Centre of Excellence R&D Project

WORK PACKAGES:

1. Structure and interactions in solid state, polycrystallinity, polymorphism (Gregor Mali)
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Work-package title:

Structure and interactions in solid materials, polycrystallinity, polymorphism (Gregor Mali)

During the last two decades we have witnessed a rapid development of solid-state nuclear magnetic resonance (NMR) spectroscopy [1]. The problem of low resolution in spectra of powdered samples was overcome by the development of superconducting magnets that provide static magnetic fields larger than 10 T and magic-angle sample spinning probes that allow spinning rates of several tens of kHz. A rapid sample spinning often averages out anisotropic interactions like chemical shift anisotropy and magnetic dipolar coupling, which are the source of broad overlapping spectral lines. On the other hand, the magnitude of the dipolar coupling bears information about the internuclear distances within molecules or crystals. By suppressing dipolar interactions magic-angle spinning thus suppresses also some information about a microscopic structure of solid material. Recently a number of methods were developed, in which the dipolar coupling in rotating samples is reintroduced by aid of radiofrequency fields. This allows one to extract information on internuclear distances even from high-resolution solid-state NMR spectra. New methods are already being employed for structural studies of proteins, polypeptides, nucleosides, polymers, etc.

In the field of pharmacy the determination of structure by solid-state NMR spectroscopy has not commonly been applied so far. NMR spectra have, however, already become indispensable for an analysis of crystallinity, polymorphism and hydration of samples [2]. Solid-state NMR measurements are very important especially due to the fact that the majority of pharmaceutical products are in a solid form. Moreover, the NMR signals that belong to drugs usually do not overlap with NMR signals that belong to excipients, which means that NMR can really allow for studies of products in their final form, e.g. in pills.

Within the last few years so called high-resolution solid-state NMR spectroscopy started to develop in Slovenia as well. Up to now, new techniques were prepared and employed mostly for an analysis of solid inorganic (micro- and mesoporous zeolitic) materials, though, lately, the NMR centre at National Institute of Chemistry started to collaborate with Slovenian pharmaceutical companies in the field of solid materials, as well. We believe that the

knowledge, obtained so far, allows us to prepare demanding techniques for an analysis of structure and interactions in materials that are interesting for biochemistry or pharmacy.

Work within the proposed work-package would roughly consist of three consecutive steps. Within the first step some modern NMR methods for structural characterization of solid pharmaceutical or biochemical samples should be introduced. The methods would comprise of those that could additionally improve resolution of carbon and hydrogen spectra, and of those that would enable qualitative and perhaps even quantitative analyses (determination of distances) of homo- and heteronuclear correlations. The methods should afterwards be extensively tested on adequate test samples. For the first two steps approximately 12 – 15 months would be needed. Within the third step the above described methods would be applied to problems emerging from solid pharmaceutical samples from two Slovenian pharmaceutical companies. For measurements and interpretations of spectra roughly 9 months would be needed. Some measurements from the complete set of prepared NMR techniques could become routine characterization methods for solid pharmaceutical samples and could in the future be regularly performed by a person, who would gain the required knowledge about solid-state NMR spectroscopy during the course of the proposed project. In addition to NMR analyses all pharmaceutical samples would be subjected also to X-ray diffraction investigation. In such a way one could find out to what extent the information obtained by two techniques overlap and to what extent the information is complementary.

The feasibility (or the success) of the proposed work-package partly depends on the upgrade of the equipment of NMR centre at National Institute of Chemistry. A purchase of a new 800 MHz NMR spectrometer along with a fast magic-angle sample spinning probe (spin rates up to 25 or 30 kHz) could impact the development of high-resolution solid-state NMR in two ways. The new spectrometer equipped with the new probe would enable application of the most demanding solid-state NMR techniques – including high resolution measurements of hydrogen spectra, which present one of the very demanding problems in solids. A sufficient resolution of signals in carbon spectra is achieved already on an existing 600 MHz spectrometer. However, the latter spectrometer is currently used mostly for original scientific research and only little for pharmaceutical applications. With a planned purchase of an 800 MHz spectrometer most of the research would transfer to the new spectrometer, which would allow a much longer experimental time for research of pharmaceutical solids on the 600 MHz spectrometer, even for some more demanding routine measurements.

To summarize, with the proposed work-package we would like to reach a current state-of-the-art in the high-resolution solid-state NMR spectroscopy of biologically and especially pharmaceutically interesting materials. For requirements of Slovenian pharmaceutical industry we wish, in tight cooperation with them, to prepare the necessary methods and to train a person for a detailed high-resolution NMR characterization of solid samples.

