cellular mitochondrial functionality of the tissue under investigation. In other word, by measuring the concentration of the main phosphorous metabolites involved in the cell energy transductions such as ATP, Pi, phosphocreatine (PCr), ADP, phoshomonoesters, it is possible to assess the capability of the cell to produce the energy required for its functioning. In addition, ^{31}P offers the possibility to measure the intracellular pH and free $[\text{Mg}^{2+}]$, giving also information on the cellular ionic metabolism.

^1H MRS, is performed almost exclusively on brain, where the detectable metabolites are: N-acetyl-aspartic acid (NAA), creatine-phosfocreatine (Cr), choline-containing compounds (Cho), mio-inositol (mI), and lactic acid (Lac). Each of these molecules can be regarded, in first approximation, as marker of specific metabolism: NAA as neuronal marker, Cho as a glial cellularity marker, MI is involved in cell osmoregulation and Lac is a marker of ischemic processes.

Therefore, any abnormal metabolism involving directly or indirectly the above molecules is, in principle, assessable by ^{31}P and/or ^1H MRS. In this light MRS offers a powerful diagnostic adjunct able to disclose biochemical defects underlying or even preceding the pathological status in living tissues.

**BIOENERGETIC AND ENZYMATIC ASPECTS OF KIDNEY DAMAGES:
AN NMR AND HISTOCHEMICAL STUDY**

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Ischaemia and drug toxicity are among the most important causes of kidney damages and have relevant clinical implications: renal ischaemia is an issue for the transplantation and toxicity is an issue for the chemotherapy.

Before the implant the kidney remains in ischaemic conditions up to 72 hours at low temperature in a suitable preservative medium like (EC) or (UW) and histology demonstrates just modest signs of tubular necrosis or of glomerular lesions. The ³¹P NMR of intact rodent kidney instead shows marked differences between the media: in EC the catabolism of the phosphorylated metabolites toward the end-product Pi is slower than in UW and most of the Pi remains inside the organ while, in UW, most of the Pi is exported. In EC phosphorylated metabolites exchange slowly on the NMR time-scale among the organ compartments, while in UW the exchange is faster and gives rise a resolved spectral pattern. The cytosolic pH, evaluated through the Pi chemical shift, in UW appears to be a unique fairly defined value (7.2 ± 0.1) while in EC the apparent pH spans over a range of 1.7 units. GPC and GPE levels remains higher in EC than in UW suggesting that EC preserves better than UW the renal medulla. In UW, NAD(H) and NADP(H) are depleted in about 30 hours, while in EC, after 72 hours the 50% of their initial levels are still visible. This depletion is confirmed by the NADPH diaphorase reaction demonstrating NOS. Moreover, in UW, the PME region shows an higher level of 1–6 fructose diphosphate than in EC. These two pieces of informations are related and indicate that apoptosis is taking place in UW. Lastly, being NAD(H)/NADP(H) precursors of the PARP, their depletion deprives the kidney of the substrates for the DNA repair and for the organ regeneration. Thus we may infer that EC shows a more favourable protective effect on cortical and medullary mitochondria and on the kidney regenerative capability.

Similar situation occurs in kidney of rodents treated with a potent anticancer drug (cis-Pt): the ³¹P NMR spectrum shows similarities with the spectrum of the ischaemic kidney in UW. In this case the histochemical techniques are more informative giving hints on specific targets of the drug toxicity. This is reasonable: the ischaemic kidney is morphologically well preserved while the kidney of a cis-Pt treated rat is severely damaged in the S3 segment of proximal tubules, as demonstrated by morphological observations and histochemical studies. Significant alterations occur also in the distal tubule where increased activities of both K⁺ pNPPase and SDH are apparent and in the macula densa where NOS activity is absent.

Na⁺/K⁺ ATPase, acting as a sodium pump, consumes ATP as energy source. Therefore, it can be reasonably supposed that tubules with strong Na⁺/K⁺ ATPase activity consume more ATP. Cisplatin impairs mitochondrial functions and the SDH suggests that mitochondria are altered in those tubules displaying weak SDH activity. In other tubules an increased SDH activity is observed because of the increased energy demand to support the Na⁺/K⁺ dependent pump.

Comparing these two cases of renal damages we may conclude that when the kidney is morphologically and ultrastructurally well preserved the ³¹P NMR spectroscopy is quite informative and the histochemical techniques are of use to explain the observed features. But, in case of severe morphological and ultrastructural alterations the ³¹P NMR may be of use just to support the histochemical findings.

MRI IN ONCOLOGY RESEARCH: EXPERIENCE WITH “IN VIVO” MODELS AND MONITORING OF THERAPEUTIC RESPONSE

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Magnetic Resonance Imaging (MRI) is a non-invasive imaging technique, which is widely used in the field of medical imaging with great potentials for the diagnosis and therapy of human tumors in both the clinical and experimental setting. Very similar methods of assessment can be applied to humans and animal models, making MRI a unique tool in preclinical studies. By using a dedicated system for small animals it is possible to visualize solid tumors *in vivo*, follow their progression and monitor response to therapeutic interventions. Data will be presented on morphological characterization of tumor models, with the *in vivo* MRI findings validated with histology. Changes in tumor growth rate or volume are usually the first indication of treatment success, but these changes occur late in the course of therapy and the discovery of an early indicator of treatment response would be of great value for experimental and clinical trials. There is also the need to understand more about tumor internal morphology and changes that occur during tumor growth and regression. Examples of the identification by MRI of tumor response to pharmacological treatment will also be shown. In addition to a nice visualization and anatomical insight, MRI is also capable of quantifying a variety of physiological and “functional” parameters. By using MR contrast agents, for example, valuable information can be obtained on microvasculature features, such as density and permeability of blood vessels and microcapillaries. Tumor characterization after injection of a contrast agent is based on differences in the spatial distribution of the rate and extent of contrast enhancement, which reflects changes in the density and permeability of blood vessels and microcapillaries.

Recent results will be presented showing a reduction in vascular permeability caused by an anti-angiogenesis compound (SU6668) and detected by contrast enhanced MRI as early as 24 hours after treatment.

STRUCTURAL AND FOLDING STUDIES ON LIPOCALINS

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The lipocalins, the fatty acid binding proteins (FABPs) and the avidins are three families of hydrophobic ligand binding proteins which together form the calycin superfamily. Lipocalins and FABPs share a related beta-barrel structure. Lipocalins are mostly extracellular proteins displaying a wide variety of biological functions, while FABPs are predominantly intracellular proteins involved in lipid metabolism. Beyond some functional similarities (hydrophobic ligand binding) these families are characterised by a similar folding pattern (an antiparallel β -barrel dominated by a largely +1 topology), within which large parts of their structures can be structurally equivalenced, although the families share no global sequence similarity.

We have investigated and are investigating the structural, folding and binding properties of beta-lactoglobulins from different species, which are important representatives of the lipocalin family. Recently we have undertaken the structural characterization of Chicken Liver Basic FABP, belonging to the basic type FABPs.

A comparative analysis of the structural, folding and interaction properties of these proteins is currently in progress in our laboratory and will be presented here.

Modified Sequences in the HIV-1 gp160 Cleavage Site Investigation.

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Proteolytic activation of the HIV-1 envelope glycoprotein precursor gp160 is a prerequisite for the virus infectivity, as the mature glycoproteins gp120 and gp41 play a key role in the fusion of viral and cellular lipid membranes. Furin and other proproteins convertases (PCs) selectively cleave the HIV-1 gp160 at the carboxyl side of the sequence R508-E-K-R511 (site 1), in spite of the presence of another consensus sequence: K500-A-K-R503 (site 2). We already reported on the solution structural analysis of the 19-residues synthetic peptide p498, spanning the gp160 sequence P498-G516 around the processing sites 1 and 2, and properly digested by furin at the site 1. There we found a N-terminal helix, enclosing the site 2, and a C-terminal loop, exposing the physiological site 1.^[1] A loop had already been suggested as a common structural feature for physiological cleavage sites in retroviral envelope glycoproteins.^[2] We hypothesized that the N-terminal helix, enclosing the secondary processing site 2, could play a key role in regulating the exposure and accessibility of the loop containing the physiological cleavage site 1.

A series of modified sequences were then designed and synthesized to exhibit:

- a) at the C-terminal side, the native gp160 sequence: R508-G516, containing the site 1;
- b) at the N-terminal side, helical or random model sequences.^[3,4]

Furthermore, mutations in proline residues were introduced in positions P3 and/or P2' as structural constraints. Digestion experiments with furin, PC5 and PC7 were performed on the synthetic analogues. Here the NMR conformational analysis of the peptides and the homology models of the proteolytic enzymes are presented and related to the different exhibited activities.

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Ni(II) AND Cu(II) BINDING TO A 20- AND 30-AMINOACIDS SEQUENCE OF CAP₄₃ PROTEIN

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A novel gene Cap43 was specifically induced by Ni(II) compounds. Cap43 expressed a 3.0-kb mRNA encoding a Mr 43,000 protein where 10 aminoacids sequence TRSRSHSTSEG was three times repeated on the C-terminal part of the protein. The protein was not found in the nucleus, but was localized in the perinuclear region and cytoplasm. (1, 2) Although Ni(II) is known to induce heat shock proteins (HSPs), metallothioneins (MTs) and acute phase reactant proteins (APRs), none of these genes were specific to nickel and other heavy metals such as cadmium, mercury, lead and zinc also induced these genes. (3) It has been suggested that the function of these three families of genes is in the detoxification and protection against oxidative stress induced by metals. Most of them have been proven to contain metal-binding domains. For example, MTs have high affinity for metals due to their high cysteine content (4); purified *Limulus* C-reactive protein, a member of the APR family, has sulphhydryl groups that bind strongly to mercury (5) and HSP_A from *Helicobacter pylori*, features a series of cysteine and histidine residues, resembling anchoring binding site for metal ions. (6) Although, Cap43 had no cysteine or histidine-rich motifs for metal binding, it had a mono-histidine fragment, 10 aminoacids (Thr-Arg-Ser-Arg-Ser-His-Thr-Ser-Glu-Gly) repeated three times, resembling a nickel binding motif at the COOH terminus.

The finding of the Cap43 protein makes it an interesting candidate for studies of molecular mechanisms of nickel carcinogenesis.(7) Ni(II) is able to bind histidines in the 20- (TRSRSHSTSEG-TRSRSHSTSEG) aminoacids sequence giving NiH₃L and Ni₂H₆L as the major species from the reaction 1:1, 1:2 (L:Ni(II)), respectively; with the 30-aminoacids sequence (TRSRSHSTSEG-TRSRSHSTSEG-TRSRSHSTSEG) species NiH₃L, Ni₂H₆L and Ni₃H₉L and Ni₃H₆L from the reaction 1:1, 1:2, 1:3 (L : Ni(II)), respectively, were obtained.

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NEW TECHNIQUES FOR INCREASING THE SENSITIVITY OF NMR EXPERIMENTS FOR ORGANIC MOLECULES STRUCTURE DETERMINATION

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It is well known that even though NMR is the most powerful technique for the structure elucidation of organic compounds, it suffers from very low sensitivity. In recent years several new techniques have appeared that increase the sensitivity of NMR experiments by almost one order of magnitude. These techniques are:

- a. The use of small volume probes like the nano probes. In these probes the sample is confined in a small volume and spun at very high speeds at an angle to the magnetic field.
- b. The use of cryogenically cooled probes. In these probes the receiver coil is cooled by gaseous He to temperatures around 25 K. This way the thermal noise on the coil is reduced and the overall signal to noise ratio is increased.
- c. The use of specialized software. Software that searches the multidimensional spectra with very strict and well-defined mathematical algorithms in order to identify correlation trends is used to bring up peaks that are very low to the noise level.

A review of these new developments as well as examples of structure elucidation of organic natural products will be presented.

POSTERS

EXAMPLES OF APPLICATION OF SELECTIVE EXPERIMENTS TO STRUCTURAL ELUCIDATION OF SMALL ORGANIC MOLECULES

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For structural elucidation of many organic compounds, 2D experiments can often be replaced by their one-dimensional (1D) counterparts, which use selective excitation of the resonance of interest (1-3).

This approach is attractive when only a restricted amount of spectral information is required but may also have advantages in terms of simplified structural assignment/identification, shortened acquisition and processing time, and increased sensitivity for low-level components of a mixture. Moreover selective excitation experiments are easier to interpret and allow precise determination of chemical shifts and coupling constants especially in case of signal overlap.

Examples of application of different 1D selective experiments (all working with SLP), together with some practical aspects, will be discussed.

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NMR STUDIES ON COPPER COMPLEXES OF AMINOGLYCOSIDIC ANTIBIOTICS

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Aminoglycosides form a very important class of antibiotics, particularly active against Gram-negative bacteria. The mechanism of their action has been studied very extensively since the discovery of streptomycin, their first representative.

Aminoglycoside antibiotics, such as kanamycin A and B, amikacin, tobramycin, etc., interfere with protein synthesis by binding to the ribosome and perturbing several steps of translation. The role of transition metal ions in the biological activity of aminoglycosidic antibiotics is not completely understood. Recently several studies have shown that Cu(II) complexes of aminoglycosidic and related antibiotics present oxidative properties, that may contribute to biological mechanisms of their antibacterial activity.

Aminosugars bind Cu(II) quite effectively: the anchoring of the cupric ion to their molecules is accomplished through amine functions. The stability and stoichiometry of resulting complexes depend, however on the formation of chelate rings using appropriate hydroxyl groups of the ligand. Therefore, aminoglycosides can be a very interesting model for studying the Cu(II) coordination and reactivity preferences.

In this communication, we report NMR studies that allow a comprehensive delineation of structure and dynamics in water solution of the Cu(II) complexes of kanamycin A and lincomycin, an antibiotic structurally similar to aminoglycosides.

NMR INVESTIGATIONS OF SAPONINS AND SAPOGENINS FROM *MEDICAGO* SPECIES

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Triterpenic saponins from *Medicago* spp. are attracting the attention of researcher not only for their implication in animal nutrition, but recently also as biologically active substances (1).

