Developing novel applications of Dynamic Nuclear Polarization in solid-state NMR spectroscopy

Alessandra Lucini Paioni

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Developing novel applications of Dynamic Nuclear Polarization in solid-state NMR spectroscopy

**Ontwikkeling van nieuwe toepassingen van Dynamische
Nucleaire Polarisatie in vaste-stof NMR spectroscopie**

(met een samenvatting in het Nederlands)

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Alla mia famiglia

(To my family)

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LIST OF ABBREVIATIONS

CE	Cross Effect
CSP	Chemical Shift Perturbations
CP	Cross Polarization
DE	Direct excitation
DNP	Dynamic Nuclear Polarization
2Q-1Q	Double-Quantum Single-Quantum
DRS	Diffuse Reflectance Spectroscopy
EPR	Electron Paramagnetic Resonance
ETH	Ethanol-to-Hydrocarbon
GC-MS	Gas chromatography-mass spectrometry
HSQC	Heteronuclear Single Quantum Coherence
INEPT	Insensitive Nuclei Enhanced by Polarization Transfer
LAC	Level Anti-Crossing
MAS	Magic Angle Spinning
MD	Molecular Dynamics
MISSISSIPI	Multiple Intense Solvent Suppression Intended for Sensitive Spectroscopic Investigation of Protonated Proteins
MRI	Magnetic Resonance Imaging
MS	Mass Spectrometry
MTH	Methanol-to-Hydrocarbon
NMR	Nuclear Magnetic Resonance
PA	Polarizing agent
PARIS	Phase-alternated recoupling irradiation scheme
PISSARRO	Phase-Inverted Supercycled Sequence for Attenuation of Rotary Resonance
PDSD	Proton-driven Spin Diffusion
ssNMR	Solid State Nuclear Magnetic Resonance
r.f.	radio-frequency
TN	Trityl-Nitroxide
TOBSY	TOTAL through Bond correlation Spectroscopy
UV-vis	Ultraviolet-visible

CHAPTER 1

General Introduction

Last section of this chapter titled “DNP and cellular NMR” has been adapted from the following review article:

“DNP and cellular solid-state NMR” - in *eMagRes*, 7 (3) 2018, pp. 51-62

Alessandra Lucini Paioni, Marie Renault, and Marc Baldus

Introduction

Nuclear Magnetic Resonance (NMR) is a powerful spectroscopic technique able to provide atomic-level information on the structure and dynamics of different systems, whether they are solids, liquids or gases. After the first successful observation of NMR signals in bulk materials reported by Bloch^[1] and Purcell^[2], the technique has experienced impressive developments, enabling the study of increasingly complex objects. Nowadays, NMR is recognized as a major analytical tool in a variety of disciplines, ranging from physics to archeology, biology, and material science. Moreover, a specialized type of NMR known as magnetic resonance imaging (MRI) has become a reliable diagnostic tool in both human and veterinary science and medicine. Such ubiquity would not have occurred without innovation in magnet technologies, that created stronger homogeneous magnetic fields. In addition, the discovery of Fourier-Transform NMR (FT-NMR) by Ernst and the subsequent development of multi-dimensional NMR techniques^[3] became indispensable for structural studies in solutions and solids. As a result, NMR is one of the primary analytical methods currently used in modern chemistry and structural biology. This being said, research in the NMR field is constantly expanding. Continuous improvement in instrumentations and techniques are fostering important novel applications. Broadly speaking, efforts towards improving the quality of NMR experiments address two key challenges; enhancing resolution and obtaining higher sensitivity. As such, the latest advances include the development of increasingly stronger magnetic fields, and the world record of 28 T was set by an ultra-high field NMR magnet just this year. At the same time, important progress has been made thanks to the development of the *Hyperpolarization Techniques*. These techniques allow us to enhance the nuclear spin polarization, increasing the signal-to-noise ratio and therefore the sensitivity of NMR experiments. Among these hyperpolarization techniques, Dynamic Nuclear Polarization (DNP) stands out due to its broad applicability in material science and biological solid-state NMR^[4].

In this thesis, novel solid-state NMR methodologies and DNP approaches are presented with their related applications. A variety of systems have been studied; from in-cell samples, where specific molecules are probed directly in their native environment in whole cells, to the molecular elucidation of organic species produced during the zeolite-catalyzed methanol-to-hydrocarbon (MTH) process. Before presenting these studies, the following introductory sections provide the fundamental aspects of (solid-state) NMR spectroscopy^[5-8] and Dynamic Nuclear Polarization^[4, 9-11].

Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) spectroscopy can provide detailed information about the chemical environment as well as structure and dynamics of molecules. Nuclei that are observable using NMR must possess a property called spin (I); only these magnetically active nuclei have a magnetic moment (μ) which can interact with a magnetic field. Since most species have at least one naturally occurring magnetic isotope, NMR can be applied to almost any element. For instance, ^1H , ^2H , ^{13}C , ^{15}N , ^{19}F and ^{31}P , are common isotopes probed in biological NMR^[12, 13]. Alternatively, ^{13}C , ^{17}O , ^{27}Al , and ^{29}Si are commonly used for applications in material science^[14-16] (see Table 1.1). The non-invasiveness of NMR is undoubtedly one of its most useful and unique characteristics. This quality is due to the fact that nuclear magnetic moments are extremely sensitive to their surroundings but at the same time interact very weakly with them. The magnetic moment μ of a nucleus is determined by its specific gyromagnetic ratio (γ) and the spin angular momentum (I), as shown in eq. 1.1

$$\mu = \gamma I \quad (1.1)$$

Nucleus	Spin I	Natural Abundance (%)	Gyromagnetic ratio (γ) $10^6 \text{ rad} \cdot \text{T}^{-1} \cdot \text{s}^{-1}$
^1H	$1/2$	99.98	267.522
^2H	1	0.016	41.065
^{13}C	$1/2$	1.108	67.283
^{15}N	$1/2$	0.366	-27.116
^{17}O	$5/2$	0.037	-36.264
^{19}F	$1/2$	100	251.662
^{27}Al	$5/2$	100	69.763
^{29}Si	$1/2$	4.683	-53.190
^{31}P	$1/2$	100	108.291

Table 1.1 NMR Properties of selected nuclei.

Since the spin is an intrinsic quantum mechanical quantity, a full description of the NMR phenomenon requires the use of a quantum mechanical approach. The magnitude of the spin quantum number I is quantized in units of $\hbar = h/2\pi$ (where h is the Planck constant). A nucleus with spin I can have $2I+1$ non-degenerate energy levels, when placed in a magnetic field $\mathbf{B}_0$. The energy levels can be distinguished by their magnetic quantum number m_I , which has $2I+1$ values running from $-I$ to $+I$ in steps of $+1$.

In the absence of a magnetic field, all $2I+1$ levels have the same energy, but this degeneracy is removed when a magnetic field is applied. The energy E of a magnetic moment ($\boldsymbol{\mu}$) in a magnetic field $\mathbf{B}_0$ is given by

$$E = -\boldsymbol{\mu} \cdot \mathbf{B}_0 \quad (1.2)$$

This energy term is called the Zeeman interaction and the splitting into different energy states upon application of an external magnetic field is referred to as Zeeman splitting. The energy of the individual states can be derived by eq. 1.2 and is proportional to the magnetic field strength (B_0) and the gyromagnetic ratio (γ)

$$E(m) = -m_I \hbar \gamma B_0 \quad (1.3)$$

Transitions can occur between these energy levels according to the selection rule $\Delta m = \pm 1$, therefore only transitions between adjacent energy levels are allowed. Hence, the NMR resonance condition is

$$\Delta E = \hbar \gamma B_0 = \hbar \omega_0 \quad (1.4)$$

$$\nu_0 = \omega_0/2\pi = \gamma B_0/2\pi \quad (1.5)$$

Where ΔE is the energy difference between adjacent states and ν_0 , called the Larmor frequency, is the frequency of the electromagnetic radiation, which depends on the nature of the nuclei and the strength of the applied magnetic field.

For example, in the case of a spin $1/2$ (which represents the majority of the nuclei conventionally probed in NMR, as shown in Table 1.1) there are only two permitted orientations when placed in an external magnetic field (fig.1.1). The spin angular momentum can be either parallel (low energy state) or antiparallel (high energy state) with respect to the magnetic field $\mathbf{B}_0$.

Figure 1.1 Zeeman energy levels for a spin $I=1/2$ in an external magnetic field B_0 applied along the z -direction. In the sketch, the nuclear gyromagnetic ratio is assumed positive.

Therefore, a nucleus with a spin of $1/2$ will experience a transition between the energy levels when a radiofrequency irradiation is applied at frequency ν_0 , and the absorbed energy is registered as a NMR signal frequency.

Conventional field strengths used in NMR are in order of tens of Tesla, roughly 10^5 times stronger than the Earth's magnetic field. As a consequence, typical resonance frequencies in NMR (eq. 1.5) fall in the radiofrequency region, in contrast to most other spectroscopies (e.g. Raman, FTIR, UV-Vis, X-ray or Mössbauer) which make use of higher frequencies.

A typical NMR experiment probes an ensemble of nuclei, and each nuclei's spin can align either parallel or antiparallel to the external magnetic field to occupy one of the two energy states. Each state will have a different population and it is known that nuclei in an ensemble populate the Zeeman energy levels according to the Boltzmann distribution (eq. 1.6):

$$n_{upper}/n_{lower} = e^{-\frac{\Delta E}{k_B T}} \quad (1.6)$$

$$\begin{aligned} p &= \frac{n_{upper} - n_{lower}}{n_{upper} + n_{lower}} \\ &= \tanh\left(\frac{\Delta E}{2k_B T}\right) \end{aligned} \quad (1.7)$$

Where $\Delta E = \hbar\gamma B_0$ is the energy gap, k_B the Boltzmann's constant, T the temperature and p the spin polarization.

In a macroscopic sample at thermal equilibrium there will be a slight excess of spins in the lower energy level, giving rise to a *net* or *bulk magnetization* $\mathbf{M}$, which is measured by NMR. The macroscopic magnetization $\mathbf{M}$ created in a sample in the presence of an external field will be aligned parallel to magnetic field $\mathbf{B}_0$, usually oriented along the z-axis. The bulk magnetization can be rotated away from its equilibrium position by the effect of a radiofrequency pulse (r.f.), which is an oscillating magnetic field applied in the x,y-plane with an oscillation frequency equal to the Larmor frequency. The effect of a r.f. pulse is to rotate the magnetization vector, as shown in fig. 1.2.

Figure 1.2 The effect of a radiofrequency pulse. (A) Before the application of the pulse the magnetization vector $\mathbf{M}$ is in its equilibrium position, along the z-axis. (B) Upon a 90° x-pulse the magnetization is rotated away from the equilibrium position and is aligned along the y-axis. (C) After the application of the pulse the net magnetization relaxes back to the initial equilibrium state. (D) The NMR signal is recorded as a free induction decay (FID).

Depending on the r.f. phase and duration, the net magnetization will be reoriented at different positions and it will begin to evolve in the x,y-plane, where the detection of the NMR signal occurs. In fact, the precession of the nuclear spins in the x,y-plane will induce an electric field and the NMR signal is measured as an oscillating voltage on the same coil that delivered the r.f. pulse(s).

In the absence of r.f. irradiation, the net magnetization will return to the initial equilibrium state through a process called nuclear spin relaxation. The magnetization in the x,y-plane will therefore decay with time and the NMR signal is recorded as a free induction decay (FID). Finally, a Fourier-transformation is applied to the FID to obtain the NMR spectrum (in frequency units).

The signal intensity of an NMR experiment is ultimately determined by the population difference between the levels. However, the energy gap (ΔE) is usually small compared to the thermal energy term ($k_B T$) at room temperature and therefore the population difference ($n_{upper} - n_{lower}$) is extremely small relative to the total number of nuclei ($n_{upper} + n_{lower}$), resulting in a low spin polarization (eq. 1.7). For instance, assuming an external magnetic field of 9.4 T and temperature of 298 K, the relative excess of ^1H spins in the lower energy state is only 32 in 10^6 . This is the reason for the low sensitivity of NMR in comparison with other spectroscopies. However, the sensitivity increases at lower temperatures and higher magnetic fields. Moreover, it can be enhanced by special approaches as will be discussed in the following section that covers hyperpolarization methods, with a particular focus on the Dynamic Nuclear Polarization technique.

Spin Interactions in NMR

Nuclear spins in an NMR sample are not isolated. In addition to interacting with externally applied magnetic fields and r.f. pulses, they interact with their local environment. These internal spin-spin interactions are mediated via chemical bonds or through space and greatly affect the NMR spectra via different phenomena such as: chemical shift, spin-spin coupling, quadrupolar coupling and dipolar coupling. Nuclear spins are typically weakly coupled to their surroundings and therefore the Hamiltonians describing these interactions are orders of magnitude weaker than the Zeeman Hamiltonian, which represents the interaction of the nuclear spin with the external magnetic field. Nonetheless, these internal Hamiltonians are extremely important for obtaining detailed information about the structure, chemical environment and dynamics of the observed nuclei. The strength of these interactions may vary depending whether the studied sample is a solution or a solid.

Spin Interaction	Hamiltonian
Chemical Shift	$\hat{H}_{CS} = \gamma_I \hat{\mathbf{I}} \cdot \boldsymbol{\sigma} \cdot \mathbf{B}_0$
J-Coupling	$\hat{H}_J = \hat{\mathbf{I}} \cdot \mathbf{J} \cdot \hat{\mathbf{S}}$
Dipole-Dipole Coupling	$\hat{H}_D = \frac{\mu_0}{4\pi} \frac{\gamma_I \gamma_S \hbar}{r_{IS}^3} \left(\hat{\mathbf{I}} \cdot \hat{\mathbf{S}} - \frac{3(\hat{\mathbf{I}} \cdot \mathbf{r}_{IS})(\hat{\mathbf{S}} \cdot \mathbf{r}_{IS})}{r_{IS}^2} \right) = \hat{\mathbf{I}} \cdot \mathbf{D} \cdot \hat{\mathbf{S}}$

Table 1.2 Hamiltonians of internal spin interactions between spin $\mathbf{I}$ and $\mathbf{S}$. $\boldsymbol{\sigma}$, $\mathbf{J}$ and $\mathbf{D}$ represent the chemical shift tensor, the J-coupling and the dipolar tensor, respectively. r_{IS} is the distance between the spins.

The most prevalent internal interactions in solid-state NMR are presented in Table 1.2 and a brief description of each is given below^[17].

Nuclei with spins greater than 1/2 exhibit another important interaction called the quadrupolar coupling. These nuclei possess an electric quadrupole moment, in addition to the magnetic moment. Consequently, the electric quadrupole moment of the nucleus interacts strongly with the electric field gradient generated by surrounding electron clouds. It is therefore important to account for this interaction when dealing with quadrupolar nuclei. In fact, the quadrupolar interaction affects the nuclear spin energy levels and can be much stronger than the other magnetic interactions. However, a detailed description of quadrupolar couplings is beyond the scope of this thesis.

Chemical shift

The actual field experienced by a nucleus in an atom or a molecule differs slightly from the externally applied field $\mathbf{B}_0$ due to its interaction with surrounding electrons. In fact, the external magnetic field $\mathbf{B}_0$ induces currents in the electron clouds in a molecule, which in turn generate an additional local magnetic field ($\mathbf{B}_{loc}$) that contributes to the total magnetic field felt by the nucleus (eq. 1.8). The interaction of the nucleus with the field produced by the electrons is known as the shielding interaction and causes a shift in the resonance frequency ω (eq. 1.9).

$$\mathbf{B}_{tot} = \mathbf{B}_0 + \mathbf{B}_{loc} = \mathbf{B}_0 + \boldsymbol{\sigma} \cdot \mathbf{B}_0 \quad (1.8)$$

$$\omega = \omega_0 + \omega_{cs}(\theta, \varphi) \quad (1.9)$$

The magnitude and direction of the local field on a given nucleus depend on the orientation of the molecule with respect to the external field and on the location of the nuclear spin within the molecule. This is why the resonance frequency of a nucleus is highly sensitive to its environment. The characteristic shift in frequency of the signal in the NMR spectrum caused by this interaction is defined as the *chemical shift*.

This is an anisotropic interaction and the chemical shift tensor $\boldsymbol{\sigma}$ describes how the size of shielding varies with molecular orientation and takes into account the fact that the local field induced by the electrons can have a different direction to the external field. Consequently, the chemical shift frequency also depends on the molecular orientation (eq. 1.9) (θ, φ are the polar angles defining the orientation of the $\mathbf{B}_0$ field with respect to the chemical shift tensor). The effect of the chemical shift anisotropy (CSA) is displayed in fig. 1.3. In a typical powder sample the molecules can adopt all possible orientations,

and each molecular orientation has a different chemical shift associated with it. The solid-state NMR spectrum is therefore a superposition of the different signals resulting in a broadening of the lineshape called the “powder pattern”.

The situation is different in liquids, where the molecules can tumble rapidly, assuming every orientation with equal probability. This leads to the averaging of the anisotropic interaction and only the isotropic component of the chemical shift contributes to the NMR spectrum.

In practice, it is common to define the chemical shift in terms of the difference in resonance frequencies between the nucleus of interest (ν) and a reference signal (ν_{ref}), by means of a dimensionless parameter δ

$$\delta = \frac{\nu - \nu_{\text{ref}}}{\nu_{\text{ref}}} 10^6 \quad (1.10)$$

where δ is given in *parts per million*, or ppm, and is independent of the magnetic field. This allows comparison of NMR spectra recorded using different spectrometers.

Figure 1.3 Schematic illustration of the CSA Effect. On the left, the influence of the orientation on the chemical shift for a single crystal in a static magnetic field B_0 . On the right, the formation of the powder pattern observed for solid samples.

J coupling

J-coupling, otherwise known as scalar coupling, is an indirect coupling between nuclear spins mediated by the bonding electrons. The J-coupling therefore provides a direct spectral manifestation of the chemical bonds. The size of the J coupling depends on the bonding electron orbitals and it is independent of the applied magnetic field. Moreover, it is an isotropic interaction and therefore visible in solids and liquids, where it causes characteristic splitting of NMR signals.

The J-coupling originates from the hyperfine interaction between nuclei and electrons; specifically, the Fermi contact interaction. Fermi contact refers to the interaction between the nucleus and the electron in the s-orbital, whereby the electron can sense the orientation of a nearby nuclear spin. Fermi contact also causes hyperfine splittings which are visible in the EPR (electron paramagnetic resonance) spectra of paramagnetic molecules.

In summary, J coupling originates from the interaction of the nucleus with a nearby electron, which in turn interacts with the electron it shares a chemical bond with. Since the Pauli principle forbids two electrons with parallel spins to be in the same orbital, the second electron must assume an opposite spin orientation. As a further consequence, the second nucleus in the bond will adopt an orientation opposite to the electron spin due to the hyperfine interaction. The overall effect results in an indirect coupling between the two nuclei, where the state in which the two nuclei have opposite polarizations is energetically favourable.

A typical NMR experiment used in solution- and solid-state NMR, which makes use of J-coupling, is the INEPT (Insensitive Nuclei Enhanced by Polarization Transfer) experiment^[18]. It is a method for enhancing the intensity of NMR signals for nuclei with a low gyromagnetic ratio (e.g. ^{13}C , ^{15}N) by using the magnetization of a J-coupled high- γ spin (e.g. ^1H). The INEPT pulse sequence, shown in fig. 1.4, is commonly used in many multi-dimensional experiments. In these experiments an NMR signal is only recorded for spins that are linked through a chemical bond and thus interact via (heteronuclear) J-coupling.

Figure 1.4 The INEPT pulse sequence. The grey boxes indicate the pulses. The interval time τ is dependent on the J-coupling between the high- γ spin *I* and the low- γ spin *S* and the decoupling is used to remove the effect of unwanted couplings.

Dipole-Dipole coupling

Since nuclear spins possess a magnetic moment, they can directly interact with each other through space. Each spin, in fact, generates a magnetic field which affects nearby nuclei. This interaction is called direct dipole-dipole coupling and may be either intramolecular or intermolecular. The magnitude of this interaction is given by the dipolar coupling constant d (in units of rad s^{-1})

$$d = \frac{\mu_0}{4\pi} \frac{\gamma_I \gamma_S \hbar}{r_{IS}^3} \quad (1.11)$$

As shown in eq. 1.11, the strength of the coupling is directly proportional to the gyromagnetic ratios of the two spins and it rapidly decreases with the cube of the internuclear distance r_{IS} . Therefore, determining the strength of dipole-dipole coupling allows calculation of the distance between coupled nuclei and the overall molecule geometry. Dipolar couplings are typically much larger than J-couplings. For instance, a ¹H-¹³C spin pair separated by a distance of 1.15 Å experience a dipolar interaction equal to $d/2\pi = 20 \text{ kHz}$ (where the J-coupling is usually in the order of $\sim 140 \text{ Hz}$).

One important characteristic of the dipolar interaction is its dependence on the orientation of the internuclear *I* – *S* vector with respect to the static magnetic field $\mathbf{B}_0$.

To explicitly observe the orientation-dependent nature of the dipolar interaction, the dipole-dipole Hamiltonian $\hat{H}_D$ (table 1.2) is expressed using polar coordinates, together with the raising and lowering operators, ($I_+ = I_x + iI_y$) and ($I_- = I_x - iI_y$). Following this procedure, $\hat{H}_D$ can be written in a form known as the “dipolar alphabet” (eq. 1.12)

$$\hat{H}_D = \frac{\mu_0}{4\pi} \frac{\gamma_I \gamma_S \hbar}{r_{IS}^3} (A + B + C + D + E + F) \quad (1.12)$$

$$A = (1 - 3 \cos^2 \theta) I_z S_z$$

$$B = \frac{1}{2} (1 - 3 \cos^2 \theta) (I_z S_z - \mathbf{I} \cdot \mathbf{S})$$

$$C = -\frac{3}{2} \sin \theta \cos \theta e^{-i\varphi} (I_z S_+ + I_+ S_z)$$

$$D = -\frac{3}{2} \sin \theta \cos \theta e^{i\varphi} (I_z S_- + I_- S_z)$$

$$E = -\frac{3}{4} \sin^2 \theta e^{-i2\varphi} I_+ S_+$$

$$F = -\frac{3}{4} \sin^2 \theta e^{i2\varphi} I_- S_-$$

Moreover, eq. 1.12 can be simplified by only taking the terms that make significant contributions to the energy of the spin system (the components of $\hat{H}_D$ which commute with the Zeeman Hamiltonian for the $I - S$ spin system). In the case of a *homonuclear* system, where I and S are the same species, the expression reduces to

$$\text{homonuclear} \quad \hat{H}_D = -d \cdot \frac{1}{2} (3 \cos^2 \theta - 1) (3 I_z S_z - \mathbf{I} \cdot \mathbf{S}) \quad (1.13)$$

Similarly, the Hamiltonian for a *heteronuclear* system, where I and S are different, will be

$$\text{heteronuclear} \quad \hat{H}_D = -d (3 \cos^2 \theta - 1) 2 I_z S_z \quad (1.14)$$

Where d is the dipolar coupling constant (eq. 1.11).

As shown in eq. 1.13 and 1.14, the dipolar interactions exhibit a $3 \cos^2 \theta - 1$ dependence with respect to the orientation of the $I - S$ vector relative to the applied field $\mathbf{B}_0$, where θ is the angle between the internuclear vector and the $\mathbf{B}_0$ direction, as depicted in fig. 1.5.

Figure 1.5 (A) Orientation of the internuclear vector $I - S$ with respect to the magnetic field B_0 which is along the z-axis, as defined by the polar angles θ and φ . (B) The powder lineshape for a spin-1/2 in a heteronuclear two-spin system. The splitting is equal to the dipolar coupling constant.

In solution state the dipole-dipole interaction is averaged out by molecular tumbling. In fact, molecules in solutions rotate rapidly with frequent changes in their axis of rotation, resulting in a rapid modulation of the angle θ and, therefore, the dipolar coupling. Consequently, the NMR spectra of isotropic liquids are not affected by the dipolar coupling, although it can play a crucial role in nuclear spin relaxation. The situation is different for static solids, where dipole-dipole couplings are not averaged and strongly influence the NMR signal. As in the case of the Chemical Shift Anisotropy (CSA), the NMR spectrum is the sum of the NMR signals of different crystallites with a random distribution of θ orientations, resulting in the characteristic “powder spectrum” shown in Fig. 1.5.

Magic Angle Spinning (MAS)

Solid-state NMR generally deals with powder samples; that is, samples consisting of many crystallites with random orientations^[17]. As mentioned earlier, since the molecules cannot tumble freely anisotropic interactions like dipolar coupling and CSA dominate the spectra, dramatically broadening the NMR signals. To overcome this limitation, in solid-state NMR samples are spun to introduce motional averaging. Spinning the samples at a specific angle, known as the *magic angle* ($\theta_R = 54.74^\circ$), removes (or strongly attenuates) the anisotropic interactions effectively improving the spectrum resolution (fig. 1.6). The $(3 \cos^2 \theta - 1)$ dependence of the dipolar interaction shown in eq. 1.12 is

also true for CSA^[17], where θ describes the orientation of the dipolar or shielding tensor with respect to the applied field $\mathbf{B}_0$.

Figure 1.6 (A) Schematic representation of the Magic Angle Spinning (MAS). A rotor containing the sample rotates at high speed (several kHz) at the magic angle $\theta_R = 54.74^\circ$ with respect to the external field $\mathbf{B}_0$. (B) Illustration of the effect of the MAS. From the bottom, a static sample with the characteristic powder spectrum. In the middle, the effect of a slow magic-angle spinning. A set of spinning sidebands appears, with a centreband at the isotropic chemical shift and further lines equally spaced at a distance given by the spinning frequency^[17]. At the top, the effect of high magic-angle spinning, where the isotropic signal is obtained.

For an angle $\theta = \theta_R = 54.74^\circ$ the condition $3 \cos^2 \theta_R - 1 = 0$ is satisfied, effectively cancelling out the $(3 \cos^2 \theta - 1)$ dependence. Under this condition, the interaction anisotropy averages to zero, provided the frequency at which the sample is spinning exceeds the strength of the chemical shift anisotropy and/or of the dipole-dipole coupling. Typically, in solid-state NMR experiments a spinning rate is selected that best removes the effect of the chemical shift anisotropy. Usually, this serves towards averaging out the ^{13}C - ^{13}C and ^{13}C - ^1H dipolar couplings. The situation is rather different for ^1H - ^1H couplings, where the time-dependency of the spin system and the strength of the coupling (~ 120 kHz at 1 Å distance) lead to an inefficient averaging of the interaction. Consequently, detection in solid-state NMR is usually performed on nuclei like ^{13}C , while the ^1H spins are typically decoupled during acquisition using r.f. pulse schemes. However, great efforts have recently been made towards removing the effect

of ^1H dipolar couplings. Nowadays, thanks to the development of improved decoupling sequences^[19, 20], ultra-fast MAS speeds^[21, 22] and deuteration schemes^[23-25], ^1H detection has emerged as a very effective technique to acquire high-resolution spectra.

A typical NMR experiment extensively used in MAS solid-state NMR which employs dipolar couplings is the Cross Polarization (CP)^[26]. In a CP experiment the sensitivity of low- γ species is enhanced by transferring polarization from abundant spins with a higher gyromagnetic ratio (usually protons) via heteronuclear dipolar couplings. To successfully transfer polarization between the coupled species, both nuclei need to be irradiated at the same time with irradiation frequencies that satisfy the Hartmann-Hahn condition^[27]:

$$\omega_x = \omega_{1H} \pm n\omega_{MAS} \quad \text{with } n=(1,2) \quad (1.15)$$

Through cross-polarization, the magnetization of the low- γ spin X is increased by a factor γ_{1H}/γ_X (i.e. $\gamma_{1H}/\gamma_X = 4$ for ^{13}C spins, or $\gamma_{1H}/\gamma_X = 10$ for ^{15}N spins). Moreover, the repetition time of a CP experiment is determined by the proton relaxation time, which is generally shorter than the ^{13}C or ^{15}N relaxation, effectively reducing the total experimental time. In light of these advantages, the CP pulse sequence (fig. 1.7) is usually employed as a first building block in many multi-dimensional experiments probing dipolar coupled nuclei.

Figure 1.7 Schematic representation of the cross polarization (CP) experiment. During the CP step, the two channels are spin-locked at the Hartmann-Hahn matching condition (eq. 1.15) and the magnetization is transferred from ^1H nuclei to the X spins (i.e. ^{13}C). The decoupling is used to remove the effect of unwanted couplings. Note that this experiment can also be used to enable the magnetization transfer from spin X to a different spin Y.

Hyperpolarization

Due to the low sensitivity of NMR experiments, the characterization of complex molecular systems, hybrid materials, or samples with a very low concentration of target molecules, is sometimes impossible. The weak NMR signal intensity is related to the intrinsically low nuclear polarization (eq. 1.7), which is defined as the fraction of spins directed along the external magnetic field $\mathbf{B}_0 \parallel z$ [28]. The polarization of nuclear spins can be seen as the result of two competing phenomena: the magnetic energy ($\Delta E = \hbar\gamma B_0$), which tends to arrange all spins on the lower energy level, and the thermal energy ($2k_B T$), which dominates at increasing temperatures and tends to equipopulate available energy levels. Consequently, nuclear polarization is low at thermal equilibrium ($<0.005\%$ for the most sensitive element, ^1H). Even though significant technological improvements such as higher magnetic fields and cryo-probes have increased NMR sensitivity, the application of hyperpolarization techniques represent the most powerful method to overcome the main limitation of NMR. The general concept behind these experimental strategies is to create a non-equilibrium polarization state by forcing all the nuclear spins to occupy a single Zeeman level. In this way, a much larger non-thermal population difference can be obtained, as shown schematically in figure 1.8.

Figure 1.8 Pictorial description of the distribution of the nuclear population at thermal equilibrium and in the hyperpolarized state. In the sketch, the magnetic field $\mathbf{B}_0$ is oriented along the z -axis and the nuclear gyromagnetic ratio is assumed positive.

The hyperpolarization techniques predominantly in use and under development include para-hydrogen induced polarization (PHIP)^[29], spin-exchange optical pumping (SEOP)^[30] (prevalently used to hyperpolarize noble gas such as ^3He and ^{129}Xe) and Dynamic Nuclear Polarization (DNP)^[31, 32].

Dynamic Nuclear Polarization

Dynamic Nuclear Polarization (DNP) is the most well-established and widespread NMR hyperpolarization technique. The main advantage of DNP is undoubtedly its broad versatility. Every NMR active species can theoretically be polarized via DNP, which can enhance NMR signals from samples in the solid or liquid state. Consequently, it is not surprising that its utility and application ranges from material science to biomedicine. The basic concept of DNP is to exploit the considerably larger electron polarization. In fact, due to its higher gyromagnetic ratio γ , the electron spin polarization is ~ 660 times larger than the ^1H nuclear polarization, as shown in fig. 1.9.

Figure 1.9 Temperature dependent polarization (eq. 1.7) at 9.4 T for different spins: electron (red), proton (blue) and carbon (green). Note: the polarization difference between ^1H and ^{13}C spins is exploited in cross-polarization experiments.

The Dynamic Nuclear Polarization technique involves the transfer of high spin polarization from the electrons to the nuclei by means of microwave irradiation with frequencies close to the Electron Paramagnetic Resonance (EPR) frequency. The DNP effect therefore relies on the presence of species containing unpaired electrons. For example, paramagnetic metal ions or organic groups are often added to the samples as a source of free electrons. The idea was first proposed by Overhauser^[31] in 1953 and soon after experimentally verified by Carver and Slichter, who showed a sizable NMR enhancement on lithium metal after irradiation of the electron frequency^[32].

The DNP mechanism exploited in these experiments employs specific electron-nuclear relaxation pathways known as the Overhauser Effect (OE). Later, Abragam and Proctor^[33] proposed a new mechanism, named the Solid Effect (SE), which uses the electron-nuclear transitions induced by microwave irradiation at frequencies equal to $\omega_S \pm \omega_I$, where $\omega_S = -\gamma_S B_0$ and $\omega_I = \gamma_I B_0$ are the EPR and NMR frequencies, respectively^[28]. Subsequently, a 3-spin Cross effect (CE) was suggested by Kessenikh and Manenkov^[34], which involves the simultaneous interaction of two electrons and one nuclear spin. In the following decade, additional innovative experiments were performed in liquids and solids. In particular in the 1980's important work was undertaken towards implementing Magic Angle Spinning (MAS) in DNP setups. One of the first successful applications was presented by Wind et al^[35], where the enhancement of ^{13}C NMR signal from coal species was obtained using the radicals present in the sample. More recently, a generally applicable method was developed by the Griffin group^[36], allowing DNP experiments at higher magnetic fields under MAS conditions, which represented a breakthrough in the MAS solid-state NMR community. The success of these high-field MAS DNP experiments is largely due to the technological developments of high-frequency microwave sources^[36], transmission lines, and low temperature multiple resonance MAS DNP probes^[37, 38]. Additionally, important advances were reported in the design of paramagnetic polarizing agents, necessary for increasing the DNP efficiency^[39-41]. With the commercialization of DNP instrumentation, DNP-enhanced NMR experiments have become more accessible and the technique has evolved into one of the most effective methods for increasing the nuclear spin polarization in inorganic, organic and biological samples^[42-44]. Moreover, an interesting type of DNP, known as dissolution-DNP^[45], was recently developed and has found important medical applications in the field of metabolic imaging^[46], with its most prominent employment in early cancer diagnosis^[47].