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Work-package title:

Structural determination and analysis of organic compounds in solution using NMR spectroscopy (Janez Plavec)

The vast majority of active ingredients in drugs on the market are still small organic compounds with the molecular mass below 1000 g/mol. That is the reason why the organic synthesis of such compounds is of utmost importance for the pharmaceutical industry. Crucial part in the organic synthesis is the analysis and structural determination of main and side products. Nowadays different spectroscopic techniques are used for analysis and structural determination of organic compounds.[1] NMR spectroscopy is certainly one of the most important methods as it allows to follow the time-course of the reaction, analysis and identification of main and side products, determination of their structures and studies of conformational equilibria in the solution.[2] This is necessary in order to optimize synthetic procedures and to choose the optimal synthetic route.[3]

NMR spectroscopy provides information necessary for the aforementioned studies through examination of chemical shifts, coupling constants and NOE and ROE effects. The chemical shift depends on the chemical environment of magnetically active nuclei. In organic compounds diverse protons such as aromatic or aliphatic protons exhibit their characteristic chemical shifts that are structure dependent and are informative of an overall 3D structure. Characteristic chemical shifts offer first indication about compounds that have formed during synthesis. Assignment of individual proton and heteroatom resonances is performed with the use of several one- and two-dimensional NMR techniques. Scalar coupling constants ($^{n}J_{XY}$, where n denotes the number of chemical bonds between coupled nuclei x and y) are another very important source of structural information. We can distinguish between homonuclear ($^{n}J_{HH}$ proton–proton coupling constants) and heteronuclear coupling constants ($^{n}J_{PH}$, $^{n}J_{CP}$, $^{n}J_{NH}$, *etc.*). Analysis and interpretation of multiple bond coupling constants gives information about torsion angles between coupled nuclei. The most important structural information is still obtained through the measurement of NOE and ROE effects.[4] These enable the extraction of distance restraints between pairs of protons that are in close spatial proximity and are indispensable in the determination of 3D structures of molecules. Structural data obtained from NMR experiments can be used as an input for the molecular modelling in order to build three-dimensional models of compounds under investigation.[5,6]

Although organic synthesis deals mainly with smaller molecules in certain cases very complicated NMR spectra are obtained (*e.g.* complex reaction mixtures) with heavy overlap of signals or signals could not be detected because of low concentrations. Such cases, which are by no means rare, can only be resolved using high quality NMR equipment. This in the first place means NMR spectrometers with high resolution and high sensitivity. Slovenian NMR centre that is located at National Institute of Chemistry is currently equipped with two 300 MHz and one 600 MHz instruments. Upgrading the existent equipment with 800 MHz spectrometer that offers high resolution and high signal/noise ratio should enable us to resolve aforementioned problems of complex reaction mixtures as well as studies of samples with very low concentrations.

Slovenian NMR centre already collaborates with the industrial partners Lek and Krka. They are co-owners of the equipment located at the NMR centre and are using it on regular basis for routine measurements as well as in research and development. The purpose of this R&D project is to further strengthen collaboration between NMR centre and both industrial partners. NMR centre can provide highly qualified personnel in the field of NMR spectroscopy in order to help industrial partners to solve structural problems that can occur during organic synthesis (*e.g.* structural determination of diastereoisomers, cis-trans isomers *etc.*). In this way transfer of knowledge from the scientific institution (National Institute of

Chemistry) to industrial partners (Lek and Krka) could be achieved. The benefit of such collaboration is mutual. Scientific co-workers from NMR centre on one hand will come in touch with real problems that occur in the industry, industrial partners, on the other hand, will have a constant access to a highly qualified personnel and high-quality equipment in the field of NMR spectroscopy. Successful transfer of knowledge between academic sphere and industry could help in more rapid solving of problems that can come about in the pharmaceutical industry. This can have direct positive economic influences on both industrial partners as well as on National Institute of Chemistry.

As the work-package: "Structural determination and analysis of organic compounds in solution using NMR spectroscopy" involves continuous following of organic synthesis which is an iterative process (analysis and identification of products from current synthetic procedure provides the information about possible improvements of the synthetic procedure *etc.*) it is almost impossible to divide it into individual time-phases. Our estimate is three to seven months for the individual problem. This time estimate includes sample preparation, recording of NMR spectra and interpretation of data as well as preparation of final report.

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Work-package title:

Characterization of structure and interactions of biologically important molecules, rational drug design (Simona Golič Grdadolnik)