The base structure of these saponins is the 2 β -hydroxy Δ^{12} oleanane skeleton (C₃₀); variations of the base structure are due to the substitution of the hydroxyl and carboxyl groups on the molecule (see figure below where R, R₁, R₂, R₃, R₄, R₅ = H, OH, COOH or sugars). Several sugars and sugar chains can be present.

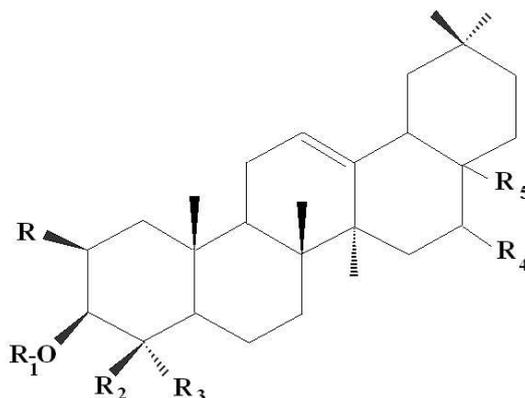
The problem of structure determination of these biomolecules has been carried out with several techniques including hydrolytic studies followed by the characterization of the aglycone and oligosaccharide moieties. NMR techniques can be used in all the steps of the structural investigation as well as in the determination of the complete picture of the saponin structure, with or without prior structure knowledges.

The NMR chemical shifts and coupling constants are the easiest parameters to be measured for the saponins and they have been recognized to carry a wealth of structural information.

Several saponins were extracted and purified from *M. sativa*, *M. arabica*, *M. arborea* and *M. hybrida*, and then analysed, after their dissolution in Py-*d*₅, using conventional NMR techniques (1H, 13C, DQF-COSY, HSQC, HMBC, 1D-TOCSY, 2D-HOHAHA, ROESY).

The following aspects were investigated:

- 1- Determination of the molecular structure of the triterpenic sapogenin moiety (substitution pattern and stereochemistry)
- 2- Determination of the molecular structure of the oligosaccharide moiety (number and type of monosaccharide residues, interglycosidic linkage and sequence, localization of the sapogenin-oligosaccharide linkage).



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Conformational Stereodynamics of Tetraethylmethane and Analogous C(CH₂X)₄ Compounds

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Variable temperature NMR studies of tetraethylmethane (**1a**), tetrapropylmethane **1b**, tetrachloromethylmethane **1c**, tetrabromomethylmethane **1d**, tetracyclopropylmethylmethane **1e**, and tetrabenzylmethane **1f** show a range of dynamic behaviour. Separate signals for two types of conformation are observed for **1a**, **1c**, and **1d** at low temperatures, with more than 95% of molecules in a time-averaged D_{2d} conformation, and the S₄ conformation as the minor populated alternative. Compound **1e** populates only S₄ type conformations but equilibrates slowly between degenerate versions of these at low temperatures. Compounds **1b** and **1f** show a temperature dependent spectrum but the low-temperature limit spectrum could not be observed. *Ab initio* calculations agree well with experiment on the conformational equilibria and suggest in particular that compounds **1b** and **1f** behave similarly to compounds **1a** and **1e** respectively. A crystal structure of compound **1f** is reported (2).

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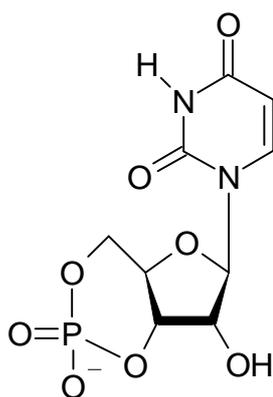
HETERONUCLEAR NMR PARAMETERS OF ORGANIC MOLECULES:
REPRODUCING COMPLEX SPECTRAL PATTERNS BY MEANS OF DENSITY
FUNCTIONALS METHODS

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High-field NMR spectrometers are nowadays giving a fundamental contribution to the study of complex organic molecules, both by providing high-resolution spectra and by simplifying the outcoming spectral patterns. However, these patterns can still be so difficult to rationalize that they are often taken as “fingerprints”, discarding useful underlying informations.

Following the results of previous works (1), we have investigated the ability of density functional theory (DFT) calculations to provide accurate heteronuclear spectral parameters (${}^nJ_{CH}$ and δ) which can be used to reproduce hetero-correlated and J -resolved ${}^1\text{H}$ - ${}^{13}\text{C}$ spectra. DFT calculations have been performed at various level of theory: B3LYP/cc-pVTZ, using Gaussian98 (2) program, which allows the calculation of the shielding constants and the Fermi Contact (FC) contribution to the spin-spin coupling constants; PW86/IGLO-III, using deMon-NMR (3) program, which allow the calculation of shielding constants and, in addition to the FC term, the diamagnetic and paramagnetic spin-orbit contributions to the coupling constants.

The surveyed model-systems include a series of dichlorophenols (2,3- 2,4- 2,5- and 3,4-) along with *o*-bromochloro benzene, *o*-dichloro benzene, furan and naphthalene. For each of these species, heteronuclear J -resolved spectra were collected and the measured ${}^nJ_{CH}$ were compared with their DFT-calculated values. Good agreement between experimental and calculated data have prompted us to also study a more complex molecule of biological relevance such as cyclic 3'-5' uridine monophosphate (cUMP). Moreover, since cUMP in aqueous solution has (at least) two exchanging conformations, care has been taken in order to account for this structural “flexibility”, as it is well known that small conformational changes can produce significant shifts in terms of J and δ values.



cUMP

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**A ^1H AND ^{13}C NMR STUDY OF MEISENHEIMER-TYPE ADDUCTS FROM
2-NITRO- AND 3-NITRO-BENZO[*b*]THIOPHENES**

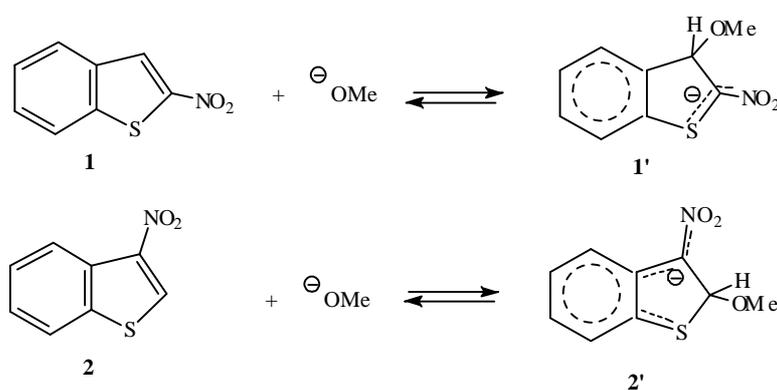
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2-Nitrobenzothiophene **1** and 3-nitrobenzothiophene **2** are found to react with sodium methoxide in either DMSO-*d*₆ or methanol-*d*₄ to give Meisenheimer-type adducts **1'** and **2'** respectively.



The formation of the adducts is accompanied by:

- (i) a 47÷48 ppm shielding for the carbons switching from sp^2 to sp^3 hybridization;
- (ii) a marked shielding for the carbons bearing a nitro group (41 ppm for adduct **1'** and 23 ppm for **2'**) which has been interpreted (1) on the basis of a marked fractional negative charge on the relevant carbons;
- (iii) only slight changes in the other ^{13}C chemical-shift values.

The possible interdependence between chemical-shift changes (ii) and (iii) (and then the corresponding π -electron-density changes) and the stability constants of the relevant adducts will be discussed in the poster.

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3₁₀-HELIX IN AIB-RICH PEPTIDES CONTAINING LACTAM-BRIDGES SIDE CHAINS: A CONFORMATIONAL STUDY, BY CD AND NMR, IN TFE

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Aib-rich side chain lactam-bridges oligomers Ac-(Glu-Aib-Aib-Lys)_n-Ala-OH with n=1,2,3 were designed and synthesised as putative models of the 3₁₀-helix. The lactam bridge between the side chains of L-Glu and L-Lys in (i)-(i+3) position was introduced in order to enhance the structural preference toward the right-handed 3₁₀-helix. The peptides conformation was previously characterised in aqueous solution containing SDS micelles by CD, NMR and computer simulations (1). The NMR results clearly indicated that there is an increase of 3₁₀-helix formation upon chain elongation.

In this work, we have been studied the Aib-rich peptides in TFE solvent.

The study of Aib containing peptides by NMR suffers from several disadvantages. The lack of scalar coupling between protons precludes the use of homonuclear scalar correlation spectra. Furthermore, the very narrow range of resonances of the non-exchangeable protons causes severe overlap problems in any two-dimensional spectrum. Hence, the complete resonances assignment for the analogs, was reached by means of heteronuclear long-range coupling and taking advantage of selective excitation, allowing us to overcome the two aforementioned problems.

The complete assignment of all resonances was achieved and structural information has been derived mainly from the analysis of NOESY spectra. The detection of all the possible sequential NH-NH connectivities in the NOESY spectra of each peptide is a strong indication of the presence of a helical structure even in the shorter peptide. The presence of $\alpha\text{N}(i, i+2)$ or $\beta\text{N}(i, i+2)$ and of $\beta\text{N}(i, i+3)$ cross-peaks and the absence of $\alpha\text{N}(i, i+4)$ or $\beta\text{N}(i, i+4)$ indicate that these peptides fold into a 3₁₀-helix rather than into a α -helix. Once the conformational features of the peptides were determined, stereospecific assignment of the Aib group methyls was possible.

The results of such experiments and of the subsequent MD demonstrated that, in trifluoroethanol, the 3₁₀-helix is well defined and stable in all the oligomers.

The CD spectra of the oligomers are similar each other but differ from the CD spectra reported for this secondary structure.

DOSY DETECTION OF A π - π COMPLEX IN SOLUTIONStéphane Viel,^a Luisa Mannina,^{a,b} and Annalaura Segre^a^a Istituto di Metodologie Chimiche, C.N.R., C.P. 10, 00016 Monterotondo Stazione, Rome, Italy^b Università degli Studi del Molise, Facoltà di Scienze MM.FF.NN., Dip. S.T.A.T., Via Mazzini 8, 86170 Isernia, Italy

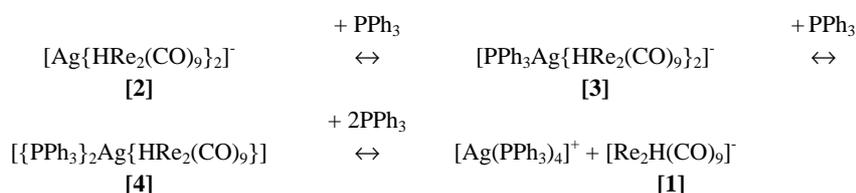
The formation of complexes through π - π stacking interactions is of the utmost importance in the study of biological systems and in organic chemistry. NMR-based techniques used for the study of such interactions have mainly relied on chemical shifts variations and on NOE effects (NOESY and ROESY). Here, a new way of detecting the presence of π - π stacked complexes in solution by means of diffusion-ordered NMR spectroscopy (DOSY) is reported. Diffusion-ordered NMR (DOSY) experiments yield 2D spectra with NMR chemical shifts in one dimension and diffusion coefficients in the other one; therefore, they allow the resolution of compounds with respect to their effective size. DOSY has been used in the investigation of the behaviour of a specific herbicide, Metolachlor, in aqueous solutions. Metolachlor is an optically active molecule that has a low solubility in water. Its molecular structure bears an aromatic ring which, in particular conditions, can give rise to π - π stacking interactions. In aqueous solutions, at concentration higher than the reported solubility, Metolachlor molecules tend to group together and form π - π stacked complexes of high molecular weight. The presence of such high molecular weight complexes revealed by DOSY is confirmed by means of a set of NOESY experiments recorded at various mixing times.

**^1H , ^{109}Ag AND ^{31}P NMR INVESTIGATION OF ADDUCTS BETWEEN
 $[\text{NET}_4][\text{Re}_2\text{H}(\text{CO})_9]$, PPh_3 AND SILVER(I).**

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The ability of the monodentate hydrido-carbonyl rhenate $[\text{Re}_2\text{H}(\text{CO})_9]^-$ to react with the group 11 electrophiles (either as M^+ or MP^+) has been recently established in our laboratories. Stable adducts like $[\text{Ag}\{\text{HRe}_2(\text{CO})_9\}_2]^-$ **[2]** and $[\text{Ph}_3\text{P}(\mu\text{-H})\text{Re}_2(\text{CO})_9]$ have been characterised both in solution and in the solid state. We report here on the competition reactions between hydride and phosphine ligands towards silver cation (1), used as AgOTf ($\text{OTf} = \text{CF}_3\text{SO}_3$). Changing the stoichiometric ratios between the three starting reagents, $[\text{NET}_4][\text{Re}_2\text{H}(\text{CO})_9]$ **[1]**, AgOTf and PPh_3 , we observed the formation in THF of several species in which the central silver atom is found to be tri- or tetra-coordinated, due to the following equilibria:



Scheme 1

Even at low temperature the ^1H NMR spectrum showed a series of broad resonances that could be partially resolved at 156 K. Only through $[\text{H}-^{109}\text{Ag}]$ HMQC experiments the different species present in solution were identified. These experiments allowed the formulation of compounds **[3]** and **[4]**, since the first one exhibits a doublet along the F1 axis and the latter one a triplet (respectively one and two PPh_3 bonded to Ag), and these couplings were lost when the same 2D $[\text{H}-^{109}\text{Ag}]$ HMQC experiments were performed decoupling ^{31}P during the acquisition period or during all the pulse sequence. A modified $[\text{H}-^{109}\text{Ag}]$ HMQC experiment (2) confirmed the number of hydrides directly bonded to silver in **[3]** and **[4]**. Trigonal co-ordination of silver was also suggested by the values of the coupling constants between $^1\text{H}-^{109/107}\text{Ag}$ and $^{31}\text{P}-^{109/107}\text{Ag}$ (3), observable only at low temperature due to the high kinetic lability of monodentate phosphine ligand (4). Spectra of samples obtained with different experimental procedures, recorded at different temperatures, showed that the equilibria depicted in **Scheme 1** are concentration and temperature dependent. At the lowest temperature, the main hydridic species is the free ligand **[1]**. At room temperature, the presence of free PPh_3 in the ^{31}P NMR spectra suggested that, on rising the temperature, the equilibria are shifted to left and the main hydridic species becomes **[2]**. ^1H and ^{31}P NMR variable temperature experiments showed the presence of dynamic processes involving all the Re-Ag species, that eventually gave rise, in the ^1H spectra at room temperature, to a single broad resonance due to fast exchange between all the species. The comparison between the hydridic regions of 1D spectra in the range 183-156 K indicated that the $[\text{2}] \leftrightarrow [\text{3}]$ exchange rate is higher than the $[\text{3}] \leftrightarrow [\text{4}]$ rate.