Cross-Effect Dynamic Nuclear Polarization

The transfer of polarization from electrons to nuclear spins can occur via different mechanisms depending on the experimental conditions (such as magnetic field, temperature, radical type and concentration). The continuous wave DNP transfer mechanisms are the Overhauser Effect (OE), the Solid Effect (SE), the Cross Effect (CE) and the Thermal Mixing (TM). The latter is closely related to the CE, but it usually employs high radical concentrations and is applied on static samples at very low temperatures (typically $<10\text{ K}$)^[48, 49]. Although the potential of the OE for MAS NMR

applications has recently been discovered^[50, 51], current MAS DNP experiments on insulating solids are usually based on either the SE or the CE. The most efficient polarization transfer mechanism for MAS applications to date is the CE, which is the focus of this thesis and is discussed in more detail below.

In the case of static samples, the electron-nucleus systems can be described via the Hamiltonian with general form:

$$\hat{H} = \omega_e S_z - \omega_I I_z + \hat{H}_{ee} + \hat{H}_{nn} + \hat{H}_{en} \quad (1.16)$$

Where the first two terms are the Zeeman interactions for the electron and nuclear spins, respectively (with S_z and I_z the electron and nuclear spin operators). The terms $\hat{H}_{ee}$ and $\hat{H}_{nn}$ describe the electron-electron and nucleus-nucleus coupling, and the $\hat{H}_{en}$ term corresponds to the hyperfine electron-nucleus interaction, which plays a major role in the DNP transfer.

The Cross-Effect is defined as a 3-spin mechanism, involving two dipolar coupled electrons and a hyperfine coupled nucleus, which satisfy the relation:

$$\omega_{0e2} - \omega_{0e1} = \omega_{0I} \quad (1.17)$$

Where ω_{0e1} and ω_{0e2} indicate the EPR frequencies and ω_{0I} is the NMR Larmor frequency.

When the matching condition (eq. 1.17) is met, the saturation of one of the electron transitions leads to a simultaneous spin flip of the other electron and the coupled nucleus (fig 1.10A). The energy level diagram for the system is schematically shown in fig. 1.10B. A microwave field is applied to the Zeeman states that are coupled by electronic and hyperfine interactions and the electron-nuclear polarization transfer is maximized when the central energy levels are degenerate. Degeneracy occurs when the condition in eq. 1.17 is fulfilled and it leads to the saturation of the connected levels and therefore results in enhanced nuclear polarization^[10, 52, 53].

The CE requires the inhomogeneous width of the EPR lines to be larger than the nuclear Larmor frequency ω_{0I} ensuring two effective EPR frequencies separated by ω_{0I} . At the same time, the homogeneous width (δ) must remain small ($\delta < \omega_{0I}$). Biradicals such as TOTAPOL^[39] and AMUPol^[40] are employed to meet these conditions, where the presence of a dipolar coupling between the two electrons dramatically improved the CE DNP efficiency (see below). However, since the EPR linewidth scales with the field, the matching condition becomes harder to satisfy at higher magnetic fields, resulting in lower DNP enhancement. The field dependence of the cross-effect DNP mechanism will be discussed further in chapter 5.

Figure 1.10 (A) Illustration of the three spin process. Under microwaves irradiation electron flip-flop transitions are induced causing a simultaneous flip of the coupled nucleus. (B) Energy diagram illustrating DNP via Cross-Effect. Energy scheme of the three-spin system at equilibrium (left). Under the matching condition, the saturation of transitions near the first EPR line give rise to a positive DNP enhancement (right).

Cross-Effect under MAS

The molecular orientation of species with unpaired electrons influences their EPR frequencies because of chemical shift anisotropy. Hence, under static conditions, only a small fraction of molecules presents the right orientation to fulfil the matching condition (eq. 1.17) and is therefore able to participate in the polarization transfer. However, in solid-state NMR, magic angle spinning is usually applied to the sample. MAS introduces a time dependence, modulating the EPR resonance frequencies and allowing the energy levels to fluctuate during sample rotation. Hence, irradiation at ω_{0e1} or ω_{0e2} and the matching condition $\omega_{0e2} - \omega_{0e1} = \omega_{0I}$ occur at different time points during the MAS

cycle. At certain rotor positions, the energy states will mix and therefore polarization can be exchanged between electron and nuclear spins. This phenomenon is known as level anti-crossing (LAC) and a combination of these LACs is what eventually leads to the DNP signal enhancement. An extensive description of the CE mechanism under MAS has been introduced by Thurber and Tycho^[54], which considers the time evolution of the anisotropic Hamiltonian. In their theory, the spin system periodically undergoes three separate types of LAC events; microwave events, CE matching events and electron-electron events^[4]. Microwave events correspond to the irradiation of one of the electron transitions. The microwave frequency has to match one of the two electron Larmor frequencies, while the other is far off-resonance. Consequently, only the on-resonance EPR transition is saturated, creating a polarization difference between the two electrons which is transferred to the coupled nucleus during a CE matching event. This level anti-crossing allows the transfer of polarization from the electrons to the nuclear spins. Although the probability of a single CE event is quite low, they act in a cumulative manner, resulting in a net nuclear polarization enhancement factor $\varepsilon > 10^2$. Finally, the electron-electron crossings (when $\omega_{0e1} = \omega_{0e2}$) lead to an exchange of magnetization between the two electrons. Each event is regulated by a different relaxation time and all level anti-crossing events must occur during the MAS cycle in order to create a net CE enhancement.

Nuclear Spin Diffusion

As described previously, the enhancement of NMR signals under DNP conditions is obtained by transferring the large polarization of electrons to nuclei (usually ^1H) via microwave irradiation. However, magnetization is only exchanged between electron spins and the hyperfine-coupled nuclei in their direct vicinity. After this initial polarization step, the polarization is spread through the sample via a process known as nuclear spin diffusion^[55-57], which is mediated by the dipolar interaction between the nuclear spins. The electron spin concentration is orders of magnitude smaller than the nuclear spin concentration ($N_e/N_n \sim 10^{-3}$) and the electron-nucleus hyperfine interaction decreases with the separation distance cubed. Consequently, only a small fraction of the nuclei, also called *core nuclei*, are directly polarized by the electrons. *Core nuclei* subsequently transfer the enhanced polarization to the *bulk nuclei* via homonuclear spin diffusion^[4, 56]. However, not every nucleus takes part in this process. The nuclei closest to the polarizing agent experience a fast relaxation and a strong shift of their Larmor frequency caused by the large hyperfine coupling, effectively quenching the

diffusion to the other nuclear spins. The cut-off distance between the core nuclei and the bulk nuclei is known as spin-diffusion barrier^[58, 59] (fig 1.11). Nuclei within the barrier cannot communicate with the bulk nuclei via spin-spin transitions. Instead, the nuclei residing just outside the barrier are polarized via CE and can spread the polarization through the sample, leading to the observed DNP enhancement. The size of the spin diffusion barrier depends on the type of DNP radical. The efficiency of the spin diffusion is determined by different experimental factors, such as temperature, solvent and protonation level. Additionally, for nuclear spin diffusion to be effective it is important that the paramagnetic agents are uniformly distributed throughout the sample.

Figure 1.11 Nuclear spins that have a stronger coupling to the electron spin than the bulk nuclei are the *core* nuclei and they reside within the spin diffusion barrier (in red). Nuclei that are strongly dipolar-coupled with the bulk homonuclear bath are the *bulk* nuclei and they are outside the barrier (in blue).

Optimization of DNP conditions

The DNP efficiency is strongly influenced by experimental conditions (including temperature, magnetic fields and microwave power) as well as sample preparation. Tuning of these features is often crucial for practical applications, where obtaining high nuclear polarization in a relatively short amount of time is desirable.

For instance, the interplay between the radical, solvent, and solute relaxation times plays

a significant role in determining the final DNP enhancement. In fact, the hyperpolarized state has a limited lifetime: after hyperpolarization is achieved, the polarization seeks to return to the thermal equilibrium level at a rate governed by the nuclear T_1 longitudinal-relaxation time. This relaxation time strongly depends on the chemical structure and environment of the hyperpolarized compound. Moreover, the electron spin relaxation is a crucial parameter for an efficient CE DNP. The electron spin-lattice relaxation time (T_{1e}) and the spin-spin relaxation time (T_{2e}) play an important role in the saturation of the EPR transition and excitation of CE under microwave irradiation^[53, 60].

Lower temperatures improve the CE enhancement, mainly due to the longer electron and proton relaxation times. This is why conventional DNP experiments are performed at temperatures of ~ 100 K. To perform DNP NMR experiments, specialized instrumentation has been developed, which includes microwave sources, waveguides and a new specialized ssNMR probe capable of performing MAS below 110 K. Gyrotrons are high-frequency vacuum electronic devices generally used as the microwave source, and are capable of producing sufficient power in the 140-590 GHz frequency range for electrons. A microwave waveguide is employed to transmit the microwaves from the gyrotron to the NMR probe. Efficient delivery of the microwave irradiation is highly important. Nowadays corrugated waveguides are used to achieve this because they have negligible loss and therefore exhibit an increased microwave transmission efficiency.

The optimization of each individual piece of instrumentation has enormously contributed to the development and success of the DNP technique^[11, 61-63].

Design and concentration of the polarizing agent

Generally, the DNP polarizing agents are evaluated based on several parameters, such as their solubility in aqueous or organic media (depending on the type of radical used) and their chemical stability. Moreover, the polarizing agents can either be exogeneous, a mono or biradical or metal ion added to the sample, or endogenous^[64], for example a stable radical present in a protein, although higher enhancements have been achieved with exogeneous biradicals.

The mechanism governing a DNP experiment depends strongly on the polarizing agent used. For initial high-field DNP experiments, TEMPO^[65], BDPA^[36, 66, 67] and trityl^[68] monoradical were used. Subsequently, biradical polarizing agents were implemented to improve the CE DNP efficiency, where a 20-30MHz intramolecular electron dipole coupling yielded ~ 4 -fold larger enhancements than monoradicals such as TEMPO^[10]. In particular, nitroxide based biradicals are suitable polarizing agents for CE DNP due to

their inhomogeneous EPR linewidths. Over the years, optimal inter-electron distance and relative orientation of the g-tensors have been investigated, with the aim to design more efficient biradicals which better satisfy the CE matching condition^[39, 41]. This has led to the development of highly efficient biradicals such as AMUPol^[40] and TEKPol^[69], which exhibit stronger electron dipole coupling due to their short linker. Additionally, the longer T_{1e} and T_{2e} electron relaxation times allow for a higher saturation of the EPR transition. An additional important advantage is the lower concentration of polarizing agents needed when using biradicals. In fact, a high radical concentration can be deleterious because it causes unfavourable bleaching effects which reduce the number of detectable spins and therefore result in lower sensitivity^[70, 71]. Additionally, high concentrations of paramagnets can dramatically broaden the NMR linewidths, leading to a loss in spectral resolution and information^[72]. Instead, using nitroxide-based biradicals achieves higher enhancements, with a concurrent 4-fold decrease in the electron concentration^[10]. However, these biradicals still present some drawbacks, especially for high-field applications. For instance, nitroxide biradicals present an unfavourable field dependence, and CE enhancements decrease with increasing field strength^[52, 73, 74]. Moreover, high MAS frequencies can negatively influence the DNP enhancements by inducing depolarization effects^[70, 75]. The research community is therefore very active in the field of radical optimization^[76], with the main goal of developing new polarizing agents better suited for high-field applications^[77, 78]. Hybrid trityl-nitroxide biradicals in particular have drawn the attention of the community as promising candidates for high-field DNP^[75, 79]. Chapter 6 presents a new class of trityl-nitroxide biradicals and illustrates new strategies to further improve these promising polarizing agents.

Figure 1.12 Chemical structures of polarizing agents frequently used in high-field DNP.

Sample preparation

Optimal sample preparation is a key factor for DNP and plays a decisive role in the success of an experiment. This is especially true when considering that enhancements measured on biomacromolecules (and the same holds true for samples in material science applications) are often reduced when compared to model substances^[4]. The difference between real world application and model situations is due to the sample complexity and internal dynamics that affect their relaxation behaviour.

Generally, a DNP sample is made from three components: the electron paramagnetic agent, the target molecule and a glassing solvent. Regarding the former; as previously discussed, the concentration of the DNP agents has to be carefully adjusted such that optimal enhancements, sensitivity and resolution are obtained. Experimental findings indicate that optimum radical concentrations lie between 10 and 20 mM^[10].

Moreover, the solvent plays a fundamental role, because it has to ensure a uniform radical distribution and an amorphous glassy structure of the frozen sample. The crystallization of the solvent upon sample freezing could otherwise cause phase separation between the target molecules and the polarizing agents, leading to strongly reduced DNP enhancements^[80]. To prevent crystallization, the solvent is usually a good glass-forming agent, which additionally offers cryoprotection for the target molecules upon freezing. So-called “DNP Juice”^[63], composed of 60% deuterated-glycerol, 30% D₂O and 10% H₂O, has experimentally been discovered as an optimal glass-forming matrix for biomolecular applications^[81]. Alternative sample preparations have been developed for material science applications. For instance, the “incipient wetness impregnation”, a well-established technique in the material science field, is specifically suited for porous samples; whereby a small volume of the solution is added to the sample, saturating the pores and the surface while avoiding excess volume. This technique has been applied to DNP experiments on porous zeolites, as shown in Chapter 3 and 4.

Finally, the deuteration of the solvent is a crucial aspect in sample preparation. ²H labelled solvent improves the ¹H DNP enhancement by attenuating the relaxation processes. Even with 90-95% solvent deuteration, the ¹H-¹H spin diffusion is sufficient to provide a homogeneous distribution of polarization over the whole sample^[82-84]. Moreover, it has been reported that deuteration of the target system can also lead to higher DNP enhancements^[85, 86]. Chapter 2 investigates the influence of deuteration in our *in-cell* DNP applications.

DNP and cellular NMR

In recent years, significant progress has been made in developing solid-state NMR (ssNMR) methods and protocols that make use of DNP for studying cellular protein preparations^[87] (mostly membrane-embedded) and in this section a brief overview on the topic is given.

In 2012, DNP-supported ssNMR studies were undertaken on ¹³C,¹⁵N-labelled cellular preparations of *E. coli* containing a recombinant membrane protein^[88]. Bacterial cells and cell envelope extracts were typically suspended in a matrix consisting of a well-defined aqueous buffer, containing deuterated-glycerol as cryoprotectant and TOTAPOL as polarizing agent. As mentioned earlier, for optimal DNP analysis, radical agent concentration must be adjusted to obtain an optimal balance between DNP enhancement and degradation of spectrum sensitivity through line broadening, signal

quenching, and enhanced relaxation caused by paramagnetic effects. Additionally, line broadening -caused by conformational disorder- becomes challenging at lower temperatures. This is particularly true for cellular components like peptidoglycans, which may exhibit a high degree of motion at room temperature. In the most critical case, line broadening cancels out the gain acquired by DNP. Furthermore, specific interactions of the radical molecule with the surface of proteins, lipids and polysaccharides may lead to a non-uniform distribution of active radicals inside the sample. This property can be exploited to enhance signals associated with a particular compartment or protein target by linking the radical to lipids^[89, 90], ligands or the protein itself^[91].

Currently, DNP-NMR instruments can operate at 400 MHz, 600 MHz, 800 MHz and up to 900 MHz ¹H Larmor frequency. Recent works show that the maximum achievable DNP enhancement varies, depending on sample preparation^[92] and choice of the polarizing agent^[79]. However, the enhancement generally reduces with increasing magnetic field in the case of biradicals that utilize the cross effect^[70, 74].

In addition to sensitivity, spectroscopic resolution is another critical factor for DNP applications on complex (bio)molecules. Previous work shows that high-field DNP facilitates in-depth structural investigations of complex molecules such as membrane proteins. For example, the spectral resolution was examined by comparing two-dimensional (¹³C, ¹³C) and (¹⁵N, ¹³C) correlation data sets on fully ¹³C, ¹⁵N labelled variants of the KcsA potassium channel at 800 MHz DNP conditions using soluble^[92] and tagged^[91] AMUPol variants to data obtained at ambient temperatures. Moreover, high field conditions were shown to significantly enhance spectral resolution^[92, 93]. Additionally, the intrinsic paramagnetic properties of the polarizing agents can be used as direct structural probes for the spectroscopic analysis. For instance, in the case of the membrane embedded KcsA channel^[92], the strong paramagnetic effects exhibited by the heavily solvent-exposed surface shell helped to pinpoint the channel's water accessible pore.

Figure 1.13 DNP enhancement factors seen at the 800MHz DNP conditions. Data are shown for the case of proteoliposomal preparations of KcsA and the type IV secretion system core complex (T4SScc), as well as EGFR-rich plasma membrane vesicles of A431 cells. In addition, it contains DNP enhancement factors measured on type III secretion needles and a FCC catalyst that exhibits significantly lower proton densities. Finally, we have included published literature on very high DNP enhancements obtained with TEMTriPol-1, a DNP polarizing agent formed by a nitroxide (TEMPO) tethered to a trityl radical, together with the results recently obtained in our group with a variant called NATriPol-3, which is a more promising DNP radical for biomolecular applications (as it will be discussed in Chapter 6).

Figure 1.13 shows a summary of several DNP enhancement factors obtained in our laboratory using a 800MHz DNP setup. The enhancement factors were recorded during studies of proteoliposomal preparations of KcsA^[91, 92] and the type IV secretion system core complex^[94] (T4SScc), as well as EGFR-rich plasma membrane vesicles of A431 cells^[95]. To investigate these larger systems, such as cell envelopes-associated complexes and eukaryotic membrane proteins, sample preparation is carried out by adapting the existing cellular ssNMR protocols. The type IV secretion system core complex (T4SScc) is a 1-MDa protein machine consisting of 14 copies of 3 proteins (VirB7, VirB9 and VirB10). T4SScc is part of a larger machine (T4SS) that spans the periplasm and is

embedded in both the inner and outer membranes of Gram-negative bacteria. Either uniformly ^{13}C , ^{15}N -labelled or selectively labelled T4SScc preparations were used for *in situ* structural studies. The well-established correlation between NMR resonance frequencies and protein secondary structure was exploited to obtain structural information about the backbone fold of cell-embedded T4SScc using 3D and 2D ssNMR data sets obtained under DNP conditions. Using a similar DNP-supported approach and dedicated sample preparation methods, structural and dynamical aspects of the EGF receptor in A431 vesicles have been investigated. In particular, specific sequential protein correlations before and after binding of the natural EGFR ligand EGF were monitored by 3D DNP-supported ssNMR, which led to the understanding of the receptor dynamics during the activation step of EGFR. In addition, Figure 1.13 contains DNP enhancement factors measured on type III secretion needles^[93] and a FCC catalyst that exhibits significantly lower proton densities^[96]. Finally, we have included recently published literature^[75, 79] on very high DNP enhancements obtained with DNP polarizing agents formed by a nitroxide (TEMPO) tethered to a trityl radical. These compounds exhibit a stronger exchange interaction and an improved electron-electron coupling that changes the magnetic field profile of the DNP enhancement. Moreover, recent work^[75] shows that these compounds exhibit low depolarization effects, which increase their DNP performance. Thus far, studies on these compounds are limited to model systems and work to make them broadly suitable for life and material science applications is ongoing, as presented in chapter 6.

Outline of the thesis

In this thesis we present novel applications and methodological investigations of DNP-enhanced solid state NMR. After a general introduction to solid-state NMR and Dynamic Nuclear Polarization (DNP) techniques presented in this chapter, the remaining sections are organized as follows.

In the first chapters solid-state NMR and DNP are employed to answer specific questions in life and material science.

Chapter 2 presents a new approach for *in-cell* DNP experiments, which allows the study of protein structures at atomic level in their natural setting. Innovative *in-cell* ssNMR studies are presented and specific aspects of the DNP sample preparation are discussed. Particular attention is given to radical incorporation in the cells and the effect of background deuteration on DNP enhancements.

Chapter 3 and **4** describe how solid state NMR and DNP approaches reveal mechanistic insights into the zeolite-catalysed Methanol-to-Hydrocarbon and Ethanol-to-Hydrocarbon processes. In particular, solid-state NMR magnetization transfer techniques, previously developed for spectral separation of biomolecules on the basis of their mobility, are adapted to distinguish between mobile species and rigid molecules trapped in the zeolite. These studies provide spectroscopic evidence of a distinctive host-guest chemistry during catalysis. Moreover, the distance-dependent DNP enhancements are utilized to investigate the spatial distribution of the reaction products.

Chapter 5 examines multiple parameters that affect the DNP efficiency, thus highlighting areas of the technique that can be improved. Emphasis is given to the study of DNP at high magnetic fields, a current hot topic within the DNP community. In light of these results, a new family of Trityl-Nitroxide biradicals has been developed. These novel radicals are described in **Chapter 6**, along with an investigation on their potential for biomolecular applications.

Finally, **Chapter 7** summarises the thesis' findings, details its conclusions and discusses promising prospective work.

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CHAPTER 2

DNP Preparations for *in-cell* solid-state NMR spectroscopy

Based on:

“DNP preparations for *in-cell* solid-state NMR spectroscopy” – *in preparation*

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Abstract

Solid-state NMR (ssNMR) can provide structural information at the most detailed level and, at the same time, is applicable in highly heterogeneous and complex molecular environments, largely irrespective of solubility or crystallinity. Revolutionary developments in the field of Dynamic Nuclear Polarization (DNP) have greatly enhanced ssNMR sensitivity. Here, we discuss ssNMR concepts and applications that make use of these advancements and enable the study of protein structures inside whole cells at atomic level under high-sensitivity DNP conditions. The method is demonstrated in human cells using Ubiquitin, a globular protein critically involved in cellular functioning. The protocol is then optimized using isotope-labelled Ubiquitin in *E. coli* cells, where the parameters determining the maximum DNP enhancement are investigated.

Introduction

The influence of the cellular context on protein structure is widely recognized. Therefore, structural studies of proteins in their native contexts are becoming increasingly important^[1, 2].

In recent years, *in-cell* (solution-state) NMR has become a powerful method to study molecules in living bacterial or mammalian cells^[3-5]. Such studies have been limited to molecules that rapidly tumble in their natural environment, putting significant constraints on the molecular size and complexity that can be investigated. Solid-state NMR (ssNMR) is a method that can be applied to gain atomic-level insight into heterogeneous molecular systems, largely irrespective of molecular size^[6]. Applications to collagen^[7], membrane proteins^[8] and even entire bacterial cells^[9, 10] have been shown. In addition to the well-established use of synthetic bilayer settings, cellular ssNMR concepts have been developed in recent years that permit extension of such studies to cellular preparations^[10-15], where NMR is able to provide high-resolution details in complex cellular settings. In these preparations, proper sample optimization is crucial for the acquisition of high quality ssNMR spectra and their interpretation. Virtually all NMR studies conducted today on proteins or other biomolecules use isotope labelling to enhance NMR signals and to allow for multi-dimensional ssNMR correlation spectroscopy. In the case of proteins, tailored isotope-labelling approaches in prokaryotes and eukaryotes are usually required. In practice, cellular NMR applications involve further dedicated procedures to control the concentration of the protein target and limit background labelling artefacts^[4]. Yet, with increasing size and molecular complexity, isotope labelling and protein overexpression may not be sufficient to detect ssNMR signals of a particular protein in its natural biological environment. Fortunately, ssNMR has profited from revolutionary enhancements in sensitivity, mainly due to the advent of Dynamic Nuclear Polarization (DNP)^[16]. Herein, we extend such cellular studies and we present a solid-state NMR approach to investigate the native cell states of proteins. The main challenge is the large dilution of the target system, compared to a purified sample, which is the reason for the extremely low sensitivity of these preparations. Moreover, in MAS ssNMR, the high G-force during sample spinning could pose a significant problem. Performing DNP experiments represents an effective strategy to address both issues. In fact, the high signal enhancement achievable with DNP can greatly increase ssNMR sensitivity, therefore overcoming the limitations associated with low protein concentrations. Additionally, DNP experiments are performed at ~100 K where the samples are cryogenically frozen and hence the G-force

experienced upon sample spinning has a negligible effect. The following chapter shows one of the first successful applications of *in-cell* DNP and the method is demonstrated in human and bacterial cells using ^{13}C , ^{15}N -labelled Ubiquitin. In both cases the sample preparation is discussed, with particular attention given to the radical incorporation. Firstly, the application of the method in human cells is presented, where the high DNP signal enhancement allowed the detection of Ubiquitin at endogenous level by means of multidimensional ssNMR experiments. Subsequently, the method is adapted to bacterial cell preparations and the parameters influencing the DNP enhancements, such as signal build-up times and sample deuteration, are investigated, with the aim to further optimize the sensitivity of *in-cell* DNP NMR.

Application to human cells

We demonstrate the feasibility of the method using Ubiquitin, a post-translational modifier that regulates a large variety of cellular functions, particularly protein degradation^[17, 18]. This soluble globular protein has already been examined in NMR studies. However, although relative small (8.6kDa), wild-type Ubiquitin has remained elusive to in-cell solution state NMR. As observed in an earlier work^[19], this is due to the numerous molecular recognition events and protein-protein interactions in which Ubiquitin is involved and to the high viscosity of the cell interior. Therefore, these observations further strengthen the need for ssNMR approaches.

Sample preparation

To prepare the sample for the DNP NMR measurements, ^{13}C , ^{15}N -labelled Ubiquitin was first expressed in E. coli BL21 Rosetta (R2) cells and subsequently delivered into HeLa cells by electroporation^[20]. In particular, about 8 million HeLa cells were electroporated with the isotope-labelled protein at a final concentration of 1200 μM in the electroporation buffer. The cells were let to recover for a period of five hours and successively harvested.

Importantly, at every stage biochemical and biophysical methods can be applied to monitor cellular processes and the proper functionality of the exogenously delivered Ubiquitin in HeLa cells. Confocal microscopy, SDS-PAGE analysis and the results of the proteasome inhibition assay confirmed that exogenous Ubiquitin can be introduced into HeLa cells and that it is functional, as evident from conjugate formation in response to proteasome inhibition. Moreover, control experiments using solution-state NMR on the cell lysate did not shown any sign of protein unfolding or degradation.

Only at the last stage of the sample preparation the DNP agents are introduced into the cells by resuspension of the pellet in 50 μL of DNP solution, to minimize the exposure of the DNP radical to the reducing cell environment. In this case, the DNP solution comprises of 60% deuterated and ^{12}C -enriched glycerol, 30% D_2O and 10% protonated HBSS buffer. The biradical AMUPol^[21] is used as the DNP source at a concentration of 15-30 mM. Afterwards, the 3.2mm DNP rotor is filled with the suspension of cells in DNP juice and, to prevent the DNP agent from quenching, this procedure is carried out as quickly as possible. In multiple steps, the cells are spun down into the DNP rotors by mild centrifugation (lower than 1000 x g) and the supernatant containing excess DNP juice is removed, for a total filling time of 10-15min. Once the filling procedure is completed, the rotor is snap frozen in liquid nitrogen and finally transferred in the pre-cooled DNP MAS probe.

By following this procedure, it is estimated that a 3.2mm DNP rotor can hold about 5-8 million cells, corresponding to approximately 10-30 μg of labelled Ubiquitin in our ssNMR preparation. Such quantities underline the need for efficient DNP for in-cell ssNMR studies.

Solid-state DNP NMR experiments

We conducted solid-state NMR experiments under low temperature (100 K) DNP conditions using 3.2 mm triple-resonance (^1H , ^{13}C , and ^{15}N) magic-angle-spinning (MAS) probe head in a static magnetic field of 9.4 T, corresponding to proton/electron resonance frequencies of 400 MHz/263 GHz and 3.2 mm double-resonance (^1H , ^{13}C) magic-angle-spinning (MAS) probe head in a static magnetic field of 18.8 T, corresponding to proton/electron resonance frequencies of 800 MHz/527 GHz (Bruker BioSpin). All spectra were recorded at a MAS rate of 8 kHz using SPINAL-64 proton decoupling^[22] at 84 kHz. A recycle delay of 2 s was used for all experiments.

To measure DNP enhancements, ^1H - ^{13}C cross polarization (CP)^[23] experiments were employed, where 50-70 μs contact times were used for H-C CP transfer. DNP enhancements were obtained by scaling the signal intensities of spectra measured under DNP with the corresponding spectrum obtained without microwave irradiation at 100 K. For the spectral processing, 100/200 Hz line-broadening were applied prior to Fourier transformation.

The build-up of ^1H polarization, with time constant T_1 , was measured via a ^1H saturation recovery experiment and determined indirectly by detecting the ^{13}C CP signal. The polarization build-up curves were fitted with mono exponential curves to determine the time constant.

Results and discussion

The DNP enhancements at 400 and 800 MHz DNP conditions are shown in fig. 2.1. To our surprise, we measured very high signal enhancements, up to 130 times compared to the case without DNP.

A - 400MHz

B - 800MHz

Figure 2.1 DNP Enhancements observed in 1D ^1H - ^{13}C cross-polarization (CP) spectra of HeLa cells containing ^{13}C , ^{15}N Ubiquitin at 400MHz (A) and 800MHz (B) DNP conditions.

These results are significantly higher than previous studies on in situ systems, including human-cell vesicles^[24] or fully labelled mammalian cells^[25] and suggest the correct incorporation and functionality of the DNP agent inside the cells. In addition, the known unfavourable field dependence of the DNP enhancement is also recorded for these preparations, where we measure a 4 fold decrease in signal enhancement upon doubling the field strength, in agreement with values reported in the literature^[26, 27].

Moreover, we also measured polarization build-up curves that report on the distance dynamics of polarization transfers. T_1 relaxation of the protein signals under DNP condition was found to be relative fast, with build-up times ranging around 5 s. This observation suggests that DNP agents are in close proximity to the target protein. In fact, as shown by previous calculations^[28], to establish efficient DNP inside mammalian cells,

the DNP agent must be localized inside the cells, for example as a result of rapid diffusion to minimize the effect of the reducing cell environment^[29]. To further prove this observation, the location of the DNP agents was tracked by confocal microscopy by using the biradical PyPOL-MTSSL^[21, 30], a DNP biradical variant of AMUPol, conjugated to the fluorophore TAMRA. As shown in figure 2.2, the water-soluble DNP agents are present in both nuclear and cytoplasmic cell compartments after a period of 10-15 minutes, therefore confirming the uniform distribution of the radicals inside the cells.

Figure 2.2 Z-slices of confocal microscopy images showing that the radical (PyPOL-TMR, a variant of AMUPol) is well distributed in both nuclear and cytoplasmic compartments of the cell.

The findings discussed in the previous section allowed us to conduct a series of 2D ssNMR experiments to detect Ubiquitin in our DNP-ssNMR preparations including ^{13}C , ^{13}C proton-driven spin diffusion (PDSF) experiment, double quantum-single quantum (2Q-1Q) ^{13}C , ^{13}C and N to C_α (NCA) 2D experiments. Moreover, the high

sensitivity of these sample preparations enabled the use of 3D ssNMR (2Q-1Q-1Q) ^{13}C , ^{13}C spectroscopy^[31] to further improve spectral resolution [*Note: the spectra are only shown in the next section dealing with Ubiquitin in bacterial cells*]. With the combined analysis of 2D and 3D spectra, we could unambiguously trace back 25 residues, which matched with the in-vitro assignments. When plotted on the 3D structure of Ubiquitin, these residues for which correlations were identified unambiguously in the 2D and 3D experiments were located throughout the protein sequence, including the N-terminus as well as the five beta sheets and the central alpha helix. This strongly suggests that uniformly ^{13}C -, ^{15}N -labelled Ubiquitin remains folded after delivery into cells and proves the feasibility of our DNP-supported solid-state NMR approach for conducting in-cell experiments. Our results pave the way for structural studies of larger proteins or protein complexes inside human cells, which have remained elusive to in-cell solution-state NMR spectroscopy due to molecular size limitations.

Application to bacterial cells

We now extend *in-cell* ssNMR studies to prokaryotic systems. Specifically, we study isotope-labelled Ubiquitin in *E. coli* cells with the aim to further optimize sample preparation. Ubiquitin, a soluble eukaryotic protein, was chosen because it is robust and inert in bacterial cells. In fact, since we anticipate that Ubiquitin is not involved in protein-protein interaction or protein complexes formation in bacteria, it represents an ideal model study. An optimal protocol to perform DNP experiments on isotope-labelled proteins in *E. coli* cells is developed, where DNP signal enhancement up to 100 times is achieved. These findings allow for the study of biomolecules in bacterial cells, which have so far remained inaccessible to in-cell solution NMR.

Materials and Methods

Sample preparation

Labelling of Ubiquitin in *E.coli* cells

A suitable method has to be implemented in bacterial cells such that the protein of interest is labelled whilst avoiding any background labelling. This is a strict requirement

for DNP-ssNMR experiments in order to eliminate signal ambiguities and therefore exclusively detect the target molecule without spurious contributions from unwanted signals stemming from the cell environment.

