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Nuclear Magnetic Resonance, a Powerful Tool for the Study of Biomolecular Dynamics

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The COST Action D6 has the general aim of investigating 'Chemical Processes and Reactions under Extreme or Nonclassical Conditions'. The usage of such conditions can be motivated by chemical engineering considerations when desired products can be produced only under extreme conditions, such as high pressure or extreme temperatures. On the other hand, the behavior of molecules under extreme conditions may reveal better their inherent structural or dynamical properties. In other words, investigations under extreme conditions can be quite fruitful for an understanding of molecular processes. It is this last aspect which decided for our participation in the COST Action D6. Being interested in the dynamical features of (bio)molecules, the variation of environmental parameters such as temperature and pressure is desirable, with high pressure being the main focus in our studies within COST Action D6.

Perhaps the most powerful presently available analytical tool for studying molecular dynamics under realistic chemical and physiological conditions is nuclear magnetic resonance. It is also well-suited for high-pressure (and high-temperature) studies. The unique power of NMR relies on the function of nuclear spins as local reporters in molecules and on their sensitivity to a wide range of dynamical time scales from picoseconds to seconds and more. Depending on the relevant time scale, different measurement techniques can be used:

- Real time observation after initial perturbation: for extremely slow processes, $t \gtrsim 1$ s.
- 2D exchange spectroscopy (EXSY): for very slow processes, 30 ms $\gtrsim t \gtrsim$ 5 s.
- Line shape effects, exchange broadening and narrowing: for slow processes, 1 ms $\leq t \leq$ 300 ms.
- Rotating frame relaxation measurements: for medium fast processes, 1 µs $< t <$ 10 µs.
- Laboratory frame relaxation measurements: for fast processes, 30 ps $< t <$ 1 µs.
- Average geometric parameter values: for extremely fast processes, $t <$ 100 ps.

We have investigated a variety of intramolecular motional processes by NMR. Three of them shall be mentioned briefly:

Pressure Dependence of Hydrogen Tunneling in a Niobium-Trihydride Complex

It is known that in some nonclassical transition-metal hydrides slow and coherent tunneling processes occur which can be visualized directly by NMR line splittings. The question, whether the tunneling frequency is pressure-dependent was investigated in $[Cp(Si(CH_3)_3)_2]_2NbH_3$ dissolved in methylcyclohexane [1]. It was found that the tunneling frequency decreases by *ca.* 15–20% when increasing the pressure from 1 bar to 5 kbar, whereby the tunnel frequencies themselves are strongly temperature-dependent with 25 Hz at 254 K and 86 Hz at 307 K. The decrease of the tunneling frequency with increasing pressure can be explained by the stronger interaction with the solvent at high pressures which influences the ease of access of vibrationally excited states [2]. There is also a direct correlation with the pressure-dependent density of the solvent [1].

Intramolecular Dynamics in the Cyclic Decapeptide Antamanide

The cyclic decapeptide antamanide ($-V^1-P^2-P^3-A^4-F^5-F^6-P^7-P^8-F^9-F^{10}-$) is at the same time structurally well-defined and dynamically highly mobile. Numerous studies of our research group have been devoted to a detailed characterization of its motional properties. At first, there is a relatively slow backbone torsional mode that leads to a flip between two, possibly four, conformations with an activation energy of 20 kJ/mol and a time constant of ~ 25 µs at 320 K. It has been studied by rotating frame relaxation measurements that are motionally sensitive in this range of time constants [3].

In addition, it has been found that two of the four proline rings, Pro^2 and Pro^7, undergo a rapid ring flipping in the range of 20–30 ps at room temperature, while the other two prolines, Pro^3 and Pro^8, are rigid [4]. This has been studied by $T_1(^{13}C)$ relaxation measurements and can be explained by the different strain imposed by the peptide ring upon the proline rings. Molecular dynamics simulations [5] lead to nearly perfect agreement with the experimental results.

The phenylalanine side chains show two rotational degrees of freedom in the χ_1 and χ_2 angles. Again, $T_1(^{13}C)$ relaxation measurements, combined with molecular dynamics simulations [6][7] allow one to obtain detailed insight into the dynamical processes, and it is possible to distinguish between rotational diffusion and rotational jump processes.

Finally, antamanide binds water molecules, particularly inside of the peptide ring. NMR allows one to study the exchange between free and bound water which seems, under the used experimental conditions, to be a slow process proceeding in the 35-µs range [8].