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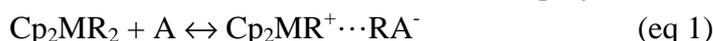
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SOLUTION STRUCTURE AND DYNAMICS OF THE ION PAIRS [(4,7-Me₂Ind)₂ZrCH₃]⁺[CH₃B(C₆F₅)₃]⁻ AND [*meso*-C₂H₄(4,7-Me₂Ind)₂ZrCH₃]⁺[CH₃B(C₆F₅)₃]⁻

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In the last decade many B or Al containing Lewis acids (e.g. B(C₆F₅)₃ (1, 2)) have been extensively used as activators of the metallocene single-site catalysts for olefin polymerisation (2, 3). The Lewis acid (A) extracts an R⁻ group from the metallocene giving an ion pair with a coordinatively unsaturated cation (eq 1) whose stability, structure in solution and dynamics greatly influence the effectiveness and characteristics of the polymerisation process.



Previous studies (3e) have shown the occurrence in solution of two dynamic processes. The first one is the dissociation of the RA⁻ anion followed by terminal R group flipping and the cation-anion reassociation on the opposite side of the molecule (**ips** = ion pair separation). The second one involves the migration of the Lewis acid A from one R group to the other (**dr** = dissociation-recombination). These two mechanisms can be distinguished by the 2D ¹H EXSY experiment. Indeed, while the **ips** mechanism produces exchange cross peaks only between signals of the π ligand, the **dr** one leads also to the exchange of the σ bound R groups.

In this work we have investigated by multinuclear NMR the solution structure and the dynamics of the ion pairs formed in toluene-*d*₈ by the interaction of B(C₆F₅)₃ with (4,7-Me₂Ind)₂Zr(CH₃)₂ (**1**) and *meso*-C₂H₄(4,7-Me₂Ind)₂Zr(CH₃)₂ (**2**).

For compound **1** the solution structure has been determined by 2D ¹H NOESY experiments at low temperature (200 K), where dynamic processes are frozen on the NMR time scale. ¹H band shape analysis and 2D ¹H EXSY experiments showed that at temperatures in the range 243-300 K only the **ips** mechanism is active, while the **dr** mechanism becomes NMR detectable at temperatures higher than 300 K. The activation parameters for the **ips** mechanism (ΔH[‡] = 66 (1) kJ mol⁻¹ and ΔS[‡] = -3 (2) J K⁻¹ mol⁻¹) and for the **dr** mechanism (ΔH[‡] = 88 (2) kJ mol⁻¹ and ΔS[‡] = 31 (5) J K⁻¹ mol⁻¹) have been estimated. When non-stoichiometric ratio between B(C₆F₅)₃/zirconocene ratios were used, the dynamic behaviour of the species in solution is modified and some preliminary results will be presented.

In compound **2** (4) an ethylenic loop is present, that forces, in the *meso* isomer, the two indenyl ligands in an “eclipsed” conformation and differentiates the two Zr-bonded methyls, as “inward” and “outward” with respect to the bis-indenyl pocket. 2D ¹H EXSY indicated that the main species present in solution has the CH₃B(C₆F₅)₃⁻ moiety in the outward position (**2a**), as expected for steric reasons. However, a very minor isomer (**2b**, in ratio 3:100) was detected, which is in slow exchange with the major isomer **2a** through the two processes (**ips** and **dr**) described above. The two mechanisms can be differentiated on the basis of their different exchange pattern in the ¹H 2D NOESY maps. In this case, the exchange was more hindered than in compound **1**, the **ips** mechanism being detected only at T > 300 K, while still higher temperatures were necessary to detect also the **dr** mechanism.

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Solid State ^{13}C NMR and FT-IR spectroscopy of the cocoon silk of two common spiders

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Individual spiders generate up to seven mechanically distinct silk fibers by drawing liquid crystalline proteins from gland-spinneret complexes. The different types of silk are produced for correspondingly different functions (web construction, cocoon, etc.). It is not well known, however, how spiders can modulate the mechanical properties of silk.

Silks are macromolecular composites formed by amorphous protein domains crosslinked and reinforced by β -sheet microcrystals: the degree of crosslinking and reinforcing determines the mechanical properties.

The dragline silk of various spiders has been tested for mechanical properties in many studies and its microstructure has been investigated by various solid state techniques like Electron Diffraction, X-Ray Diffraction, FT-IR and Raman Spectroscopy as well as Solid State ^{13}C NMR. A model has been proposed in which the polyalanine regions forming β -sheets stack to form crystals in an amorphous glycine-rich matrix.

In this work solid state ^{13}C NMR and FT-IR spectroscopies are used to investigate the supramolecular structure of cocoon silk of two spiders: a) *Araneus Diadematus* (garden spider) and b) *Achaeranea tepidariorum* (house spider).

The extensive use of deconvolution techniques allow a qualitative and quantitative comparison of the two types of silk to be made. On the basis of these results the structure and the morphology of the cocoon silk of the two spiders are discussed.

DYNAMICS OF A FERROELECTRIC LIQUID CRYSTAL BY MEANS OF ^2H
RELAXATION MEASUREMENTS

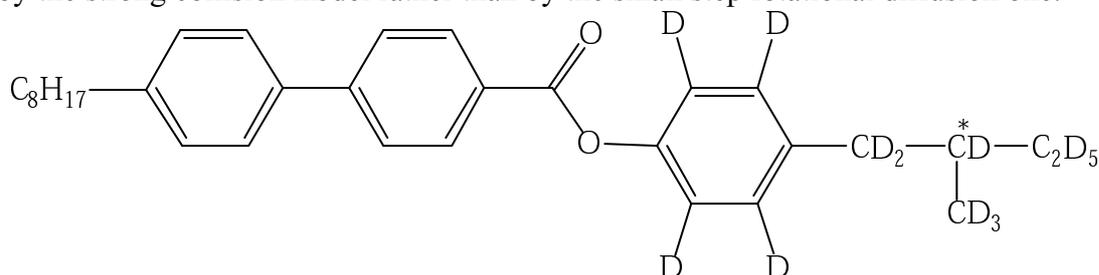
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The dynamics of the chiral polymorphic liquid crystal smectogen (S)-[4-(2-methylbutyl) phenyl]-4'-*n*-octylbiphenyl carboxylate (8BEF5), partially deuterated on the aromatic core and on the chiral chain, has been investigated in its smectic A and C* phases. The ^2H T_{1Q} and T_{1Z} spin-lattice relaxation times, and, consequently, the spectral densities $J_1(\omega)$ and $J_2(2\omega)$ have been determined for the aromatic as well as methylene deuterons of the chiral chain closer to the aromatic core.

The interpretation of the relaxation behaviour has been carried out qualitatively in the tilted smectic phases and by means of the available motional models in the SmA phase, making use of the order parameters determined by the deuterium dipolar and quadrupolar splittings. The diffusional coefficients of the internal rotations of both the phenyl ring and the methylene group have been found to be more than one and two orders of magnitude higher than the diffusional coefficients relative to the overall molecular spinning and tumbling, respectively. Contrary to what usually observed for other mesogens, in the case of 8BEF5 the internal rotations are much better described by the strong collision model rather than by the small step rotational diffusion one.



I 410 K **BPIII** 409 K **N*** 408 K **SmA** 351 K **SmC*** 339 K **SmI*** 336 K
SmJ* 334 K **SmG*** 319 K **Cr**

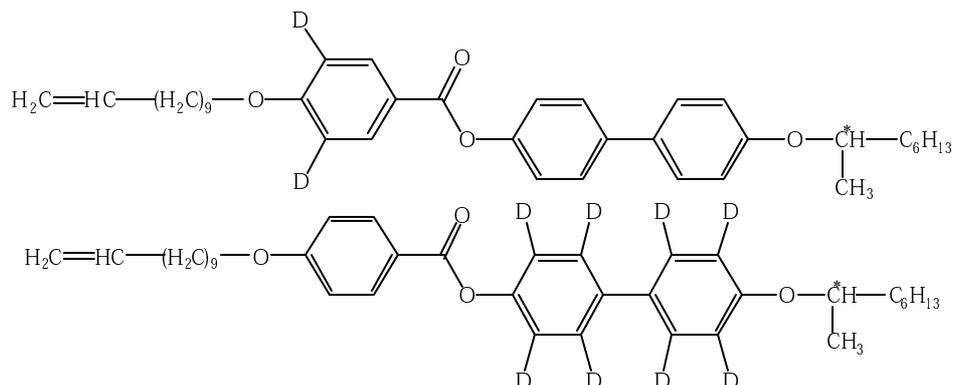
SYNTHESIS AND ^2H NMR STUDY OF A DEUTERIUM LABELLED FERROELECTRIC LIQUID CRYSTAL MONOMER

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Labelling of ferroelectric liquid crystal monomers by stable isotopes permits one to prepare labelled polymers therefrom for studies by neutron scattering and ^2H NMR spectroscopy. On the other hand, the monomers can exhibit interesting mesogenic polymorphism on their own. As a part of our continuing interest in the synthesis and characterization of deuterated ferroelectric liquid crystals (1) and ferroelectric liquid crystal polymers, in this work we have developed a new procedure for the synthesis of (+)-(S)-4-[4'-(1-methylheptyloxy)]biphenyl 4-(10-undecenyloxy)benzoate (11EB1M7). 11EB1M7 was labelled on alternatively the phenyl or the biphenyl moieties of the mesogenic core, thus allowing to obtain information on the order and dynamics of the labelled parts of the molecule, as well as the whole liquid crystal molecule.



The liquid crystal phase sequences found were:

CrX 72.2°C SmI* 66.9°C SmC* 91°C SmA 102°C TGBA 108°C N* 113.2°C BPI 115.9°C I for 11EB1M7-d₂, and

CrX 76.4°C SmI* 68°C SmC* 90°C SmA 103°C TGBA 107°C N* 109°C BPI 111°C I for 11EB1M7-d₈.

By ^2H NMR measurements we obtain the order parameters (S_{zz} and $S_{xx}-S_{yy}$) for the two rigid rings and tilt angle of the director in the SmC* phase, while local order parameters ($S_{z'z'}$) of all carbon sites have been calculated by means of ^{13}C NMR. The dynamics of the monomers is investigated by ^2H NMR and data relative to the SmA phase are analysed in term of theoretical models describing the overall molecular and internal motions.

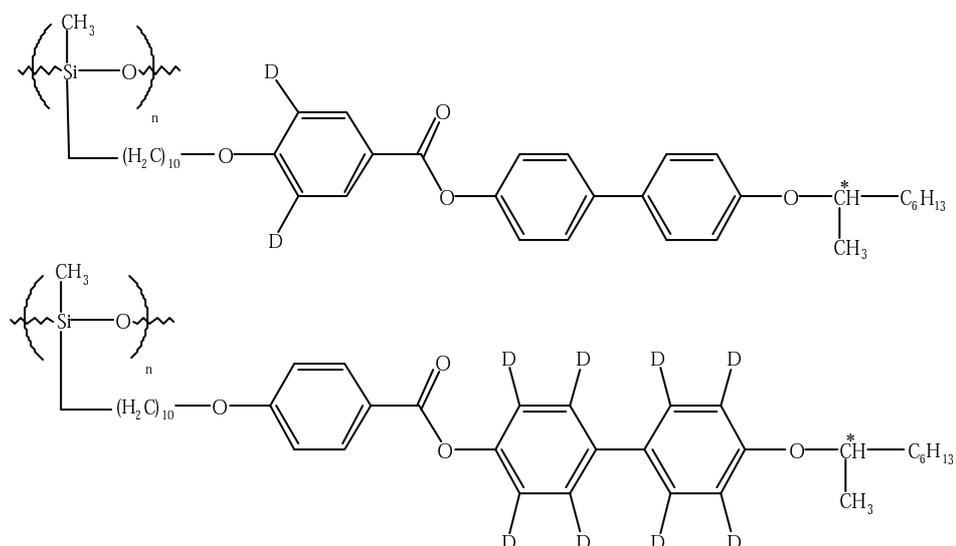
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SYNTHESIS AND ^2H NMR STUDY OF DEUTERIUM LABELLED FERROELECTRIC LIQUID-CRYSTALLINE POLYMERS

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In this work we present the synthesis and characterization of ferroelectric liquid-crystalline polysiloxanes derived from the mesogen (+)-(S)-4-[4'-(1-methylheptyloxy)]biphenyl 4-(10-undecenyloxy)benzoate (11EB1M7). The labelling on the phenyl and biphenyl fragments allows us to study the orientational order of the labelled moieties highlighting the different behaviour between the polymer and the relevant monomer under the applied magnetic field. From the analysis of the deuterium line-shapes it is also possible to determine the effect of the magnetic field on the mesogen alignment and ferroelectric helix distortion. In particular deuterium spectra show a contribution due to aligned mesogens summed to an isotropic one, which can be justified by considering helix isotropic distribution, helix distortion and temperature-dependent translational diffusion rate.



The liquid crystal phase sequences found (above the glass transition temperature) were:

SmI (or F) 58°C SmC* 141°C I for Poly11EB1M7- d_2 , and

SmI (or F) 65°C SmC* 170°C I for Poly11EB1M7- d_8 .