The targeted labelling of Ubiquitin in Lemo cells is achieved by expressing the protein in ^{13}C -labelled Glucose and ^{15}N -labelled Ammonium Chloride or by using an ^{13}C -, ^{15}N Algal Amino Acid Mix to label the protein. Moreover, the antibiotic rifampicin is utilized to suppress native *E. coli* RNA-polymerase dependent protein expression^[14]. When utilizing ^{13}C -Glucose, the background labelling of lipids and sugars could not be fully suppressed and dominated the NMR spectra (Supporting Information, fig. S1 & Fig. S3). However, with the use of an Algal Amino Acid mixture as isotope source^[32] the lipid and sugar resonances could be removed (except for signal contributions of phosphatidylethanolamine, whose head group is derived from serine). Molecule-specific isotope labelling is achieved with minimal background labelling, as visible in the 1D and 2D solid-state spectra (Supporting Information, fig. S2 & Fig. S3). Moreover, control solution-state NMR experiments of the cell lysate can be performed. The DNP-ssNMR experiments on the labelled protein were found to exhibit good spectral resolution.

DNP radical incorporation

After protein expression, the cells are harvested and the preparation follows the same steps as described in the case of mammalian cells. Again, preparation should be rapid to minimize the exposure of the DNP radical to the reducing cell environment. The DNP agents are introduced into the cells by resuspension of the pellet in 50 μL of DNP solution, which in this case is composed of 30% deuterated and ^{12}C -enriched (^{13}C -depleted) glycerol, 60% D_2O and 10% protonated M9 salts. It is important to note that isotope depletion of the solvent eliminates background glycerol signals that might otherwise overlap with the analyte spectrum. The biradical AMUPol^[21] is used as the DNP source at a concentration of 15-30mM. Moreover, due to the high DNP enhancements observed, we can assume that the nitroxide biradical is stable in our biological samples. The cells are packed into the DNP rotors by centrifugation (4500 x g) and the supernatant containing excess DNP juice is removed. The rotor is then snap frozen in liquid nitrogen and finally transferred into the pre-cooled DNP MAS probe.

Solid-state DNP NMR experiments

Solid-state NMR experiments were conducted under low temperature (100 K) DNP conditions using a 3.2 mm triple-resonance (^1H , ^{13}C , and ^{15}N) magic-angle-spinning (MAS) probe head in a static magnetic field of 9.4 T. This corresponds to

proton/electron resonance frequencies of 400 MHz/263 GHz (Bruker BioSpin). All spectra were recorded at a MAS rate of 8 kHz using SPINAL-64 proton decoupling^[22] at 84 kHz. A recycle delay of 2 s was used for all experiments.

^1H - ^{13}C and ^1H - ^{15}N cross polarization (CP)^[23] experiments were employed to measure DNP enhancements, where 200 and 900 μs contact times were used for H-C and H-N CP transfer, respectively. DNP enhancements were obtained by scaling the signal intensities of spectra measured under DNP with the corresponding spectrum obtained without microwave irradiation at 100 K. For the spectral processing, 100 Hz line-broadening was applied prior to Fourier transformation.

The build-up of ^1H polarization, with time constant T_{DNP} , was measured via a ^1H saturation recovery experiment and determined indirectly by detecting the ^{13}C or ^{15}N CP signal. The polarization build-up curves were fitted with mono exponential curves to determine the time constant. (See Supporting Information, Fig. S4 and S5).

[Experimental parameters of the 2D and 3D ssNMR spectra are given in the Supporting Information]

Results and discussion

Background deuteration

In the last 10-15 years, different studies have shown the beneficial effect of matrix deuteration on DNP enhancement. The deuteration of the solvent was observed to result in significant gain in DNP efficiency^[33] and subsequently many experiments have employed ^2H -labelled DNP solutions^[34-36] (as is the case in this work). The approximate deuteration level of 90% increases the proton relaxation time and reduces the number of bulk ^1H spins. Consequently, more target ^1H spins are polarized and less polarization is lost to the surroundings. At the same time, the 10% protonation level is sufficient to ensure efficient ^1H - ^1H spin diffusion, distributing the enhanced polarization throughout the sample. Deuterated protein^[33] and deuterated radicals^[37] have also been used to further enhance the sensitivity of DNP experiments. Moreover, large effects of DNP buffer deuteration are also reported for labelled target protein in cell lysates^[2, 38]. In light of these results, we investigated the effect of deuteration of the cellular background as a strategy to boost DNP enhancements. To achieve background deuteration, the cells were grown in deuterated unlabelled M9 medium supplied with deuterated glucose. Following this procedure, fully deuterated ($\sim 90\%$) cells were obtained, while Ubiquitin is the only isotope labelled and strongly protonated species in the cells. The enhancement

factors and the DNP build-up times for Ubiquitin with a fully deuterated background are presented in Table 2.1, alongside a comparison with the fully protonated sample.

	DNP enhancement (ϵ_{DNP})	DNP build-up time (T_{DNP})
^{13}C CP protonated sample	~20 (CO protein) ~25 (sugars) ~29 (lipids)	7.5 s (CO protein) 10.5 s (sugars) 8.5 s (lipids)
^{13}C CP deuterated sample	~100	6-7s
^{15}N CP protonated sample	~20	8s
^{15}N CP deuterated sample	~90	7s

Table 2.1. ^{13}C and ^{15}N DNP enhancements (as measured from CP experiments) and build-up times for the *in-cell* samples in a fully protonated (blue values) and fully deuterated (orange values) cellular background. Experimental parameters, together with the spectra and T_{DNP} build-up curves, are reported in the Supporting Information (see fig. S1-S2 and S4-S5).

Signal enhancements up to 100 were found in fully deuterated cells, which are among the highest DNP enhancement factors observed for bacterial cells. In contrast, experiments performed using a fully protonated cell background exhibited significantly lower enhancements (~20). These findings clearly demonstrate the beneficial effect of the background deuteration for whole cells preparations, in agreement with earlier results obtained in cell lysates^[2, 38]. We attribute the high DNP enhancement to the more efficient polarization transfer from electrons of the radical to the protein protons, as less polarization is lost to the background protons.

However, comparing the absolute signal intensity of the spectra recorded with DNP (Fig. 2.3) shows that the deuterated and protonated sample have similar sensitivities, while the DNP-off spectrum of the protonated sample reveals higher ^{13}C and ^{15}N intensities. A similar behaviour was reported by the group of Mei Hong when studying the effect of membrane deuteration on a transmembrane peptide^[39], and was attributed to the reduced ^1H - ^{13}C CP efficiency due to the lower lipid ^1H density in the deuterated membrane. This implies that protons from the background transfer their magnetization to the protein during Cross-Polarization (CP) experiments with protonated samples, whereas their contribution is filtered out when using the deuterated equivalent. By scaling the signals to the CO peak (~180ppm) in the ^{13}C -detected spectra and to the backbone peak (~120ppm) in the ^{15}N -detected experiments for the DNP-off condition, we estimate that

only approximately 15-20% of the signal effectively belongs to labelled Ubiquitin. Hence, background deuteration is greatly advantageous for *in-cell* DNP experiments, with DNP enhancement more confined to the protein of interest and reduced signal from the cellular background serving to increase selectivity towards the molecule of interest.

I - DNP OFF

II - DNP ON

Figure 2.3. Comparison of 1D ^1H - ^{13}C cross-polarization (CP, a) and 1D ^1H - ^{15}N cross-polarization (CP, b) recorded without (panel I) and with (panel II) microwave irradiation between ^{13}C , ^{15}N -labelled Ubiquitin *in-cell* samples in fully protonated (blue) and fully deuterated (orange) cellular background. [Note: the different peak profile between protonated and deuterated samples is due to the different labelling strategy, see SI, Fig S1 and S2].

It is well known that solvent deuteration causes an increase in the nuclear relaxation times^[34, 35]. In fact, deuteration dilutes proton dipolar couplings, which are the main source of relaxation. Moreover, deuteration increases the radicals' relaxation time, thus contributing to a higher saturation factor and larger DNP enhancement, and reduces the pool of protons within the so-called spin diffusion barrier. However, deuteration can also slow down the build-up of hyperpolarization, effectively increasing the total experimental time^[33, 37]. For this reason, we measured polarization build-up curves in both cases and the respective time constants are reported in Table 2.1. The relaxation

times did not increase upon deuteration, suggesting that ^2H -labelling does not interfere with the spreading of polarization via spin diffusion in our preparations. Indeed, Hovav et al.^[40] demonstrated that the rate at which hyperpolarization is transferred to the bulk nuclei is strongly dependent on the strength of dipolar coupling between the core nuclei and the electron, whereas it is less susceptible to the dipolar coupling strength between the bulk nuclei, which is the parameter mainly influenced by the background deuteration. Moreover, uniform relaxation values were measured in the case of fully deuterated cells, further confirming that only protein signal is detected. Conversely, different relaxation times were measured for different species, such as lipids and sugars, in the case of protonated cells - where ^{13}C -Glucose is used as labelling source. (see Supporting Information, Fig. S3 and S4).

Polarization build-up curves can also indicate the distance dynamics of polarization transfers. As demonstrated in the case of a peptide crystal^[41], a classical pseudo-1D diffusion model based on the Fick's law^[42] can be used to describe the spin diffusion process. Under the assumption of uniform polarization of the nuclei nearby the radical, the nuclear spin diffusion can be modelled as a function of the distance; following this procedure, the DNP enhancement can be correlated with various properties, such as molecular size or variation in proton density^[28]. In this model, the local DNP enhancement is ultimately determined by the longitudinal nuclear relaxation time (T_{DNP}) and the diffusion constant (which depends on the average ^1H - ^1H distance^[41]). The effect of these parameters on the propagation of the DNP enhancement is shown in fig. 2.4.

Figure 2.4. One-dimensional diffusion model calculations of DNP enhancement factors as a function of the distance. (A) the polarization is computed for an average ^1H - ^1H distance of 1 Å (red), 5 Å (blue) and 10 Å (green). (B) The polarization is computed for different relaxation times, namely 1 s (red), 5 s (blue) and 10 s (green). [Note: details of the calculation are given in Chapter 4, Supporting Information section SIV]

The DNP enhancement decreases visibly as the distance from the hyperpolarization source is increased. The situation is especially accentuated in the case of short relaxation times and a large average ^1H - ^1H distance, which is the condition of our deuterated sample. Therefore, the high DNP enhancement obtained in our *in-cell* preparations indicates a close proximity between the radicals and the analyte, as was observed in the case of human cells. Finally, the sample produced with a fully protonated background resulted in a higher enhancement for the labelled lipids when compared to the protein. This suggests that the radical might locate preferentially in the proximity of the lipid membrane, in line with studies reported in the literature^[39, 43]. The higher enhancement is also associated with a longer relaxation time (T_{DNP}), in agreement with the model calculation (Fig. 2.4B).

Further studies need to be conducted to fully elucidate the spin diffusion process responsible for the propagation of DNP enhancement in *in-cell* samples. For instance, direct detection of ^{13}C or ^{15}N signal enhancement, where the measured signal is not affected by the cross-polarization efficiency is yet to be performed. Moreover, measuring the exact location of the DNP agent in the bacterial preparations will provide invaluable information and contribute to the fundamental understanding of the effective enhancement radius of the biradical.

Preliminary results on the glycerol percentage for optimal sample preparation

In this section, preliminary results on the optimal composition of the DNP solution for *in-cell* preparations are illustrated. These studies were conducted on a different protein, namely LmrR, a *Lactococcus lactis* derived transcription repressor that controls the transcription of the ABC type multidrug transporter, LmrCD^[44]. This system is of particular interest because it can be functionalized into an Artificial Metalloenzyme (ArM), able to catalyse novel reactions in live cells^[45]. We intend to repeat these studies on Ubiquitin, however, we do not expect the results to be specific to the protein probed and hence we anticipate a similar outcome.

The optimal concentration of the DNP agent and glycerol amount in our *in-cell* sample preparations are investigated. As previously discussed, the standard DNP solution^[46] generally adopted for biomolecular DNP applications is composed of 60% deuterated glycerol, 30% D_2O and 10% H_2O , with a radical concentration varying between 10 to 60 mM^[26, 29, 39, 47, 48]. However, the radical and the target molecule are now in the cell interior, therefore the requirements to achieve homogenous radical distribution may differ.

In our preparations, using a radical concentration of 15mM or 30mM did not influence the signal enhancement, possibly indicating that DNP performance is limited by the fraction of biradicals that remain DNP-active in the cellular environment. Since low concentrations of DNP agents are typically beneficial, due to the reduced quenching effect, a 15mM radical concentration was chosen. Figure 2.5 (A) shows the dependence of the DNP signal enhancement on the glycerol percentage. (also see Supporting Information, fig. S6-S7).

A 50% drop in the DNP enhancement is observed for the DNP buffer devoid of any glycerol. Thus, also for *in-cell* preparations, the presence of glycerol minimizes ice formation at low temperature, which is detrimental to the DNP enhancements, and facilitates the radical mixing. However, the reduced viscosity of the sample prepared with lower glycerol concentration facilitates packing the sample in the NMR rotor. Consequently, despite the final sample volume being the same as in previous experiments, more cells could be packed – as less volume was occupied by the glycerol – resulting in a higher signal intensity, as shown in fig. 2.5 (B). Moreover, the increase in relaxation time appears to correlate with the increase in glycerol percentage. Therefore, reducing the amount of glycerol results in an effective shortening of the experimental time. Based on these results, a 30% glycerol percentage was successively utilized for *in-cell* preparation, balancing optimal sample cryoprotection and maximum DNP efficiency.

Figure 2.5. (A) Comparison of ^{13}C and ^{15}N DNP enhancement and build-up times (T_{DNP}) is shown for *in-cell* samples prepared in the same conditions, with the only difference being the d_8 -glycerol amount added to the DNP solution. A comparison of the ^{13}C enhancement values measured for Ubiquitin in human cell is also presented. The much lower values measured for LmrR samples are due to the different radical used. The biradical TOTAPOL was used for LmrR in E.coli cells, instead AMUPol was used for Ubiquitin samples. (B) Overlay of DNP-off 1D ^1H - ^{13}C CP spectra of LmrR with (blue) and without (black) glycerol.

2D and 3D NMR spectra of Ubiquitin in *E.coli* cells with deuterated cellular background

Finally, thanks to the optimized sample preparation, 2D and 3D ssNMR spectra could be recorded on the isotope labelled Ubiquitin in fully deuterated *E.coli* cells. It is worth noting that the high signal enhancements ($\sim 100\times$) achieved with this sample preparation reduces experimental time by a factor of 10^4 when compared to conventional ssNMR. The sensitivity gain achieved with DNP permits multi-dimensional experiments that would otherwise be unfeasible. 2D ^{13}C , ^{13}C spin diffusion spectrum and 3D double quantum-filtered ^{13}C , ^{13}C correlation spectrum are shown in Fig. 2.6 and 2.7, respectively. Analysing these spectra allowed unambiguous identification of 25 residues, matching the in-vitro assignments. The results therefore demonstrate the feasibility and the great potential of *in-cell* experiments conducted in bacterial cells using DNP.

Figure 2.6. The ^{13}C , ^{13}C spin diffusion spectrum of Ubiquitin in fully deuterated *E.coli* cells. The black crosses indicate the in vitro NMR assignments at room temperature. Resolved peaks are assigned as indicated. Dashed boxes show the head group of phosphatidylethanolamine (PE).

Figure 2.7. 2D (F_2 , F_3) slices from the 3D double quantum-filtered ^{13}C , ^{13}C correlation spectrum with unambiguously identifiable peaks. The corresponding F_1 frequency is indicated on the right.

Conclusions

A specific DNP-NMR approach has been developed and successfully implemented on a eukaryotic and a prokaryotic system, allowing direct observation of the protein of interest in its native cellular context. High DNP sensitivity enhancements are essential to perform such studies, due to the extreme dilution of the target analyte in these conditions. Our results reveal that full deuteration of the cellular background leads to improved DNP efficiency in bacterial cells. However, the polarization-transfer is non-selective, resulting in signal enhancements for all molecules in the sample. Therefore it becomes necessary to selectively polarize the molecule of interest over the cellular background. By electroporating the labelled protein in unlabelled cells or by employing labelled amino acid mixtures and optimal cellular background deuteration, the targeted isotope labelling of proteins in mammalian and bacterial cells, respectively, could be achieved for DNP-ssNMR studies. We have demonstrated that DNP biradicals are compatible with *in-cell* studies and sample preparations must be carefully adjusted to achieve optimal DNP efficiency towards the target molecule. Thus, this approach offers effective means to study proteins at atomic resolution in native environments. In the future, this strategy could be combined with targeted-DNP techniques where, by attaching the biradical to the specific molecule of interest^[30, 38], the enhancement can be largely localized to the target system. Furthermore, selective isotope labelling techniques can be implemented to increase the spectral resolution. With these advances, investigations at an atomic scale of target molecules at low concentrations in a crowded background can be envisaged. Such studies could significantly expand our understanding of cellular processes that strongly depend on the molecular environment, such as ligand binding, protein-protein interactions and protein folding.

Supporting Information

I. ^{13}C and ^{15}N DNP Enhancements

$[^{13}\text{C}, ^{15}\text{N}]$ -Ubiquitin in fully protonated background

Figure S1. DNP enhancements on *in-cell* NMR samples of uniformly labelled Ubiquitin with protonated cellular background and the conventionally ^{13}C , ^{15}N -labelled medium as the labelling source. The spectra are scaled on the protein signal, indicated by blue arrows. a) ^1H - ^{13}C CP spectra- Lipid signals (both tails, sugars and head groups) show higher enhancements compared to the protein. b) The ^1H - ^{15}N CP spectrum showing an enhancement of ~ 20 .

[^{13}C , ^{15}N]-Ubiquitin in fully deuterated background**a) ^{13}C CP****DNP ON**
DNP OFF **DNP ON**
DNP OFF Scaled: ~100x
(on protein signal) **b) ^{15}N CP****DNP ON**
DNP OFF **DNP ON**
DNP OFF Scaled: ~90x
(on protein signal)

Figure S2. DNP enhancements on *in-cell* NMR samples of uniformly labelled Ubiquitin with deuterated cellular background and labelled algal amino acid mix as the labelling source. The spectra are scaled on the protein CO signal. a) ^1H - ^{13}C CP spectra- Protein signals clearly dominate the spectra b) The ^1H - ^{15}N CP spectrum showing an enhancement of ~90.

II. Labelling source

Figure S3. Aliphatic region of the ^{13}C , ^{13}C spin diffusion spectrum of Ubiquitin. A comparison between cell prepared using the conventionally ^{13}C , ^{15}N -labelled medium (blue) and a medium containing ^{13}C , ^{15}N -algal amino acid mixture (orange) is given. The black crosses indicate the in vitro NMR assignments at room temperature. The spectra show that the background lipid and sugar signals are strongly suppressed when algal amino acid mixture is used as labelling source.

III. Relaxation data

- Protonated sample

Figure S4. (A) ^1H - ^{13}C cross-polarization spectrum of Ubiquitin with fully protonated cellular background. (B-E) Relaxation profiles and respective mono exponential fitted values for the indicated peaks. (F) Relaxation time as measured in the ^1H - ^{15}N saturation recovery experiment.

- Deuterated sample

Figure S5. (A) ^1H - ^{13}C cross-polarization spectrum of Ubiquitin with fully deuterated cellular background. (B-E) Relaxation profiles and respective mono exponential fitted values for the indicated peaks. (F) Relaxation time as measured in the ^1H - ^{15}N saturation recovery experiment.

IV. Glycerol study

• LmrR in *E.coli* cells

(A) - No glycerol

 ^1H - ^{13}C CPDNP ON
DNP OFF ^1H - ^{13}C CPDNP ON
DNP OFF Scaled: ~5x
(to protein CO signal) ^1H - ^{15}N CPDNP ON
DNP OFF ^1H - ^{15}N CPDNP ON
DNP OFF Scaled: ~5x

(B) - 60% glycerol

 ^1H - ^{13}C CPDNP ON
DNP OFF ^1H - ^{13}C CPDNP ON
DNP OFF Scaled: ~10x
(to protein CO signal) ^1H - ^{15}N CPDNP ON
DNP OFF ^1H - ^{15}N CPDNP ON
DNP OFF Scaled: ~10x

Figure S6. ^{13}C and ^{15}N DNP Enhancements of LmrR in *E.coli* cells in the absence (A) and presence (B) of deuterated glycerol in the DNP buffer.

- **Ubiquitin in human cells**

(A) - No glycerol

(B) - 60% glycerol

Figure S7. ^{13}C and ^{15}N DNP Enhancements of Ub in human cells in the absence (A) and presence (B) of deuterated glycerol in the DNP buffer.

V. Experimental details for 2D and 3D spectra

2D- ^{13}C , ^{13}C Proton-driven spin diffusion (PDSD) experiments

For 2D proton-driven spin diffusion (PDSD) experiments, a mixing time of 30 ms was employed and the H-C CP contact time was set to 200 μs . 80 scans were acquired with acquisition times of 10 ms and 5 ms for the direct and indirect dimensions, respectively. The 2D spectra were processed using a 0.25π shifted sine squared window function on both dimensions.

3D- double quantum-filtered ^{13}C , ^{13}C correlation spectrum

The 3D 2Q-1Q-1Q ^{13}C , ^{13}C correlation spectrum, was obtained using a 2.5ms SPC5^[49] mixing period for generation and subsequent reconversion of double quantum coherences. A contact time of 200 μs was used for H-C CP and continuous wave (CW) ^1H decoupling was applied during the SPC5 sequence with a field strength of 85 kHz. The spin diffusion mixing time was set to 30 ms. 64 scans were acquired with a 2 s recycle delay. Acquisition times of 10 ms in the F3 1Q direct dimension, 1.5 ms for the F2 1Q indirect dimension and 0.85 ms for the F1 2Q indirect dimension were used. The 2D spectra were processed using a 0.28π shifted sine squared window function on all dimensions.

For spectral analysis, NMRFAM-SPARKY software package was used^[50]. ssNMR assignments were obtained from BMRB ID: 7111 of microcrystalline Ub. For Ub loops for which ssNMR assignments were not available, we supplemented assignments from solution NMR (BMRB ID: 15410).

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CHAPTER 3

Zeolite Shape Selectivity during the Methanol-to-Hydrocarbons process as Revealed by Solid-State NMR Spectroscopy

Based on:

“Elucidating Zeolite Channel Geometry–Reaction Intermediate Relationships for the Methanol-to-Hydrocarbon Process” – Published in *Angew. Chemie - Int. Ed.* 59, 2–9 (2020)

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Abstract

The shape or topology of the internal pore structure of a zeolite material can strongly affect the reaction pathways it is catalysing. It is generally believed that the host-guest chemistry between the inorganic zeolite host and organic guest species determines the product selectivity of the methanol-to-hydrocarbons (MTH) process. However, precise spectroscopic or experimental evidence is yet to be achieved. Herein, we elucidate the host-guest chemistry between zeolite channel shape and reaction intermediates during MTH over anisotropic zeolite crystals using advanced magic angle spinning (MAS) solid-state nuclear magnetic resonance (ssNMR) spectroscopy. These experiments revealed that subtle differences in zeolite channel topologies drastically alter the configuration of the reaction intermediates formed. The slightly more rigid sinusoidal channels of zeolite ZSM-5 favour the olefin cycle by promoting the homologation of alkanes, while the extended straight zeolite ZSM-5 channels foster the aromatic cycle with a higher degree of alkylation of aromatics. Dynamic nuclear polarization (DNP) ssNMR experiments further suggested the preferential formation of heavy aromatics at the sinusoidal channel dominated surface, providing insights into catalyst deactivation.

Introduction

The shape or topology of internal pore structures of zeolites strongly affects the product selectivity by permitting the configuration of different reactants/products/intermediates, thereby contributing to highly cost-effective processes with well-defined product distributions^[1-3]. One of the prototypical case is the already commercialized methanol-to-hydrocarbons (MTH) process of converting biomass- and municipal waste- or coal-derived methanol to a range of products from olefins to gasolines, depending on the frame-work topology of the zeolite material chosen^[4-7]. As a result, significant research efforts have been focused on elucidating the influence of zeolite topology, i.e., channels, cages and cavities, on the ultimate product distributions, and hence the zeolite topology-reaction intermediates relationships could be derived^[8-23]. Yet, no direct experimental evidence has been presented for the effect of channel geometry on the reaction intermediates within a single zeolite framework, which is crucial to the design of catalyst materials with maximized product yields and minimized costs.

Figure 3.1 Schematic illustrations of A) the hydrocarbon pool (HCP) concept during the MTH process and B) the morphology of a-oriented and b-oriented zeolite ZSM-5 crystals. The surface of the a-oriented and b-oriented crystals are dominated by sinusoidal channels (blue) and straight channels (orange), respectively.

There is a consensus that the MTH reaction proceeds through a dual cycle hydrocarbon pool (HCP) mechanism (Figure 3.1A), which consists of two inter-dependent reaction cycles based on aromatic and olefinic species, i.e., aromatic and olefin cycles, respectively^[4, 24, 25]. These aromatic cycle species are repeatedly methylated and subsequently split off light alkenes, contributing to the production of ethylene. The olefin

cycle involving methylation and cracking of olefins primarily produces larger alkenes. Consequently, it is concluded that an active MTH catalyst can be viewed as an organic-inorganic “hybrid” material of “supramolecular nature” consisting of the inorganic zeolite and the organic hydrocarbon pool^[26, 27]. Recent research further highlighted the complexity and significance of the host-guest chemistry during the MTH process^[28-33]. Therefore, it is of fundamental importance to obtain a mechanistic understanding of the zeolite geometry-reactive intermediates relationships by examining the distinctive host-guest chemistry of MTH^[2].

Herein, we have utilized complementary solid-state NMR magnetization transfer schemes (Figure 3.2) to separate reactive MTH intermediates based on their differential mobility^[34] over anisotropic zeolite ZSM-5 crystals (possessing the MFI framework topology, Figure S5). We obtained novel molecular insights into the host-guest chemistry between the zeolite channels and reaction intermediates in terms of their reactivity and mobility. Next, we applied dynamic nuclear polarization (DNP)^[32, 35-39] to improve the sensitivity and examine the spatial distribution of the hydrocarbons within the MTH-reacted materials.

Materials and Methods

Methanol-to-Hydrocarbons Reactions

The methanol-hydrocarbons (MTH) reactions of the anisotropic zeolite ZSM-5 crystals were performed at 623 K in a quartz, rectangular fixed-bed reactor (ID= 6 mm× 3 mm). In-situ UV-Vis diffuse reflectance spectroscopy (DRS) was applied simultaneously to monitor the entrapped hydrocarbon pool (HCP) species during the MTH^[40, 41] (Figure S8). In-situ UV-Vis DRS spectra were obtained using an AvaSpec 2048L spectrometer connected to a high-temperature UV-Vis optical fiber probe, which was used to collect spectra in reflection mode. The measurements were performed in the wavelength range of 200-1000 nm (11000-50000 cm⁻¹ in wavenumber). Typically, ca. 100 mg of catalysts was loaded and calcined under a flow of pure O₂ with a ramp of 15 K/min to 823 K and hold for 120 min. The MTH reaction was then performed by first cooling the catalysts to 623 K and switching to He atmosphere. A weight hourly space velocity (WHSV) of 5 h⁻¹ was obtained with a ¹³C-methanol saturation of ca. 14.5% by flowing the carrier gas through a saturator containing ¹³C-methanol at 293 K. After 2 min of reaction the flow was switched back to He, and the heating furnace was removed. The reaction was quenched by rapid cooling of the reactor using compressed air.

Solid-state NMR experiments

All solid-state nuclear magnetic resonance (ssNMR) experiments were performed at room temperature (298 K) and MAS frequency of 16 kHz on a Bruker 500MHz wide-bore magnet with an AVANCE-III console and equipped with a 3.2 mm HXY probe in double channel mode.

The ^1H and ^{13}C NMR experiments were performed at room temperature (298 K) and MAS frequency of 16 kHz. Note that effective sample temperatures can be 5-10 degrees higher due to frictional heating. Referencing of ^1H and ^{13}C chemical shifts was done externally to adamantane. Hard ^1H and ^{13}C pulses were applied with field strength of 88 kHz and 45 kHz, respectively. A 88 kHz SPINAL64 ^1H decoupling was applied during acquisition for the 1D and 2D ^{13}C detected spectra^[42]. *1D ssNMR*: The 1D ^1H - ^{13}C cross-polarization (CP) spectrum^[43] was recorded using a 1.5 s recycle delay, 12 ms acquisition time and an accumulation of 4096 scans. CP was achieved using a 38 kHz ^{13}C field and 78 kHz 70-100% ramped ^1H field and a contact time of 0.7 ms. The 1D ^{13}C DE spectrum was recorded using a 2 s recycle delay, 14 ms acquisition time and an accumulation of 4096 scans. The 1D ^1H - ^{13}C insensitive nuclei enhanced by polarization transfer (INEPT)^[44] spectrum was recorded using a 1.5 s recycle delay, 33 ms acquisition time and an accumulation of 4096 scans. 1D CP, DE and INEPT spectra were processed using 150 Hz line-broadening.

2D ssNMR for probing rigid molecules. The dipolar based 2D ^{13}C - ^1H correlation spectrum was obtained using a 0.7 ms ^1H - ^{13}C CP period and a 0.4 ms ^{13}C - ^1H CP period. Background signals from catalyst protons were suppressed by a 8 kHz MISSISSIPPI block ($\tau = 5$ ms, $N = 2$) prior to the last CP^[45]. ^{13}C PISSARRO (Phase-Inverted Supercycled Sequence for Attenuation of Rotary Resonance) decoupling was applied during acquisition^[46]. The recycle delay was 1.5 s, acquisition times 10 ms (F2, ^1H) and 6 ms (F1, ^{13}C) and 128 scans. The spectrum was processed using a 0.33π shifted sine squared window function in both dimensions. 2D ^{13}C - ^{13}C spectra were recorded using a 1.5 s recycle delay, 10 ms (F2) and 3.5 ms (F1) acquisition time and an accumulation of 1024 scans. ^{13}C - ^{13}C mixing was achieved through proton-driven spin-diffusion using phase-alternated-recoupling-irradiation-schemes (PARIS) for 30 ms^[47]. Spectra were processed using a 0.33π shifted sine squared window function in both dimensions.

2D ssNMR for probing mobile molecules. A 2D ^{13}C - ^1H HSQC (Heteronuclear Single Quantum Coherence)^[48] spectrum was recorded using 155 Hz for J in the INEPT delay. A 1.5 s recycle delay was used, acquisition times were 30 ms (F2, ^1H) and 4 ms (F1, ^{13}C) ms and 256 scans were accumulated. 6 kHz GARP ^{13}C decoupling was used during detection^[49]. Prior to Fourier transformation, 0.5π shifted sine squared function was

applied in both dimensions. 2D ^1H - ^{13}C spectrum was recorded using a 1.5 s recycle delay, 15 ms (F2) or 4 ms (F1) acquisition time and an accumulation of 256 scans. 155 Hz for J was used in the INEPT delay. A 10 kHz WALTZ ^1H decoupling was used during detection^[50]. Prior to Fourier transformation, a window function corresponding to a 0.5π shifted sine squared function was applied to both dimensions. 2D ^{13}C - ^{13}C TOBSY^[51] was recorded using a mixing time of 4 ms, a 2 s recycle delay and 16 ms (F2) or 3 ms (F1) acquisition time using 384 scans. The spectrum was recorded using 155 Hz for J in the INEPT delay and processed using 200 Hz line-broadening in the F2 dimension and a 0.5π shifted sine squared function to the F1 dimension. 10 kHz WALTZ ^1H decoupling was used during detection.

Dynamic Nuclear Polarization (DNP) MAS NMR. DNP experiments were conducted at low temperature (100 K) using 3.2 mm triple-resonance (^1H , ^{13}C , and ^{15}N) MAS probe heads in static magnetic fields of 9.4 T, corresponding to proton/electron resonance frequencies of 400 MHz/263 GHz (Bruker BioSpin). DNP samples were prepared by wetting, using a solution of 16 mM bTbk in tetrachloroethane^[35, 52, 53]. The ^1H - ^{13}C cross polarization DNP experiments presented in Figure 3.10 were recorded using a 2 s recycle delay, 15 ms acquisition time and an accumulation of 1024 scans. A CP contact time of 0.6 ms was used an 83 kHz SPINAL-64 proton decoupling was applied during acquisition. The spectra were processed with 75 Hz line-broadening. All spectra were recorded at a MAS rate of 8 kHz. DNP enhancements were obtained by scaling the signal intensities of spectra measured under DNP with the corresponding spectrum obtained without microwave irradiation at 100 K. Spinning sidebands were identified by varying spinning speeds. The DNP samples were prepared following the same procedure in both cases^[54].

Analysis of ssNMR Data. All NMR spectra were processed with Bruker Topspin3.5 and analysed using SPARKY^[55].

Research methodology of this work

Figure 3.2. Schematic representation of the application of specific magnetization transfer techniques to probe the different reaction products.