Pressure-Induced Unfolding of Proteins

Folding and unfolding of proteins are key processes in molecular biology which, however, are still not understood in all details. The unfolding can be induced by a number of external perturbations, such as increasing temperature and the addition of guanidinium hydrochloride or urea. In many cases, the denatured random coil protein undergoes agglomeration, and the unfolding process becomes irreversible.

Sometimes, unfolding by high pressure (3–5 kbar) is less destructive and refolding is possible in a reversible manner. We have studied the protein human ubiquitin and, indeed, found that the pressure unfolding is reversible and allows one to study the process in detail on a molecular scale by high-resolution NMR [9]. Other research groups involved in the COST Action D6 are investigating folding/unfolding processes with optical spectroscopy techniques and obtain complementary information.

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The Proton Sponge: a Trick to Enter Cells the Viruses Did Not Exploit

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Abstract. Several non-permanent polycations possessing substantial buffering capacity below physiological pH, such as lipopolyamines and polyethylenimines, are efficient transfection agents *per se*, *i.e.* without the addition of lysosomotropic bases, or cell targeting, or membrane disruption agents. These vectors have been shown to deliver genes as well as oligonucleotides both *in vitro* and *in vivo*. Our hypothesis is that their efficiency relies on extensive endosome swelling and rupture that provides an escape mechanism for the polycation/DNA particles.

Introduction

Gene transfer is the weak link of gene therapy because DNA is a pro-drug rather than the therapeutic effector molecule itself. The cascade of events leading to the synthesis of a large number of therapeutic protein molecules from a single gene begins in the nucleus. Therefore, vector systems are required to carry the exogenous DNA through the plasma and nuclear membranes. Most often, recombinant viral vectors are used for this task [1], since viruses have evolved sophisticated break-in ways [2] that can now be exploited. These include efficient cell-membrane rupture mechanisms and nuclear targeting. Membrane rupture can occur either directly at the cell surface or after endocytosis. In any case, the viral fusogenic protein becomes 'informed' of the cell proximity and undergoes a major conformational change induced either by binding to a cell surface receptor or by the acidic nature of the endosomal compartment. Chemists examining such complex molecular sequences may well be daunted. Yet synthetic nonviral gene transfer systems, however basic, will be of great potential to the gene therapy field just as soon as they show sufficient *in vivo* transfection capacities. We insist on the term *sufficient*, – the same adjective is apparently used to describe the Rolls-Royce engine power in the technical notice accompanying the car! – as it suggests an adequate performance. However, as emphasized by the recent British and American attempts to apply gene therapy to cystic fibrosis or melanoma patients, we know that this criterion is far from being satisfied [1b].

Chemistry is not constrained by the need for replication characteristic of a biological system and can therefore explore and exploit a much wider spectrum of candidate molecules for a given task. With some imaginative leads and a great deal of 'evolutionary' trial and error, two classes of synthetic vectors have been developed over the last decade. These compounds, whether lipids [3] or polymers [4], are all cationic like their classical predecessors used for *in vitro* transfection (calcium phosphate, DEAE-dextran). On complexing with DNA, they cause several plasmid molecules to condense together into submicrometric particles.

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In vitro Transfection with Cationic Lipids [5]

When a cationic lipid is used at an excess ratio of cationic charges to nucleic-acid phosphates, the resulting nucleolipid particles will fix to the cell surface. Indeed, electrostatic interactions between the positively charged DNA/lipid complexes and anionic heparan sulfate proteoglycans of the cell membranes are enhanced by increasing the overall charge of the complexes, which is in turn achieved by increasing the ratio of lipid to DNA. This interaction between the particle and the cell membrane is spontaneously followed by endocytosis. Cationic lipids give variable transfection efficiencies that depend both on the chemical structure of the vector and on the cell type. Even so, irrespective of the cell type, the lipopolyamines constitute one of most efficient vector classes [3][6][7]. This general efficiency is an intrisic property of the charged head-group, and the addition of neither fusogenic lipids or of nuclear localization signals can increase it, suggesting that the polyamine head group may carry in itself these multifunctional properties [8]. Moreover, when the potentiometric protonation states of the amines were measured, it was found that at physiological pH only three of the four nitrogens in the spermine head were cationic (*Fig. 1*). The pK_a of the last amine is 5.5, halfway between the extracellular and intralysosomial pH values, a clue to a possible buffering property that could well be exploited, and a point we shall return to later.

In vitro Transfection with Cationic Polymers

Compared to the lipopolyamines, most members of the other class of cationic