DIELECTRIC AND ^2H NMR RELAXATION STUDIES OF THE SAME
SMECTOGENIC LIQUID CRYSTAL

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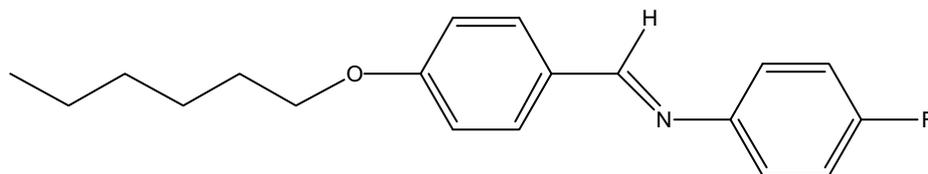
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The dynamics of Liquid Crystals is characterized by a superposition of internal and overall molecular diffusion and collective fluctuations. By using different techniques the various motions can be studied over a wide dynamic range.

Dielectric spectroscopy is a powerful tool to study the stochastic reorientation of molecules and collective motions of molecules having a permanent dipole moment. A wide frequency range in this method (from sub-hertz to several gigahertz) enable one to follow very slow as well as very fast rotations of molecules and their polar part. ^2H NMR relaxation is also a powerful technique for the investigation of molecular dynamics of partially Deuterated Liquid Crystals, in the range $10^{-11} - 10^{-4}$ s. The Redfield theory directly relates the Zeeman and Quadrupolar relaxation times, which can be measured by suitable pulse sequences, to the spectral densities of motion (which are the Fourier transform of the correlation functions) at the Larmor frequency and at twice this frequency. As a part of our continuing interest in the study of the molecular motions in liquid crystals, in this work we have applied both techniques to the same smectogen, (FAB-OC6), in its isotropic, Nematic, SmA and SmB_{cryst} phases.



Cr 325.1K SmB_{cryst} 329.6K SmA 334.3K N 335.8K I

Comparison between the results from the two techniques reveals very interesting features about the merits and the drawbacks of the two methods and allows us a better insight in the evaluation of the dynamics of FAB-OC6.

ETHYLENE–NORBORNENE COPOLYMERS FROM SINGLE–SITE CATALYSTS: MICROSTRUCTURE, REACTION KINETICS AND COPOLYMER PROPERTIES

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There has been considerable interest in the last few years in the study of ethene–norbornene copolymerization by means of single–site catalysts.¹ This is due to the novel properties of these cycloolefin copolymer materials, such as high glass transition temperatures, excellent transparency and good heat/chemical resistance. These copolymer characteristics depend on several parameters, such as the comonomer composition, the distribution of comonomers within the chain, and also the chain stereoregularity. Metallocene structure is the key for producing controlled copolymer microstructure. In turn, a detailed knowledge of the effectiveness of catalysts in producing a given polymer structure can be achieved provided that a methodology for the detailed determination of the polymer microstructure is available.² A large number of ethylene–norbornene (E–N) copolymers were synthesized with catalyst precursors composed of racemic isospecific metallocenes as *rac*–Et(indenyl)₂ZrCl₂ (**1**), *rac*–Me₂Si(indenyl)₂ZrCl₂ (**2**), and *rac*–Me₂Si(2–Me–[e]–benzindenyl)₂ZrCl₂ (**3**), or a constrained geometry catalyst (CGC) as Me₂Si(Me₄Cp)(NⁱBu)TiCl₂ (**4**).

Some of our relevant achievements in the elucidation of E–N copolymer microstructure will be reported. Copolymer microstructure will be related to catalyst structure. Examples of use of a detailed ¹³C NMR analysis of copolymer microstructures to test first–order and second–order Markov copolymerization statistics will be given. Results will also be presented on the reaction kinetics,^{4,5} on the growth of the molecular weight of copolymers with reaction time, and on the E–N copolymer characterization by size–exclusion chromatography (SEC) and rheology to determine the presence of long chain branching (LCB).

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EVALUATION OF THE THERMO-OXIDATIVE DAMAGE IN TIRES USING SOLID STATE NMR, NMR-MOUSE AND HR NMR SPECTROSCOPY.

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During competitions, tires undergo severe damages due to mechanical and heat stresses. Our goal was to identify the kind of chemical damage and the conditions of less damage as function of modified gas mixtures. To this aim we analyzed 11 tire samples using the NMR spectroscopy approach. The 11 sample were obtained from tires of the same brand and rubber mixture: SBR (styrene-butadiene copolymer), NR (natural rubber, *cis*-polyisoprene) and small quantities of *trans*-polyisoprene. All the tires were inflated with different gas mixture (X_n , $n=9$) and air, and subjected to the following bench test: after the imposition of a 163 kg weight, the tire was spun for 20 min at 115 km/h and the speed was increased by 10 km/h every 10 min until the tire broke down.

We used solid state NMR and NMR-Mouse to evaluate the overall conditions of the tires and to select the less damaged ones. In addition we used high resolution NMR spectroscopy applied to swollen samples of the tires to detect the integrity of cross-linked structures. The combination of these methodologies represents a good approach to obtain information on the thermo-oxidative damage of the chemical components of the rubber mixture and allows us to identify the best gas mixture to be used.

ANISOTROPIC MOBILITY OF PE CHAINS AS CONFINED TO TPP NANOCOMPOSITES CHARACTERIZED BY ^2H NMR

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Molecular confinement is receiving remarkable interest by the scientific community. The properties of the confined molecules and macromolecules can be set according to the space available. We already described unusual conformational properties for single polymer chains when surrounded by cylindrical aliphatic nanochannels (1). Here fully deuterated n-hexatriacontane (C36) in tris(o-phenylenedioxy)cyclotriphosphazene (TPP) and fully deuterated polyethylene (PE) included in the same matrix are characterized by ^2H NMR. The structure of the nanocomposites can be depicted as in Figure 1b, the aromatic paddles of the TPP molecules constitute a regular cylindrical channel with 0.5 nm cross-section, the walls are relatively smooth and compel the included aliphatic chain to assume an all-trans conformation (2). The dependence on the temperature of ^{13}C T₁s demonstrate that at room temperature the mobility of the chains inside the channel is liquid-like with a correlation time of 1.2 ps. Anyway ^{13}C NMR cannot provide a complete description of the molecular motion of the chain, and therefore we exploited ^2H NMR spectroscopy. Variable temperature ^2H NMR spectra acquired using quadrupolar echo are reported in Figure 1

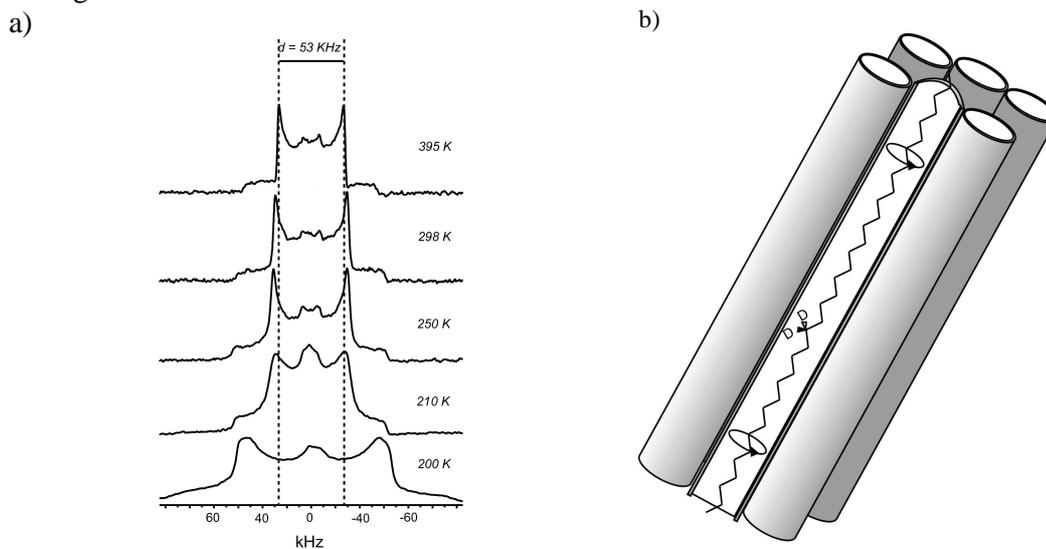


Figure 1) a) Variable temperature static ^2H NMR spectra of TPP/C36 IC with the C36 molecule fully deuterated. b) Cartoon of C36 molecule included in the nanochannel rotating as a rigid body (only two ^2H are highlighted).

The deuteron line-shape is determined by the reorientation of the C-D bond, in the case of a fixed C-D vector a Pake powder pattern is shown with two singularities separated by 125 kHz, instead a C-D vector freely diffusing gives rise to a single peak. In the case of TPP/C36 inclusion compound (IC) at room temperature a Pake powder pattern with the singularities separated only by 53 kHz is recorded. Lowering the temperature a moderate broadening is highlighted, and only at 200 K the powder pattern of a fixed C-D vector is recorded. These results and the similar results for the TPP/PE IC support the model of methylenic chains rotating inside the channels.

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NMR CHARACTERIZATION OF POLYELECTROLYTE / SURFACTANT INTERACTIONS

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Flocculation of colloidal particles due to the introduction of a charged polymer into a liquid suspension is an important solid-liquid separation process in various industrial mineral processing and in municipal water and waste-water treatment (1-5). In fact, adsorption of polyelectrolytes at the particle/liquid interface has a deep effect on the flocculation and the stabilization behaviour of colloidal suspensions.

At low surfactant doses flocculation can occur, while at high surfactant doses the suspension can be resolubilized. Very long and extended charged macromolecules can become adsorbed on more than one charged particle producing a bridging mechanism (6), resulting in a marked flocculation of the entire system.

In this work (7), due to the industrial interest on charged copolymers, the influence of a different charge density in the interaction with an oppositely charged surfactant was tested, using copolymers purposely synthesized. Interaction between a cationic copolymer (acrylamide-trimethylaminoethyl acrylate) P(AAm-TMA) and an anionic perfluorinated surfactant (lithium perfluorooctanoate) (LiPFO) was studied using ¹H and ¹⁹F NMR. In order to clarify the effect of the charge density, copolymers having a different composition of comonomers have been used.

In each copolymer-surfactant system the presence of the polyelectrolyte in solution strongly modifies the ¹⁹F chemical shifts. Consequently a strong polymer-surfactant interaction is evidenced. A complete saturation of the cationic charges of the polymer is clearly detectable, followed by a partial precipitation, and subsequent re-dissolution of the system. The chemical shifts and line widths observed have been interpreted as dependent on two main factors, that is the aggregation state of the system and the rate of exchange among different sites.

¹H spectra, that run at a constant concentration of polymer and different concentrations of surfactant, appear rather broad and poorly defined. The spectral broadening becomes extremely marked at certain value *C* of the surfactant concentration, where a strong superlorentian line appears, surmounted by weak sharp lines in which the chemical shift is still visible. Increasing the charge density the critical concentration *C* increases. A further increase in the surfactant concentration causes a sharp precipitation which is accompanied by the disappearance of the superlorentian resonance. This critical concentration value *C* may be interpreted as being due to the so called "CAC", i.e. the concentration of critical aggregation. At a higher surfactant concentration, after the re-solubilization, a superlorentian ¹⁹F line is observed superimposed again by a sharp spectrum. The presence of the superlorentian ¹⁹F line can be interpreted as an indicator of the incoming gelification of the mixture. A model of interaction has been proposed based on the results obtained.

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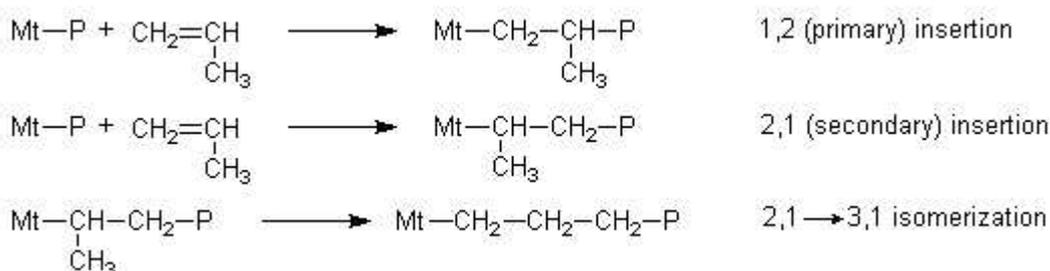
ISOTOPE EFFECT ON 2,1 → 3,1 ISOMERIZATION REACTION
IN PROPENE POLYMERIZATION

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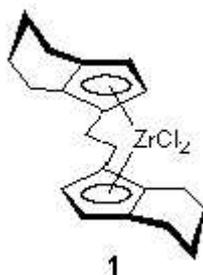
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NMR techniques represent a fundamental tool in the mechanistic study of both regiochemical and stereochemical features of propene polymerization. It is well known that metallocene catalyzed olefin insertion is largely predominantly primary (Scheme 1). However isolated secondary propene units are often detected in many polypropylene samples (Scheme 1). In some cases these 2,1 units isomerize leading to the formation of tetramethylene sequences (3,1 units, Scheme 1). The mechanism of this 2,1 → 3,1 isomerization reaction is yet to be clarified (1).



Scheme 1

Since the methyl group is involved in the isomerization, a possible way of investigation is to monitor the presence of a CD₃ on respect to that of a CH₃ in the monomer. The compound obtained by polymerization of propene with **1**/MAO was compared to that achieved with the **D**-labeled derivatives (CH₂=CH-CD₃) in the same reaction conditions.



Qualitative and quantitative analyses were performed on the bases of ¹H and ¹³C NMR spectra. The amount of 2,1 → 3,1 isomerization product resulted to be strongly affected by the presence of deuterium. A significant isotope effect of $k_H/k_D = 2.7 \pm 0.5$ was indeed found for this reaction. These experimental results indicate that the 2,1 → 3,1 isomerization proceeds through a β-H transfer which represents the rate determining step of the reaction.