Using magic angle spinning (MAS) solid-state nuclear magnetic resonance spectroscopy (ssNMR), the reactive MTH intermediates formed in the different zeolite channels over the anisotropic zeolite ZSM-5 crystals were separated and identified on the basis of their mobility. The rigid and mobile hydrocarbons trapped within zeolites could be spectrally separated using different ssNMR experiments that invoke through-space (dipolar transfer such as in cross-polarization experiments, CP) or through-bond (scalar interactions such as in insensitive nuclei enhanced by polarization transfer experiments, INEPT) magnetization transfer schemes, respectively^[28, 29]. The spatial distribution of hydrocarbons formed in the anisotropic zeolite ZSM-5 crystals was identified using dynamic nuclear polarization surface enhanced NMR spectroscopy^[32, 35, 53].

Results and discussion

^{13}C -enriched MTH reactions were performed over anisotropic zeolite ZSM-5 crystals with different micropore orientations dominating the surface. Scanning electron microscope (SEM, Supporting Information Figures S5) as well as MAS ^{29}Si and ^{27}Al ssNMR (Figures S2-S3) demonstrated that the anisotropic crystals have comparable physicochemical properties^[56]. Molecular dynamics simulations (see details in Supporting Information, Section S3-S5) revealed that methanol molecules are accumulated into the zeolite channels with short dimensions at the beginning of the reactions (Figure S7). This finding suggests that the MTH reactions occurred primarily in the sinusoidal and straight zeolite channels for the *a*-oriented and *b*-oriented zeolite ZSM-5 crystals in the initial stage (*i.e.* 2 min) of the reaction, respectively, as corroborated by the *in-situ* UV-Vis DRS (Figure S8)^[40]. Furthermore, the use of the isotope-enriched reactant drastically enhanced the ssNMR signal, and allowed us to distinctly determine the zeolite-trapped hydrocarbons in different environments using multi-dimensional ssNMR correlation experiments^[29, 57].

Figure 3.3. Magic angle spinning (MAS) solid-state nuclear magnetic resonance (ssNMR) spectra of the trapped MTH products in the A) *a*-oriented and B) *b*-oriented zeolite crystals. 1D ^1H - ^{13}C cross-polarization (CP, blue) to probe rigid molecules, ^1H - ^{13}C insensitive nuclei enhanced by polarization transfer (INEPT, green) to probe mobile molecules and ^{13}C direct excitation (DE, red) to probe all chemical species, including those that exhibit intermediate dynamics.

In the 1D ^{13}C -spectra (Figure 3.3) two main features can be recognized in both MTH-reacted *a*-oriented and *b*-oriented ZSM-5 crystals: (i) 9-55 ppm aliphatic moieties and methyl groups, (ii) 110-155 ppm (methylated) aromatic/olefinic groups. Overall, we

noticed that the olefinic/aromatic species are either immobilized (detected by cross polarization, CP) or have restricted mobility (visible in the direct excitation, DE). In contrast, mobile molecules (visible in scalar-based INEPT experiments) are predominantly saturated/aliphatic in nature. Moreover, the broadening of the ^{13}C DE spectra is due to fast relaxing species with intermediate dynamics.

2D solid-state NMR spectra of the adsorbed methanol and dimethyl ether within the anisotropic zeolite ZSM-5 crystals

2D ssNMR experiments were performed to elucidate in more detail the molecular signatures of the zeolite-trapped hydrocarbon species and the binding structures of both the rigid and mobile methanol molecules were identified (Figure 3.4). In the experiments probing the rigid methanol, two different motifs, i.e., side-on ($\eta_2:\eta_2$) and end-on ($\eta_1:\eta_1$) conformations, were found in both a-oriented and b-oriented zeolite crystals, indicating comparable adsorption environments in the two distinct crystals^[30]. Moreover, the assignments are confirmed by examining the mobile molecules. As shown in Figure 3.4c-d, the intensity of the end-on conformation of methanol is enhanced in both zeolites. In contrast, the intensity of the side-on conformation is sharply decreased or absent, as in the a-oriented zeolite crystals. This demonstrates a lower mobility of the side-on conformers and a stronger H-bonding, suggesting a more rigid nature in the a-oriented zeolite crystals. Additionally, dimethyl ether (DME), a well-known intermediate of the MTH reaction, is observed in the INEPT-based ^1H - ^{13}C correlation spectra for both anisotropic crystals (Figure 3.4c-d). Interestingly, compared with the mobile reactant, the chemical shifts of surface adsorbed methanol are all shifted downfield, indicating that the zeolite channels altered the electronic state of the immobilized methanol^[28].

Figure 3.4. 2D magic angle spinning (MAS) ssNMR spectra of the surface adsorbed a,b) rigid and c,d) mobile methanol molecules in the anisotropic zeolite ZSM-5 crystals after 2 min of the methanol-to-hydrocarbons (MTH) at 623 K. a, c) and b,d) are the ssNMR spectra for the adsorbed molecules trapped in the a-oriented and b-oriented zeolite ZSM-5 zeolites, respectively. e) Overview of the surface adsorbed molecules. A contact time of 400 μs was used for the ^{13}C - ^1H correlation experiment. All ssNMR spectra were recorded at 290 K, using 16 kHz MAS.

2D solid-state NMR spectra of the rigid moieties trapped within the anisotropic zeolite crystals

The rigid molecules present were further examined by 2D ^{13}C - ^{13}C ssNMR, as shown in Figure 3.5. Clear correlations between methyl groups at 20-25 ppm (^{13}C) and aromatic at 130-140 ppm (^{13}C) moieties revealed the presence of several HCP-type intermediates^[29]. For the a-oriented zeolite crystals (Figure 3.5a), only one methyl resonance at 21.1 (^{13}C) ppm was detected, while two interacting methyl resonances at 18.4 and 21.7 (^{13}C) ppm correlated with aromatic signals for the b-oriented zeolite crystals (Figure 3.5b).

Figure 3.5. 2D ^{13}C - ^{13}C and ^{13}C - ^1H magic angle spinning (MAS) ssNMR spectra of the rigid methylated aromatics molecules in the a) a-oriented and b) b-oriented zeolite ZSM-5 crystals after 2 min of the methanol-to-hydrocarbons (MTH) at 623 K. c) Assignment of the observed hydrocarbons trapped within the anisotropic zeolite crystals. The spectra were recorded at 290 K, using 16 kHz MAS. For the ^{13}C - ^{13}C correlation experiment, polarization transfer among ^{13}C spins was achieved by initial cross-polarization (CP) and a 30 ms phase-alternated recoupling irradiation scheme (PARIS)^[47] mixing period. A CP contact time of 400 μs was used for the ^{13}C - ^1H spectra. Resonances coloured in grey in the top panel show species that are present only in the a-oriented ZSM-5 crystals.

This suggests the formation of only symmetric methylated aromatics within the a-oriented crystals, while asymmetric methylated aromatics were formed within the b-oriented analogue. This finding is confirmed by further experiments with longer ^{13}C - ^{13}C

mixing time (Figure S10). In fact, even with a longer mixing time (120ms) the cross-peaks between ~ 18 ppm and ~ 21 ppm resonances are absent in the a-oriented zeolite crystals. Instead, the cross-peaks become more prominent in the b-oriented zeolite crystals at a longer ^{13}C - ^{13}C mixing, therefore confirming the presence of asymmetric methylated aromatics in the sample with a higher accessibility for the straight zeolite channels. Moreover, the 2D CP-based ^{13}C - ^1H spectra (right panels, Figures 3.5a-b) showed clear correlations between aliphatic at ~ 2 (^1H) ppm and aromatic at 7-8 (^1H) ppm protons for the a-oriented zeolite crystals, while they are absent in the b-oriented zeolite crystals. This result demonstrates a higher degree of alkylation on the aromatics/olefinic HCP species in the b-oriented zeolite crystals, which does not allow such ^{13}C - ^1H correlation as the aromatic protons are substituted by methyl groups.

Figure 3.6. a) Zoom-in of the 2D ^{13}C - ^{13}C magic angle spinning (MAS) ssNMR spectra of the rigid methylated aromatics molecules in the a-oriented and b-oriented zeolite ZSM-5 crystals after 2 min of the methanol-to-hydrocarbons (MTH) reaction at 623 K. b) 1D projection spectra (as defined in topspin) of the aromatic resonances in the direct dimension. The projections are normalized to the 131ppm resonance. The spectra were recorded at 290 K, using 16 kHz MAS. The polarization of ^{13}C atoms was achieved through cross-polarization (CP), and a 30 ms phase-alternated recoupling irradiation scheme (PARIS) mixing period was used.

A further evidence is given by the observation of a higher ratio of ~ 136 ppm to ~ 130 ppm species for the b-oriented zeolite crystals, compared to the a-oriented counterpart (Figure 3.6). In fact, the substitution of the aromatic proton by a methyl group causes a shift of the connected aromatic carbon to higher values, therefore increasing the signal at 136 ppm.

The higher degree of alkylation reveals the promotion of the aromatic cycle in the straight zeolite channels. It should be noted that the high ratio of ~ 136 to ~ 130 ppm could also be attributed to the fast formation of H-deficient coke deposits, e.g.,

naphthalene and anthracene, within the straight zeolite channels^[40, 58]. Additionally, fewer resonances and a weaker signal intensity are found in all the proton-detected ^{13}C - ^1H correlation spectra (Figures 3.5, 3.8) for the b-oriented zeolite ZSM-5 crystal compared to the a-oriented analogue, demonstrating the preferential formation of H-deficient hydrocarbons in the b-oriented zeolite crystals.

Figure 3.7. Overview of the reaction intermediates detected from the ssNMR experiments along with their assigned chemical shifts. Note that toluene was also probed in the products from the sinusoidal zeolite channels in oriented zeolite thin-films by mass spectrometry (MS), as demonstrated in a previous work^[40].

Collectively, it can be concluded that the straight zeolite channels favour the aromatic cycle due to the fast formation of internal coke deposits^[40, 58], with the formation of a high degree of alkylated aromatics and asymmetric molecules, e.g., 1, 2, 4-trimethylbenzene (Figure 3.7) that are believed to be responsible for ethylene production^[59]. Therefore, more ethylene can be produced in the straight channels of zeolite ZSM-5. Previously, it was shown that the introduction of alkaline-earth metals could dramatically suppress the aromatic cycles^[31, 32, 41]. We speculate that these promotor species might be primarily located within the straight zeolite channels.

2D solid-state NMR spectra of the mobile moieties trapped within the anisotropic zeolite crystals

In the next stage, we probed mobile molecules by 2D ssNMR measurements using through-bond magnetization transfer schemes (Figure 3.2). In these ssNMR spectra (Figures 3.8 and S9) certain resonances appear to be broadened or some signals show more than one peak for the same species, clearly indicating that the same molecule exists in different molecular environments inside the zeolite framework^[29].

Figure 3.8. 2D scalar-based ^{13}C - ^1H magic angle spinning (MAS) ssNMR spectra of mobile molecules trapped in the a-oriented (blue) and b-oriented (orange) zeolite crystals after 2 min of the methanol-to-hydrocarbons (MTH) reaction at 623 K. The spectra were recorded at 290 K using 16 kHz MAS. The black box indicates the methyl resonances corresponding to the species shown in the right panel of Figure 3.5. The blue box highlights the hydrogen-transferred species only present in the a-oriented zeolite ZSM-5 crystals^[60].

As shown in Figures 3.8, the ^{13}C - ^1H correlation spectra identified ethane at 9.15 ppm (^{13}C) and 0.98 ppm (^1H) as well as propane at 18.9 ppm (^{13}C) and 0.7 ppm (^1H) in the a-oriented zeolite crystals, while only ethane (8.97 (^{13}C) and 0.99 (^1H)) was observed

in the b-oriented analogue. Moreover, the resonances at ~ 24 and 26 ppm (^{13}C) and 0.9 ppm (^1H) are only visible in the a-oriented crystals and are compatible with butane and/or tetramethylethane signal. Based on the literature and by combining the spectra of the scalar-based ^{13}C - ^1H and ^{13}C - ^{13}C correlations experiments (Figure S9), the signals were tentatively assigned to butane moieties. Interestingly, the results (Figure 3.8) also reveal that mobile olefinic species are only present in the a-oriented zeolite crystals. Taken together, these findings demonstrate the homologation reactions in the a-oriented zeolite crystals, and thereby the preference of the olefin cycle in the sinusoidal zeolite channels^[61].

Spatial distribution of the hydrocarbons within the MTH-reacted materials as probed by DNP-enhanced solid-state NMR experiments

Finally, we examined the spatial distribution of the hydrocarbons formed in the anisotropic crystals using dynamic nuclear polarization (DNP), a method used to improve ssNMR sensitivity. In particular in the field of heterogeneous catalysis, DNP is recognized as a powerful technique for studying catalytic sites both at the outer surface and in the pores of zeolites as well as detecting confined intermediates, such as carbocations^[32, 39].

Figure 3.9. a) Simplified illustration of the hyperpolarization of carbon atoms of the hydrocarbons (in dark grey spheres) using DNP ssNMR. The light grey background represents the solvent matrix containing the DNP agent (red-white ellipsoids) within the post-impregnated zeolite. As a result of the microwave (μW) irradiation, electron polarization is transferred from the biradical (bTbK) to the zeolitic Brønsted acid sites and protons in the solvent matrix. Next, ^1H - ^1H spin-diffusion is able to occur at cryogenic temperatures, then cross-polarization (CP) is used to transfer enhanced polarization to the dilute spins of the medium (i.e., zeolite-trapped hydrocarbons in the present case). b) The molecular structure of the DNP agent, i.e., bTbK. Note that the illustration is adapted from literature^[32].

In a typical DNP experiment, the nuclear signal is enhanced by microwave induced polarization transfer from unpaired electrons to nuclei (usually protons). For DNP, the porous samples are impregnated with a solution of stable radicals as electron source^[36, 62]. These DNP agents are in direct contact with the surface of the material, allowing the polarization of the protons on the surface to be highly enhanced and then transferred to the probed heteronuclei (e.g., ^{13}C , ^{27}Al , ^{29}Si , ^{119}Sn)^[54, 63]. In this work, samples for DNP experiments were prepared by impregnating the MTH-reacted zeolites with a 16 mM solution of bis-TEMPO-bis-ketal (bTbK, ca. $6 \text{ \AA} \times 13 \text{ \AA}$) in tetrachloroethane (TCE)^[53]. The bTbK radical is too large, e.g., similar to benzantracene, to diffuse into 10-membered ring channels (ca. $5.3 \text{ \AA} \times 5.6 \text{ \AA}$), which will be filled with TCE and/or water from ambient moisture.

The results are illustrated in Figure 3.10. The ^{13}C DNP spectra show significant enhancements for aromatic and methylene species. The negligible enhancement for methyl species is most likely due to their fast rotational dynamics even at cryogenic temperatures, which provides an efficient relaxation sink under typical DNP MAS experimental conditions^[64]. For the aromatics at ~ 150 ppm, 22 and 18 times DNP enhancement were observed for the a-oriented and b-oriented zeolite crystals, respectively. This indicates the formation of a greater fraction of the large aromatics at the sinusoidal channel dominated surface of the a-oriented zeolite. Furthermore, a two-fold enhancement of the solvent was observed for the a-oriented zeolite crystals, compared to the b-oriented analogue. As the DNP enhancement is largely restricted to the surface, the above result suggests that the solvent remains more confined at the surface of the a-oriented zeolite particles, while in the b-oriented crystals it can more easily penetrate into the pores^[54]. Hence, we propose that more aromatic species are formed at the surface of the sinusoidal channel dominated particles, with respect to the b-oriented crystal where the surface is dominated by the straight channels.

This finding is consistent with the results obtained from oriented zeolite thin-films^[40].

Figure 3.10. ^1H - ^{13}C cross polarization (CP) dynamic nuclear polarization (DNP) ssNMR spectra measured with the 400 MHz DNP system at 100 K for the A) a-oriented and B) b-oriented zeolite crystals after 2 min of the methanol-to-hydrocarbons (MTH) reaction. Black and red lines represent the spectra without and with irradiation of microwaves, respectively. Magic angle spinning (MAS) sidebands are indicated by *. The numbers denote the DNP-enhancements computed from the intensity ratios of the spectra from on- and off-DNP experiments.

Conclusions

Figure 3.11. Schematic representation of the proposed reaction and deactivation roadmap of the zeolite catalysed methanol-to-hydrocarbons (MTH) process.

In conclusion, detailed insights into the effect of channel geometries on the reaction intermediates of MTH within a single zeolite framework were obtained using a combination of anisotropic zeolite crystals and multi-dimensional ssNMR. The results unequivocally show that subtle differences in the zeolite channel geometry dramatically alter the configuration of reaction intermediates. In particular, the more constrained sinusoidal channels in the a-oriented zeolite ZSM-5 crystals favour the olefin cycle with the promotion of homologation of alkanes. Instead, the more extended straight channels in the b-oriented catalysts facilitate the aromatic cycle, as given by the higher degree of alkylation. This study further promotes the concept of the hybrid inorganic-organic nature of an active MTH catalyst, where the mobility-dependent distinct host-guest chemistry between the shape and topology of the internal zeolite pore structure and the trapped hydrocarbons plays an important role in control of the ultimate product selectivity. Such knowledge will not only be useful for the development of superior and/or improved catalysts for the MTH reaction (via designing zeolite-based catalysts to mimic the transition states of the reactions catalysed)^[65], but will also contribute to our understanding of the host-guest chemistry and shape selectivity.

Supporting Information

S1. Materials

Figure S1. Molecular structures of the employed structure-directing agents (SDAs). a) Tetrapropylammonium cation (TPA⁺) and b) trimer-tetrapropylammonium (trimer-TPA³⁺) for the synthesis of b-oriented and a-oriented zeolite ZSM-5 crystals, respectively.

The a-oriented and b-oriented zeolite ZSM-5 crystals were prepared with the method adjusted from literatures using trimer-tetrapropylammonium cation (trimer-TPA⁺) and tetrapropylammonium cation (TPA⁺) as the structure-directing agents (SDA, Figure S1), respectively^[66, 67]. The chemical compositions of the solutions were adjusted to obtain anisotropic zeolite ZSM-5 crystals with comparable framework Si/Al ratios.

Materials characterization. The crystallinity and morphology of the prepared anisotropic zeolite ZSM-5 crystals were measured using X-ray diffraction (XRD) and scanning electron microscope (SEM), respectively. The XRD patterns were collected using a Bruker D2 Phaser (2nd Gen) instrument using a cobalt radiation source, Co $k\alpha = 1.789\text{\AA}$. The samples were rotated at 15 rad/min. The SEM images of the zeolite ZSM-5 crystals were examined using an XL-30 (Philips) microscope operating at an accelerating voltage of 5 kV. Before measurement the surface of the film was coated with Pt of *ca.* 5 nm thickness to avoid charging effects. The framework Si/Al ratios of the anisotropic zeolite ZSM-5 crystals were examined using magic angle spinning (MAS) solid-state nuclear magnetic resonance spectroscopy (Figures S2-S3).

S2. Solid-state NMR spectroscopy of the framework Si⁴⁺ and Al³⁺ for the anisotropic zeolite ZSM-5 crystals

All solid-state nuclear magnetic resonance (ssNMR) experiments were performed at room temperature (298 K) and MAS frequency of 16 kHz on a Bruker 500MHz wide-bore magnet with an AVANCE-III console and equipped with a 3.2 mm HXY probe in double channel mode. For the ²⁷Al NMR experiments, a radio frequency (r.f.) of 50 kHz was used for the $\pi/12$ pulse to excite only the central transition, followed by an acquisition time of 4.5 ms. 10240 scans were accumulated using an inter-scan delay of 1 s. The ²⁷Al chemical shift was externally referenced to an aluminium nitrate solution (Al(NO₃)₃(aq)). The 1D NMR spectra were processed using a line-broadening of 100 Hz. ²⁹Si NMR experiments were performed with a MAS frequency of 12 kHz and referencing was done externally to tetramethylsilane (TMS). ²⁹Si NMR spectra were recorded using direct excitation (DE). A r.f. field of 54 kHz was used for the measurement of ²⁹Si DE spectra, acquired with 1024 scans and an inter-scan delay of 10 s. A line broadening of 50 Hz was applied for spectral processing. All spectra were processed and analysed with Bruker Topspin 3.5. The silicon to aluminum (Si/Al) ratio was estimated using Lowenstein's rule and using *eq. S1*, where I denotes the intensity of the ²⁹Si NMR signal and $n_{\max} = 1$ in the present case^[68].

$$\frac{Si}{Al(framework)} = \frac{\sum_{n=0}^4 I_{Si(nAl)}}{\sum_{n=0}^4 0.25n * I_{Si(nAl)}} \quad (Eq. S1)$$

In order to explore the steric effect of the zeolite channels on the reaction behaviour of the methanol-to-hydrocarbons (MTH), the coordination environments of Si and Al were evaluated by ²⁹Si and ²⁷Al ssNMR, as shown in Figure S2 and Figure S3, respectively. The ²⁹Si magic angle spinning (MAS) ssNMR spectra of the zeolite samples and their deconvolution curves are shown in Figure S2^[68, 69]. Five peaks can be observed in both the *a*-oriented and *b*-oriented zeolite ZSM-5 crystals after deconvoluting with Gaussian curves. The peaks at ~ -116 and ~ -112 , ~ -108 and ~ -105 , and ~ -102 ppm are assigned to Si(0Al), Si(1Al) and silanol groups, respectively. The deconvoluting results are listed in Table S1. It shows that the two zeolite crystals with different orientations have comparable Si/Al ratios with 32 and 34 for the *a*-oriented and *b*-oriented zeolite ZSM-5 crystals, respectively.

Figure S2. Direct-excitation ^{29}Si magic angle spinning (MAS) ssNMR spectra of the a) a-oriented and b) b-oriented zeolite ZSM-5 crystals. The spectra were deconvoluted using a Gaussian function. The Si-species are identified as Q4 (−112 and −116 ppm), Q3 (−106 ppm and −108 ppm) and Q2 (−102 ppm).

Moreover, ^{27}Al ssNMR spectroscopy was used to elucidate the coordination environment of different Al species. As shown in Figure S3, two peaks are observed for both samples at ~ 56 and ~ 0 ppm, being assigned to the four-coordinated framework Al^{3+} and the six-coordinated extra-framework Al species, respectively. The intense peak at ~ 56 ppm and less intense peak at ~ 0 ppm as well as similar peak ratios of four- to six-coordinated Al, show the predominance of framework Al^{3+} species and similar Al environment distributions in the two samples. Additionally, it has been demonstrated that the position of framework Al^{3+} is heavily determined by the locations of the structure directing agents (SDAs), and that the addition of inorganic mineralizers will modify the locations due to charge balance effect^[70, 71]. Considering the comparable structures of TPA^+ and trimer- TPA^{3+} (Figure S1), we expect that the locations of framework Al^{3+} are also comparable in the anisotropic zeolite ZSM-5 crystals. Therefore, these results confirm that the effect of steric structure can be isolated by preparing samples with different morphologies.

Figure S3. Normalized ^{27}Al direct excitation (DE) magic angle spinning (MAS) ssNMR spectra of the *a*-oriented (blue) and *b*-oriented (green) zeolite ZSM-5 crystals. The chemical shift values are reported for each peak.

Table S1. Parameters for the deconvolution of ^{29}Si direct excitation (DE) magic angle spinning (MAS) solid-state nuclear magnetic resonance (ssNMR) spectra of the *a*-oriented and *b*-oriented zeolite ZSM-5 crystals.

	<i>a</i> -ZSM-5			<i>b</i> -ZSM-5		
	Peak	Area	I	Peak	Area	I
Si(0Al)	-116	1.49E7	19.05	-116.15	1.29E7	12.50
Si(0Al)	-112.41	5.34E7	68.26	-113.05	7.76E7	75.19
Si(1Al)	-108.13	4.91E6	6.28	-109.99	4.82E6	4.67
Si(1Al)	-105	3.07E6	3.92	-105.27	6.49E6	6.29
Si-(OSi) ₃ (OH)	-101.49	1.95E6	1.36	-102.46	1.40E6	1.36
Si/Al	34.08			32.00		

S3. Computational Methods

Molecular dynamics simulation (MD). The commercial software Materials Studio (Accelrys, Inc.) was used to calculate diffusivity of methanol molecules inside pure-Si zeolite ZSM-5 (MFI framework, Figure S4). The COMPASS force field was chosen to describe the interactions between atoms in the simulations. The diffusion coefficients (D) were evaluated from the mean squared displacement (MSD), according to the Einstein relation.

Figure S4. Molecular dynamics (MD) model for methanol molecules in a pure-Si zeolite ZSM-5.

Continuum model for the diffusion. A 2D model was constructed for the diffusion of small molecules inside porous ZSM-5 by using COMSOL Multiphysics, the software of finite element method. The entire model covers 2150 nm by 642 nm, comparable to the aspect ratio of the anisotropic zeolite ZSM-5 crystals. Methanol molecule moves from right and top to left and bottom across the geometry. The flow in the pores does not penetrate the solid grains. The inlet and outlet fluid pressures are known. The time-dependent concentration follows the equation:

$$\frac{\partial c}{\partial t} + \nabla \cdot (-D \nabla c) = 0 \quad (\text{Eq. S2})$$

where c denotes concentration (mol/m³ using SI units) and D the diffusion coefficient (m²/s) of the small molecules.

The Stokes equation (Eq. S3) was used to calculate the flow in the channels. The incompressible assumption together with the stationary condition reads:

$$0 = -\nabla p + \nabla \cdot \mu(\nabla \mathbf{u} + \nabla \mathbf{u}^T), \quad \nabla \cdot \mathbf{u} = 0 \quad (\text{Eq. S3})$$

Here, p is the pressure (SI unit: Pa), $\mathbf{u}$ is the velocity field (SI unit: m/s), and μ is the dynamic viscosity of the fluid (SI unit: Pa·s). At the physical boundaries, the inlet pressure and the outlet pressure are known. Velocities are zero at the boundaries, which implies a no-slip condition. Tables S2 and S3 summarize the boundary conditions and the simulation results obtained from the model, respectively.

Table S2. Boundary conditions for the continuum diffusion model.

Boundary type	Boundary condition	Value
Inlet	Pressure, no viscous stress	$p=p_0$
Outlet	Pressure, no viscous stress	$p=0$

Note: p_0 is a specified pressure drop.

Table S3. Simulation results obtained from the continuum diffusion model.

Quantity	Value	Description
ρ_0	10 kg/m ³	Fluid density
μ_0	0.001 Pa·s	Fluid dynamic viscosity
p_0	1000 Pa	Pressure drop
D	1×10^{-13} m ² /s	Diffusion coefficient

S4. Crystallographic details for the anisotropic zeolite ZSM-5 crystals

Figure S5. a,b) Schematic illustrations of a) the hydrocarbon pool (HCP) species trapped within a MFI framework (2D projection) during the methanol-to-hydrocarbons (MTH) and b) the morphology of the a-oriented and b-oriented zeolite ZSM-5 crystals. The surface of the a-oriented

and b-oriented zeolite crystals are dominated by the sinusoidal channels (blue) and straight channels (orange), respectively. c,d) Scanning electron microscopy (SEM) images of the c) a-oriented and d) b-oriented zeolite ZSM-5 crystals. e) X-ray diffraction (XRD) patterns of a-oriented (blue) and b-oriented (orange).

S5. Validating reaction locations within the anisotropic zeolite ZSM-5 crystals

Initially, a molecular dynamics (MD) simulation was applied to qualitatively study the effect of morphology on the diffusion behaviours of molecules in zeolite channels. The diffusivity of the representative molecule, *i.e.*, methanol, within pure-Si zeolite ZSM-5 (MFI framework) was first calculated by fitting the mean squared displacement (MSD) curve (Figure S6). The effect of morphology on the diffusion behaviours was further investigated using the obtained diffusion coefficient in a two-dimensional (2D) model. The 2D model is constructed with the similar aspect ratio to the anisotropic zeolite ZSM-5 crystals. The results show that the channels with short dimensions (SD) were preferentially filled with the molecules at the early stage of diffusion. Furthermore, no apparent diffusion of the molecules from the SD channels into the channels with long dimensions (LD) was observed, while diffusion within the SD channels was prompt at the same time. These results suggest that the molecules will enrich into the SD channels in the beginning of the reactions, although both channel dimensions are equivalently filled with the molecules eventually. Therefore, short time MTH reaction was performed over the synthesized anisotropic zeolite ZSM-5 crystals with different morphologies.

Figure S6. The mean squared displacement (MSD) profile of methanol in the pure-Si zeolite ZSM-5. The calculated diffusion coefficient $D=1.11 \times 10^{-13} \text{ m}^2/\text{s}$.

Figure S7. Time-dependent concentration profiles in the 2D pore network model. Note that diffusion starts at t_0 . The results from t_1 to t_4 suggest that methanol molecules preferentially diffuse into the channels with short dimension (SD), with the diffusion direction indicated by the arrow. Long-time diffusion will result in uniform distribution of methanol molecules in both channel orientations. These results demonstrate that the molecules will enrich into the SD channels in the beginning of the reactions, although both channel dimensions will eventually be filled with the molecules equivalently at long diffusion times. Therefore, short time MTH reaction over the anisotropic zeolite ZSM-5 crystals with different morphologies could produce hydrocarbons trapped in single channel orientations. Specifically, hydrocarbon species will be primarily formed in the sinusoidal and straight zeolite channels over the a-oriented and b-oriented zeolite ZSM-5 crystals, respectively.

To prepare zeolite ZSM-5 crystals with HCP species primarily trapped into certain channel orientations, the MTH reaction was closely followed using *in-situ* UV-Vis DRS^[40, 72]. We have demonstrated different reaction behaviours within the sinusoidal and straight zeolite ZSM-5 channels with the combination of *operando* UV-Vis DRS and uniformly oriented zeolite ZSM-5 thin-films^[40]. Distinct UV-Vis DR spectra of HCP species were observed from the two different channel orientations.

Specifically, HCP species with absorption band above 560 nm will primarily be formed in the straight channels during the MTH reaction^[40]. Therefore, those species were used as a marker to locate the reactions within zeolite channels of the anisotropic zeolite ZSM-5 crystals. As shown in Figure S8, negligible UV-Vis absorbance in the range above 560 nm was observed for the *a*-oriented zeolite ZSM-5 crystals during the first 2 min MTH reactions, suggesting that the reaction primarily occurs in the sinusoidal zeolite channels. Conversely, fast evolution and strong absorbance from HCP species were observed in the same range for the *b*-oriented analogue during the first 2 min MTH reactions, showing that methanol molecules preferentially diffused into and were catalysed primarily in the straight channels of the *b*-oriented zeolite ZSM-5 crystals.

S6. UV-vis diffuse reflectance (DRS) measurements

Figure S8. In-situ UV-vis diffuse reflectance spectra (DRS) of the a) *a*-oriented and b) *b*-oriented zeolite ZSM-5 crystals during 2 min of the ^{13}C methanol-to-hydrocarbons (MTH) at 623 K.

S7. 2D scalar-based ^1H - ^{13}C and ^{13}C - ^{13}C ssNMR spectra of mobile molecules within the anisotropic zeolite ZSM-5 crystals

Figure S9. 2D scalar-based ^1H - ^{13}C (top) and ^{13}C - ^{13}C (bottom) magic angle spinning (MAS) ssNMR correlation spectra of the mobile molecules trapped in the a) a-oriented and b) b-oriented zeolite ZSM-5 crystals. The black box indicates the mobile methyl resonances, which are equivalent to the immobile species shown in the right panels of Figure 3.5. The blue box highlights the hydrogen-transferred species only present in the a-oriented ZSM-5 crystals. For the ^{13}C - ^{13}C correlation experiment a total through-bond correlation (TOBSY) mixing time of 4 ms was used. The ^1H - ^{13}C correlation spectra identified ethane at 9.15 ppm (^{13}C) and 0.98 ppm (^1H) as well as propane at 18.9 ppm (^{13}C) and 0.7 ppm (^1H) in the a-oriented zeolite ZSM-5 crystals, while only ethane (8.97(^{13}C) and 0.99 (^1H) ppm) was observed in the b-oriented analogue.

S8. 2D ^{13}C - ^{13}C spin-diffusion ssNMR spectra of the rigid molecules within the anisotropic zeolite ZSM-5 crystals using different mixing times

Figure S10. 2D ^{13}C - ^{13}C magic angle spinning (MAS) ssNMR spectra of the rigid molecules in the a,c) a-oriented and b,d) b-oriented zeolite ZSM-5 crystals after 2 min of the methanol-to-hydrocarbons (MTH) reaction at 623 K. The spectra were recorded at 290 K, using 16 kHz MAS. For the ^{13}C - ^{13}C correlation experiment the polarization of ^{13}C atoms was achieved through cross-polarization (CP). A a,b) 30ms or c,d) 120ms phase-alternated recoupling irradiation scheme (PARIS) mixing period was used. Note that even with a longer mixing time (120ms) the cross-peaks between ~ 18 ppm and ~ 21 ppm resonances are absent in the a-oriented zeolite crystals. Instead, the cross-peaks become more prominent in the b-oriented zeolite crystals at a longer ^{13}C - ^{13}C mixing, therefore confirming the presence of asymmetric methylated aromatics in the sample with a higher accessibility for the straight zeolite channels.