High-resolution NMR spectroscopy is a powerful technique for investigation and design of drug molecules. Its high ability in determination of three-dimensional structure and dynamics of molecules in solution is utilized for comparative studies of conformation and biological activity of drugs, which lead to rational design of new synthetic molecules with better physicochemical properties [1,2]. Several NMR methods for studies of drug-receptor complexes have been developed and applied in modern research of novel drugs [3]. The conformation of the bound drug can be separately determined by application of isotope edited proton NMR or NOE-difference experiments. Such studies allow identification of functional groups of the drug important for binding and determination of their spatial orientation, which is vital for design of analogues with higher potency and better physical properties. For the NMR studies of tightly bound drugs one of the members of the complex must be isotopically labelled. The most detailed structural information for the drug design is obtained from the studies of the complete three-dimensional structure of the drug-receptor complex, which require application of modern three- and four-dimensional heteronuclear NMR methods and isotopically labelled proteins. These methods are designed to overcome the NMR

spectroscopic problems, which are associated with the large molecular mass of proteins. Conformational studies of the complex provide information about the drug and protein functional groups, their interactions and about the steric and electronic properties of the receptor-binding pocket. Knowledge of the protein binding site and better appreciation of interaction energies enables design of analogues with the improved binding affinity and increased specificity. A great advantage of conformational studies using the high resolution NMR spectroscopy is the solution environment, which is close to physiological conditions in living cells. The proper environment can be selected even for the studies of drugs, which reach the receptor-binding site after the diffusion through the membrane bi-layers. The drug-membrane interactions and their influence on biological activity can be studied by NMR methods [4].

Recently, the NMR spectroscopy has been very efficiently applied at an earlier stage of drug design, as a new screening technique [5]. The so-called SAR (structure-activity-relationships) by NMR is a linked-fragment based approach, where a new lead compound is constructed from individual fragments, which bind weakly to the protein. In particular, the ability of NMR spectroscopy in identification of weakly binding ligands through the observation of chemical shift changes of the protein signals is utilized. The molecules, which are found to interact with the protein, are further improved on the basis of knowledge of their interactions with the protein target. Finally, the linkers are selected, which can combine the individual fragments giving rise to potent lead compounds. A great advantage of SAR by NMR is acquirement of structural information. The fragments that bind to the protein are identified before linking and linkers are selected on the basis of structural information. Thus the number of compounds in the library, which have to be synthesized, is reduced and there is no need for investigation of a huge number of compounds by random screening. SAR by NMR is a very reliable and robust screening technique originally developed in Abbott Laboratories, which allows investigation of binding of a large number of compounds per day. We are using this method for investigation of novel DNA gyrase inhibitors [6]

At National Institute of Chemistry several research projects, which involve investigation of biologically important molecules with modern NMR techniques are currently in progress. In the field of drug research and development we collaborate with Slovenian pharmaceutical industry, Faculty of Pharmacy and Faculty of Chemistry and Chemical Technology for many years. With the new 800 MHz spectrometer our capacities for drug research would be greatly improved and expenses reduced. In particular, the higher sensitivity of new NMR equipment would allow measurements at lower concentrations of expensive isotopically labelled proteins and would reduce the measurement times. We would be able to cover the present needs of Slovenian industrial and academic sphere. Moreover, new options for collaboration of experts in structural and synthetic chemistry would be opened, which could enlarge the progress in drug research and development in the state.

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Work-package title:

Characterization of recombinant proteins and biological macromolecules in solution (Primož Pristovšek)

High-resolution biomolecular nuclear magnetic resonance (NMR) has recently expanded dramatically and is now established as one of the most important methods for structural and functional characterization of recombinant proteins and their complexes with other proteins or DNA and RNA. Most useful in this respect is the chemical shift of the ^{1}H, ^{13}C and ^{15}N nuclei being strongly dependent on the nature of their chemical environment. Simple one- and two-dimensional experiments (*e.g.* NOESY with isotopically unlabelled samples, and HSQC with ^{15}N labelled samples) provide data on the native state of the protein (whether it is denatured or folded; in the latter case dispersed resonances are expected) and presence of secondary structures. HSQC spectra are in this respect characteristic for proteins at a given temperature, ionic strength and pH that they can be used for its identification and determination of percentage of denaturation. It is also most useful to detect interactions with other molecules, *e.g.* other proteins, DNA/RNA or smaller molecules [1],[2]. In the first case effects of interactions of (isotopically unlabelled) protein A on (isotopically labelled) protein B can be detected, and *vice-versa*; based on these independent data a reliable model of the complex of both proteins can be calculated if both assignments of HSQC signals are known [3]. Such a method can also be applied for short-lived complexes [4].

NMR is also the only method for determination of three-dimensional structure and dynamics of proteins in solution [5],[6], *i.e.* in conditions that correspond most closely to their natural environment. It does not rely exclusively on NOE signals anymore, but also on residual dipolar couplings that occur in anisotropic media and provide structural information based on their dependence on the angle between the bond vector and the magnetic field [7]. The limitation on the size of proteins that can be studied with NMR has recently been significantly diminished with the development of TROSY sequences that enable insight even into 900 kDa systems [8]. The TROSY effect, however, becomes significant only at magnetic fileds above 700 MHz; the acquisition of an 800 MHz spectrometer would therefore enable the Slovenian NMR centre to tackle larger proteins and complexes of pharmacological importance. At present we depend on access to equipment in large infrastructural centres abroad, *e.g.* the Large scale facility in Frankfurt; in these cases, however, we have to partially disclose the subject and method of our study, and have to wait for access for months; additionally, the samples do not always survive the transport.