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**MORPHOLOGICAL AND STRUCTURAL FEATURES OF ACTIVATED
Fe-SILICALITES: A ^{129}Xe - NMR AND EPR INVESTIGATION.**

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^{129}Xe -NMR and EPR spectroscopies have been employed in the characterization of the activation of two iron silicalites, with different iron loading. The ^{129}Xe chemical shift was recorded as a function of the gas pressure and revealed to be extremely sensitive to the state of the system and in particular to the hydration and oxidation state of extraframework iron ions. The thermal activation process brings about a progressive dislodgment of iron ions from the framework positions and their partial reduction to Fe(II). Upon activation at 973 K particularly large oxo-iron moieties are formed that (at least in the case of the higher loaded sample) prevent penetration of Xe atoms in the silicalite channels. The steric hindrance is removed both by further reduction in hydrogen or by rehydration of the sample.

The description of this complex phenomenology has been possible by comparing the ^{129}Xe -NMR data with EPR ones (which directly monitor the oxidation state of the systems and the effect of hydration-dehydration) and by performing parallel experiments on a Fe exchanged ZSM-5 zeolite containing exclusively extraframework iron.

NMR STUDY OF MEAT MEALS

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A ban on ruminant-derived proteins in ruminant feeds has been introduced as a preventive measure to avoid the spread of bovine spongiform encephalopathy (BSE), as well as to minimize any potential risk of BSE transmission from bovines to humans. Thus, the feeding to any farmed animal of proteins derived from mammals is currently prohibited in the EC (1,2). The EU recommendations include processing conditions for meat and bone meal (MBM) preparation which are considered effective for inactivation of *scrapie* and BSE agents (heat treatment > 133 °C for 20 min, 3 bars of pressure and particle size lower than 50 mm) (3,4).

Thus the “mad cow crisis” in Europe, produced the storage of many tons of slaughter waste without an alternative use, causing severe environmental and economic costs. It is now of extreme importance to find new and eventually non-food use for those MBM which are found to be free from the BSE agents.

Here, we present a preliminary NMR investigation of a MBM produced from beef, pork and turkey meat. This study was carried out in order to characterise the whole sample and MBM fractions extracted with chloroform, methanol and water. The experiments were carried out using both solid state and high-resolution NMR at different magnetic fields.

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NMR CHARACTERIZATION OF OLIVE OIL

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¹H and ¹³C NMR together with a multivariate statistical analysis can be used usefully to establish the geographical origin, the cultivar, the quality and the freshness.

Geographical classification of Italian olive oils ¹H NMR spectroscopy allows to analyze minor components present in olive oil such as aldehydes, sterols and terpenes. These minor components are very useful for the determination of the geographical origin of Italian olive oils.

Varietal classification of Italian olive oils ¹³C NMR and GC techniques combined with a multivariate statistical procedure allows to group monovarietal olive oils belonging to particular cultivars. Grouping of olive oils according to their cultivars occurs specifically for ¹³C resonances belonging to fatty chains in the *sn* 1,3 position of the glycerol moiety.

Pedoclimatic effect in olive oil composition In order to find out which cultivar is the best to be grown in extreme climatic conditions, the composition of olive oils obtained from few matched Mediterranean cultivars grown in experimental fields located in Italy and in the Catamarca region of Argentina has been checked. Olive oils were analysed by NMR, Gas Chromatography and statistical analysis; Mediterranean cultivars less affected by the climatic conditions present in Catamarca have been selected. The selected cultivars produce olive oils which keep their Mediterranean characteristics and which can thus be proposed as colonizing plants in wild areas.

Olive oil authentication ¹H NMR can be used to analyze oils of different botanical origin such as hazelnut oil and seed oils using NMR and Gas Chromatography. This procedure allows to determine a possible fraud on olive oil down to a relative hazelnut oil contents of 5%. Moreover, the ¹H NMR spectrum can be used to measure the amount of di-glycerides in olive oil; the total di-glycerol profile and the 1,2/1,3 ratio are strongly related to the good quality and freshness of olive oils. Finally, ¹³C NMR technique provides information about the fatty acid composition and the acyl distribution on the glycerol moiety; this information can be of crucial importance in the analytical characterization of edible oils and in the prevention of frauds such as the addition of esterified oils.

PROTON HIGH FIELD NMR STUDY OF TOMATO JUICE

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According to recent publication (1, 2) high resolution proton NMR spectra of tomato juices can be used as an input for chemical fingerprint analysis even without detailed assignment of signals. Here we report the first detailed analysis of proton high-field (600 MHz) NMR spectra of tomato juices.

Sample preparation: fresh tomatoes were grinded using an electrical blender, tomato juices were centrifuged and a phosphate buffer (pH value was 6.5) was added (1:2 v/v). In order to assign each spin system and the components of complex patterns overlapped in 1D proton spectra, a combination of J-resolved, COSY and TOCSY 2D sequences were used. Assignment of a spin system to a particular compound was made by comparison with literature data and, when possible, confirmed by direct addition of standards (this is the case of sugars, some amino acids and some organic acids).

The potential usefulness of proton high resolution magic angle spinning (HR-MAS) NMR spectroscopy of tomato pulp in comparison with standard proton NMR spectroscopy of juices in the liquid state is discussed.

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EFFECTS OF ACCELERATED AGING OF WHEAT SEEDS ON FLOUR AS STUDIED
BY ^1H AND ^{13}C SOLID STATE NMR

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Seeds of soft wheat (*Triticum aestivum* cv. Centauro) were artificially aged by storage at 40 °C and 100% relative humidity for periods ranging from 0 to 10 days. The effects of accelerated aging on structural and dynamic properties of flour at a molecular level were investigated by solid state NMR techniques. Structural information was obtained through ^{13}C SPE, CP, NQS and other selective experiments performed under MAS and Dipolar Decoupling conditions, as well as ^1H -fast MAS spectra. FID analysis and wideline measurements of proton spin-lattice relaxation times in both the laboratory (T_1) and rotating frames ($T_{1\rho}$) were performed in order to get insight into the changes induced by the aging process in the molecular dynamics. To this aim, we used two recently developed procedures: one allowing the proton relaxation to be completely interpreted in terms of dynamics, disregarding the multi-exponential behaviour, thus eliminating the spin diffusion effects; the other correlating the different exponential components of the $T_{1\rho}$ decay to the different FID components. Static and MAS ^1H spectra at different spinning rates were also recorded to investigate the nature of the magnetic interactions responsible for spectral line broadening. NMR results were discussed in relation to biomolecular properties of flour determined by other techniques.

MECHANISMS OF PROTEIN-PEPTIDE INTERACTIONS

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Peptide and antigen fragment libraries obtained by the phage display technology have been extensively used to study antibody-antigen interaction in order to develop vaccines and diagnostic tools. Moreover, the same technology has been proposed, in general, for investigating any protein-protein or peptide-protein interaction for the design of selective ligands of potential pharmaceutical interest. Understanding the fine details of these intermolecular processes is crucial to design peptide ligands with improved affinity and activity. In this respect, it is now well established that surface plasmon resonance (SPR) and nuclear magnetic resonance (NMR) are playing a major role in elucidating dynamics and structural aspects of protein-protein and peptide-protein complex formation. SPR yields accurate estimates of kinetic parameters of the intermolecular interaction, such as k_{on} and k_{off} , while NMR offers precious information on the structure stability and the affinity between protein subdomains has been found from SPR data and a combined use of SPR and NMR measurements has been proposed for investigating peptide-antibody and virus-receptor interactions. Among the various peptide-protein interactions, the complex formation between acetylcholine receptor (AChR) mimotopes and α -bungarotoxin (α -btx) has been analyzed in detail both by SPR and NMR. Thus, the structure of three slightly different complexes are available in the Protein Data Bank (PDB) with the 1JBD, 1HAA and 2BTX codes, where α -btx is bound respectively to the peptides WRYYESLKPYP, HRYYESLEPWYP and MRYYESLKSYP, henceforth called HaPep, p6.7 and LLPep. In the present report the favorable opportunity to discuss a good wealth of dynamic and structural data for such similar molecular systems will be exploited in order to correlate complex affinity with structural features. A good agreement of SPR derived K_A values with IC_{50} and K_A conventional methods has been recently discussed for the investigated system. The SPR characteristics of these mimotopes and of some of their analogs will be also analyzed. At least in the case of the considered α -btx-mimotope interactions, the complex stability cannot be fully explained in terms of molecular compactness or number of van der Waals and H bonding interactions. From the analysis of the toxin conformational changes of the three complexes and the corresponding kinetic parameters, it can be suggested that entropic contributions may play a relevant role. The reduced conformational freedom observed for the α -btx carboxy terminus, when bound to LLPep and p6.7 in respect to HaPep, seems to yield also a relevant contribution of this type. Thus, it is apparent that the here proposed SPR/NMR combined approach offers new possibilities to investigate fine details of the peptide-protein interaction. These findings can be exploited to design ligands with improved affinity by means of amino acid substitutions, which can be suggested by the specific roles exhibited by each residue separately in association and dissociation processes. Affinity maturation of ligands, derived from peptide libraries on a rational structural and kinetic basis, appears the main application of our approach.

INVESTIGATION OF METMYOGLOBIN CAVITIES BY ^{129}Xe NMR SPECTROSCOPY

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Since the discovery of a xenon binding site in the interior of sperm whale myoglobin by Schoenborn et al. (Nature, 1996, 25, 495) by X-ray crystallography, the interactions of proteins with xenon have been studied with the goal of using the inert gas as biomolecular probe. In this study, the interaction of xenon with horse and pig metmyoglobin in aqueous solution is investigated by ^{129}Xe NMR spectroscopy. Horse and pig myoglobin differ by 15 aminoacids. Among these residues only one (H19) is at internal position in heme contact. Chemical shifts of both proteins (1mM) are measured as a function of xenon concentration. In these systems, xenon is in fast exchange between all possible environments.

The experimental data for horse and pig metmyoglobin were interpreted using a thermodynamic model which supposes that xenon forms a 1:1 complex with the protein and exchanges between a cavity in the proteins and all the other environments. At low xenon concentration (1 atm) horse metmyoglobin shows an upfield shift for the xenon resonance relative to the xenon resonance in the solvent, while in pig metmyoglobin a downfield shift is observed. As the xenon concentration increases, the xenon resonance shifts downfield in horse and upfield in pig metmyoglobin. The two proteins are characterized by ^{129}Xe spin lattice relaxation time (0.3 s in horse and 0.7 s in pig metmyoglobin) much shorter than that measured in water (~500 s). These observed differences in the direction and magnitude of the paramagnetic shift and in the magnitude of the spin lattice relaxation time can be attributed to differences in the relative orientation and proximity of Xenon to the unpaired electron in these proteins.

SOLUTION STRUCTURE OF A BOWMAN BIRK INHIBITOR ISOLATED FROM SNAIL MEDIC SEEDS (*MEDICAGO SCUTELLATA*)

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A trypsin inhibitor (MsTI), belonging to the Bowman-Birk inhibitor family, was purified from snail medic seeds (*Medicago Scutellata*) (1). The Bowman-Birk protease inhibitors (BBI) are small serine protease inhibitors (6-9 kDa), found in seeds of legumes and many other plants, containing seven disulphide bonds that help stabilising their active configuration. All BBIs present two regions of tandem homology; each region comprising a consensus motif of three beta-strands with a kinetically independent reactive site on the outermost loop. Most BBIs inhibit trypsin at the first reactive site (N-terminal) and chymotrypsin at the second reactive site (C-terminal), while MsTI can inhibit the catalytic activity of bovine β -trypsin, but exhibits no anti-chymotrypsin activity.

Despite extensive studies of BBIs, only a few three-dimensional structures have been solved by X-ray and NMR (2,3) and many questions about the relationship between structure and function are still open.

In the present work the high resolution three dimensional structure of MSTI has been determined in solution by ¹H NMR spectroscopy at pH 5.5 and 27°C.

The assignment of MSTI spectra was not straightforward due to the presence of two symmetrical motives; extensive overlap was observed in the spectra acquired at 500 MHz and the complete assignment of all spin systems was possible only at 800 MHz.

Temperature coefficients for amide protons and amide exchange rates have been used as complementary techniques to predict hydrogen bond donors.

Experimentally derived restraints (NOEs, hydrogen bonds from amide exchange rates, ϕ angles restraints from J coupling measurements and 7 disulphide bridges restraints) were used as input for restrained molecular dynamics, using DYANA program, followed by a restrained minimisation using Discover (AMBER force field). The structure of MSTI comprises two distinct domains composed by a three stranded beta sheet and the active sites, located in a VIb type loop. The combination of the tight turn and the antiparallel beta sheet forms the characteristic beta-hairpin inhibitory domain common to other Bowman Birk inhibitors.

MsTI was also studied at different protein concentrations ranging from 0.6 to 2.0 mM: at 2.0 mM protein concentration a few amide shifts higher than 0.2 ppm were observed, probably due to self-association. Most of these residues are localised in the region connecting the two domains and a variation of their reciprocal position upon association might be hypothesised.

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NMR characterization of interaction between the copper transport protein CopZ and the N-terminal domain of the copper ATPase CopA from *Bacillus subtilis*

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NMR spectroscopy represents a unique tool to obtain structural and dynamics information in solution and experiments are continuously developed and improved to make NMR characterization of larger and larger molecules and to derive new different classes of constraints. In this work the interaction of cytoplasmic CopZ and the N-terminal domain of the CopA ATPase from *Bacillus subtilis* has been studied by NMR through ^{15}N HSQC experiments in an attempt to understand the role of the two proteins in the whole copper trafficking mechanism of the bacteria. It appears that the two proteins do interact in a similar fashion as the yeast ortholog proteins do¹, although the surface potential are reversed. A structural model for the interaction is proposed. ^{15}N mobility studies on the free proteins and on their adduct is presented. From these data, it appears that copper is largely transferred from CopZ to CopA, thus indicating their involvement in a detoxification process.

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NMR studies of the multifunctional protein Sso7d from *Sulfolobus solfataricus*

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Sso7d is a small basic protein, consisting of 62 amino acids, isolated from the thermoacidophilic archeobacterium *Sulfolobus solfataricus*. The protein is endowed with DNA binding properties, RNase^{1,2} activities and capability of rescuing aggregated proteins in the presence of ATP. The small size of the protein allowed us to express with relative ease the recombinant protein² and several mutants³, hence this protein was found to be a suitable tool for investigating thermal, pressure and pH stability of small thermophilic proteins, endowed with a single domain.