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CHAPTER 4

Solid-State NMR and DNP studies on the Reaction Mechanism of the Zeolite-Catalyzed Ethanol-to-Hydrocarbons Process

Adapted from:

“Unraveling the Homologation Reaction Sequence of the Zeolite-Catalyzed Ethanol-to-Hydrocarbons Process” – Published in *Angew. Chemie - Int. Ed.* 58, 3908–3912 (2019)

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The DNP data presented here are preliminary results and were not included in the cited publication.

Abstract

Although industrialized, the mechanism of catalytic upgrading of bio-ethanol over solid-acid catalysts, i.e., ethanol-to-hydrocarbon (ETH) reaction, is not yet fully understood. Moreover, the mechanistic understanding of the ETH reaction relies primarily on its well-known ‘sister-reaction’, the methanol-to-hydrocarbon (MTH). Understanding the complete reaction mechanism at the molecular level is not only important from a fundamental perspective, but also offers the possibility to maximize the catalytic performance to the benefit of economic affordability and environmental sustainability. Herein, by employing a tailored solid-state NMR approach, together with operando UV-visible diffuse reflectance spectroscopy and on-line mass spectrometry, the reaction and deactivation mechanism of the zeolite H-ZSM-5 catalysed ETH process is elucidated. This approach allows the accurate molecular identification of the involved reaction products and intermediates of the ETH process and establishes the existence of a homologation reaction sequence through the pattern analysis of the identified reactive and deactivated species. Furthermore, the distribution of the reaction products inside the zeolite particles is investigated by DNP-supported solid-state NMR spectroscopy. This combined spectroscopic approach provides new insights into the host-guest chemistry between the zeolite and trapped-organics, in terms of their surface mobility, reactivity, and products’ distribution.

Introduction

Our society is experiencing growing concerns over climate change and the depletion of natural resources. This concern has gradually steered the chemical industry towards the development of sustainable chemical processes. The conventional production of (C₂-C₄) olefins, one of the most important raw materials in the petrochemical industry, is currently primarily based on the cracking of natural gas and naphtha^[1, 2]. Since the direct carbon emissions from fossil feedstocks are considerably higher than with renewables, the economic and environmental feasibility of replacing fossil feedstock with sustainably resourced biomass and waste is steadily increasing every year^[1, 3]. For instance, ethylene is also currently manufactured from bioethanol derived from different sugary and starchy feedstocks (i.e. sugar cane, maize, corn, ligno-/hemicelluloses)^[3-6]. In fact, the catalytic upgrading of the ethanol-to-hydrocarbon (ETH) process using solid-acid catalysts has recently captured the attention of both academic^[4-11] and industrial^[3, 6, 12-14] sectors.

Despite the industrial advancements, the reaction and deactivation mechanisms of the ETH process are yet to be established. Moreover, the mechanistic insights available to date are principally built on the understanding of the more studied methanol-to-hydrocarbon (MTH) process^[2, 15-17]. However, it is unreasonable to assume that all zeolite-catalysed conversion processes of alcohols have identical mechanisms. The importance of understanding the characteristics of the ETH reaction should not be underestimated, as it is crucial to maximizing yields and developing new or improved porous solid catalysts^[18].

With the aim to clear the ambiguity around the zeolite-catalysed ETH process, we have established an in-depth fundamental mechanistic understanding of its reaction sequence, primarily through the identification of multiple surface-adsorbed active catalytic species (i.e. surface alkoxy and carbonylated species), C₂-C₄ olefins, homologated governing HCP-species (i.e. hydrogen-transferred alkylated aromatics and smaller paraffin), and deactivated polyaromatic 'coke' species. These new insights were obtained by using advanced multi-dimensional solid-state magic angle spinning (MAS) nuclear magnetic resonance (NMR) spectroscopy and corroborated with the operando UV-visible diffuse reflectance spectroscopy (DRS) coupled with on-line mass spectrometry (MS). In addition to the elucidation of the (homologation dominated) reaction and deactivation mechanism, the mobility dependent distinctive host-guest chemistry between the zeolite and the trapped hydrocarbons during catalysis were also identified^[19-21]. Finally, to obtain further information on the spatial distribution of the zeolite-trapped reaction intermediates, we employed DNP-supported ssNMR spectroscopy. The technique has been applied to the characterisation of a broad range of materials including pharmaceuticals^[22-24], polymers^[25-27], biomaterials^[28, 29], catalysts^[30, 31], and inorganic

materials^[32-35]. For the latter, DNP has proven to be extremely important to study surface species on functional materials^[36-38]. In the current study, we utilize DNP enhancements in combination with a model for polarization dynamics to obtain insight into the macromolecular distribution of the reaction products. The selectivity of these experiments has allowed us to attain information on the location of the reaction intermediates in the zeolite particles.

Materials and Methods

The ethanol-to-hydrocarbon (ETH) reaction was performed using millimetre-sized alumina-bound H-ZSM-5 catalyst extrudates, using ethanol as a reactant at a reaction temperature of 573 K for 30 min. Details on the catalyst materials, experimental conditions as well as on the details of the methods employed in this study can be found in the Supporting Information.

Solid-state NMR Experimental Details

All experiments were performed on a Bruker 500MHz wide-bore magnet with an AVANCE-III console and equipped with a 3.2 mm triple channel Efree HCN probe. All experiments were performed at room temperature (298 K) and MAS frequency of 16 kHz. Note that effective sample temperatures can be 5-10 degrees higher due to frictional heating. Referencing of ¹H and ¹³C chemical shifts was done externally to adamantane.

Hard ¹H and ¹³C pulses were applied with field strength of 90 kHz and 53 kHz, respectively. 90 kHz SPINAL64^[39] ¹H decoupling was applied during acquisition for the 1D and 2D ¹³C detected spectra. The 1D ¹H-¹³C cross-polarisation (CP)^[40] spectrum was recorded using a 2 s recycle delay, 16 ms acquisition time and an accumulation of 8192 scans. Cross-polarization was achieved using a 46 kHz ¹³C field and 69 kHz 70-100% ramped ¹H field during 0.7 ms. The 1D ¹³C direct excitation (DE) spectrum was recorded using a 5 s recycle delay, 14 ms acquisition time and an accumulation of 8192 scans. The 1D ¹H-¹³C insensitive nuclei enhanced by polarization transfer (INEPT)^[41] spectrum was recorded using a 2 s recycle delay, 33 ms acquisition time and an accumulation of 8192 scans. 1D CP, DE and INEPT spectra were processed using 25 Hz line-broadening.

2D ¹³C -¹³C spectra (both CP and DE) were recorded using a 2 s recycle delay, 10 ms (F2) and 5ms (F1) acquisition time and an accumulation of 256 scans. ¹³C -¹³C mixing was achieved through proton-driven spin-diffusion using Phase-alternated-recoupling-

irradiation-schemes (PARIS) for 30ms and 150ms^[42]. The 2D CP ^{13}C - ^{13}C spectra were processed using a 0.25π shifted sine squared window function in both dimensions, instead 0.4π shifted sine squared window function was used in both dimensions for the 2D DE ^{13}C - ^{13}C spectrum.

The dipolar based ^{13}C - ^1H correlation spectrum was obtained using a 0.7 ms long ^1H - ^{13}C CP period and a 0.9 ms long ^{13}C - ^1H CP period. Background signals from catalyst protons were suppressed by a 8 kHz MISSISSIPPI^[43] block ($\tau = 5$ ms, $N = 2$) prior to the last CP. ^{13}C PISSARRO (Phase-Inverted Supercycled Sequence for Attenuation of Rotary Resonance) decoupling^[44] was applied during acquisition. The recycle delay was 2 s, acquisition times 10 ms (F2, ^1H) and 7 ms (F1, ^{13}C) and number of scans 48. The spectrum was processed using a 0.29π shifted sine squared window function in both dimensions.

To probe mobile molecules, a 2D ^{13}C - ^1H HSQC (Heteronuclear Single Quantum Coherence)^[45] spectrum was recorded using 155 Hz for J in the INEPT delay. A 2s recycle delay was used, acquisition times were 7 ms (F2, ^1H) and 5 ms (F1, ^{13}C) and 256 scans were accumulated. GARP ^{13}C decoupling^[46] was used during detection. Prior to Fourier transformation, 20 Hz line-broadening was used in the F2 dimension and a 0.29π shifted sine squared function was applied to the F1 dimension. 2D ^{13}C - ^{13}C TOBSY^[47] was recorded using a mixing time of 5 ms, a 2 s recycle delay and 14ms (F2) or 4 ms (F1) acquisition time using 256 scans. The spectrum was recorded using 155 Hz for J in the INEPT delay and processed using 50 Hz line-broadening in both dimensions. 11 kHz WALTZ ^1H decoupling^[48] was used during detection.

Dynamic Nuclear Polarization (DNP) MAS NMR

DNP experiments were conducted at low temperature (100 K) using 3.2 mm triple-resonance (^1H , ^{13}C , and ^{15}N) MAS probe heads in static magnetic fields of 9.4 T, corresponding to proton/electron resonance frequencies of 400 MHz/263 GHz (Bruker BioSpin). DNP samples were prepared by incipient wetness impregnation^[49, 50], using 20 μL a solution of 15 mM AMUPol^[51] in $\text{D}_2\text{O}/\text{H}_2\text{O}$ (60:40 v:v) and an impregnation time of approximately 30 min. The ^1H - ^{13}C cross polarization DNP experiments were recorded using a 2 s recycle delay, 15 ms acquisition time and an accumulation of 512 scans. A CP contact time of 0.25 ms was used and SPINAL-64 proton decoupling at 83 kHz r.f. strength was applied during acquisition. The spectra were processed with 25 Hz line-broadening. For 2D proton-driven spin diffusion (PDSD) experiments, a mixing time of 50 ms was employed and the H-C CP contact time was set to 200 μs . 128 scans were acquired with acquisition times of 15 ms and 4.5 ms for the direct and indirect

dimensions, respectively. The 2D spectra were processed using a 0.4π shifted sine squared window function on both dimensions.

All spectra were recorded at an MAS rate of 9.5 or 7 kHz. DNP enhancements were obtained by scaling the signal intensities of spectra measured under DNP with the corresponding spectrum obtained without microwave irradiation at 100 K. Spinning sidebands were identified by varying spinning speeds.

Analysis of solid-state NMR Data

All NMR spectra were processed with Bruker Topspin3.5 and analysed using SPARKY^[52]. Assignments of the identified spin-systems in the NMR spectra are summarized in Table S1. Additional spin-systems identified in the 2D spectra that could not be unambiguously assigned to a molecular structure are listed in the Table S2. For the identification of unique spin-systems of rigid molecules, ^{13}C - ^{13}C correlations were collected in the CC PARIS spectra, using cross-polarization (CP) and direct-excitation (DE) to create polarization on ^{13}C atoms. Frequencies of the ^{13}C spins were first obtained from a CP-based 2D CC correlation experiment with short mixing time (30 ms) and subsequently confirmed by tracking spin systems in the experiment using longer mixing times (150 and/or 250 ms), where more correlations become visible (Figure S3). Note that the overall correlation pattern in CP-based experiments exhibits asymmetry relative to main diagonal. This effect (not visible in the DE-based 2D correlation set) is due to the fact that cross peak intensities reflecting transfer from protonated to non-protonated spin species will depend on the protonation level of source and target spin, because of the preparatory CP step. Such considerations do not apply to DE-based correlation experiments where cross peaks are readily observable such as shown in Figure S3d.

The identification of unique spin-systems of mobile molecules was more challenging because of the high rigidity of the sample, however the 2D ^{13}C - ^1H HSQC experiment was analysed through comparison of 2D ^{13}C - ^{13}C TOBSY and a few molecular species could be recognized. Assignment of the identified spin-systems was based on the observed chemical shifts and signal intensity ratios (Table S1 & S2).

Results and discussion

Initially, the operando UV-visible diffuse reflectance spectroscopy (DRS) with mass-spectrometry (MS) was used to identify and differentiate between neutral and carbocationic zeolite-trapped organic species, as well as gas-phase products formed during the ETH reaction conducted for 30 minutes at 573 K over millimetre-sized alumina-bound H-ZSM-5 extrudates (See Supporting Information, Section II)^[20]. The UV-visible DRS were dominated primarily by the presence of multiple HCP-species, ranging from the active neutral (alkylated) aromatic species to the deactivated polyaromatics, including their carbocationic/ σ -complex counterpart. The levelling off of the intensity of the UV-region bands after 7 min of reaction is indicative of the autocatalytic part of the reaction, whereas the continuous increase in the intensity of the visible region bands could be explained by the formation of larger-sized poly/fused aromatics (Figure S1c) during the deactivation of the catalyst. The simultaneously measured on-line MS data reveal the predominant existence of diethylether (DEE) and C₂-C₄ olefins (Figure S1d), along with C₄-C₇ hydrocarbon species at relatively lower quantities (Figure S2b). The simultaneous decrease and increase of ion-current of ethylene and butylene (>10 min, Figure S1d), respectively, essentially demonstrates the ethanol-assisted dimerization of ethylene to butylene^[8], and hence the existence of a homologation reaction mediated C-C bond coupling sequence.

Solid-state NMR Spectroscopy

Next, advanced MAS solid-state NMR was performed on the post-reacted material resulting from the ETH reaction using ¹³C₂-ethanol, with the aim to identify (homologated) zeolite-trapped products and hence establish the reaction and deactivation mechanism of the ETH reaction. The use of isotope-enriched ethanol not only significantly increased the NMR sensitivity, but also allowed us to perform multi-dimensional NMR correlation experiments for the accurate structural determination of the homologation products. ¹H-¹³C cross-polarization (CP), ¹H-¹³C insensitive nuclei enhanced by polarization transfer (INEPT), and ¹³C direct excitation (DE) solid-state NMR spectra show the following five features: (i) 7-37 ppm aliphatic groups, (ii) 58-70 ppm alkoxy groups, (iii) 125-160 ppm aromatic moieties, (iv) 180-187 ppm carbonyl groups, and (v) additional ~227 ppm peaks for carbocationic carbons (Figure 4.1 & Table S1). The strongest aliphatic and aromatic signals at 20-32 and 129-144 ppm, respectively, are mostly from the zeolite-trapped ethylated benzenes. This is consistent with the concept of both HCP species formation and the homologation reaction. The dominating alkoxy peak at ~63 ppm is due to the surface adsorbed ethanol, while its neighbouring small peaks at ~69 ppm could be attributed to surface-ethoxy species (SES)^[20, 53].

The applied different NMR magnetization transfer techniques also allowed us to distinguish their spectral appearance on the basis of mobility^[19-21, 54]. As a result, both mobile (i.e. molecule/group with fast tumbling or local rotation) and rigid (i.e. physisorbed in/on zeolite) species of zeolite-trapped organics could be distinguished. Assignments of the identified spin-systems in the NMR spectra are summarized in Table S1. Additional spin-systems identified in the 2D spectra that could not be unequivocally assigned to a molecular structure are listed in Table S2.

Figure 4.1. 1D ^{13}C solid-state NMR spectra of the molecules trapped within a post-reacted alumina-bound H-ZSM-5 extrudates obtained after the ETH reaction for 30 minutes at 573 K. In blue ^1H - ^{13}C CP (16kHz MAS, NS=8k), in red ^{13}C DE (16kHz MAS, NS=8k) and in green ^1H - ^{13}C INEPT experiments (16kHz MAS, NS=8k, intensity scaled by a factor of 4 for the sake of clarity) [*=spinning sideband, MAS=magic angle spinning, NS=number of scans].

In NMR, spectral separation of mobile and rigid species could be achieved using magnetization transfer schemes that invoke either through-bond (scalar interactions such as in INEPT) or through-space (dipolar transfer such as in CP), respectively. All chemical species, including those that exhibit intermediate dynamics (for which either an INEPT or CP transfer would be less efficient), were visualized using DE. Comparing the 1D spectra (Figure 4.1) shows that the mobile molecules/groups were of purely saturated/aliphatic nature (INEPT spectrum in Figure 4.1). In contrast, unsaturated molecules (aromatics/carbonyls) were mostly immobilized (CP spectrum in Figure 4.1)

and/or exhibited restricted mobility (DE spectrum in Figure 4.1).

To probe the rigid molecules, 2D ^{13}C - ^{13}C dipolar-based correlation spectra were acquired (Figures 4.2-4.5, S3-S8). The carbons were polarized through CP and ^{13}C - ^{13}C mixing was achieved through proton-driven-spin-diffusion (PDS) using phase-alternated-recoupling-irradiation-schemes (PARIS). In Figure 4.2, we identified two non-identical binding motifs of the unreacted reactant, ethanol (**1a**), and the ethylating/homologation agent, surface-ethoxy species (SES, **1b**). The peaks at 63.0 and 18.1 ppm were due to the methylene ($-\text{CH}_3\text{CH}_2-$) and methyl ($-\text{CH}_3\text{CH}_2-$) carbon (side-on, $\eta^2:\eta^2$) of ethanol, respectively^[20, 55]. In another spin-system, slightly upfield methylene carbon at 58.9 ppm with an identical methyl chemical shift could be attributed to the end-on (i.e. $\eta^1:\eta^1$) orientations of ethanol on the Brønsted acid site of H-ZSM-5^[20]. Interestingly, we observe broader resonances for the ‘side-on’ with respect to ‘end-on’ conformation, indicating that the former exists in different molecular environments (i.e. heterogeneity) within the zeolite. Therefore, a ‘side-on’ conformation of the ethanol adduct is more firmly adsorbed due to the stronger H-bonding, which led to its more downfield signal. Such a deshielding effect could be attributed to the removal of local electron density through a hydrogen-bond network with the zeolite surface^[20].

Figure 4.2. Zoom-in of the 2D ^{13}C - ^{13}C MAS solid-state NMR spectra of rigid zeolite trapped molecules in the surface adsorbed alkoxy region. Spectra were recorded at 295K, using 16 kHz MAS. Polarization of the ^{13}C atoms was achieved through CP and a 150 ms PARIS mixing period was used. See Figure S3 for the full spectrum.

In the carbonyl region, the correlations observed at 187.1/186.5 ppm exhibited cross-peaks with a ^{13}C -methylene signal at 31.8/32.0 ppm and another ^{13}C -methyl signal at 24.5/25.5 ppm, which is due to the surface-propionate ($1'$) species (Figure 4.3). The slightly different chemical shifts of $1'$ could reflect heterogeneous arrangements of the chemisorbed propionate group on zeolite. Simultaneously, two more carbonyl signals at 182.7 and 183.9 ppm correlate with two ^{13}C -methyl signals at 23.1 and 23.0 ppm, respectively, which we attributed to the surface-acetate ($1''$) and acetic acid (i.e. hydrolysed product of $1''$), respectively^[21].

Figure 4.3. Zoom-in of the 2D ^{13}C - ^{13}C MAS solid-state NMR spectra of rigid zeolite-trapped molecules in the carbonyl region. Spectra were recorded at 295 K, using 16 kHz MAS. Polarization of the ^{13}C atoms was achieved through CP and a 150 ms PARIS mixing period was used. See Figure S3 for the full spectrum.

The presence of carbonylated surface species is consistent with the existence of the Koch-carbonylation based direct C-C bond forming route during the zeolite-catalysed methanol-to-hydrocarbon (MTH) process^[19, 21, 56].

For the identification of unique spin-systems in the aromatic regions of rigid molecules, we collected ^{13}C - ^{13}C correlation spectra using different PARIS mixing times. Resonance frequencies of the directly attached ^1H spins were obtained from the two-dimensional ^{13}C - ^1H CP-based spectrum, as shown in Figures S5-S6. Using experimental data recorded at short mixing time (30 ms), our molecular identification started from the methyl resonances (as observed in Figure 4.4) and concomitant connections to other resonances via the observed cross peaks. For example, an intense cross peak of the

methyl resonance at 10.4 ppm with a resonance at 37 ppm was observed (green cross in Figure 4.4) and these aliphatic resonances further correlated with aromatic carbons through a strong peak with 144.6 ppm (green dotted line in Figure 4.4). This signal was assigned to diethylbenzene (**2a**) moieties. Further correlations of these aliphatic carbons with aromatic carbons and corresponding intra-aromatic correlations could be observed (Figure 4.4, S4, S7 and Table S1). Similarly, additional multiple ethylated benzene species were identified (blue and red crosses correspond to tri- (**2b**) and tetra-ethylbenzenes (**2c**), respectively, in Figure 4.4). These resonances were then confirmed by tracking spin systems in experiments using longer mixing times (120ms and 250ms). Under these conditions, additional correlations were observed (see Figure S4). Simultaneously, several methylated benzenes were readily identified too (Figure S7), which we putatively assigned to the different isomers of tetra-methylbenzenes (**3a**, i.e. isomerized from **2a**). The more intense peak speaks in favour of one dominant conformation (**3a**) which is visible in the correlation experiment with shorter mixing (30 ms) period. This observation may be consistent with an increased stabilization of one isomeric form compared to other forms.

Figure 4.4. 2D ^{13}C - ^{13}C solid-state NMR (16kHz MAS) correlation experiments probing rigid zeolite trapped ethylated aromatics measured at 295K. Polarization of the ^{13}C atoms was achieved through CP and a 30 ms PARIS mixing period was used. Dotted diagonal line indicates a spinning sideband.

The identification of such homologated hydrocarbons (i.e., **2a/2b/3a**) inside the post-reacted extrudate materials was further confirmed by so-called Guinset's method of

zeolite-material dissolution (Figure S10, see also Supporting Information section SV for more details)^[57].

Moreover, the formation of poly/fused-aromatic species could be observed with the UV-visible DRS, where their formation was indicated during the course of the ETH reaction (cf. continuous rise in intensity of ≥ 575 nm band in Figure S1a-c).

In 2D solid-state NMR experiments probing through-bond connectivities, mobile molecules such as ethanol as well as hydrogen-transferred species from olefins, i.e. C₂-C₄ alkanes, were detected (Figure S8 & Table S1). In the ¹³C-¹H correlation spectrum, three different conformers of ethane (**5a**: 8.9 (¹³C)/0.40 (¹H), 8.8 (¹³C)/0.25 (¹H), and 8.7 (¹³C)/0.17 (¹H) ppm) signals could readily be identified, which provide further evidence in support of the existence of non-identical local environments, i.e. heterogeneity, within the zeolite framework. The 17.4 (¹³C)/0.64 (¹H) resonance indicate the presence of propane (**5b**) moieties, where the correlations between -CH₂ and -CH₃ carbons are difficult to identify because of spectral crowding, probably due to its presence in different local environments (analogous scenario for the ethane).

Figure 4.5. Zoom of 2D magic angle spinning (MAS) solid-state nuclear magnetic resonance (NMR) spectra of rigid zeolite trapped molecules in the carbocation region (**1g**). Spectra were recorded at 295K, using 16 kHz MAS. Polarization of the ¹³C atoms was achieved through cross-polarization (CP) and a 250 ms PARIS mixing period was used. The grey strip indicates an artefact from spinning sidebands.

Finally, weak signals at ~227 ppm in the 1D DE (Figure 4.1) and their cross-peaks with ^{13}C -methylene carbons at 32-37 ppm in the ^{13}C - ^{13}C correlation spectra with 30ms and 150ms mixing time (Figure S3), indicate the formation of alkylated carbenium species/ σ -complex within a six-membered ring framework^[16, 20]. Indeed, the assignments were confirmed and the complete spin-system was revealed in the long ^{13}C - ^{13}C correlation experiment (250ms mixing time), where the cross peaks at 227-44 ppm and 159-44 ppm were identified (Fig. 4.5). Moreover, the formation of such carbenium HCP species is in agreement with our UV-visible DRS experiments (Fig. S1-S2).

Based on the above-mentioned results, a catalytic (homologation dominated) reaction pathway for the ETH process is proposed in Scheme 1.

Scheme 1. Proposed mechanism of the homologation reaction dominated carbon-carbon bond coupling routes during the zeolite-catalysed ethanol-to-hydrocarbon (ETH) process. The double arrow indicates the plausible existence of multiple reaction steps. ZeOH represents the zeolite acid site. For the complete description of the reaction mechanism the reader is referred to the publication <https://doi.org/10.1002/anie.201814268>

DNP-NMR Studies

Dynamic Nuclear Polarization (DNP) enhanced ssNMR is based on the transfer of the substantial spin polarization of unpaired electrons to nearby nuclei, driven by microwave irradiation near the electron paramagnetic resonance (EPR) frequency^[58, 59]. The use of DNP can strongly enhance the NMR signals by multiple orders of magnitude, resulting in spectra with improved signal-to-noise (S/N) ratios and thereby leading to a very significant reduction in experimental time^[58, 60, 61]. The recent developments in high-field DNP-NMR have significantly improved the sensitivity of this technique and broadened its application to the field of catalysis. The sensitivity enhancement provided by DNP has been employed to detect insensitive spins with low natural abundance, such as ^{29}Si ^[62, 63] or ^{17}O ^[64]. Currently, MAS-DNP has enabled the structural characterization of a broad range of materials including organic, hybrid, and inorganic materials^[31, 36, 37, 65]. In particular in heterogeneous catalysis, this approach offers the possibility to selectively detect catalytic sites on the surface or in the pores of the catalysts^[33, 34, 66-68], including surface-enhanced NMR on mesoporous silica^[36], Sn^{VI} -active sites in the $\text{Sn-}\beta$ zeolite^[33, 34] and Brønsted acid sites of aluminosilicates^[66]. Recently, the DNP technique has also been applied to MTH-reacted microporous zeolites^[30, 35].

In MAS DNP experiments on materials, transfer of the DNP-enhanced ^1H polarization to the detected isotope is often employed in an approach coined “indirect DNP”. The subsequent transfer of DNP enhanced magnetization to the bulk relies on ^1H - ^1H spin diffusion. However, in the case of heterogeneous samples, spin diffusion is limited by the low proton density. In such cases, indirect DNP can be used for the selective observation of nuclei near the surface, since cross-polarization from the surface protons to the nuclei of interest results in the excitation of only (near) surface species. This approach has been demonstrated in hybrid materials such as functionalized silica^[36, 63] or alumina.^[69] Moreover, surface species often represent a small fraction of the total number of nuclei and the sensitivity gain provided by DNP is necessary to detect surface NMR signals. Furthermore, it has been shown that domain sizes in complex materials can be determined using DNP, where a distribution of signal enhancement is obtained by locally enhancing polarization.^[23, 50, 70, 71] For instance, a DNP-approach combined with models for polarization dynamics was applied to determine the pore length of a mesoporous silica material where a length of 750 nm was obtained^[71]. In these systems, the heterogeneous distribution of polarization within the sample causes a variation in the observed DNP enhancements and relative polarization build-up curves, which is found to correlate with the sample size and geometry.

Based on the difference in DNP enhancements measured for the different zeolite-trapped species present in our sample, a similar approach was used to model the polarization

dynamics in our sample, as explained below.

To obtain insight into the spatial distribution of ETH reaction products in the H-ZSM-5 zeolite particles, we recorded one-dimensional ^{13}C ssNMR experiments using cross polarization (CP), with and without DNP (fig.4.6A). The material was wetted with 20 μL of a solution containing a DNP polarizing agent (AMUPol^[51], 15mM) by incipient wetness impregnation^[49, 50]. The ^{13}C signal enhancements are presented in Figure 4.6A. The polarization is transferred from the unpaired electrons of the biradical polarizing agent to the nearby ^1H nuclei of the solvent and then hyperpolarization diffuses into the target via homonuclear proton spin-diffusion. Subsequently, the polarization is transferred to the ^{13}C nuclei of the reaction intermediates via CP-MAS experiments.

(A) - DNP results

(B) - spin diffusion model

Figure 4.6. (A) DNP Enhancements observed in 1D ^1H - ^{13}C cross-polarization (CP) spectra of zeolite trapped molecules. Spectra were recorded at 100 K and 9.5 kHz MAS on a 400 MHz DNP system. The enhancement of the different molecules is indicated. (B) Simulated DNP enhancements as a function of the distance calculated using $T_{1\rho}=0.8\text{s}$; the maroon curve represents the normalized enhancement for a diffusion constant (D) of $32\text{ nm}^2/\text{s}$ based on a proton-proton distance of $6.5\text{ \AA}$, while the grey curve represents the normalized enhancement for a diffusion constant (D) of $138\text{ nm}^2/\text{s}$ based on a proton-proton distance of $4.5\text{ \AA}$. In the top right corner a simplified illustration of the system. The orange ellipsoids represent the DNP agents transferring the polarization to the protons of the solvent. Next, CP is used to transfer the enhanced polarization to the surface (in purple) and aromatic (in green) species which exhibit different locations within the zeolite.

Contrary to the *in-cell* dnp results presented in Chapter 2, which revealed uniform enhancements, here we observe a hyperpolarization gradient with remarkably different DNP signal gains for the different molecular species. In particular, carboxylic carbon resonances exhibit the largest enhancement factor ($\epsilon_{\text{DNP}} = 8$), which agrees with

previously identified surface-adsorbed species (see fig. 4.3). We speculate that these moieties are located at the solvent-accessible surface and are on average closer to the polarizing agents (PAs). The lower signal enhancement obtained for the (hydrogen-enriched) aromatic species ($\epsilon_{DNP} = 1.2$) may be related to their larger distance from the polarizing agent, for which diffusion into the microporous zeolite is restricted^[29, 33, 34, 68, 72]. Moreover, these enhancement values were also confirmed by 2D ^{13}C - ^{13}C correlation experiments, where a better spectral separation between the different aromatic species can be obtained (See Fig. S9). Interestingly, a new correlation at ~ 65 ppm and ~ 185 ppm is visible under DNP, possibly indicating the spatial proximity between the reactant (ethanol) and the surface carbonylated species (reactive intermediate). Previous works have shown that under these conditions DNP enhancements can be used to estimate molecular dimensions on the nanometer scale^[28, 70, 71]. In fact, since the radical is confined to the surface, polarization transfer to the interior of the sample via proton spin diffusion can be described classically using Fick's law. Following these approaches, we used a classical one-dimensional spin diffusion approximation and correlated the relative DNP enhancement of distinct molecular species to their local distribution. In this description, a uniform polarization of the nuclei nearby the radical at the surface is assumed, given by the DNP enhancement factor ϵ_0 . The local DNP enhancement ϵ is ultimately determined by the characteristic spin diffusion length $\sqrt{DT_{1n}}$, where D is the diffusion constant (which depends on the average ^1H - ^1H distance and the MAS rate)^[70] and T_{1n} the longitudinal nuclear relaxation time. A phenomenological relation between the enhancement and the distance can thus be determined:

$$\epsilon = \frac{\epsilon_0 \sqrt{DT_{1n}}}{w} \left[\text{Coth} \left[\frac{a}{\sqrt{DT_{1n}}} \right] - \text{Cosh} \left[\frac{a-w}{\sqrt{DT_{1n}}} \right] \text{Csch} \left[\frac{a}{\sqrt{DT_{1n}}} \right] \right]. \quad (4.1)$$

Where a is the distance at which the nuclei do not experience any DNP enhancement and w is the layer size. (See Supporting Information, section SIV for the full derivation)

Build-up time and steady state enhancement of the radical solution depend on the proton concentration at 100 K. Using the experimentally observed longitudinal relaxation time, after normalising the DNP enhancement to the value measured for the surface species, we obtained an approximate distance between 30 to 50 nm for the aromatic moieties from the surface exposed species (Fig. 4.6B). Note that, due to the fact that the average ^1H - ^1H distance is difficult to predict for this system, we simulated the curve for two possible values and therefore we obtain a range of distances. Such a structural analysis could not be applied to ethanol or aliphatic moieties, due to the dynamic nature of these species. The fast rotational dynamics experienced by the methyl groups even at cryogenic temperatures heavily affect their relaxation and their final DNP enhancement. Moreover, the mobile species and the ethanol moieties exist in different molecular

environments within the zeolite. Therefore, a simple one-dimensional diffusion model to predict the average measured DNP enhancement cannot be employed as a reliable indication of their location. However, the more rigid nature of the aromatic species allows for such analysis. We could therefore conclude that, under our reaction conditions, the aromatic species reside in the interior of the pores at a depth of approximately 30-50 nm. However, additional studies are needed to fully rationalize this result. For instance, experiments with different cross-polarization contact times could be performed to observe the build-up of the polarization. Additionally, longer impregnation times could be tested to follow the spin diffusion within the micropores of the zeolite. In fact, it was shown that the diffusion of the solvent into these microporous materials could continue over a period of days^[30]. Nonetheless, this preliminary analysis shows that this type of studies can be performed on post-reacted materials and may provide valuable structural information. Moreover, future analysis could be extended to include different geometries where the diffusion is modelled in two or three dimensions, following the formalism introduced by Pinon *et al.*^[71]. Having access to this type of information is undoubtedly important for the design of improved catalysts. It is well-known that the physical properties of these materials are strongly related to their pore geometry and architecture^[73-79]. Therefore, studies such as the one presented here can complement research on the effect of zeolite topology on the final product distribution, with the aim to help determine optimal catalyst geometry for the development of novel materials with improved reactivity and/or selectivity of the reaction.