The time table of a typical project of structural characterization of biological macromolecules consists of the following steps:

- Sample preparation, where optimal conditions for acquisition (concentration, temperature, ionic strength, pH, etc.) have to be determined, can take a few weeks even with established biotechnological production and has to be followed with spectroscopic methods constantly,
- NMR acquisition, up to 3-4 weeks of continued measurement time in case of double ($^{13}C/^{15}N$) labelled samples,
- Processing and analysis of spectra (assignment of resonances, NOE signals etc.), in case of large systems a few months if access to modern computer hardware and software is provided,
- Final modelling of the structure that is usually finished within a few weeks, and is in many cases expanded with molecular modelling using docking and/or molecular dynamics protocols.

New knowledge on the recombinant proteins that is acquired in this way are 3D structure [2,6], dynamics on a microsecond or nanosecond time scale [6], data on the structure of the

complex of two macromolecules [2,4] and the identification of changes upon chemical or biochemical modification and/or transformation.

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Work-package title:

Research of complex mixtures in solution - impurity profile in drugs, degradation products, metabolites (Roman Jerala)

Demands on purity of bioactive pharmaceuticals are increasing constantly. It is essential to determine the total content of impurities for each active substance, which should not exceed 1% and on the other side to determine the identity of those impurities (treated also in work-package on Structural determination and analysis of organic compounds). Analysis of impurities is today mainly performed by chromatographic methods (mainly HPLC and GC) which are interfaced to mass spectrometer. Setting up proper separation conditions is time consuming. In many cases, particularly when the impurities contain NMR-active nuclei (^{1}H, ^{19}F,…) detection and identification of impurities can be performed by high-resolution nuclear magnetic resonance, particularly aided by signal suppression of dominating species in solution. High-field NMR spectrometers provide better signal dispersion, preventing signal overlap of a number of compounds in the complex mixture. This method is also applicable for continuous monitoring of the progress of chemical reaction and detection of produced impurities.

High-field NMR spectrometry is particularly advantageous for the analysis of complex mixtures, characteristic for biological systems, where we can either quantify the content of each component or on the other side determine the profile of low molecular weight components, which is particularly important for the metabolite analysis of industrial production microorganisms. NMR methods are used to monitor change of the physiological state of the producing organism in living cells through metabolite conversion, change of the intracellular pH, etc. This information is essential for understanding of physiological processes, which are responsible for biomass production and production of desired compounds. Those compounds in case of industrial microorganisms often represent secondary metabolites, such as statins as one of the most frequently prescribed drugs, which are produced by streptomycetes. Most common metabolites can be identified through characteristic chemical shifts and in case of good separation of signals we can also determine their concentrations. The alternative option is the use of the fingerprint approach, where we can compare samples of different mixtures (natural products, metabolites, biological fluids) without having to identify separate components. Chemometric analysis based on the

fingerprint can be used to identify or select production strains with particular trait or production conditions.

Perfusion systems, with continuous flow of cultivation media between the living cells and probe of the NMR spectrometer is used to analyze extracellular components produced by living cells. NMR allows the following measurements on intact cells:

- determination of the concentration of major intracellular metabolites,
- accurate, continuous monitoring of specific extracellular metabolites,
- determination of phosphorylated compounds, based on ^{31}P signal,
- determination of intracellular pH and analysis of cultivation conditions, which affect intracellular pH,
- monitoring of metabolism of particular minerals,
- transport of specific molecules across the cell membrane.

Additional opportunity for the use of NMR in biotechnology represents the possibility to follow the time course and quantification of conversion of nutrients (*e.g.* glucose, nitrogen sources, aminoacids) into final products, where we add isotopically enriched compounds, such as ^{13}C into the medium. Analysis of the results provides the data for mass balance analysis and process or strain optimization.

Compounds in the complex mixture can be separated based on the diffusion coefficient in addition to chemical shift, which translates to the size of the molecular species in solution. DOSY (diffusion ordered spectroscopy) technique provides two-dimensional spectra where in one dimension we separate components according to their chemical shift and in the second dimension according to their diffusion coefficient. This is also used to identify formation of complexes or aggregates in solution.

High sensitivity instrument is needed for the sensitivity necessary for analysis of complex mixtures. This sensitivity is achieved in the instrument at high field and by application of cryo-probes, which can increase the sensitivity by as much as an order of magnitude. Automatic sample exchanger allows processing of a large number of samples and continuing analysis of biological processes such as fermentation.

Precision of the analysis of impurities in drugs, their degradation products, metabolites, biological fluids, etc. would increase by investment into the 800 MHz spectrometer and simultaneously decrease the required experimental time.

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