In order to explore the stability determinants for this protein, a number of mutants has been produced and characterized by means of NMR spectroscopy. In particular, thermal stability was characterised through denaturation studies performed by monitoring the chemical shift variation of the methyl resonances of the two methionines localized in positions 28 and 57. Pressure resistance was determined by monitoring by FT-IR the frequency variation of the beta structure at 1635 nm. pH stability was characterised through pH titration studies by determining experimentally the pKa values of a large number of ionisable residues in the pH range from 2 to 12.

Ribonuclease activity has been characterized by producing series of single and double mutants based on the NMR results. In some cases, the catalytic activity and the stability at extreme conditions of mutants obtained were strongly modified compared to the WT³.

Stability and activity of selected mutants are discussed in terms of NMR tri-dimensional structure⁴.

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¹⁹F AND ¹H NMR STUDIES ON BINDING AND STABILITY OF CHICKEN LIVER (BASIC) FATTY ACID-BINDING PROTEIN COMPLEXES

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Fatty acid-binding proteins are a family of low-molecular weight intracellular proteins that associate non-covalently with fatty acids, retinoids and steroids (1). Although the specific biological function of each of these proteins has not been established yet, they are generally considered to be involved in fatty acid transport and metabolism (2). Binding of fatty acids to proteins belonging to this family might provoke modifications in the proteins themselves; in particular, side-chains of the aminoacids that lean inside the binding cavity can change their position. These changes may be different depending on the nature of the ligand (a fatty acid or a different hydrophobic molecule), on the presence of heteroatoms and their position.

We have previously studied the interaction of chicken liver basic fatty acid-binding protein (Lb-FABP) towards palmitic (PA) and oleic acid (OA) (3) and report here on the differences in binding and affinity of Lb-FABP towards 2-F palmitic acid (2FPA) with respect to other fatty acids, and on denaturation features of the protein itself and its complexes. Lb-FABP/fatty acids interactions were investigated through ¹H and ¹⁹F NMR spectroscopy and, in the case of denaturation experiments, the results were compared with those from fluorescence spectroscopy.

¹⁹F NMR shows that Lb-FABP binds 2FPA with 1:1 stoichiometry in ca 30 minutes at 300 K. The binding of 2FPA to Lb-FABP affects the protein ¹H 1D NMR spectra differently from PA and OA. Indeed some resolved signals at high field (0.5/-0.4 ppm), in the region around 2 ppm (2.0/1.8 ppm) and one peak in the aromatic region (7.7/7.6 ppm) are shifted at different extents for non-fluorinated and fluorinated fatty acid.

Relaxation data of fluorine in the Lb-FABP/2FPA complex, measured at different temperatures, indicate that this fatty acid is not bound tightly to the protein but it moves in a motional regime in which $\omega^2 \cdot \tau_c^2 < 1$.

Equilibration experiments between *holo* Lb-FABP and 2FPA or PA showed that, at 300K, after ca 3 hours 2FPA reaches equilibrium with Lb-FABP/PA complex, whereas PA cannot significantly displace 2FPA from the binding site even after 60 hours of incubation.

Unfolding and/or ligand release upon urea or guanidinium chloride addition to the Lb-FABP/2FPA complex solutions was monitored through ¹⁹F and ¹H NMR spectroscopy. NMR results are in agreement with those obtained through fluorescence experiments on the same complex. Fluorescence denaturation experiments were performed also on Lb-FABP in its *apo* form or complexed to PA. Collected data show that binding of 2FPA or PA to Lb-FABP does not change significantly the protein stability to denaturants.

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EXHAUSTIVE METABOLISM ANALYSIS OF BIOLOGICAL PREPARATIONS BY COMBINED ^{31}P AND ^1H MRS

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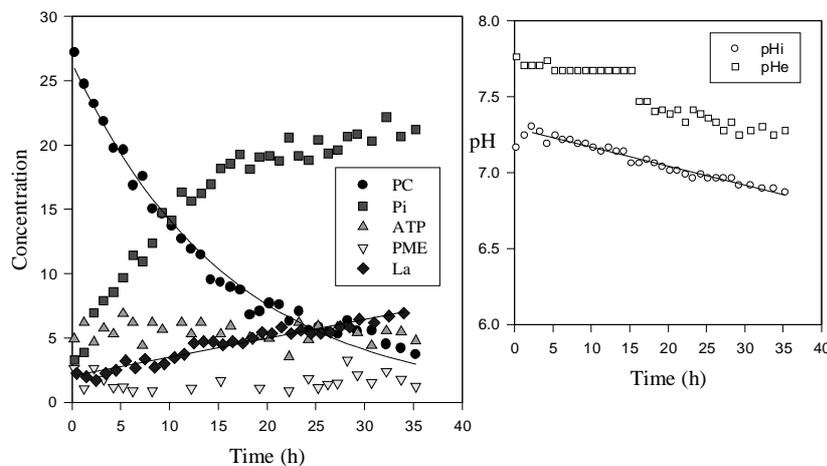
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Nuclear magnetic resonance spectroscopy (MRS) has been widely used for non-invasive studies of metabolism in several biological systems. Combined ^{31}P and ^1H MRS allows to gain a dynamic insight into metabolic pathways. The assessment of the kinetics of the most relevant metabolites by the use of adequate standards (1) makes it possible to describe the energetics of a tissue in quite different conditions, even in the presence of environmental stresses such as low temperature, extracellular acidosis and anoxia that are known to play a key role in metabolic regulation.

Serial biochemical assays quantitatively estimate tissue metabolites. However all require disruption of the cells. Thus, extracellular pH cannot be estimate, time-dependent changes in metabolite concentration within given cell populations cannot be obtained. The application of MRS to intact tissues offers the possibility of monitoring time-dependent changes of cellular metabolites.

In the present study the time course of anaerobic metabolism in the resting isolated muscle, in

anoxic conditions, was investigated by means of joint ^{31}P and ^1H MRS. The rate of exergonic processes occurring in an anoxia tolerant tissue, as skeletal muscle was determined. As shown in the figure, the rate of phosphocreatine (PC) hydrolysis, inorganic phosphate (Pi) accumulation,



adenosinetriphosphate (ATP), phosphomonoesters (PME) and both intracellular and extracellular pH (pHi, pHe) time course could be obtained by collecting ^{31}P spectra. By collecting ^1H spectra the rate of lactate accumulation (La) could be determined too. The study was carried out by varying both temperature (15, 20, 25°C) and extracellular pH (7.9, 7.3, 7.0) to reach a better insight in the mechanisms involved in metabolism and pH regulation.

In conclusion, MRS is nowadays the technique of choice to obtain a complete view of tissue metabolism in order to study regulation mechanisms at cellular level in most physiological (physical exercise, aging) paraphysiological (acute and chronic hypoxia) and pathological conditions (mitochondrial myopathies, cardiovascular disease, etc.).

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**BACKBONE DYNAMICS OF HUMAN PARATHYROID HORMONE IN A
MEMBRANE-MIMETIC ENVIRONMENT STUDIED BY TWO-DIMENSIONAL
PROTON-DETECTED ^{15}N NMR SPECTROSCOPY**

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Previous studies on human parathyroid hormone (1-34) [hPTH(1-34)] and many of its analogs indicated the presence of two helices located at the N- and C-terminus, respectively, separated by a region of undefined conformation. A legitimate doubt remained, whether the scarce definition of the structure in the central portion of all the analogs studied so far is due to actual mobility or to lack of sufficient experimental constraints.

We decided to tackle this problem with an independent NMR technique, *i.e.*, utilizing ^{15}N relaxation measurements to characterize the internal motions of hPTH(1-34). The relaxation of such nuclei as amide ^{15}N is mediated by the overall rotational tumbling of the molecule and by internal motions of the X-H bond vector; consequently, heteronuclear NMR spectroscopy is a powerful technique to investigate dynamics in biological macromolecules.

Here we describe the results of proton-detected ^{15}N - ^1H 2D NMR experiments and their interpretation in terms of backbone dynamics of hPTH(1-34). The uniformly ^{15}N enriched peptide sample used was produced as GST-fusion protein. ^{15}N relaxation parameters of the 33 backbone amides were determined in the presence of dodecylphosphocholine (DPC) micelles. Results of the present study indicate that in the presence of DPC micelles the motion of the peptide is slower with respect to our previous study in water at high salt concentrations, although a higher mobility centered at position 12 is still maintained. Moreover, the presence of micelles causes an increased stability of the N-terminal helix relative to the C-terminal one and the presence of a new point of local mobility at residues 16-17.

These results support the hypothesis that the relative orientation of the two N- and C-terminal helices is not defined until a positive interaction with the receptor is established. The correct disposition of the two helices is favored by the flexibility in the central part of the molecule.

COPPER BINDING TO OCTAREPEAT PEPTIDES OF PRION PROTEIN

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Prions are the cause of neurodegenerative diseases including Creutzfeldt-Jacob disease in humans and scrapie and bovine spongiform encephalopathy in animals. A variety of experiments have indicated the accumulation of an abnormal isomer (PrP^{sc}) of host-derived cellular prion protein (PrP^c) as a critical pathogenic event in prion diseases.

Up to now, the only reported consistent differences between the two isoforms are in chain conformation and state of assembly, but the molecular mechanism of the conformational transition and the subsequent neurodegeneration remains unknown. Until recently, little has even been known about the normal function of PrP^c in the brain but there are now growing evidences to indicate a functional role for the prion protein in copper metabolism. Most current work suggests that PrP is a copper binding protein and that copper binding takes place in a highly conserved octapeptide repeat region (sequence PHGGGWGQ) spanning residues 60-91. This region selectively binds bivalent copper ions *in vivo* and the high histidine content seems to promote this binding.

In order to define the stoichiometry, site and mode of binding of Cu(II) to PrP, the copper-binding properties of peptides corresponding to 1- and 2-octarepeat sequences were tested using circular dichroism and NMR. Both peptides are unstructured in water solution in the absence of Cu(II). The changes in the far UV CD spectrum upon addition of metal indicates a distinctive structuring of the octarepeat regions on Cu(II) binding which are characteristic of turns and structured loops.

CD titration experiments demonstrate a 1:1 copper/peptide binding stoichiometry for the octamer sequence and at least the binding of two ions for hexadecamer.

Although the structure of the octarepeats is not affected by pH changes in the absence of Cu(II), copper binding is strongly pH-dependent. Circular dichroism spectra indicate no structural changes at pH below 6; as the pH is raised, clear structural perturbation is induced. The center of the transition suggests the involvement of histidine in the coordination of copper; this is confirmed by NMR data. Proton NMR histidine and copper titration experiments were carried out, indicating histidine coordination to the Cu(II) ion and pH dependent binding.

THE EFFECT OF Ce(III) IONS ON THE CONFORMATION OF RAT ANGIOTENSIN II IN AQUEOUS SOLUTION AS PROBED BY ^1H -NMR SPECTROSCOPY

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Angiotensin II (AngII) is a linear octapeptide hormone, which plays a critical role in the regulation of cardiovascular homeostasis.

AngII was postulated to act on a receptor located on the plasma membrane of its target cells. Two types of receptor (AT_1R and AT_2R) have been recognized. AngII binds to $\text{AT}_{1\text{A}}$ receptors to cause vasoconstriction and fluid retention, both of which lead to an increase in blood pressure.

The conformation of AngII has been studied by NMR and CD spectroscopy: various conformational models of AngII in aqueous solution have been proposed, but no consensus has been reached yet about its three-dimensional conformation.

The structural features of Ang II bound to the $\text{AT}_{1\text{A}}$ receptor have been recently determined under conditions mimicking the natural environment of the $\text{AT}_{1\text{A}}$ receptor.

The transition of Ang II from the free to the $\text{AT}_{1\text{A}}$ receptor-bound state can be assimilated to a process of conformational selection where a single structure of the bound peptide is picked out from a wide spread of structures available in the free solution state.

Here we present ^1H -NMR studies on cerium(III) complex with Ang(II) in aqueous solution at pH 4.0.

The complex of AngII with Cerium(III) was investigated due to the chemical similarities of this metal with Ca(II) ion, which may play a relevant biological role in the interaction between angiotensin II and the receptor by selecting the active conformation among many of the flexible peptide.

The peptide forms a 1:1 complex with Ce(III). Although the measured dissociation constant is rather high (0.0338 M), the obtained results could be very useful to understand the role that calcium may play in the mechanism of the interaction with the receptor.

NMR STUDIES OF THE INTERACTIONS BETWEEN A FRAGMENT OF HUMAN
ADENOSINE A_{2A} RECEPTOR AND ITS ANTAGONIST SCH58261

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This study is aimed at characterizing through NMR the interactions between a 12-aminoacid fragment of the human adenosine A_{2A} receptor and its antagonist SCH58261.

Adenosine is a nucleoside acting on the cardiovascular system by controlling the functions of cardiac and vascular tissues. It is released from cells as a result of metabolism and its release can increase in case of metabolic stress.

Its effects in blood vessels are mediated by membrane-bound receptors (A₁, A_{2A}, A_{2B}, A₃), that are therefore potential therapeutic targets. They belong to the family of G-protein-coupled receptors, which transfer signals by activating G proteins. In particular, adenosine-induced vasodilatation is mediated by A_{2A} and A_{2B} receptors. SCH58261 is a selective A_{2A} receptor antagonist.

The structure of the free A_{2A} fragment has been determined by the assignment of 2D spectra (NOESY, COSY, TOCSY), while its interactions with SCH58261 have been investigated through the analysis of longitudinal relaxation rate (T₁) measurements.

The results obtained so far indicate that the A_{2A} fragment adopts in DMSO a structure similar to that predicted for the whole receptor sequence.

Study of the conformational changes in the catalytic mechanism of the *Azotobacter vinelandii* rhodanese by NMR spectroscopy.