Conclusions

In this work, we have examined the reaction and deactivation pathways for the catalytic upgrading of ethanol over zeolite via the so-called ethanol-to-hydrocarbon (ETH) process by employing a combined spectroscopic approach. We illustrate here the use of a dedicated solid-state NMR method, with application of one and two-dimensional MAS solid-state NMR experiments in combination with Dynamic Nuclear Polarization (DNP)-supported solid-state NMR to investigate the ETH reaction products. In addition to the numerous surface-adsorbed species (e.g. surface-ethanol, -ethoxy, -methoxy, -propionate, and -acetate species), hydrogen-transferred hydrocarbon pool species (e.g. aromatics and alkanes) have also been identified. The homologation reaction dominated hydrocarbon-pool mechanism is found to be operative during the zeolite catalysed ETH process, where the HCP species in the ETH process are constituted by multiple (m)ethylated aromatics.

Furthermore, the possibility to distinguish between rigid and mobile zeolite-trapped species highlights the important role of mobility dependent distinctive host-guest chemistry during catalysis. Additionally, the heterogeneous features of catalytic active sites within the zeolite framework have been evaluated. Therefore, the knowledge established from reaction mechanism and structure-reactivity relationship studies in the heterogeneous catalysis will not only be useful for the development of superior porous materials for the catalytic up-grading of ethanol (via designing suitable zeolite catalysts to prevent/decelerate deactivation and/or via doing co-feeding experiments to yield desired hydrocarbon range), but also contribute to the understanding of the zeolite-catalysed hydrocarbon conversion chemistry. Finally, a DNP-ssNMR study provides structural insight into the spatial location of the reaction products. Surface signals are strongly enhanced whereas aromatic moieties exhibit weaker DNP enhancements. These differences can be interpreted using a classic spin diffusion model to describe the diffusion of polarization within the porous sample and the variation in DNP enhancement is correlated to the size and lengths in the nanometer scale. Such studies could be extended to different systems and the structural information obtained may help the design of novel zeolite materials.

Supporting Information

I. Sample preparation

The H-ZSM-5 (Type: P-38, CAS Number: 1318-02-1), $^{12}\text{C}_2$ -ethanol ($\geq 99.8\%$, CAS Number 64-17-5), and $^{12}\text{C}_2$ -ethanol (99%, CAS Number: 70753-79-6) were purchased from ACS materials, Acros Organics and Cambridge Isotope Laboratories, respectively. Catapal D (alumina) was received from SASOL. Zeolite extrudates were prepared using a Caleva Mini-Screw Extruder. A Caleva Mixer Torque Rheometer was used to optimize the solid-to-liquid ratio. The preparation along with characterization details of alumina-bound zeolite H-ZSM-5 extrudates are included in an earlier publication^[20]. All catalytic reactions were performed using a Linkam cell (THMS600) equipped with a temperature controller (Linkam TMS94). The UV-visible diffuse reflectance spectroscopy (DRS) measurements were performed with a CRAIC 20/30 PVTM UV-Vis-NIR micro-spectrophotometer using a 15X objective. A 75 W Xenon lamp was used for illumination. The on-line gas phase product analyses were performed by a Pfeiffer OmniStar GSD 320 O3 (1-300 amu) mass spectrometer. The mass spectrometry database from the National Institute of Standards and Technology was consulted for assignments purposes. The optical images were taken using an Olympus BX41M microscope. For solid-state NMR measurements, extrudates were crushed and transferred into a 3.2 mm rotor for the magic-angle spinning (MAS) solid-state NMR experiments.

II. Operando Experimental Setup and Reaction Conditions.

Operando UV-visible diffuse reflectance spectroscopy (DRS) reactions were performed using ~40 mg of the alumina-bound zeolite H-ZSM-5 extrudate catalyst material. Initially it was placed on the heating stage of a Linkam cell, which was further connected to a water cooler. The inlet of the reactor was connected to the N_2 gas line, via a reactant saturator containing ethanol, whereas the outlet is either connected to the Pfeiffer mass spectrometer or vented out. The lid of the Linkam cell is equipped with a quartz window to monitor the reaction by UV-visible DRS. Details of the setup can be found in previous manuscripts^[19-21]. Prior to each UV-visible DRS measurement, the calcined extrudates were further pre-treated according to the following procedure under N_2 -atmosphere (flow rate of 20 mL/min): heating to 673 K at 15 K/min and keeping the sample at this temperature for the next 1 min, then heating the sample to 823 K at a rate of 5 K/min and held there for the next 30 min. The sample was then cooled down to 573 K (the

reaction temperature) with a rate of 10 K/min under a flow of N₂ gas. Finally, a N₂ flow of 20 mL/min was introduced at 573 K to a saturator containing ethanol. The UV-visible DRS were recorded every 30 s during the operando experiments, which typically took 30 min. The reaction was finally quenched by rapid cooling of the Linkam cell, by using a Linkam TMS94 temperature controller.

Figure S1 shows spectral features during the first 10 min of the ethanol dehydration reaction, as the absorption bands at ≤ 300 , 336, 355, 421 and 576 nm increase in intensity as a function of reaction time (Figure 2b-c). The observed absorption bands at ≤ 300 , 330-375, 421, and ≥ 575 nm are attributed to π - π^* transitions of neutral alkylbenzenes, carbenium ions with alkyl side chain carbocations (up to four alkyl groups)/neutral poly-aromatics, alkylated areniums, and poly-areniums/ π -complexes between the zeolite and aromatic species, respectively^[8, 19-21, 80].

Figure S1. Operando UV-visible DRS measurements during the ETH reaction over alumina-bound H-ZSM-5 extrudates at 573 K for (a) 0-10 and (b) 10-30 min. Time-resolved changes of (c) absorbance bands and (d) mass spectral profiles for diethyl ether and C₂-C₄ olefins as a function of reaction time.

Figure S2. Time-dependent operando UV-visible diffuse reflectance spectroscopy (DRS) measurements during the ethanol-to-hydrocarbon (ETH) reaction conducted at 573 K over alumina-bound H-ZSM-5 zeolite extrudates: (a) 3D plot of the UV-visible DR spectra and (b) on-line mass spectral profiles of C₄-hydrocarbon (cf. C₄H₁₀ with $m/z=58$, e.g. butane and/or its structural isomer), C₅-hydrocarbon (cf. C₅H₉ with $m/z=69$, e.g. pentene and/or its structural isomer), C₆-hydrocarbon (cf. C₆H₈ with $m/z=80$, e.g. cyclohexadienyl type species), C₇-hydrocarbon (cf. C₇H₇ with $m/z=91$, possibly toluene, as a fragmented product of higher alkylated benzenes), and C₈-hydrocarbon (cf. C₈H₁₀ with $m/z=106$), as a function of reaction time. (c) 2D plot of the UV-visible DRS for 7-20 min, revealing the presence of two clear isosbestic points at 343 and 365 nm, presumably suggesting a direct transformation from a dienylic carbenium species (characterized by an absorption band at 336 nm) to higher alkylated benzenium ions (absorbs at 421 nm), through its lesser alkylated counterpart (characterized by an absorption band at 355 nm). (d) Ex-situ plot of the UV-visible DRS of edge/top surface (black) and middle (red) of the cross-section. (e) Microscopic images of the cross-section of the post-reacted extrudate. Although the cross-section of the post-reacted extrudate appeared to have a uniform color, their ex-situ UV-visible DRS ‘spot-check’ measurements reveal the absence of the 336 nm band (explained by the presence of dienylic carbocationics/smaller alkylated carbeniums) in the core of the post-reacted extrudate. This observation could again be correlated to the deactivation of the catalyst during the ETH process, as the relatively bulkier species tend to be situated towards the edge of the extrudate and hence, blocking its external pores and assisting in the deactivation step^[6].

III. Supplementary NMR figures

Figure S3. 2D ^{13}C - ^{13}C magic angle spinning (MAS) solid-state NMR correlation spectra using dipolar CP (a, b, c) and direct excitation (d, yellow) obtained at 295 K, 16 kHz MAS, 500 MHz. Mixing was obtained using the PARIS scheme for 150 ms (a, d) 30 ms (b) and 250 ms (c). Solid boxes in a, b and c contain correlations above 200 ppm. Dashed inserts relate to main figures. Grey strips indicate spectral regions containing spinning sidebands.

Figure S4. 2D ^{13}C - ^{13}C magic angle spinning (MAS) solid-state nuclear magnetic resonance (NMR) correlation experiments where the indicated correlations are compatible zeolite-trapped hexaethylbenzene signal measured at 295K, using 16kHz MAS. Polarization of the ^{13}C atoms was achieved through cross-polarization (CP) with a short (30 ms, orange) and long (120 ms, blue) PARIS mixing period. The dotted diagonal line in the left panel indicates a spinning sideband.

Figure S5. 2D magic angle spinning (MAS) solid-state nuclear magnetic resonance (NMR) spectra of rigid zeolite trapped molecules, i.e. di- (**2a**) and tri-(**2b**)ethylbenzene as well as surface-adsorbed ethanol (**1a**), measured at 295 K, 16 kHz MAS, 500 MHz. Zooms from 2D ^{13}C - ^{13}C (left panel in blue) and ^{13}C - ^1H (right panel in pink) solid-state NMR spectra with a long mixing (150 ms) or CP contact time (700us), respectively.

Figure S6. 2D magic angle spinning (MAS) solid-state nuclear magnetic resonance (NMR) spectra of rigid zeolite trapped molecules, i.e. diethylbenzene (**2a**) and surface-acetate species ($1''$), measured at 295 K, 16 kHz MAS, 500 MHz. Zooms from 2D ^{13}C - ^{13}C (left panel in blue) and ^{13}C - ^1H (right panel in pink) solid-state NMR spectra with long mixing (150 ms) or CP contact time (700us), respectively. The dotted diagonal line in the left panel indicates a spinning sideband.

Figure S7. 2D ^{13}C - ^{13}C magic angle spinning solid-state nuclear magnetic resonance (NMR) correlation experiments probing rigid zeolite trapped methylated aromatic molecules measured at 295K, using 16kHz MAS. Polarization of the ^{13}C atoms was achieved through cross-polarization (CP) with a short (30 ms, orange) and long (120 ms, blue) PARIS mixing period. The dotted diagonal line in the bottom panel indicates a spinning sideband.

Figure S8. 2D ^{13}C - ^1H (left, INEPT) and ^{13}C - ^{13}C (INEPT-TOBSY) magic angle spinning (MAS) solid-state nuclear magnetic resonance (NMR) correlation experiments using scalar transfer. These experiments probe mobile molecules, i.e. hydrogen-transferred alkanes and unreacted ethanol (inset at the bottom), and were conducted at 295 K and a MAS rate of 16 kHz. The TOBSY mixing time was set to 5 ms. We attribute the line broadening compared to other studies of the correlations in both ^1H and ^{13}C dimensions as well as limited intensities of TOBSY cross-peaks to enhanced T_1 and T_2 ^{13}C relaxation. ZeOH represents the zeolite-acid site.

Concurrently, C_2 - C_4 alkanes (5a-5c) are formed as a result of the hydrogen-transfer reactions from the corresponding olefins during the ETH process. Such simultaneous detection of saturated paraffins, in addition to aromatics, indicates that the hydrogen-transfer reaction mechanism is concomitantly operating with the homologation reaction during the ETH process.

IV. DNP Spin diffusion model

For the analysis of the relative DNP enhancements factors ε_N (Fig. 4.6B, main text), we utilized a classical one-dimensional diffusion model (following van der Wel *et al.*^[70] and Jantschke *et al.*^[28]) to describe the polarization dynamics. The process by which the enhanced polarization diffuses into the core is assumed to be dominated by 1D nuclear spin diffusion and described by Fick's law^[81]:

$$\frac{\partial P}{\partial t} = D \frac{\partial^2 P}{\partial x^2} - \frac{P}{T_{1n}} \quad (S1)$$

$P(x, t)$ represents the polarization that depends on time (t) and the distance (x). D is the diffusion constant and T_{1n} stands for the longitudinal nuclear relaxation time.

In the steady state $\frac{\partial P}{\partial t} = 0$, therefore eq. (S1) can be rewritten as:

$$D \frac{\partial^2 P}{\partial x^2} = \frac{P}{T_{1n}} \quad (S2)$$

If we assume that the nuclei at the surface are closest to the radical and have reached a steady-state polarization $P(0)$ while $P(a)$ represents the polarization of nuclei that are located at a distance a from the biradical where they do not experience any DNP enhancement, we obtain the following boundary conditions:

$$P(0) = \varepsilon_0 P_0, \quad P(a) = 0 \quad (S3)$$

Where ε_0 is the steady state enhancement factor and P_0 is the Boltzmann polarization at thermal equilibrium.

The solution of eq (S2) with boundary conditions given in eq. (S3) is:

$$P(x) = \varepsilon_0 P_0 \operatorname{Csch} \left[\frac{a}{\sqrt{DT_{1n}}} \right] \operatorname{Sinh} \left[\frac{a-x}{\sqrt{DT_{1n}}} \right] \quad (S4)$$

Experimentally the average polarization is observed, therefore we integrate the polarization over a desired region with size w (assuming linear symmetry):

$$\frac{1}{w} \int_w^w P(x) dx = \frac{\varepsilon_0 P_0 \sqrt{DT_{1n}}}{w} \left[\text{Coth} \left[\frac{a}{\sqrt{DT_{1n}}} \right] - \text{Cosh} \left[\frac{a-w}{\sqrt{DT_{1n}}} \right] \text{Csch} \left[\frac{a}{\sqrt{DT_{1n}}} \right] \right] \quad (\text{S5})$$

And thus the measured enhancement factor ε is defined as:

$$\varepsilon = \frac{\varepsilon_0 \sqrt{DT_{1n}}}{w} \left[\text{Coth} \left[\frac{a}{\sqrt{DT_{1n}}} \right] - \text{Cosh} \left[\frac{a-w}{\sqrt{DT_{1n}}} \right] \text{Csch} \left[\frac{a}{\sqrt{DT_{1n}}} \right] \right] \quad (\text{S6})$$

Figure S9. Zoom-in of the 2D ^{13}C - ^{13}C DNP-ssNMR spectra probing zeolite-trapped species measured at 100 K, using 7 kHz MAS. On the left the 1D projection in the direct dimension corresponding to a) aromatic resonances and b) surface species. Polarization of the ^{13}C atoms was achieved through CP and a 50 ms PDS mixing period was used. In red are the spectra recorded with DNP (i.e. under microwave irradiation) and in grey without DNP (without microwave irradiation). The dotted box indicate the 65/185 ppm cross-peak only observable under DNP conditions.

V. Characterization of Retained Hydrocarbon

Hydrocarbons trapped inside the post-reacted catalytic material after the ETH reaction were analyzed by dissolving the extrudate material in hydrofluoric acid (HF), followed by extracting the hydrocarbons using CH_2Cl_2 (Figure S10)^[57, 82]. Typically, ~100 mg of spent catalyst was dissolved in 5 mL of a 45% HF solution in a Teflon container. The

organic compounds were extracted from the water phase two times by the addition of 7mL CH_2Cl_2 . Analysis of the extracted phase was performed on a Shimadzu GCMS-QP2010 GC/MS system, equipped with an Agilent VF-5 ms column as well as ESI-MS (Agilent, 1100 Series LC/MSD Trap). It should be noted in this regard that used alumina bound zeolite extrudate also contains micropores (see section S1.1). Therefore, all detected larger species could be trapped in the macropore-region (interface between zeolite and alumina) of the post-reacted extrudate material.

Figure S10. The analysis of retained hydrocarbon on/within the post-reacted extrudate material, by (a) GC-MS and (b) ESI-MS, after the ethanol-to-hydrocarbons (ETH) reaction was performed for 30min at 573K. The identification of multiple homologated hydrocarbons (i.e., **2a/2b/3a/4a/4b**) inside of the post-reacted extrudate materials is consistent with the solid-state NMR observation. Additionally, the existence of non-homologated products as a result of cracking has also been confirmed.

VI. SUPPLEMENTARY TABLES

Table S1. Overview of the assigned solid-state nuclear magnetic resonance (NMR) spin-systems from the reaction intermediates observed in this work. Atoms, as observed in our solid-state NMR experiments along with their assigned chemical shifts (in ppm), are given in this table only. Due to the overlap of signals, even in the 2D spectra, some signals could not be assigned to a specific atom.

<p><i>end-on_1a</i></p> <p>18.1 (C_a), 63.0 (C_b), 3.75 (H_b)</p>	<p><i>side-on_1a</i></p> <p>18.2 (C_a), 58.9 (C_b)</p>	<p>Rigid_1b: 15.5 (C_a), 69.4 (C_b)</p> <p>Restricted_1b: 14.9 (C_a), 68.7 (C_b)</p>	<p>1'</p> <p>25.5 (C_a), 32.0 (C_b), 186.5 (C_c)</p>	<p>1''</p> <p>24.5 (C_a), 31.8 (C_b), 187.1 (C_c)</p>
<p>1'''</p> <p>23.1 (C_a), 182.7 (C_b), 1.6 (H_a)</p>	<p>2a</p> <p>23.0 (C_a), 183.9 (C_b)</p>	<p>2b</p> <p>10.4 (C_a), 37.0 (C_b), 144.6 (C_c), 130.7 (C_d), 1.75 (H_a), 2.05 (H_b)</p>	<p>2c</p> <p>17.0 (C_a), 31.0 (C_b), 147.0 (C_c), 128.4 (C_d), 1.1 (H_b), 6.7 (H_d)</p>	<p>2d</p> <p>19.0 (C_a), 29.0 (C_b), 136.0 (C_c), 129.8 (C_d), 2.02 (H_b)</p>
<p>3a</p> <p>21.2 (C_a), 29.7 (C_b), 138.0 (C_c)</p>	<p>3b</p> <p>14.1 (C_a), 21.8 (C_b), 129.4 (C_c), 126.7 (C_d)</p>	<p>3c</p> <p>16.8 (C_a), 22.2 (C_b), 130.6 (C_c), 128.0 (C_d), 1.05 (H_a), 7.31 (H_d)</p>	<p>4a</p> <p>19.0 (C_a), 31.7 (C_b), 138.0 (C_c), 130.3 (C_d)</p>	<p>4b</p> <p>13.7 (C_a), 29.6 (C_b), 146.4 (C_c), 128.8 (C_d)</p>
<p><i>mobile_1a</i></p> <p>226.7 (C_a), 158.8 (C_b), 37.3 (C_c), 44.1 (C_d), 32 (C_e)</p>	<p>5a</p> <p>17.2 (C_a), 62.5 (C_b), 0.42 (H_a), 3.25 (H_b)</p>	<p>5b</p> <p>8.8 (C_a), 0.25 (H_a)</p>	<p>5c</p> <p>8.9 (C_a), 0.40 (H_a)</p>	<p>5d</p> <p>8.7 (C_a), 0.17 (H_a)</p>
<p>5e</p> <p>17.4 (C_b), 0.64 (H_b)</p>	<p>5f</p> <p>15.2 (C_a), 0.05 (H_a), 26.9 (C_b), 0.8 (H_b)</p>			

Table S2. Remaining spin-systems unassigned in solid-state nuclear magnetic resonance (NMR). Data for restricted mobile (denoted as DE, observed in DE ^{13}C - ^{13}C correlation spectrum), purely mobile (denoted as M, observed in INEPT-TOBSY ^{13}C - ^{13}C correlation spectrum), and rigid (denoted as R, observed in CP ^{13}C - ^{13}C correlation spectrum) molecules/fragments. The following spin-systems could not be assigned to a particular structure in a definitive manner, due to missing correlations and/or spectral crowding ^a.

Name	Chemical Shifts in ppm											
	$^{13}\text{C}_a$	$^{13}\text{C}_{a2}$	$^{13}\text{C}_b$	$^{13}\text{C}_{b1}$	$^{13}\text{C}_{b2}$	$^{13}\text{C}_c$	$^{13}\text{C}_d$	$^{13}\text{C}_{d1}$	$^1\text{H}_a$	$^1\text{H}_b$	$^1\text{H}_c$	$^1\text{H}_d$
DE1 ^b	-	-	36.6	-	-	144.0	-	-	-	-	-	-
DE2_1	14.8	-	-	-	-	-	-	-	0.56	-	-	-
M2	9.8	-	-	-	-	-	-	-	1.22	-	-	-
M9	21.3	-	-	-	-	-	-	-	1.58	-	-	-
R2	-	-	-	32.2	37.2	-	-	-	-	-	-	-
R3	-	-	30.9	-	36.9	146.4	129.2	-	-	-	-	-
R6 ^c	23.2	-	181.1	-	-	-	-	-	-	-	-	-
R4_1	22.9	-	-	-	-	-	-	-	0.18	-	-	-
R7	44.3	-	57.4	-	-	-	-	-	-	-	-	-
R9_1	17.3	-	-	-	-	-	-	-	0.24	-	-	-
R10 ^d	21.9	-	30.9	-	-	140.3	129.8	-	-	2.24	7.14	6.91
R11_1	-	-	-	-	-	-	130.5	-	-	-	-	-
R16 ^b	-	-	-	28.8	36.9	-	134.8	-	1.70	-	-	-
R17	13.1	16.4	-	-	-	147.1	140.3	-	-	-	-	-
R19	22.3	-	43.9	-	158.6	-	-	-	-	2.49	-	-
R20 ^c	19.7	-	44.5	-	-	135.9	133.4	129.4	-	-	-	-
R21_1	-	-	-	-	-	129.8	-	-	-	2.35	-	-
R22 ^f	15.8	-	24.7	-	-	-	-	-	-	-	-	-
R23 ^f	10.8	-	33.44	-	-	-	-	-	-	-	-	-
R25 ^g	37.1	-	227.2	-	-	-	-	-	-	-	-	-
R25_2 ^g	37.6	-	227.8	-	-	-	-	-	-	-	-	-
R26 ^g	33.1	-	227.8	-	-	-	-	-	-	-	-	-

R32 ^h	32.2	-	184.5	-	-	-	-	-	-	-	-	-
R34 ^h	31.9	-	183.3	-	-	-	-	-	-	-	-	-

^a Unassigned restricted mobile species (denoted by DE) were partial elucidation of the ethyl group on a rigid aromatic framework. Unassigned mobile molecules (denoted by M) were aliphatic fragments. Unassigned rigid molecules (denoted by R) were fragments/groups from either aromatics or surface-adsorbed carbonylated species. ^b Consistent with the benzylic group (C₆H₅-CH₂-).
^c Consistent with the acetyl-group, analogous to surface-acetate species (1'') and/or acetic acid.
^d Consistent with the ethylated benzene framework, possibly a structural isomer of either **2b** or **2c**.
^e Either coupled (*via* benzylic carbon of ethylbenzene) or fused bi-aromatic species (for example, analogous to 2,3-Diphenylbutane). ^f Ethyl group (CH₃-CH₂-) only. ^g Consistent with the alkylated carbenium species/ σ -type complex in a six-membered ring framework. ^h Consistent with the surface propionate species (Zeolite-CO-CH₂- ; analogous to 1').

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CHAPTER 5

Investigating the Cross-Effect DNP efficiency of Trityl-Nitroxide biradicals at high fields

The work described in this chapter has been conducted in collaboration with Dr. Yangping Liu (Tianjin Medical University, China) and Dr. Martina Huber (Leiden University)

Abstract

There is an increasing interest in the development of new polarizing agents better suited for high-field Dynamic Nuclear Polarization (DNP) applications. Currently employed water-soluble biradicals exhibit an unfavourable field dependence, where DNP enhancements decrease with increasing magnetic field strength. Recent experimental and numerical studies have identified mixed trityl-nitroxide radicals as promising candidates for high-field DNP. Here, we employ quantum mechanical simulations to examine the parameters affecting the DNP performance of trityl-nitroxide biradicals at high magnetic fields. We show that these radicals are not affected by depolarization effects at high MAS frequencies. Moreover, the interplay of the electronic dipolar and J-coupling interactions on the DNP efficiency is investigated. Finally, additional numerical simulations are employed to reproduce and explain the experimentally observed DNP enhancements for the novel trityl-nitroxide compounds under investigation.

Introduction

Magic angle spinning dynamic nuclear polarization (MAS-DNP) has become a valuable approach to enhance the intrinsic low sensitivity of nuclear magnetic resonance (NMR) spectroscopy, prompting exciting new applications in life^[1-7] and material sciences^[8-15]. In parallel to this experimental work, new advances were triggered by theoretical and numerical studies which substantially improved our understanding of the Cross-Effect (CE) DNP under MAS^[16-20], essential for further development of the technique. The simplest CE model describes a coupled three-spin system composed of two electron spins and a nuclear spin. When the difference in the electrons' paramagnetic resonance (EPR) frequencies matches the nuclear Larmor frequency, polarization transfer from the electron spins to the nuclear spin occurs via simultaneous flip-flop transitions of the three spins. Microwave irradiation is necessary to saturate one of the EPR frequencies, creating a polarization difference between the two electron spins which, in turn, can be transferred to the nuclear spins. Since the extent of polarization transfer is proportional to the polarization difference between the electrons of the polarizing agent (PA), tailored PAs have been developed with optimized electron-electron spin properties. For MAS-DNP, a variety of tethered-radicals fulfilling the CE condition have been designed with the aim to maximize the DNP efficiency^[21-23]. In particular, nitroxide-based biradicals are widely used stable PAs which have undergone important structural optimizations. For instance, studies have shown that their DNP efficiency can be improved by carefully adjusting the relative orientation of the g-tensors of the two electron spins and the inter-spin distance^[22, 24-29]. Moreover, the radical rigidity and molecular weight^[30] related to electron relaxation times^[25] have been recognized as key parameters for radical optimization, together with electron-electron dipolar couplings^[29, 31]. Based on these developments, highly-efficient polarizing agents for CE-DNP have been synthesized, such as TEKPol^[26] and AMUPol^[22]. The latter is currently one of the most widely used biradicals for biological applications, due to its water solubility, bio-compatibility and commercial availability. However, bis-nitroxide biradicals present a critical drawback: their performance deteriorates severely at high magnetic fields and faster MAS rate^[18, 32]. Under these regimes the saturation efficiency diminishes, as predicted by the decrease in the Landau-Zener transition probability, resulting in a strongly reduced DNP transfer efficiency^[17, 19]. Therefore, the full potential of high-field DNP remains unexplored.

Mixed-biradicals have been introduced to overcome the inefficiency of DNP at high fields, a current hot topic within the DNP community^[27, 29, 31]. In the case of mixed biradicals (that include a narrow-line radical), a more efficient saturation of one EPR line is achieved, creating a sizable polarization potential also at high MAS rates^[33].

Moreover, the large exchange interaction exhibited by these mixed radicals helps to maintain the large polarization difference between the two electrons, produced by the microwave irradiation^[17, 19, 31]. In particular, Trityl-nitroxide (TN) biradicals have shown great potential^[34] (see also chapter 6). These polarizing agents are composed of one narrow line (Trityl) and one broad (nitroxide) EPR line radical, where the small g-anisotropy of the narrow line species allows for a better saturation of the EPR line. Consequently, mixed radicals perform better at high fields and faster MAS rates, and further improvements of TN biradicals are currently investigated that would benefit high-field DNP. Recent numerical approaches have deeply improved our understanding of CE efficiency under MAS for bis-nitroxides^[18, 20, 35-37]. However, a theoretical characterization of the electron properties of Trityl-nitroxide biradicals still remains incomplete.

To achieve a better understanding, we performed here quantum-mechanical simulations on a three-spin system ($e_a - e_b - n$). These simulations investigate the influence of different parameters on the MAS-DNP mechanism and in particular the effect of the electron-electron J-coupling interaction. Finally, the DNP performance of different TN compounds is simulated, showing good agreement with the experimental observations.

Methodology

Numerical simulations

The CE-DNP mechanism under magic angle spinning conditions differs greatly from the static case. In fact, as theoretically described by Thurber and Tycko^[17] and Mentink-Vigier^[16] et al., the polarization transfer between the electron and the nuclear spins in rotating solids occurs through Level Anti-Crossings (LACs), with the efficiency proportional to the transition probability at the energy level crossing of two states^[38]. In this study, we performed simulations on a three-spin system composed of two coupled electron spins and one coupled proton spin under MAS conditions and microwave irradiation, following earlier work^[18]. A quantum mechanical density matrix formalism is applied, where the time evolution of the energy terms introduced by the sample rotation is taken into account. Simulations of the DNP performance of Trityl-nitroxide radicals are carried out using a Matlab-based approach introduced by Mance et al^[18] with the addition of J-coupling between the two electron spins to the total time-dependent Hamiltonian.

$$\begin{aligned}
 \hat{H}(t) = & \Delta\omega_a\hat{S}_{az} + \Delta\omega_b\hat{S}_{bz} + \omega_n\hat{I}_z + \omega_1\{\hat{S}_{ax} + \hat{S}_{bx}\} \\
 & + D_{ab}\{2\hat{S}_{az}\hat{S}_{bz} - \hat{S}_{ax}\hat{S}_{bx} - \hat{S}_{ay}\hat{S}_{by}\} \\
 & + A_{zz}\hat{S}_{az}\hat{I}_z + A_{zx}\hat{S}_{az}\hat{I}_x + A_{zy}\hat{S}_{az}\hat{I}_y \\
 & + J_{ab}\{\hat{S}_{ax}\hat{S}_{bx} + \hat{S}_{ay}\hat{S}_{by} + \hat{S}_{az}\hat{S}_{bz}\}
 \end{aligned} \tag{5.1}$$

$\Delta\omega_a$ and $\Delta\omega_b$ represent the electronic Zeeman interaction and $\omega_n\hat{I}_z$ is the nuclear Zeeman interaction. ω_1 represents the field strength of the microwave irradiation, D_{ab} the dipolar electron-electron interaction and J_{ab} the J-coupling between electron a and b . A_{zz}, A_{zx}, A_{zy} represent the hyperfine interaction (HFI) between electron a and the nucleus.

The DNP enhancement is obtained by solving the Liouville-Von Neumann equation for the spin density operator of the spin system ($e_a - e_b - n$), formed by two electrons a and b , and a nuclear spin $n=1/2$. For simplicity, we assume that the nucleus is only coupled to electron a and, following a previous publication by Mentink-Vigier *et al.*^[16], we adopt a fixed geometry of the biradical where the Principal Axis System (PAS) of the two electronic g-tensors is locked in a fixed orientation with respect to each other. This assumption is consistent with the experimental data for most biradical structures^[39]. Calculations are performed for each single orientation of PAS, given by a set $\{\alpha, \beta, \gamma\}$ of Euler angles. Results for a randomly oriented sample in the magnetic field are subsequently obtained by performing a summation over all Euler angles. In our calculations, we assume a hyperfine interaction of 0.023 MHz, as it was observed to best fit the experimental observations^[18, 37]. The g-tensor values for the nitroxide and the trityl electron spin are set to $g_a = [2.0088, 2.0061, 2.0022]$ and $g_b = [2.0032, 2.0032, 2.0026]$, respectively, as described in the literature^[34]. The longitudinal and transverse relaxation rates for electron and nuclear spins are also incorporated in the simulation. These values were obtained from experimental studies and are listed in the result section, together with all the other relevant parameters.

Since DNP experiments are conducted under MAS conditions, the relaxation rates and interactions are computed in a step wise manner where the Hamiltonian in eq. (5.1) is constant. The resulting propagators are calculated for each time step of a single rotor cycle. After the completion of a single rotor period, an evolution super-operator is defined and applied to the density matrix of the system to calculate subsequent rotor cycles. The number and duration of time steps used to simulate the evolution of a single rotor period was carefully chosen to ensure numerical stability and convergence of the results^[16, 18]. Finally, the net magnetization and its time dependence are computed for all spins. The nuclear spin polarization (P_k) is then normalized to the polarization at equilibrium conditions (P_{eq}) to obtain the DNP enhancement factor $\varepsilon_{DNP} = P_k/P_{eq}$.

Since our aim is to investigate the CE-DNP efficiency at high fields, the simulations assume a magnetic field of 18.8 T, corresponding to a ^1H nuclear Larmor frequency of 800 MHz. Our theoretical results are supplemented by experimental EPR studies to determine the electron relaxation times of the biradicals and by DNP measurements conducted at a static magnetic field of 18.8 T.

DNP measurements

DNP experiments were performed on frozen solutions of 10 mM and 15 mM biradical concentrations for TN radicals and AMUPol respectively, in $\text{d}_8\text{-glycerol:D}_2\text{O:H}_2\text{O}$ 60:30:10 v:v:v with 0.25 M $\text{U-}^{13}\text{C-}^{15}\text{N}$ proline. Samples were packed into 3.2 mm sapphire rotors with a sample volume of 25 μL . DNP experiments at 800 MHz were performed on a Bruker BioSpin 527 GHz solid-state NMR DNP spectrometer and the sample temperature was kept at 103 K. The nuclear polarization was measured through the spectrum of $^{13}\text{C-}^{15}\text{N}$ proline, which is observed via $^1\text{H-}^{13}\text{C}$ cross-polarization (CP) experiments. A CP spin-locking field of 48 kHz was applied on ^{13}C , while a ramped (80-100%) power was employed during a 50 kHz spin-locking field on ^1H . The contact time was set to 2 ms and 83 kHz SPINAL-64 decoupling^[40] was applied during acquisition.