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The sulfurtransferases are widespread enzymes, in particular the thiosulfate:cyanide sulfurtransferase (EC 2.8.1.1), an enzyme given the name rhodanese, in vitro catalyzes the production of thiocyanate, transferring the sulfane sulfur atom from thiosulphate to cyanide, by a double displacement mechanism. The physiological role of these enzymes is still unclear. Proposed roles include cyanide detoxification [1], restoration of iron-sulfur centers in Fe-S proteins, as ferredoxin [2], and sulfur metabolism [3]. In the course of catalysis the rhodanese cycles between two isolable intermediates, a sulphur-loaded (ES) and a sulphur free forms (E). Physical properties of these intermediates of the enzyme have been demonstrated to be different by a variety of solution methods, but the crystallographic data do not appear to show appreciable flexibility in the rhodanese when ES crystals are soaked with cyanide. Our studies were performed using the recombinant His-tagged *Azotobacter vinelandii* rhodanese (RhdA, MW 31 kDa) [4], that is one of the prokaryotic recently expressed enzymes, and its three dimensional structure has been recently elucidated [5]. The bovine and *Azotobacter vinelandii* rhodaneses differ in the number of cysteines, and this fact can have a profound impact on the conformational differences between the loaded and unloaded forms in the two systems. In fact, the RhdA protein has only one cysteine residue (Cys230), which is the residue involved in the catalytic mechanism and it can be a fundamental advantage in the study of the sulfurtransferase activity of the rhodanese-like proteins. ¹H-¹⁵N correlation experiments were recorded on uniformly labelled samples of RhdA in the two forms (E and ES). Both spectra show a remarkable dispersion of chemical shift and line-shape characteristics, indicating a stable fold of the protein in both forms. One form could be readily converted into the other by adding KCN or Na₂S₂O₃. A closer inspection of the position of the peaks in the two spectra reveals a high similarity. This observation indicates only slight changes in the backbone conformation of RhdA when converting the E into the ES form. To further characterise the possible differences between the two states of RhdA, ¹⁵N T₁ and T₂ relaxation times, as well as steady-state ¹H-¹⁵N NOEs were measured. As expected from the high similarity of chemical shifts observed, data obtained in the two cases were comparable. In view of the fact that only 21 peaks show some difference in chemical shift between the E and ES forms, changes in conformation are confined in a small region around the active site. It was possible to calculate a correlation time of 16.8 ns, reasonable value for a monomeric form of the enzyme. The solvent accessibility of the active site in the two forms was measured recording ¹H-¹⁵N correlation spectra at different times after dissolving the protein in D₂O and followed the intensities of the cross-peaks. Globally, the E form shows a higher rate of exchange with D₂O respect to the ES form. This result points out that the active site of the unloaded form of RhdA is more readily accessible to water. Moreover a selective labelling of the protein, with a ¹⁵N-cystein was performed and the position of catalytic Cys-230 has been univocally assigned in the two spectra. We have used the selective labelling of the protein, with a ¹⁵N-cysteine, to investigate, by NMR spectroscopy, the conformational changes of the catalytic residue in the enzymatic activity and the interaction with other ions.

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BamHI DNA Recognition Site reveals structural features different from canonical B-DNA: Nuclear Magnetic Resonance investigation and comparison of results with Unrestrained Molecular Dynamics

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ABSTRACT

The solution structure of the self-complementary DNA dodecamer 5'd(TATGGATCCATA)₂, comprising the specific target site for the restriction endonuclease BamHI, is investigated by means of Nuclear Magnetic Resonance and Molecular Dynamics simulation.

Using 2D NOESY spectra all non-exchangeable proton resonances, but the H5'/H5" sugar protons, and most of the labile protons resonances are assigned, permitting the determination of 200 approximate interproton distances.

The nuclear Overhauser study, together with P.E.COSY spectra, reveals a classical B-DNA structure for the dodecamer, with some structural and dynamical features different from canonical B-DNA for the couple of cytosines complementary to the residues where the cleavage occurs.

These sequence-dependent differences are present before complex formation and suggest that even if the left subunit of BamHI cleaves the DNA dimer on the left half-site, after G4, recognition occurs on the right half-site of DNA, where almost all of the base pair contacts take place (2).

NMR data are matched with structural and dynamical results from a 3ns Unrestrained Molecular Dynamics carried out using Amber force field and Ewald summation method, starting from classical B-DNA structure (4).

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SOLUTION STRUCTURE OF THE CONOPEPTIDE CONTRYPHAN-VN

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Advances in methods used for design and chemical synthesis of peptides has enabled the study of basic folding principles, structure-function relationships in bioactive peptides and development of novel pharmaceutical agents. Contryphans are a family of small, cyclic, well-structured peptides isolated from the venom of many species of cone snails (genus *Conus*), characterized by the presence of D-Tryptophan, a five-residue intercystine loop and other post-translational modifications. The nonapeptide Contryphan-Vn, recently extracted from the Mediterranean *Conus Ventricosus*, shares the typical disulfide-bridged fold with the other known contryphans but contains a basic Lys residue inside the seven-residue cycle and an acid Asp residue between the ring and the N-terminal Gly.

We have investigated the solution structure of this conopeptide by means of various 2D homonuclear and heteronuclear NMR techniques. The results show the presence of two stable conformations as other members of the family, due to the cis-trans isomerization of the Cys3-Pro4 peptide bond. Cis conformer is the major one and the structural elements of its loop differ considerably from the Contryphan motif. Three-dimensional structures calculated on the basis of interprotonic NOEs suggest that Lys 6 residue plays a central role for this molecule, mediated by hydrophobic interactions with other amino acid side chains and by a salt bridge with Asp2 residue. The conformational features of the trans form are currently under investigation.

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A SIMPLE PROTOCOL TO STUDY BLUE COPPER PROTEINS BY NMR

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An NMR approach based on classical two and three dimensional experiments for sequential assignment leaves unobserved in the case of oxidized plastocyanin from *Synechocystis* sp. PCC6803 14 residues out of 98 amino acids. A protocol that simply makes use of tailored versions of 2D HSQC and 3D CBCA(CO)NH and CBCANH leads to the identification of 9 of the above 14 residues. At variance with previous approaches, the proposed protocol does not involve the use of unconventional experiments designed specifically for paramagnetic systems, and does not exploit the occurrence of a corresponding diamagnetic species in chemical exchange with the blue copper form. This protocol is expected to extend the popularity of NMR in the structural studies of copper (II) proteins, because it allows researchers to increase the amount of information available via NMR on the neighborhood of a paramagnetic center without requiring a specific expertise in the field. The resulting 3D spectra are standard spectra that can be handled by any standard software for protein NMR data analysis.

CELLULAR RETINOL-BINDING PROTEIN: AN NMR STUDY OF ITS STRUCTURAL FEATURES AND STABILITY

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Retinoid-binding proteins play an important role in regulating transport, storage, and metabolism of vitamin A and its derivatives. Among the four types of cytosolic retinol-binding proteins (CRBPs) that have been identified in mammals so far, CRBP type I (134 residues, 15.7 kDa) forms the most stable complex with retinol; the protein is widely distributed in various tissues.

In our recent work, the solution structure and backbone dynamics of CRBP have been determined in the apo and holo-form (1). The global fold consists of a flattened β -barrel formed by two nearly orthogonal five-stranded β -sheets and a helix-turn-helix domain. The most relevant difference between the NMR structure ensembles of the two protein forms is a higher backbone disorder, in the ligand-free protein, of some segments that frame the putative entrance to the ligand-binding site (portal region). The internal backbone dynamics, obtained from ¹⁵N relaxation data, confirmed that there are several residues with higher mobility in the apo-form (1).

To further investigate the structural features and conformational stability of the protein, a series of multidimensional NMR experiments were recorded under different solution conditions. Hence, measurements were carried out either at acidic pH, or in the presence of increasing amounts of methanol. In addition, hydrogen/deuterium (H/D) exchange experiments were performed.

CRBP exhibits a very high stability of the hydrogen-bonding network of the β -sheet structure, as suggested by an extremely slow amide proton exchange behaviour, especially when compared to other members of the intracellular lipid-binding protein family (2). Although a detailed analysis of the data is still in progress, preliminary results seem to indicate that the holo-protein is also very stable at acidic pH and in the presence of large amounts of methanol.

Together with previous results (1) the data suggest that CRBP is characterized by a holo-form which is perfectly rigid and closed to guarantee the stability of the complex during retinol transport and the protection of the ligand from non-specific interactions. Targeted release of retinol requires a transiently open state; this is likely to be promoted, *in vivo*, by the properties of the micro-environment near the membranes surface where the enzyme molecules involved in its metabolism are embedded.

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THE SCORPION TOXIN TSTX-IV: PH DEPENDENCE OF THE NMR-DERIVED 3D STRUCTURE AND AN INVESTIGATION OF ITS BINDING TO K⁺ CHANNELS BY MOLECULAR DYNAMICS SIMULATIONS

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TsTX-IV is one of the several small proteins present in the venom of the Brazilian scorpion *Tityus serrulatus*. It blocks the high conductance Ca²⁺-activated K⁺ channels (1) and it has been indicated to form a new family of short-chain scorpion toxins together with butantoxin (2). Using mass spectrometry, besides determining the position of the four S-S bridges, we found that, differently from what previously reported (1), the toxin is composed of only 40 residues and its sequence coincides with the one of butantoxin (2).

The stability and conformational flexibility of TsTX-IV have been studied by circular dichroism (CD) and ¹H-NMR. The toxin is stable up to 90 °C, the highest temperature tested and the addition of trifluorethanol, as co-solvent, does not affect its secondary structure. The 3D solution structure consists of a small triple-stranded antiparallel β -sheet anchored to a short α -helix by two S-S bridges, resulting in a $\beta\alpha\beta\beta$ motif. Although the α/β scaffold is conserved among other known short-chain scorpion toxins, some structural differences can be identified.

An analysis of the NMR data obtained at pH 4.5 and 6.0 indicated that at the higher pH the toxin is characterized by a less rigid structure. To verify whether this finding indeed was due to an increased molecular flexibility or simply to a reduced number of the observed NOEs resulting from the fast amide exchange, we employed the molecular dynamics simulations taking into account the protonation state of TsTX-IV at those two pHs. The difference in flexibility was confirmed by calculations carried out without the NMR distance restraints. The protocol used and the obtained results will be presented.

To describe in more details the interaction of the toxin with K⁺ channels, we built a homology model of the Shaker B K⁺ channel based on the crystallographic data available for a KcsA (3). Subsequently, selected structures of TsTX-IV from the trajectories obtained at pH 4.5 and pH 6.0 were docked on the K⁺ channel model. The possible key amino acid residues responsible for the binding were identified and the importance of pH in describing the structure of the toxin-channel complex will be discussed.

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FOLDING STUDIES OF HYDROPHOBIC LIGAND BINDING PROTEINS:
β-LACTOGLOBULINS AND LIVER BASIC FATTY ACID BINDING PROTEINS

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The lipocalins, the fatty acid binding proteins (FABPs) and the avidins are three families of hydrophobic binding proteins which together form the calycin superfamily (1). Lipocalins and FABPs share a related beta-barrel structure. Lipocalins are mostly extracellular proteins displaying a wide variety of biological function (2), while FABPs are predominantly intracellular proteins involved in the lipid metabolism (3). Beyond some functional similarities (hydrophobic ligand binding), these families are characterised by a similar folding pattern (an anti-parallel beta barrel), within which large parts of their structures can be structurally equivalent, although the families share no global sequence similarity. We have investigated and are investigating the structural and folding properties of beta-lactoglobulins from different species, which are important representatives of the lipocalin family (4,5); recently we have undertaken the structural characterisation of Liver Basic FABP (Lb-FABP), belonging to the FABPs family. This work is part of a project on the folding of lipid binding proteins: prior folding studies on this family have revealed remarkable differences between the various members, and the cause of the difference is essentially unknown (6). The comparison of the folding properties of bovine beta-lactoglobulin and Lb-FABP, which share, beyond the barrel fold, a hydrophobic cavity for the interaction with palmitic acid, is relevant for the identification of the main determinants for the folding mechanism. Folding studies, performed on the recombinant FABP, compared to the results obtained for beta-lactoglobulins, will be presented in this communication.

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SOLUTION STRUCTURE OF CHICKEN LIVER BASIC FATTY ACID BINDING PROTEIN

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The fatty acid binding proteins (FABPs) are a family of intracellular, low molecular weight, nonenzymatic proteins isolated from many different sources, capable of binding small endogenous and exogenous lipophilic ligands (1,2). FABs are assumed to carry out the function of solubilisation and transport of fatty acids and their CoA derivatives, although the specific function of each member of this family has not been established yet. FABPs have been classified according to the organ from which they were initially isolated. Two different types of liver FABPs are known: the mammalian, and the basic type. Among the basic type, some of us have isolated and purified from chicken liver the "liver basic FABP" (Lb-FABP) (3), so called from the very high isoelectric point.

We report here the structural characterisation of Lb-FABP as obtained from ¹H NMR data performed at pH 5.6, 500 MHz, and molecular dynamics simulations, using DYANA program.

The overall fold of the protein comprises a 10-stranded, antiparallel β -barrel that includes two short α -helices, inserted between the first and the second strand of the β -structure. A backbone RMSD of 1.37 ± 0.19 Å was obtained for the twenty best structures. The NMR structure shows a gap between strands β_D and β_E , and a higher flexibility is observed for the N-terminal region of the protein. The structural data are discussed and compared with the structures reported in the literature for both mammalian and basic type proteins (4,5).

This structural characterisation is preliminary to the subsequent folding studies, as part of a wider project on the folding properties of β -barrel proteins belonging to the calycin superfamily.

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Ni(II) AND Cu(II) INTERACTION WITH METAL BINDING SEQUENCES OF HISTONE H4

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Nickel compounds are well known as human carcinogens. (1) The responsible molecular mechanisms remain, however, to be fully understood. The leading concepts in nickel carcinogenesis is believed to involve oxidative promutagenic DNA damage and epigenetic effects in chromatin resulting from nickel binding inside the cell nucleus. (2-7)

It is known that DNA and phospholipids of cellular membranes do not provide binding sites of high affinity for Ni (II).(8) Consequently, the nuclear proteins, and in particular the most abundant among them, the histones, reaching in somatic cells a formal concentration of 3 mM, (9) are able to compete for metal ions with even higher affinity metal binding sites in other less abundant nuclear proteins or smaller molecules. Phagocytosis of insoluble particles of Ni₃S₂ by either macrophages or epithelial cells causes buildup of very high levels of nickel inside the cells after its intracellular dissolution catalyzed by the acidic pH of endocytic vacuole, thus providing a continuous source of Ni(II) ions. (10)

The detection and structural and mechanistic description of specific Ni(II) sites in histones would provide a molecular basis for better understanding of the mechanisms underlying Ni(II)-induced carcinogenesis.

We investigated the issue of Ni(II) and Cu(II) binding within the histone octamer. Using histone sequences in conjunction with the structural data we identified a binding site for Ni(II) and Cu(II) ions located in the N-terminal tail of the histone H4.

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MEASURING DIFFUSION COEFFICIENTS WITHOUT A GRADIENT PROBE: IMPLEMENTATION OF THE OE-CTPG SEQUENCE ON AN OLD SPECTROMETER

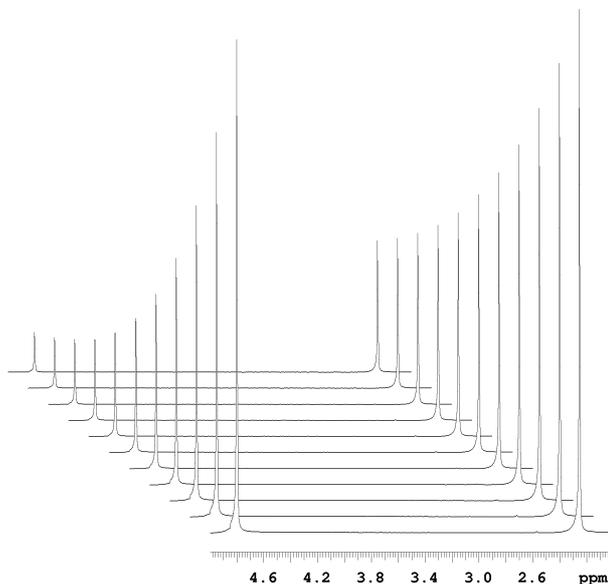
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In a perfect world every NMR scientist can access state-of-the-art NMR hardware and run the newest experiments that the NMR journals publish monthly. Unfortunately the world is not perfect and many researchers dealing with NMR keep using old or second-hand NMR spectrometers that can hardly be upgraded. This is even more true in the less developed countries where the difficulty of running modern NMR experiments may hamper chemical and pharmaceutical research, thus creating a sort of technological divide that we may name the "NMR divide".

Although some of those old-fashioned NMR spectrometers cannot do anything more than recording 1D spectra, some other machines were projected with an eye to the future and today they still surprise the passionate NMR technician with the richness of their hardware and software capabilities. Thus, some of those old spectrometers can still be used for running experiments that were not specifically supported by the producing companies (or did not exist) at the time of shipping.

As an example, we show the implementation on an old Bruker AC200 spectrometer of an high resolution OE-CTPG sequence for measuring the diffusion coefficients of the components in a liquid mixture (1). The sequence does not use gradient coils other than the Z_1 shim coil and it is thus particularly suited for being implemented on old spectrometers.



OE-CTPG diffusion spectra of a water/acetone solution
obtained with an old Bruker AC200 spectrometer

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TEST METHODS IN MRI: SENSITIVITY OF UNIFORMITY PHANTOMS

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MRI (Magnetic Resonance Imaging) is nowadays the diagnostic technique with major rise trend, despite the greater complexity with respect to other techniques like TC and angiography. The most important step in building a good RM exam is the quality assurance (QA) *i.e.* a group of test that determine the precision and reproducibility level in exams execution of RM equipments. All imperfections in equipments setup can affect the proper diagnostic pathways.

In the past years some quality assurance tests have been proposed: AAPM, NEMA and other authors proposed a standard in quality assurance. They pointed their attention on reliability of technical point of view, showing various method for determine all parameters necessities to obtain a good QA. Signal to noise ratio (S/N), uniformity of static magnetic field, uniformity of RF field, linearity of fields, slice profile/thickness, resolution, precision in T_1 and T_2 , SAR (Specific Absorption Rate), ghosting, gradient noise and presence/absence of artefacts are the main parameters checked in an acceptance and quality test.

In this work we focused our attention in uniformity test (UT). When we performed UT with different equipments at the same field (1 T) and the same phantom filled to series of solutions, a great variation was found in this parameter. These variation, despite the same level of equipment setup, force us to understand the influence of filler in UT.

NMR DATA PROCESSING: THE TNMR SOFTWARE PACKAGE

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The extraction of reliable information from NMR experiments requires a suitable processing of the NMR data set. Several algorithms have been developed to improve data quality, spectral quantification, and to reduce processing and acquisition artefacts especially in multidimensional experiments. In addition, some particular problems, such the diffusion and relaxation analysis in the presence of multi-components, require different approaches. The native software packages of the commercial NMR spectrometer rarely contains advanced processing algorithms. There are several commercial and freeware processing packages (NMRPIPE, XWINMR, GIFA, MRUI etc.) that are focused on different aspect of computing, use different user-interfaces and run on different hardware and software platforms. However, in some cases the algorithms needed for a particular processing are found in different packages and cannot easily applied to the same data set. In addition, scientific data analysis benefits from precise knowledge about processing algorithms including the individual implementation and thus from access to program sources. Even with code available the readability of program sources is often impaired by the complexity of programming languages. This problem is significantly reduced in high-level languages. The availability of some data analysis routines is often insufficient to the user without the help of a suitable graphic interface in which the results can be easily displayed.

For this reasons we have developed a new software package designed on our processing needs, which grant us for the knowledge of the algorithms used and which permit the relative rapid implementation of the new algorithms appearing in the literature. This software, named TNMR, is developed under MATLAB environment which is an high level programming language which ensure easy implementation and readability of the processing routines.

TNMR has a window based graphic user interface which permit to run the different processing functions from menu, push buttons and command line and to control processing parameters through sliders and interactive text editing. Together, with the usual routines for FT, phase and baseline correction, apodization, TNMR contains different new processing algorithms: Wavelet transform and denoise, Zoom FFT, 1D FDM, spectral deconvolution, different algorithms for inverse Laplace transform and diffusion and relaxation analysis.

Software Engineering and Virtual NMR Instruments

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We describe an object-oriented C++ implementation of a *virtual NMR instrument*, complete with everything that an intended range of NMR applications might ever need in the way of hardware and its functionality.

Any instrument can be intended as a specialized device which 'owns' other specialized devices. The result is a *rooted tree of devices* in which the root vertex is the complete *virtual instrument* while the *terminal vertices* are devices as simple as a 'switch'. In the C++ implementation *each device is a class* derived from an abstract base class *cDevice*. It is the structure and functionality of *cDevice* which makes the whole approach simple and powerful.

Interfaces are handled as *virtual interface drivers*. Each device is assigned a driver (possibly null), i.e., another device which sets/reads it. Since the driver of a device needs not be its owner, this leads to a distinct *interface drivers tree* of devices whose root vertex is usually the main control processor (or the operator who is also an optional virtualized device).

When the package is loaded, the *virtual instrument* is *instantiated* by means of a configuration script. All physical characteristics of the devices composing an *actual instrument* and of its *actual interfaces* are *registered* with the virtual instrument. The *instantiated virtual instrument* thus becomes an image of the actual machine.

During execution, the *virtual instrument* receives from the application *directives* as to what logical actions should be carried out. It tries to do so using the resources of its current instance and terminates by informing the application about whether it succeeded or not.

The concept leads to a highly logical, in-depth modularization of the software. When properly implemented, it also provides for:

- Almost total isolation of the application programmer from the actual hardware.
- Vice versa, isolation of the hardware-control programmer from application details.
- Extreme *hardware adaptability* (the same executable used on different machines).
- Easy implementation of new or improved hardware.
- Good compatibility with event-driven operating systems (Windows, Linux, Unix).
- Reduction of hardware-specific program modules to the barest possible minimum.
- Easier management of team development/maintenance.

The Author wishes to stress the fact that the concept of a ***Universal Virtual NMR Instrument*** – one of which all real NMR machines are imperfect descendants – might go well beyond single Company's software engineering and become a powerful tool of the whole NMR community whose various experts should take part in its development. It would undoubtedly help to uniform NMR hardware and software terminology, provide a *reference ideal* against which to measure the characteristics of real machines, enhance the uniformity of human interfaces, reduce the need for machine-specific User training, help to set up data formats and even uniform scripting of sequences, experiments, automation procedures, etc.

Field/offset noise effects on NMR signals. I. FID's and spectra

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NMR (Nuclear Magnetic Resonance) FID instabilities due to the most common types of magnetic field and/or RF offset fluctuations are investigated. The study explains quantitatively some of the artifacts encountered in practice and indicates methods for overcoming them.

The net effect of a random, normally distributed field noise on *averaged* FID's is shown to be their multiplication by a well-defined weighing function whose shape is intermediate between a Lorentzian and a Gaussian. While integral sensitivity of the instrument is not affected, there is a line shape distortion which is likely to be mistaken for field inhomogeneity.

The effects of periodic and quasi-periodic field perturbations on FID's and spectra are also investigated in detail. The results are directly applicable to the effects of uniformly distributed instabilities (type A) such as mains-related field brum and ripple (including noise picked up from the environment). Instabilities whose magnitudes have a non-uniform spatial distribution (type B) require an additional averaging over individual sample voxels. The study establishes the respective averaging procedures and shows that the net effect of such field fluctuations on averaged FID's is still a simple weighting by a particular function. The formalism is applicable to field fluctuations induced by sample spinning (HR-NMR), sample vibrations (all branches of NMR) and field gradients noise (MRI). Detailed analysis carried out for sample spinning artifacts in HR-NMR indicates the presence of a so far suspected but never quantified effect of *unstable* spinning on the line widths of *all* spectral lines (not just of the rotational sidebands).

Exploiting the new insights, possible methods of suppressing some of the related artifacts are proposed. This includes alternative methods of signal averaging as well as post-acquisition correction techniques for traditionally averaged data.

Field/offset noise effects on NMR signals. II. Hahn echos, CPMG and $T_{1\rho}$.

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Using the techniques developed in Part I, this study investigates the effects of various types of field fluctuations on Hahn echos and CPMG echo trains.

It turns out that, compared to an FID, the echos (both single and multiple) refocus a substantial part of the induced phase errors, provided the field noise is Gaussian and its mean correlation time is not much smaller than tau (the time interval between the 90 and 180-degree pulses). For a normally distributed field noise, the echo-phase errors never exceed those of an FID at the same total elapsed time (measured from excitation).

When the field is subject to a periodic modulation, however, there are tau values at which the refocussing is very good, alternating with regions where the echo phase errors exceed those encountered in an FID. For a single echo, the critical tau values center around odd multiples of $T/2$, T being the modulation period, while the 'good' tau values are in the vicinity of integer multiples of T .

This picture changes in a train of n echos. Increasing n beyond 3, ever more *ample regions* of tau values with excellent field-error refocussing alternate with ever *narrower regions* of severe *resonant error amplification*. The resonant, critical tau values are centered around odd multiples of $T/4$ and may all but preclude a successful application of a CPMG sequence. This, among other things, explains some striking and only partially explained CPMG artifacts noticed by virtually every experimentalist but never explained in sufficient detail (for an excellent but isolated early attempt see A. Allerhand, Rev.Sci.Instruments 41, 269, 1970).

The results provide a basis for the evaluation of errors due to field noise in those experimental techniques which use multiple spin echos and/or spin-locking mechanisms. They are directly applicable also to the $T_{1\rho}$ experiment which can be viewed as a limit case of CPMG for tau tending to 0. Due to field noise, averaged CPMG and $T_{1\rho}$ data are biased in a way which increases the apparent decay rate. Since this noise-induced decay contribution is inherently non-exponential, it can even simulate/modify non-exponentiality. Methods to estimate, detect and overcome such errors are discussed.

STUDIES OF VISCOTOXINS AND THEIR PRECURSORS: STRUCTURE–FUNCTION CORRELATIONS IN VISCOTOXINS FROM VISCUM ALBUM L. AND EXPRESSION OF VISCOTOXIN A3 PRECURSOR IN E. COLI.

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Viscotoxins are a group of small toxic proteins (MW 5KDa) found in several European mistletoe species. Many viscotoxin isoforms have been described and classified, on the basis of sequence homology, as belonging to the α and β thionins family (1). Thionins are basic, cysteine-rich plant polypeptides, well known for their toxicity to bacteria, fungi and animal cells, and are possibly involved in plant defence against phytopathogens (2). Here we report the high resolution three dimensional structure of viscotoxin C1, determined in solution by ¹H NMR at pH 3.6 and 12°C, and its comparison with the structures of i) viscotoxin A3, previously determined (3); ii) viscotoxin B, iii) viscotoxin A1, iv) viscotoxin A2 and v) viscotoxin 1-PS obtained by molecular modelling.

Experimentally derived restraints, including 893 interproton distances, 14 hydrogen bonds and 18 ϕ angle restraints were used for viscotoxin C1 as input to restrained molecular dynamics using DYANA. RMSD of 0.75±0.20 Å and 1.31±0.21 Å were obtained for backbone and heavy atoms, respectively. The overall fold of viscotoxin C1, consisting of two α -helices connected by a turn and a short stretch of anti parallel β -sheet, is in agreement with the fold reported for other thionins (4, 5). The differential biological activity of many viscotoxins is discussed in terms of the differences in the calculated electrostatic surface properties, playing an important role in the cytotoxic activity through cell membranes disruption.

We also report a study on the expression strategy for the viscotoxins A3 precursor in E. coli. Viscotoxins' precursors consist of a short signal sequence at the N-terminus, the viscotoxin domain and, an acidic domain at the C-terminus. The role of the acidic domain is to keep inactive the viscotoxin inside the plant cell, but it is still unknown whether it acts by altering the native conformation of the mature viscotoxin, or by neutralising the surface charges of the protein. The expression of these precursor protein is preliminary to subsequent studies on structure-function relationship.

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