Trityl-Nitroxide (TN) Radicals

In the following section, we examined the previously published TN radical named TEMTriPol-1^[34] as well as three novel compounds in which the trityl and a nitroxide radical are chemically tethered. The molecular structures of the Trityl-nitroxide radicals tested for CE-DNP are shown in Figure 1. These molecules were synthesized and characterized by EPR in the group of Yangping Liu (for the synthesis of NATriPol-1 the reader is referred to chapter 6). EPR measurements of dipolar and J-coupling were carried out at low temperature (~ 220 K) on a Bruker EMX-plus X-band spectrometer. Additional high-field EPR experiments to determine electronic relaxation rates were conducted in the group of Martina Huber. These EPR measurements were performed at 100 K on a spectrometer operating at 275 GHz^[41, 42].

Figure 5.1. Trityl-nitroxide radicals synthesized by the group of Yangping Liu and studied in this chapter. For further information see also chapter 6.

Results and discussion

MAS dependence for Trityl-nitroxides

In addition to their unfavourable field dependence, one of the important drawbacks of bis-nitroxides biradicals is the drop in DNP enhancement with increasing MAS frequency^[32].

The observed dependence of the enhancement is attributed to the electron nuclear polarization transfer occurring at the Cross-Effect (CE) level anti-crossings (LAC) upon sample spinning^[17]. In fact, CE-DNP under magic angle spinning can be explained as the result of three separate types of LAC events (or “rotor” events)^[16, 17]:

- i. Microwave events, which correspond to the irradiation of one of the electron transitions, creating a polarization difference between the two electron spins.
- ii. CE matching events, which allow the transfer of polarization from the electrons to the nuclear spins when the matching condition $\omega_{0e2} - \omega_{0e1} = \omega_{0n}$ is fulfilled.
- iii. Electron-electron events, which lead to an exchange of magnetization between the two electrons.

For bis-nitroxide radicals, the presence of a strong depolarization was observed under MAS conditions and it was ascribed to inefficient electron-electron events and the small mean polarization difference between the two electronic spins, which ultimately affects the polarization transfer to the nuclear spins^[33]. However, it is highly beneficial to perform DNP experiments at a high magnetic field and MAS rate due to the significant gain in spectral resolution^[43, 44]. In these conditions, mixed Trityl-nitroxide (TN) biradicals perform better. In fact, they do not show the disadvantageous field and spinning dependence, as shown by Mathies, et al.^[34] and demonstrated by Mentink-Vigier, *et al.*^[33]. Knowing the parameters for our TN compound, we computed the DNP enhancement as a function of MAS rate and compared our results to the measured experimental data. The results are presented in fig. 5.2 for TEMTriPol-1^[34]. For reference, a comparison with the data obtained for the bis-nitroxide AMUPol is also included. (Further details of our simulations are given in table 5.1).

Figure 5.2. Experimental (red circle) and simulated (red dotted line) MAS frequency dependence of ϵ_{DNP} for Trityl-nitroxide (TN) radical, in comparison to experimental data measured for the binitroxide AMUPol. The parameters used in the simulation are given in Table 5.1.

The simulated MAS frequency dependence of the DNP enhancement (ϵ_{DNP}) for the TN radical TEMTriPol-1 is in agreement with our experimental data. In particular, it shows that the enhancement remains constant with increasing MAS frequency (≥ 5 kHz). In contrast, ϵ_{DNP} decreases when increasing spinning speed for the bis-nitroxide AMUPol, in line with earlier studies conducted at lower fields^[33]. This is a consequence of the improved EPR lineshape of the trityl-nitroxide biradical, with the isotropic g -values of the narrow-line trityl and broad-line nitroxide separated approximately by the ^1H

Larmor frequency^[33]. A higher electron polarization difference is maintained and transferred to the nuclear spins, leading to an increased CE-DNP performance, especially at faster spinning rates, in case of TN radicals.

Parameter	Value
Field	18.8 T
Microwave frequency ($\omega_m/2\pi$)	526.9 GHz
Microwave strength ($\omega_1/2\pi$)	0.85 MHz
Temperature	100 K
MAS frequency	Varied from 0 to 12 kHz
Electron dipolar coupling ($D_{ab}/2\pi$)	20 MHz
Electron J-coupling ($J_{ab}/2\pi$)	36 MHz
Relaxation data	Value
T_{1e}	Trityl: 330 μ s Nitroxide: 60 μ s
T_{2e}	Trityl: 800 ns Nitroxide: 750 ns
T_{1n}	3 s
T_{2n}	1 ms

Table 5.1. Parameters used to calculate the MAS dependence of the DNP enhancement for a Trityl-nitroxide biradical. The values of dipolar and J-coupling are similar to the ones used by Mentink-Vigier *et al.*^[33]. The electronic relaxation times correspond to the values measured for the GT compound. (see table 5.3)

J-coupling dependence for Trityl-nitroxides

According to the theory of CE-DNP^[17, 35], the CE transition probability is proportional to the strength of the electron-electron spin interactions. In the Hamiltonian describing our system there are two terms of electronic spin-spin coupling: the dipolar coupling and the J coupling (also known as exchange interaction), between electrons *a* and *b*. In previous quantum mechanical simulations only the dipolar coupling was considered when calculating the DNP enhancements^[16, 18]. However, recently the importance of J-coupling has been experimentally^[29, 31, 35, 37] and theoretically^[19, 33, 35, 37] recognized. In fact, the stronger J-coupling exhibited by the bisnitroxide AMUPol, in comparison with the TOTAPOL^[24] radical, was found to be the reason of its improved DNP performance at

higher magnetic fields^[37]. This result indicates that enhancing the strength of the electron-electron J coupling constitutes a viable way to increase the CE-DNP efficiency, underlining the promising potential of biradicals with sizable J -couplings for ultra-high field DNP^[34]. Moreover, it was shown that the presence of J -coupling also mitigates the MAS dependence of the DNP enhancement at high spinning frequencies^[19, 35].

In order to investigate the influence of J -coupling on the CE-DNP enhancement of Trityl-nitroxide biradicals at high fields, specifically in relation to the value of dipolar interaction, we performed quantum mechanical simulations for a three spin system under MAS, at 100 K and 18.8 T conditions. This numerical approach offers a convenient means for studying the role of the specific interaction, since it is experimentally challenging to fully distinguish the effect of J - and dipolar coupling on the DNP enhancement.

Fig. 5.3 presents a graph of the simulated ^1H enhancement (ϵ_{DNP}) as a function of the J value, for different strengths of the dipolar interaction.

Figure 5.3. MAS-DNP simulations showing the J -coupling dependence of ϵ_{DNP} for a Trityl-nitroxide (TN) radical. Results are shown for 3 different values of dipolar coupling: 5 MHz (black), 18.5 MHz (blue) and 50 MHz (magenta). DNP simulations are based on the parameters shown in Table 5.2 and the average was done over 125 orientations.

Importantly, we observe that increasing the strength of J -coupling increases the predicted ^1H enhancement up to a threshold value, regardless of the dipolar coupling strength. Therefore, the presence of J -coupling undoubtedly improves the CE-DNP performance.

However, as visible in fig. 5.3, the DNP enhancement drops off after exceeding a critical value of J-coupling, which is about 100 MHz under our conditions.

Moreover, for a strong dipolar interaction, higher values of ε_{DNP} are obtained with smaller J-couplings, showing that the interplay between the two interactions contributes to the DNP enhancement. In fact, the exchange interaction (J) plays a similar role to the dipolar interaction (D) in contributing to the CE rotor events and hence the state mixing required for CE-DNP. In particular, the CE transition probability is proportional to the sum of the electronic spin-spin interactions ($D + J$)^[17, 45, 46], indicating that a larger D and J values allow for efficient CE rotor events. Please note that there is a factor 2 difference in the cited literature, due to the different form of the J-coupling term used in our Hamiltonian (JS_1S_2 is used instead of $2JS_1S_2$). On the other hand, the relation $|D + J|/2\pi \leq \omega_n/2\pi$ has to be satisfied for an efficient CE-DNP^[33], where ω_n is the ^1H nuclear Larmor frequency. This notion is in agreement with our numerical simulations in Fig. 5.3, where for high values of J (a few hundred MHz), a further increase in J leads to a decrease in ^1H enhancement. This result is also supported by experimental findings^[34, 47]. Interestingly, for the considered dipolar and J parameters in Figure 5.3, optimal DNP enhancements are seen in the simulations when $D+J \sim 80 \text{ MHz} \pm 25 \text{ MHz}$. Consequently, an optimal combination of dipolar and J-coupling values for TN biradicals may exist that is large enough to guarantee a highly efficient CE polarization transfer without interfering with the frequency matching condition required for CE-DNP and the CE-DNP transition probability is maximized.

Finally, it is evident that the J-coupling has to exceed a certain value relative to the dipolar interaction to obtain maximum DNP enhancement. However, further studies need to be conducted and simulations with different dipolar coupling values are necessary to investigate the existence of an optimal ratio of J/D.

Parameter	Value
Field	18.8 T
Microwave frequency ($\omega_m/2\pi$)	526.9 GHz
Microwave strength ($\omega_1/2\pi$)	0.85 MHz
Temperature	100 K
MAS frequency	8 kHz
Electron dipolar coupling ($D_{ab}/2\pi$)	5/18.5/50 MHz
Electron J-coupling ($J_{ab}/2\pi$)	Varied from 0 to 200 MHz
Relaxation data	Value
T_{1e}	Trityl: 330 μ s Nitroxide: 60 μ s
T_{2e}	Trityl: 800 ns Nitroxide: 750 ns
T_{1n}	3 s
T_{2n}	1 ms

Table 5.2. Parameters used to calculate the J-coupling dependence of the DNP enhancement for a Trityl-nitroxide biradical, for three different values of dipolar coupling.

Novel Trityl-nitroxide biradicals

Due to the effects of J-coupling described in the previous section, an ideal biradical requires both a moderately strong dipolar coupling and exchange interaction. Moreover, the two radicals should originate from different chemical species and with EPR frequencies separated by the nuclear Larmor frequency. Recently, systems like the Trityl-TEMPOs^[34] or the hybrid BDPA-TEMPOs^[31] have been developed. For example, Trityl-nitroxide (TN) variants have been synthesized by the group of Yangping Liu (see fig. 5.1) with the aim to increase their DNP efficiency as function of dipolar and J-coupling. In the following, we investigated their DNP performance on a model sample (0.25 M ^{13}C - ^{15}N proline in the standard DNP juice, d_8 -glycerol/ $\text{D}_2\text{O}/\text{H}_2\text{O}$, 60/30/10, V/V/V) and the experimental results are compared to the DNP simulations, as shown in fig. 5.4. Electronic relaxation times are known to be a key parameter in determining the CE-DNP efficiency, especially under continuous wave microwave irradiation. In particular, relatively long longitudinal relaxation times (T_{1e} of hundreds of millisecond) are required to retain a large electron polarization difference created by microwave

irradiation [18, 25, 31, 32]. The simulations used the EPR relaxation times that were experimentally determined for these compounds by Huber et al. (see Table 5.3). Fig. 5.4 reveals a good agreement between experimental data and numerical simulations, indicating that our quantum mechanical approach can reproduce the observed DNP performance for the different compounds and in particular the decrease in DNP efficiency for the GLH radical. The electron relaxation times of these TN biradicals are similar, suggesting that in our case these parameters are not responsible for the variations in DNP enhancement. The situation is different if we consider the electronic spin interactions, with the GLH biradical presenting the lowest values of dipolar and J-coupling. In fact, for the 3 compounds that exhibit the largest DNP enhancements, the dipolar and J parameters fall approximately in the range where $D+J \sim 80 \text{ MHz} \pm 25 \text{ MHz}$ seen in our simulations in Figure 5.3, whereas the values seen for GLH are significantly beyond these ranges. Having established the important role played by these interactions, we therefore ascribe the reduced CE-DNP efficiency to the lower dipolar and J-coupling strength for the GLH radical.

Figure 5.4. Experimental (black) and simulated (blue) ϵ_{DNP} for the different Trityl-nitroxide (TN) radicals. The simulations were performed with the parameters available for the TN biradicals given in Table 5.3 and the average was done over 125 orientations. Dashed lines are included to guide the eye.

Parameter	Value GT	Value GTM	Value GLH	Value GLOH
Field	18.8 T	18.8 T	18.8 T	18.8 T
μw frequency ($\omega_m/2\pi$)	526.9 GHz	526.9 GHz	526.9 GHz	526.9 GHz
μw strength ($\omega_1/2\pi$)	0.85 MHz	0.85 MHz	0.85 MHz	0.85 MHz
Temperature	100 K	100 K	100 K	100 K
MAS frequency	8 kHz	8 kHz	8 kHz	8 kHz
e dipolar coupling ($D_{ob}/2\pi$)	19.6 MHz	18 MHz	8.4 MHz	22.4 MHz
e β -coupling ($J_{sk}/2\pi$)	45 MHz	50 MHz	25.4 MHz	64.5 MHz
Relaxation data	Value GT	Value GTM	Value GLH	Value GLOH
T_{1e}	Trityl: 330 μs Nitroxide: 60 μs	Trityl: 380 μs Nitroxide: 60 μs	Trityl: 300 μs Nitroxide: 60 μs	Trityl: 330 μs Nitroxide: 60 μs
T_{2e}	Trityl: 800 ns Nitroxide: 750 ns	Trityl: 780 ns Nitroxide: 750 ns	Trityl: 760 ns Nitroxide: 750 ns	Trityl: 1050 ns Nitroxide: 750 ns
T_{1H}	3 s	3 s	3 s	3 s
T_{2H}	1 ms	1 ms	1 ms	1 ms

Table 5.3. Parameters used to calculate the DNP enhancement for indicated Trityl-nitroxide biradicals.

Conclusions

The numerical simulations and the experimental data reveal the impact of the electron J-coupling (in relation to the dipolar interaction) on the final nuclear polarization enhancement of trityl-nitroxide radicals under MAS-DNP conditions. Cross Effect DNP relies on the dipolar and J-coupling interaction between the two electron spins of the biradical and both interactions are taken into account and computed in our quantum-mechanical MAS-DNP simulations for a three-spin system. The results show that the presence of a sizable J-coupling (below a critical threshold) is highly beneficial to the DNP efficiency, increasing the CE transition probability especially at high MAS frequencies. However, a J-coupling that exceeds this critical threshold can interfere with the Cross-Effect matching condition, effectively reducing DNP enhancements. It is known that the performance of bis-nitroxide radicals used for DNP at high fields is dramatically reduced. Consequently, biradicals with relatively strong J-coupling such as trityl-nitroxides are the preferred choice. For these systems, it is crucial to maximize the electron J-coupling and dipolar interactions to achieve an optimal balance and maximize the CE-DNP efficiency.

Finally, an experimental investigation of novel trityl-nitroxide compounds showed good agreement with our numerical simulations. Interestingly, the biradical GTM (or NATRiPol-1) exhibits an improved DNP performance while maintaining parameters such as electron-electron interactions and relaxation times similar to GT (or TEMTriPol-1), which is the best trityl-nitroxide polarizing agents obtained so far. Based on this observation, a new series of biradicals has been synthesized starting from the structure of NATRiPol-1 with the aim to improve water solubility, a crucial characteristic for their biomolecular applications. The DNP performance of these novel biradicals and the results from additional experimental studies on their suitability for DNP applications to biomolecules are presented in the next chapter.

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CHAPTER 6

Reducing Hydrophobicity Enhances the High-Field Dynamic Nuclear Polarization Efficiency of Water-soluble Trityl-nitroxide Biradicals for Biomolecules

Based on:

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Abstract

Dynamic nuclear polarization (DNP) is a powerful method to enhance nuclear magnetic resonance (NMR) signal intensities, enabling unprecedented applications in life and material science. An ultimate goal is to expand the use of DNP-enhanced solid-state NMR to ultra-high magnetic fields where optimal spectral resolution and sensitivity are integrated. Trityl-nitroxide biradicals have attracted significant interest in high-field DNP, but their application to complex (bio)molecules has so far been limited. Here we report the synthesis of five trityl-nitroxide biradicals (referred as to NATriPols) that vary in hydrophobicity and are derived from TEMTriPol-1, so far the best trityl-nitroxide biradical at high fields. We observe that the electron-electron magnetic interactions of NATriPols are independent of the substituents at the α -position of the amino-acid linkers. We demonstrate that DNP enhancements at 18.8 T of these biradicals for $^{13}\text{C}, ^{15}\text{N}$ -proline in a glycerol/water matrix are inversely correlated with their hydrophobicity. Moreover, applications of this new class of biradicals to biomolecules including a globular soluble protein and a membrane targeting peptide reveal significantly improved performance compared to TEMTriPol-1 and AMUPol. Our work identifies hydrophobicity of the polarizing agents as a critical factor for DNP performance in biomolecules and paves the way for further optimization of new biradicals for biomolecular applications at ultra-high magnetic fields.

Introduction

Dynamic nuclear polarization (DNP) has evolved into a well-established and powerful technique to enhance the sensitivity of nuclear magnetic resonance (NMR) spectroscopy in the liquid^[1, 2] and solid state^[3-5] by microwave-driven transfer of polarization from unpaired electrons (i.e., polarizing agents) to nuclei. Signal enhancements by several orders of magnitude when using DNP create entirely new application areas of solid-state NMR (ssNMR) in structural biology^[6-11] and material science^[4, 12-16]. In parallel, recent innovations in DNP instrumentation (including microwave sources and low-temperature NMR/DNP probeheads)^[17, 18], have allowed to extend DNP-enhanced ssNMR to ultra-high magnetic fields (up to 21.1 T)^[19-23] and the potential to increase spectral resolution under such conditions has already been demonstrated^[20, 24].

At high fields and low temperatures (typically 100 K), the cross effect (CE) has so far proven to be the most efficient mechanism, which requires a coupled three-spin system consisting of two electrons and one nucleus^[3, 4, 25]. To fulfil the CE condition, biradicals are routinely used as polarizing agents. In the past decade, much effort has been devoted to improve nitroxide-based biradical polarizing agents by optimizing g-tensor orientations, rigidity of the linker and electron spin relaxation times^[26-31]. These efforts have led to the development of efficient nitroxide biradicals such as AMUPol^[28] and TEKPol^[29] that possess DNP enhancement factors ($\epsilon_{\text{on/off}}$) of up to ~ 250 at moderate magnetic fields (e.g., 9.4 T) under magic-angle spinning (MAS) conditions. In parallel, theoretical approaches and numerical studies have been employed to understand the factors determining the CE polarization efficiency and to guide the design of new polarizing agents^[32-36]. Ideally, MAS-DNP experiments are carried out at high magnetic fields where the spectral resolution is maximized together with further improved sensitivity. Although nitroxide biradicals exhibit satisfying performance below 10 T, their DNP enhancements rapidly decrease as the magnetic field is increased. For instance, the ^1H signal enhancements drop from 250 at 5 T to approximately 30 at 18.8 T for AMUPol (10 mM) in the conventional “DNP juice” (d_8 -glycerol/ $\text{D}_2\text{O}/\text{H}_2\text{O}$, 60/30/10, V/V/V)^[37]. These values could be further attenuated after considering nuclear depolarization and quenching effects induced by the paramagnetic polarizing agents^[32, 38-40]. The unfavourable correlation of the DNP enhancements of the nitroxide biradicals with the external field is partially due to the linear broadening of their EPR linewidths with the field and the MAS-dependence of the underlying dipolar electron-electron interactions^[33, 41]. Hence, very recently, efforts have been devoted towards the development of novel dinitroxide biradicals that show excellent DNP performances with

the $\epsilon_{\text{on/off}}$ values of up to 90 for the TinyPols series at high fields and high spinning frequencies^[22]. However, these enhancement values were obtained using 1.3 mm MAS rotors where the microwave field distribution is more favourable than in 3.2 mm rotors used in our current study. Moreover, these dinitroxides still exhibit the unfavourable field dependence albeit attenuated when compared to AMUPol.

Contrarily, trityl-nitroxide (TN) mixed biradicals^[37] (also referred to as TEMTriPols), initially developed as electron paramagnetic resonance (EPR) probes for the redox status^[42, 43], exhibit remarkable DNP properties. In contrast to nitroxide biradicals, the DNP enhancements of TEMTriPols exhibit favourable magnetic-field dependence and the optimal value is displaced towards higher magnetic fields^[37]. For example, TEMTriPol-1 shows DNP enhancement factors for ^{13}C -labelled urea of 50, 87 and 65 at 5.0, 14.1 and 18.8 T, respectively, without a significant depolarization effect at the chosen MAS settings^[44]. Thus, the signal enhancement obtained using TEMTriPol-1 in hydrophilic environments at 18.8 T still represents the highest value among the currently available biradicals using 3.2 mm MAS rotors. The distinct DNP properties of TEMTriPols can be explained by their unique physicochemical properties including (i) the ideal EPR frequency separation between the nitroxide g_{yy} component and the almost isotropic g -value of trityl radical; (ii) their favourable relaxation times, which allow simultaneous microwave saturation and polarization turnover; (iii) moderate electron-electron exchange interactions which are beneficial for their DNP performance at high fields^[41, 45].

Likewise, other hybrid biradicals (e.g., BDPA-nitroxide^[22, 46, 47] and PTM-nitroxide biradicals^[48], and asymmetric nitroxide biradicals^[34]) were developed for high-field DNP or fast dissolution DNP, although most of them are not compatible with biomolecular studies due to their high hydrophobicity. In these studies, the importance of dipolar/exchange interactions for high-field DNP properties of biradicals was also highlighted. Recently, we have confirmed the influence of the exchange interaction on the DNP enhancement using chiral TN biradicals that exhibit almost identical dipolar interactions but completely different exchange interactions^[49]. Certainly, optimal dipolar/exchange interactions for a biradical may exist. They should be large enough to maintain the polarization difference between two spins by efficient polarization transfer and, at the same time, sufficiently small to preserve the frequency matching required for DNP^[49]. Recent theoretical studies predicted that TEMTriPol-1 has approached the optimal dipolar/exchange interactions.^[34, 44] Therefore, further optimization of other physicochemical properties of TEMTriPol-1 while maintaining its dipolar/exchange interactions may be an effective method to design new powerful polarizing agents.

High hydrophilicity is critical for biomolecular applications and so far mostly involve the use of AMUPol and TOTAPol^[50] because of their excellent water solubility.

Accordingly, there is a great need to develop new water-soluble biradicals which have excellent DNP performance at high fields.

Chart 6.1. Molecular structure of NATriPols and TEMTriPol-1. Note: SPy, 2-pyridinylthio; SG, glutathionyl.

Based on the molecular structure of TEMTriPol-1, in this work we synthesized a new series of TN biradicals (NATriPols, Chart 6.1) in which α -amino acids such as L-alanine, L-phenylalanine and L-aspartic acid were used as linkers. EPR experiments confirm that these biradicals exhibit very similar electron-electron dipolar/exchange interactions.

Based on these findings, the pyridine disulfide-appended NATriPol-4 was further synthesized from which NATriPol-5 was obtained through a “click” reaction with glutathione in a quantitative manner. We measured the DNP performance of these NATriPols using standard DNP preparations at 18.8 T and investigated the relationship between DNP enhancement and hydrophobicity. Using the hydrophilic NATriPol-3 and -5 at 10 mM concentration, we observed strong absolute signal gains (ϵ_{abs}) of up to 60 for [^{13}C -, ^{15}N]-proline, achieving a new maximum of the DNP enhancement at 18.8 T using 3.2 mm MAS rotor in hydrophilic environments. Furthermore, experiments employing [^{13}C -, ^{15}N]-algal amino acid mixtures, [^{13}C -, ^{15}N]-ubiquitin and a membrane-associated peptide confirm the excellent DNP performance of NATriPol-3 and -5 and reveal their potential for biomolecular applications.

Materials and Methods

DNP-ssNMR spectroscopy of NATriPols 1-5

DNP experiments were performed on frozen solutions of 5 mM, 10 mM or 15 mM biradical in d_8 -glycerol: D_2O : H_2O 60:30:10 v:v:v with 0.25 M $U\text{-}^{13}C\text{-}^{15}N$ proline. Samples were packed into 3.2 mm sapphire rotors with a sample volume of 25 μ L. DNP experiments at 800 MHz were performed on a Bruker BioSpin 527 GHz solid-state NMR DNP spectrometer. This spectrometer is equipped with a Bruker 800 WB/RS Plus magnet with a sweep coil, an Avance III NMR console, and a low-temperature 3.2 mm double-resonance DNP MAS NMR probehead. A gyrotron microwave source emits microwaves at a frequency of 527.043 GHz. The nuclear polarization was measured through the NMR spectrum of $^{13}C\text{-}^{15}N$ proline, which is observed via $^1H\text{-}^{13}C$ cross-polarization (CP) experiments. A CP spin-locking field of 48 kHz was applied on ^{13}C , while a ramped (80-100%) power was employed during a 50 kHz spin-locking field on 1H . The contact time was set to 2 ms. During acquisition, SPINAL-64^[51] decoupling was applied at 83 kHz and a delay of $1.26 \cdot T_B$ was employed for optimal sensitivity. Each spectrum was acquired with a 4-step phase cycle and repeated three times to confirm stability and reproducibility. The MAS frequency was set to 8 kHz and the sample temperature was kept at 103 K. The time constant T_B , which describes the build-up of 1H polarization, was measured via a 1H saturation recovery experiment and determined indirectly by detecting the ^{13}C CP signal. The polarization build-up curves were fitted with mono-exponential curves and the largest error of the fit was found to be $\pm 5\%$ in the microwaves- off case.

To find the optimal CE DNP enhancement, the magnetic field was swept and at each field position the nuclear polarization was measured via the cross-polarization experiment as described above. (See Supporting Information, Fig. S3)

To perform the DNP experiments on the labelled ubiquitin, 1mg of lyophilized [^{13}C , ^{15}N] ubiquitin was dissolved in d_8 -glycerol: D_2O : H_2O 60:30:10 v:v:v, for a final protein concentration of 4 mM. In the same way, 1mg of the $^{13}C\text{-}^{15}N$ - enriched ($\geq 98\%$) algal amino acid mixture (Cortecnet, CCN070P1) was dissolved in 30 μ L of standard DNP juice (d_8 -glycerol: D_2O : H_2O 60:30:10 v:v:v).

For the 2D PDSD experiments, a mixing time of 30 ms and a $^1H\text{-}^{13}C$ CP contact time of 0.7 ms were used. A cumulative number of scans of 32 were applied and acquisition times were set to 17 ms and 10 ms for the direct and indirect dimensions, respectively. The experiments were recorded at a MAS rate of 8 kHz, using a 84 kHz SPINAL-64

proton decoupling and a recycle delay of 2 s. The 2D spectra were processed using a 0.5π shifted sine squared window function on both dimensions (Bruker software Topspin 4.0).

Quantification of enhancement, bleaching, and depolarization

The crude DNP enhancement factor is given by the ratio of the signal intensity in the presence and absence of microwave irradiation.

$$\varepsilon_{on/off} = I_{on}(\omega_r)/I_{off}(\omega_r)$$

where $\omega_r/2\pi$ is the spinning frequency (in Hz) at which the DNP-MAS NMR experiment is performed. It should be noted that in this case $\varepsilon_{on/off}$ corresponds to the real polarization gain, since the biradicals investigated in our study did not show sizable depolarization^[39].

However, the absolute signal gain is a more accurate estimate of the DNP enhancement, since it accounts not only for depolarization but also for bleaching effects. As shown previously^[44], the absolute signal gain can be described as

$$\varepsilon_{abs} = I_{on}(\omega_r)/I_{undoped} = \varepsilon_{on/off} \cdot \chi_{bleach} \cdot \chi_{depo}$$

where $\chi_{depo} = I_{off}(\omega_r)/I_{off}(0)$ is the MAS-induced contribution factor accounting for depolarization and $\chi_{bleach} = I_{off}(0)/I_{undoped}$ is the contribution factor accounting for paramagnetic bleaching.

Finally, the practical sensitivity gain introduced by Corzilius *et al.*^[52] is a DNP sensitivity enhancement factor which combines the absolute signal gain and the shortening of T_B due to the addition of the polarizing agents.

$$\Sigma = \varepsilon_{abs}/\sqrt{(T_{1,undoped}/T_B)}$$

Where $T_{1,undoped}$ is the T_1 of the undoped DNP matrix measured at cryogenic temperatures and, as described above, T_B is time constant that describes the build-up of ^1H polarization. Σ represents the sensitivity gain obtained when performing DNP experiments as compared to a MAS NMR experiment measured at the same cryogenic temperature without DNP and where no radical has been added to the sample.

Error estimation.

The errors $\Delta\varepsilon$ in the enhancements $\varepsilon_{on/off}$ were calculated according to the formula

$$\Delta\varepsilon = \varepsilon(\Delta I_{\mu W, on}/I_{\mu W, on} + \Delta I_{\mu W, off}/I_{\mu W, off})$$

Where ΔI is the error in the intensity I of the peak (with and without microwaves), as reported previously^[53]. The error is estimated based on the noise level and small imperfections in the baseline. The relative error was larger for the spectra recorded without microwaves and for this reason the spectra were recorded with a higher number of scans to obtain an acceptable signal-to-noise ratio. The highest observed error was found to be ± 2 .

Results and discussion

Room-temperature and low-temperature EPR studies of NATriPols 1-5

Fig. 6.1A and Fig. S1A show EPR spectra of NATriPols 1-3 in phosphate buffer at room temperature. The spectra are very similar and asymmetric with two partially overlapping and weak peaks at low field, one intense peak at medium field and one moderate peak at high field. In a previous study the separation between the two low-field peaks was shown to be inversely proportional to the magnitude of the exchange interaction (J) in TN biradicals^[43]. The almost identical separations (4.0–4.6 G) between the two low-field peaks for NATriPol biradicals indicate that they exhibit similar J values. EPR spectral simulations showed that both NATriPol-1 and NATriPol-3 have similar J values as TEMTriPol-1 (~ 60 G, Table 1), which are slightly larger than that of NATriPol-2 (48 G). These results indicate that the J values of NATriPols and TEMTriPol-1 at room temperature are independent of the substituents at the α -position of amino acid linkers. Recent studies have shown that both the exchange and dipolar interactions of biradicals are crucial for their DNP properties^[34, 37, 41, 44, 49]. For this reason, we recorded EPR spectra of NATriPols 1-3 in 6/4 (v/v) glycerol/H₂O glass-forming solutions at low temperature (~ 220 K) (Fig.6.1A and S1B).

It is evident that these biradicals are characterized by similar EPR spectra with almost identical overall separations (71 G) between the two outermost lines that are slightly

larger than $2A_{zz}$ (~ 70 G), indicative of their similar but weak exchange interactions. Moreover, dipolar interactions that are averaged out at room temperature are now detectable, due to restricted molecular tumbling in the frozen state. Spectral simulation using a recently developed EPR program^[43] showed that NATriPols 1-3 and TEMTriPol-1 have almost the same dipolar ($D = 5\text{--}6$ G) and exchange ($J = 17\text{--}19$ G) interactions between the two spins (Table 6.1). The J values of NATriPols in the frozen state are much smaller than those at room temperature possibly due to a change in the conformation equilibrium^[54]. Collectively, these observations demonstrate that the α -position of amino acid linkers is a suitable choice for the structural modification site of TN biradicals while preserving the optimal electron-electron interactions.

Having shown that the electron-electron interactions of TN biradicals remain constant upon substitution at the α -position of the amino acid linkers, room- and low-temperature EPR spectra of NATriPol-4 and NATriPol-5 were recorded and simulated (Fig. S1, Supporting Information), with the aim to check if the linkage of the thiol groups affects the electron-electron interactions of the disulfide conjugates. Once again, both of them have very similar J values of $56\text{--}64$ G at room temperature and $16\text{--}18$ G at ~ 220 K as well as similar dipolar interactions of $5\text{--}6$ G at ~ 220 K (Table 6.1). These values are fully consistent with those from NATriPols 1-3 and TEMTriPol-1. Thus, we could conclude that the “click” reaction of NATriPol-4 with thiols is an efficient approach to synthesize new TN biradicals with tunable physicochemical properties.

Figure 6.1. A) Experimental (black solid line) and simulated (red dotted line) EPR spectra of 300 μM NATriPols in phosphate buffer (20 mM, pH 7.4) at room temperature (Top) or in glycerol/water (v/v, 60/40) at ~ 220 K (Bottom). (B) Comparison between experimentally determined DNP values ($\epsilon_{\text{abs, 10 mM}}$ and the ratio $\epsilon_{\text{abs, 5 mM}} / \epsilon_{\text{abs, 10 mM}}$) for the NATriPols indicated. $\epsilon_{\text{abs, 5 mM}}$ and $\epsilon_{\text{abs, 10 mM}}$ represent the absolute DNP enhancements at biradical concentrations of 5 mM and 10 mM, respectively. (C) EPR spectra of 300 μM (black solid line) and 10 mM (red solid line) NATriPol 2-5 in glycerol/water (v/v, 60/40) at ~ 220 K; EPR signals of each biradical at 300 μM and 10 mM were normalized. (D) Plots correlating the octanol-water partition coefficient (LogP , black diamonds) and the retention times (RT, blue squares) with the ratio $\epsilon_{\text{abs, 5 mM}} / \epsilon_{\text{abs, 10 mM}}$. (A,C) “*” indicates signals from the trityl monoradical impurity.

Dynamic nuclear polarization studies on ^{13}C - ^{15}N proline

The DNP performance of NATriPols in 3.2mm sapphire rotors was examined using a high-field DNP setup (800 MHz/527 GHz). Firstly, ^1H - ^{13}C cross polarization experiments were carried out on frozen solutions of NATriPols in DNP juice (d_8 -glycerol/ $\text{D}_2\text{O}/\text{H}_2\text{O}$, 60/30/10, V/V/V) containing 0.25 M ^{13}C - ^{15}N proline at 103 K. The MAS frequency was set to 8 kHz which was shown to be optimal for TN biradicals (e.g., TEMTriPol-1)^[44]. To assess the DNP performance of the NATriPol biradical polarizing

agents, we computed absolute signal gains ($\epsilon_{\text{abs}} = \epsilon_{\text{on/off}} \cdot \chi_{\text{bleach}} \cdot \chi_{\text{depo}}$), which take into account depolarization and bleaching effects, and the sensitivity gain ($\Sigma = \epsilon_{\text{abs}} / \sqrt{(T_{\text{B}} / T_{\text{off}})}$). The latter parameter represents the sensitivity gain observed when performing DNP as compared to a MAS NMR experiment performed at the same (cryogenic) temperature without DNP, as reported previously^[39, 52]. At 5 and 10 mM biradical concentration, we observed paramagnetic bleaching factors (χ_{bleach}) in the range of 0.85 to 0.90 in good agreement with reported values for TEMTriPol-1^[44]. Similar to TEMTriPol-1, no depolarization was observed for these biradicals.

When comparing absolute DNP enhancement factors (ϵ_{abs}) of NATriPols (Table 6.1), we observed strong variations with values ranging from 9 to 60 for ^{13}C - ^{15}N proline at 10 mM biradical concentration. The absolute signal gains of NATriPol-1 ($\epsilon_{\text{abs}} = 51$), NATriPol-3 ($\epsilon_{\text{abs}} = 60$) and NATriPol-5 ($\epsilon_{\text{abs}} = 50$) were higher than the value found for TEMTriPol-1 ($\epsilon_{\text{abs}} = 43$). It should be noted that under similar conditions, the ϵ_{abs} values of the widely used water-soluble AMUPol and the newly synthesized AsymPolPOK were reported to be 14 and 24^[34], respectively. These results suggest that NATriPol-3 outperforms currently available water-soluble biradicals in terms of the absolute DNP enhancement at 18.8 T.

Table 6.1 Exchange (J) and dipolar (D) interactions at ambient temperature (AT) and low temperature (LT), octanol-water partition coefficient (LogP), HPLC retention time (RT, min) and water solubility (WS, mM) of NATriPols and TEMTriPol-1 as well as experimental DNP parameters of NATriPols and other biradicals in the DNP juice (ds-glycerol/D₂O/H₂O, 60/30/10, V/V/V) containing 0.25 M ^{13}C - ^{15}N proline (unless indicated otherwise) at 18.8 T and 103 K.

Biradicals	J (AT)/G	J (LT)/G	D/G	LogP	RT	WS	$\epsilon_{\text{on/off}}$	ϵ_{abs}	T_{B}	$\Sigma = \epsilon_{\text{abs}} / \sqrt{(T_{\text{B}} / T_{\text{off}})}$
TEMTriPol-1	64	17	6	-0.55	13.38	75	50 (55)	43 (50)	3.7 (8)	198
NATriPol-1	63	18	6	0.19	13.68	174	60 (80)	51 (72)	3.5 (6)	244
NATriPol-2	48	17	5	0.72	14.81	101	40 (60)	36 (54)	7 (21)	122
NATriPol-3	64	18	6	-1.70	11.29	161	70 (65, 58 ^[a])	60 (59, 48 ^[a])	4.8 (8.5, 4 ^[a])	243
NATriPol-4	64	18	6	0.85	14.92	N.D.	10 (20)	9 (18)	2 (4.7)	57
NATriPol-5	61	18	6	-1.85	11.22	190	56 (55, 43 ^[a])	48 (50, 36 ^[a])	4.5 (6, 2.2 ^[a])	213
AMUPol ^[b]							35 ^[b]	19 ^[c]	5 ^[b]	76
AsymPolPOK ^[d]								24	5.8	

The experimental parameters were obtained using 10 mM, 5 mM (in parenthesis) or [a] 15 mM biradical (in parenthesis). The overall sensitivity gain Σ is calculated for a 10mM radical concentration. [b] measured in this work under the same experimental conditions; [c] ϵ_{abs} was estimated based on the reported value of $\chi_{\text{bleach}} \cdot \chi_{\text{depo}}$ (0.54) see the reference 44. [d] see the reference 34. N.D. stands for “not detected”.

Since both dipolar and exchange interactions are almost identical for the NATriPols under investigation, factors other than magnetic interactions are responsible for the strong variation in their experimentally observed DNP performance. Indeed, previous studies suggested that the formation of high local concentration zones of polarizing agents induced by inhomogeneous dispersion in the matrix is detrimental to their DNP properties^[29, 55]. The aggregation of the trityl radical CT-03 which was used to synthesize NATriPols in this work was observed in glycerol/water mixture at low temperature^[56, 57]. Thus, we hypothesized that the self-aggregation of NATriPols driven by their hydrophobicity in the DNP matrix is mainly responsible for the difference in their DNP performances. Assuming that the self-aggregation occurs for NATriPols in the matrix, their DNP enhancements should increase when using lower radical concentrations. Hence, we measured absolute DNP enhancements of NATriPols at 5 mM biradical concentration ($\epsilon_{\text{abs, 5mM}}$) and correlated the ratio of $\epsilon_{\text{abs, 5mM}}/\epsilon_{\text{abs, 10mM}}$ with the absolute enhancements observed at 10 mM concentration. As visible in Fig. 6.1B, the NATriPol variants that exhibit lower absolute enhancements at the 10 mM concentrations have significantly larger values of $\epsilon_{\text{abs, 5mM}}/\epsilon_{\text{abs, 10mM}}$, which we tentatively ascribe to their self-aggregation tendency.

Fig. 6.1B suggests that with the exception of NATriPol-1, the $\epsilon_{\text{abs, 10mM}}$ values of the biradicals are inversely correlated with the ratios of $\epsilon_{\text{abs, 5mM}}/\epsilon_{\text{abs, 10mM}}$, indicating that the self-aggregation is a critical factor for the DNP efficiency of NATriPols. Note that, although NATriPol-1 exhibits a similar and moderate self-aggregation tendency as TEMTriPol-1, it shows a relatively higher $\epsilon_{\text{abs, 10mM}}$ value than the latter. We attribute this effect to the relatively rigid linker of NATriPol-1, which leads to the improved and narrow distribution of the dipolar/exchange interactions. However, multifrequency EPR experiments are needed to address this issue and are outside the scope of this study^[44].

To further verify the influence of self-aggregation of NATriPols, we recorded EPR spectra of NATriPols and TEMTriPol-1 at a high concentration (10 mM) and low temperature ($\sim 220\text{K}$) in DNP buffer. Interestingly, broad EPR single line signals were observed for NATriPol-4 and NATriPol-2 that exhibit the strongest self-aggregation tendency. Conversely, EPR spectral profiles of NATriPol-3 and NATriPol-5 with weak or no self-aggregation still showed well resolved hyperfine splittings, similar to the corresponding spectra obtained at 300 μM (Fig. 6.1C and Fig. S2). We attribute the broad EPR single lines of NATriPol-4 and NATriPol-2 to significant exchange couplings among neighbouring biradicals that result from self-aggregation and lead to a featureless EPR spectrum, as expected for multi-spin arrays where all spins are coupled.

Finally, we investigated factors that induce the self-aggregation of the NATriPols in aqueous solutions. For this purpose, we measured the octanol-water partition

coefficients (LogP) and retention times (RTs) of NATriPols together with TEMTriPol-1 on a reversed-phase HPLC, both of which can be used to quantitatively describe the hydrophobicity of the biradicals. Again, the ratio $\epsilon_{\text{abs, 5mM}}/\epsilon_{\text{abs, 10mM}}$ seems to correlate with both LogP and RT, indicating that the hydrophobicity is mainly responsible for their self-aggregation (Fig. 6.1D). Collectively, these results demonstrate that preventing the self-aggregation of NATriPols by reducing their hydrophobicity is an effective method for improving their DNP properties.

Dynamic nuclear polarization studies on biomolecules

Since some of the newly synthesized NATriPols exhibit excellent DNP enhancements for ^{13}C - ^{15}N proline due to their high hydrophilicity, we examined their potential for applications to complex biomolecules. First, we investigated whether the DNP enhancement seen for proline significantly differs for other amino acids. For this purpose, we tested the DNP performance of the hydrophilic NATriPol-3 and NATriPol-5 on a [^{13}C , ^{15}N] labelled Algal amino-acid mixture containing 16 amino acids and compared our results to DNP experiments using AMUPol. In 1D ^{13}C CP MAS experiments (Table S1, Supporting Information), we observed a DNP enhancement $\epsilon_{\text{on/off}} = 48$ for a sample prepared with 10 mM NATriPol-3. Notably, we measured a weaker enhancement ($\epsilon_{\text{on/off}} = 35$) for NATriPol-5, confirming the higher efficiency of NATriPol-3. Even though a decrease in enhancement ($\epsilon_{\text{on/off}} = 48$) is observed in comparison to standard proline ($\epsilon_{\text{on/off}} = 70$), the enhancements using NATriPol-3 were about 1.6-2 times higher than those seen for AMUPol ($\epsilon_{\text{on/off}} = 30$ for the labelled Algal mixture and $\epsilon_{\text{on/off}} = 35$ for proline, see Table S1). Moreover, considering the depolarization and bleaching effects, NATriPol-3 outperforms AMUPol by a factor 2.5 in terms of the absolute DNP enhancement^[34, 44]. To probe amino-acid specific DNP enhancements, we conducted 2D proton-driven spin diffusion (PDSD) experiments with and without microwave irradiation using a 10 mM NATriPol-3 concentration (Fig. 6.2 and Fig. S4). These data allowed us to separate signal intensities of different types of amino acids including threonine, serine and aspartic acid (Fig. 6.2, left). When comparing 1D slices, we however only observed minor variations in the DNP performance of the different amino acids.

The results shown in Fig. 6.2 suggest that our new NATriPol preparations can provide a significantly improved DNP enhancement compared to the current standard AMUPol.

Figure 6.2. Right panel: aliphatic region of a 2D DNP-enhanced ^{13}C , ^{13}C PDSD spectrum of the Algal amino acid mixture in d_8 -glycerol: D_2O : H_2O (60:30:10 v:v:v) and 10 mM NATriPol-3. Crosses represent chemical-shift predictions for the different amino acids, based on the respective BMRB average shift. Blue arrows (left) refer to 1D slices of isolated peaks (right) of specific amino acids that were used to calculate the relative enhancement factors.

Therefore, we next examined their potential for applications to soluble proteins by testing their performance on ubiquitin, a regulatory protein that was already examined in DNP studies^[58]. It is well known that enhancements measured on biomacromolecules are often reduced when compared to model substances^[5], possibly due to the complexity and internal dynamics of biological samples that affect their relaxation behaviour. Unlike free amino-acid mixtures, proteins can exhibit local hydrophobic pockets that often engage in protein-protein or protein-lipid interactions. For example, the surface of ubiquitin contains a hydrophobic patch (comprising residues L8, I44, and V70) that form a prominent site of molecular recognition^[59]. Thus, the biradicals may exhibit hydrophobic interactions with both the target protein and with other biradical molecules (i.e., self-aggregation). Both interactions are driven by hydrophobicity of the biradicals and are detrimental to their DNP enhancements. To investigate any local interactions between NATriPols and ubiquitin we performed solution-state NMR titration experiments in which we added increasing amounts (0, 0.01 and 0.1 mM) of biradical into a 0.1 mM solution of [^{13}C , ^{15}N] ubiquitin. This allowed us to investigate the potential

interaction between the protein and biradical, which would be detrimental to DNP enhancement. As a control, we prepared samples using TOTAPOL (Fig. S6). In Fig. 6.3, zoom-ins of ^{15}N -HSQC spectra of ubiquitin are shown using TEMTriPol-1, NATriPol-5 and NATriPol-3 at increasing biradical concentrations.

Figure 6.3. Upper panel: zoom-in on the ^{15}N -HSQC spectra of ubiquitin (0.1 mM, blue) in 90/10 $\text{H}_2\text{O}/\text{D}_2\text{O}$ and after a titration with 0.01 mM (purple) and 0.1 mM (orange) biradical concentrations for TEMTriPol-1 (left), NATriPol-5 (middle) and NATriPol-3 (right). The regions exhibiting the biggest chemical shift perturbations (CSPs) are highlighted in dashed boxes. Lower panel: ubiquitin residues are highlighted which showed the strongest chemical-shift perturbations Δcs calculated using $\Delta\text{cs} = \sqrt{\delta_H^2 + (\delta_N/6.51)^2}$

The chemical shift changes evident in the case of TEMTriPol-1 suggest a clear interaction between the biradical and the protein. Residues affected by the biradical involved Ile44 and Val70 that are part of the aforementioned hydrophobic patch of ubiquitin. These findings are in line with our earlier result^[60] that the trityl radical CT-03 is prone to bind to proteins, driven by the hydrophobic interaction. The protein electrostatic properties are likely to be responsible for the high chemical shift perturbations on the charged residues. Moreover, we detected additional paramagnetic effects in flexible parts of the protein (residues 46, 47, 49 and the C-terminal tail residues

71-75) indicating possible binding events and/or proximity to the radical (see figures S7-S9 in SI for the complete analysis of the peak intensities and chemical shift changes). In contrast, such interactions are strongly reduced when using the more hydrophilic NATriPol-5. We observed fewer chemical shift changes in the case of NATriPol-5 except for some signal intensity loss for the mobile residues K48-Q49 and the C-terminal tail residue Arg72, when compared to pure ubiquitin. Ultimately, only a marginal interaction with the protein was detected when using NATriPol-3 (Fig. 6.3, right column), similar to results obtained using TOTAPOL (Fig. S6). This result can be rationalized by the difference of their hydrophobicity (Figure 6.1D and Table 6.1). Having established on an atomic level that NATriPol-3 and NATriPol-5 reveal reduced hydrophobic interactions with [^{13}C , ^{15}N] ubiquitin in solution in comparison to TEMTriPol-1, we conducted DNP experiments on both compounds.

Figure 6.4. Left: 1D DNP-enhanced ^1H - ^{13}C CP spectra of ubiquitin (4 mM) in d_8 -glycerol: D_2O : H_2O (60:30:10 v:v:v) and 10 mM biradical concentration. Three different biradicals were tested: TEMTriPol-1 (red), NATriPol-5 (in blue) and NATriPol-3 (purple). Right: the aliphatic region of the 2D ^{13}C , ^{13}C correlated PDS experiment measuring ubiquitin with 10 mM NATriPol-3. Black crosses indicate NMR assignments (BMRB IDs 7111 & 15410).

We compared our results to those of TEMTriPol-1 and AMUPol (see Table S1). We observed that TEMTriPol-1 performs very poorly when measured on the ^{13}C , ^{15}N labelled protein, with an enhancement ($\epsilon_{\text{on/off}}$) of only 3 and a relatively short DNP build-up time ($T_B = 800$ ms) at a 10 mM biradical concentration (Fig. 6.4). The short DNP build-up time suggests close proximity between the biradical and the protein^[33], further confirming our NMR titration experiments. On the other hand, NATriPol-5 showed a superior DNP enhancement ($\epsilon_{\text{on/off}} = 15$, $T_B = 1.6$ s) for ubiquitin. The DNP signal increase achieved by NATriPol-3 was even higher, with an enhancement of 30 ($T_B =$

2.5s) (Fig. 6.4), which is 10 times larger than TEMTriPol-1 and comparable to what we observed using AMUPol ($\epsilon_{\text{on/off}} = 30$, Table S1). Considering that NATriPol-3 has a much higher contribution factor (which we define as $\theta = \chi_{\text{bleach}} \cdot \chi_{\text{depo}} = 0.85$) than AMUPol (0.54), the absolute DNP enhancement of NATriPol-3 for ubiquitin is 1.6 fold higher than for AMUPol. Moreover, a two-dimensional PDS spectrum (Fig. 6.4 right, and Fig. S10) confirmed our earlier observations^[20, 24] that conducting ssNMR experiments at 800 MHz can improve spectral resolution compared to data obtained at 400 MHz^[58] (see Fig. 6.5). In addition, the observed 2D correlation pattern was in good agreement with the NMR assignments (indicated black crosses in Fig. 6.4), indicating that our preparations contained properly folded ubiquitin. Taken together, both self-aggregation behaviour and the tendency of TEMTriPol-1 to localize to hydrophobic protein residues contribute to its markedly low DNP efficiency. Both effects are reduced for the more hydrophilic NATriPol-3 and NATriPol-5, resulting in significantly higher DNP enhancements in a biomolecular context. Therefore, these findings suggest a direct relationship between hydrophilicity and DNP enhancement when using NATriPols for biological applications.

Figure 6.5. Comparison of 400 and 800 MHz DNP 2D PDS spectra obtained on $[^{13}\text{C}, ^{15}\text{N}]$ -ubiquitin. Data were recorded at 400 (blue, AMUPol) and 800 MHz (purple, NATriPol-3). Isoleucine Cb–Ca, Proline Cd1–Ca, Serine Cb–Ca and threonine Cb–Ca regions are shown, together with 1D projections (as defined in topospin) of the Ca peaks in the direct dimension on the right.

DNP studies on the membrane targeting peptide Nisin

Finally, we tested our new biradical NATriPol-3 on the pore-forming membrane peptide Nisin^[61]. DNP is particularly crucial for the study of membrane proteins and membrane embedded peptides (see the representative references^[6, 10, 11]). As reported by Hong et al.^[62], the structural and magnetic properties of the polarizing agents and their distribution in the membranes strongly influence the DNP enhancement in a lipid environment. To better rationalize the performance of our new biradicals, we determined the DNP enhancement for the ^{13}C signals of lipids and the (isotope-labelled) Nisin peptide alongside with the respective build-up times.

In Figure 6.6, we compare spectra of the lipid II-bound state of ^{13}C -labelled Nisin in DOPC liposomes prepared using published procedures^[61] and employing a 15mM AMUPol concentration (Fig. 6.6A) as well as a 10mM NATriPol-3 concentration (Fig. 6.6B), respectively. In line with previous work^[61], we found a moderate and uniform enhancement ($\epsilon_{\text{on/off, AMU}} \approx 8$) using AMUPol. Instead, we observed a higher enhancement of the lipid signals ($\epsilon_{\text{on/off, NAT-3}} \approx 22$), as well as for peptide signals ($\epsilon_{\text{on/off, NAT-3}} \approx 11$) in the case of NATriPol-3. When taking into account the faster build-up time and the lower depolarization factor, NATriPol-3 provides an improvement of a factor 2.4 on the peptide signal compared to AMUPol. The short build-up times measured in both samples suggest proper mixing of the polarizing agents in this system.

Figure 6.6. (A): 1D DNP-enhanced ^1H - ^{13}C CP spectra of the lipid II-bound state of ^{13}C -labelled nisin in DOPC liposomes, with 15 mM AMUPol. (B): 1D DNP-enhanced ^1H - ^{13}C CP spectra of the lipid II-bound state of ^{13}C -labelled nisin in DOPC liposomes, with 10 mM NATriPol-3. Yellow and orange boxes indicate NMR frequencies in which the observed signal is dominated by lipid and peptide contributions, respectively. (See Fig. S5 in the SI for corresponding 1D ^{13}C double-quantum spectra.)

Remarkably, lipid signals are further enhanced in Figure 6.6B, which may be related to the slightly higher hydrophobicity of the TN biradical in comparison to the bis-nitroxide, that favours interactions between the TN biradical and the lipid bilayer. Previous studies on nitroxide radicals in the presence of phospholipid membranes suggest that the physical location of the radical and especially the g-tensors alignment are critical factors for the DNP enhancement^[62]. On one hand these studies demonstrate that introducing polarizing agents in the hydrophobic core of the lipid bilayer can diminish the enhancement gradient typically observed across the membrane^[63]. On the other hand, they show that the localisation of the radicals within the membrane can also be deleterious to the DNP enhancement^[64]. Hence, a more systematic study of the performance of TN biradicals for the investigation of membrane proteins and polypeptides will be required in the future. However, the current results already suggest the beneficial use of the new class of biradicals together with the improved resolution achieved at high fields for ssNMR studies on complex biomolecules including membrane proteins.

Conclusions

Ultra-high field DNP offers great potential to combine the benefits of strongly enhanced DNP signals with increased spectral resolution. Because of their favourable magnetic-field dependence, trityl-nitroxide (TN, aka TEMTriPols) mixed biradicals are ideal candidates for such studies but their practical application to biomolecules was thus far limited. We have synthesized NATriPols, structural variants of TEMTriPol-1, with various substituents at the α -position of the amino acid linkers. NATriPol-4 could also be covalently attached to the protein of interest or lipid by thiol-specific labelling, providing several potential advantages over exogenously added polarizing agents^[63, 65, 66]. NATriPols have almost identical dipolar and exchange interactions but strongly differ in hydrophobicity. We found that concentration-dependent DNP studies provide an indicator for the self-aggregation tendency, which mainly depends on the compound hydrophobicity. Next, we showed the importance of testing the polarizing agents in biological applications, to obtain a more complete picture of the radical efficiency. The experiments on ^{13}C - ^{15}N labelled amino-acid mixture and ubiquitin confirm that hydrophilic effects strongly dictate the performance of NATriPols in a biomolecular context. The optimized hydrophilic NATriPol-3 exhibits a 10-fold DNP improvement compared to TEMTriPol-1 when applied to the globular protein and shows the highest absolute DNP enhancements thus far obtained at 18.8 T in a biological context. These findings along with our results on a membrane peptide represent the first step towards a better understanding of this new class of biradicals and provide novel routes for optimization of high-field polarizing agents for biomolecular applications.

Supporting Information

I. EPR Spectroscopy and spectral simulation

EPR measurements were carried out on a Bruker EMX-plus X-band spectrometer at room temperature (298 K) or low temperature (~ 220 K). General instrumental settings were as follows: modulation frequency, 100 kHz; microwave power, 10 mW; modulation amplitude, 1 G (room temperature) and 2 G (low temperature). Measurements were performed in 50 μ L capillary tubes. Spectral simulation was performed by the program developed by Professor Rockenbauer^[43]. The exchange, dipolar and hyperfine couplings given in Gauss units can be converted to cm^{-1} by multiplying with $4.6686 \times 10^{-5} \times g$, where g is the respective Zeeman factor.

The EPR spectra recorded in phosphate buffer (20 mM, pH 7.4) at room temperature or in glycerol/water (v/v, 60/40) at ~ 220 K were simulated by EPR/ROKI program and DNP/ROKI program, respectively. EPR/ROKI program could calculate a reliable exchange interaction of TN biradicals. As for the DNP/ROKI program, magnetic resonance parameters including the principal values of the two g - and hyperfine tensors, the Euler angles between the principal directions of tensors, the polar angles of linker, exchange and dipolar interactions can be optimized to achieve the best fit of the experimental spectra.

II. Solution-State NMR Experiments

The samples for the solution-state NMR titration experiments were prepared by dissolving 1 mg of lyophilized [^{13}C , ^{15}N] ubiquitin in 90/10 $\text{H}_2\text{O}/\text{D}_2\text{O}$ solvent, for a final concentration of 0.1 mM. Increasing amounts of biradical were then subsequently added to the sample, for final radical concentrations of 0, 0.01 and 0.1 mM.

^{15}N - ^1H HSQC spectra^[67] were acquired at 298 K with a triple channel (^1H , ^{13}C , ^{15}N) cryogenically cooled-probe, at a static magnetic field of 14 T, corresponding to a proton frequency of 600 MHz with 16 scans with a delay of 1 s. Acquisition times were 66 ms and 26 for the ^1H and ^{15}N dimensions, respectively. The spectra were processed using a 0.5π sine squared window function in both dimensions.

III. Measurements of water solubility

Excess of biradical (carboxylate sodium form) was added as a solid to water. The resulting suspension was centrifuged and the supernatant fraction was separated. Then, after appropriate dilution with water, the concentration of the supernatant (i.e., the water solubility of the biradical) was estimated by UV-Vis spectroscopy according to the pre-determined molar absorption coefficient ($16.8 \text{ mM}^{-1} \text{ cm}^{-1}$) at 464 nm, assuming that this type of biradicals exhibit the same molar absorption coefficients.

IV. Determination of LogP

The aqueous solution of the biradical (100 μL , 200 μM) was mixed with octanol (100 μL) and the resulting solution was gently shaken for 24 h. Then, the aqueous fraction was separated and the concentration of the biradical in this fraction was determined by EPR double integration. Accordingly, the concentration of the biradical in the octanol layer was calculated. Finally, the octanol-water partition coefficient (LogP) of the biradical was estimated according to its concentrations in the two fractions.

V. EPR measurements

Figure S1. Experimental and simulated EPR spectra. Experimental (black solid lines) and simulated (red dotted lines) EPR spectra of 300 μM TEMTriPol-1 and NATriPols (A) in phosphate buffer (20 mM, pH 7.4) at room temperature and (B) in glycerol/water (v/v, 60/40) at ~220K.

Figure S2. EPR spectra of biradicals at different concentrations. EPR spectra of TEMTriPol-1 and NATriPols at a concentration of 300 μM (black solid lines) or 10 mM (red solid lines) in glycerol/water (v/v, 60/40) at $\sim 220\text{K}$. “*” indicates signals from the trityl monoradical impurity.

VI. DNP enhancements

Table S1. DNP enhancements. DNP enhancements ($\epsilon_{\text{on/off}}$) of NATriPol-5 and NATriPol-3 for the different samples, with a comparison to AMUPol. All samples were prepared in d_8 -glycerol: D_2O : H_2O (60:30:10 v: v: v) and measured at 103 K.

	$[^{13}C, ^{15}N]$ Proline	$[^{13}C, ^{15}N]$ Algal amino-acid mixture	$[^{13}C, ^{15}N]$ Ubiquitin
NATriPol-5 (10mM)	56	35	15
NATriPol-3 (10mM)	70	48	30
AMUPol (15mM)	35	30	30

VII. Magnetic field-dependence of the Cross-effect 1H DNP enhancement.

Figure S3. Normalized CE DNP enhancement field profile at 800 MHz for a) TEMTriPol-1, b) NATriPol-1 and c) NATriPol-5. Dashed lines indicate the x-axis at $\epsilon=0$.

The radicals exhibit very similar field profile, with the characteristic narrow line associated with the trityl moiety (the TEMTriPol-1 showing the same profile as it was previously reported in the literature)^[37]. NATriPol-1 and -5 reach their maximum enhancement at the same field position (which is identical to the TEMTriPol-1) and this field was used to calculate the maximum DNP enhancement for all NATriPols, since we do not expect a strong deviation within the NATriPol family.

VIII. 2D- ^{13}C , ^{13}C PDS spectra of a U[^{13}C , ^{15}N] Algal mixture

Figure S4. Crosses represent chemical-shift predictions for the different amino acids, based on the respective BMRB average shift. For the spectral analysis, the NMRFAM-SPARKY software package was used^[68].

IX. 1D ^{13}C -2Q1Q spectra of ^{13}C -Nisin in DOPC membranes

Figure S5. On the left, 1D DNP-enhanced ^{13}C double-quantum spectra of the lipid II-bound state of ^{13}C -labelled nisin in DOPC liposomes, with 15 mM AMUPol. On the right, 1D DNP-enhanced ^1H - ^{13}C CP spectra of the lipid II-bound state of ^{13}C -labelled nisin in DOPC liposomes, with 10 mM NATriPol-3. The on/off enhancement is reported.

X. ^{15}N -HSQC spectra of U[^{13}C , ^{15}N] ubiquitin

Figure S6. ^{15}N -HSQC spectra of ubiquitin (0.1 mM, blue) in 90/10 $\text{H}_2\text{O}/\text{D}_2\text{O}$ and after a titration with 0.01 mM (purple) and 0.1 mM (orange) biradical concentrations for TEMTriPol-1 (top right), NATriPol-5 (bottom left) and NATriPol-3 (bottom right), in comparison to a titration with TOTAPOL (top left). The sidechain amide groups of Asn and Gln are indicated with dashed lines. The sidechain amide groups of Arg are indicated with dashed circles. Backbone assignments were taken from BMRB ID: 15410.

XI. Analysis of peak intensities and CSPs

Figure S7. Analysis of peak intensities and CSPs – TEMTriPol-1. Intensity ratios and chemical shift perturbations (CSPs) per residue comparing ubiquitin prior and after the addition of the biradical at the highest concentration, 0.1mM. The mean (dashed green line) plus one (orange line) or two (red line) standard deviations are indicated. The typical error in the peak intensity and position was used to calculate the error in the intensity ratio and CSP using standard error-propagation techniques. The weighted CSPs were calculated according to $\Delta cs = \sqrt{\delta_H^2 + (\delta_N/6.51)^2}$. Backbone assignments were taken from BMRB ID: 15410.

Important drops in peak intensity (>60%) are evident in the case of TEMTriPol-1 for the regions including residues F45-Q49 and L71-G75. Moreover, in the same regions some residues present high CSP (up to 0.1ppm, so 4x times than the max CSP registered in the presence of TOTAPOL), clearly indicating a higher degree of interaction between the radical and the protein.

Figure S8. Analysis of peak intensities and CSPs – NATriPol-5. Intensity ratios and chemical shift perturbations (CSPs) per residue comparing ubiquitin prior and after the addition of the biradical at the highest concentration, 0.1mM. The mean (dashed green line) plus one (orange line) or two (red line) standard deviations are indicated. The typical error in the peak intensity and position was used to calculate the error in the intensity ratio and CSP using standard error-propagation techniques. The weighted CSPs were calculated according to $\Delta cs = \sqrt{\delta_H^2 + (\delta_N/6.51)^2}$. Backbone assignments were taken from BMRB ID: 15410.

Important drops in peak intensity are evident also in the case of NATriPol-5 for the regions including residues F45-Q49 and V70-G75, therefore indicating an interaction between the radical and the protein. However, the average and the maximum measured CSPs are halved when compared to TEMTriPol-1.

Figure S9. Analysis of peak intensities and CSPs – NATriPol-3. Intensity ratios and chemical shift perturbations (CSPs) per residue comparing ubiquitin prior and after the addition of the biradical at the highest concentration, 0.1mM. The mean (dashed green line) plus one (orange line) or two (red line) standard deviations are indicated. The typical error in the peak intensity and position was used to calculate the error in the intensity ratio and CSP using standard error-propagation techniques. The weighted CSPs were calculated according to $\Delta cs = \sqrt{\delta_H^2 + (\delta_N/6.51)^2}$. Backbone assignments were taken from BMRB ID: 15410.

The same regions still show intensity drops, even if fewer residues are involved compared to the other trityl-nitroxides. Moreover, the weighted CSPs strongly agree with the values reported for the TOTAPOL radical.

XII. 2D- ^{13}C , ^{13}C PDSB spectra of a $\text{U}[^{13}\text{C}, ^{15}\text{N}]$ ubiquitin

Figure S10. 2D- ^{13}C , ^{13}C PDSB spectra ubiquitin. Black crosses indicate NMR assignments (BMRB IDs 7111 & 15410).

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CHAPTER 7

Summary and Outlook

Summary

Nuclear Magnetic Resonance (NMR) Spectroscopy provides a unique window into the atomic world, revealing information on the structures and dynamics of molecules without altering their properties. However, the intrinsically low sensitivity of NMR imposes significant challenges for its application. Thus, NMR has greatly benefited from the advent of sensitivity enhanced methods. One such method is Dynamic Nuclear Polarization (DNP), a technique specifically used to enhance the signal in solid- and liquid-state NMR as well as MRI. The extension to Magic Angle Spinning (MAS) DNP has made the approach widely applicable to problems in a variety of fields, including structural biology, biophysics, and chemistry.

In this thesis, we aimed to contribute to the further advancement of the DNP technique. These contributions include methodological developments and novel applications of DNP-enhanced solid-state NMR. High-sensitivity NMR approaches are employed on a variety of systems to answer specific questions in life and material science; from *in-cell* structural studies, where target molecules are probed directly in their natural setting at atomic level; to zeolite-based catalytic systems, where the distinctive host-guest chemistry between the zeolite and trapped organics during catalysis is investigated. Moreover, strategies to further improve sensitivity at high magnetic fields are described and new biradical polarizing agents are presented, along with an investigation on their potential for biomolecular applications.

The main results of the thesis are further discussed in this concluding chapter, along with a glance into the possible exciting future directions of Dynamic Nuclear Polarization.

Chapter 1 introduces and reviews key aspects of solid-state NMR and provides a description of the DNP mechanism.

The benefits of applying DNP for the study of systems in which sensitivity typically plays a dominant role are shown in **Chapter 2**. In particular, the successful development of an innovative approach to study soluble proteins in cells is explained. NMR can play a decisive role in providing atomistic details in complex cellular settings and one of the major goals of cellular NMR is the investigation of structure and functions of biomolecules within their native environment, which can strongly affect their behaviour. However, such studies are often impeded by the extremely low sensitivity, due to the low concentration of the molecule of interest in the cellular environment. We addressed this issue by employing DNP and demonstrated our method by studying the Ubiquitin protein in mammalian and bacterial cells. In both cases DNP agents were introduced into the cells by rapid diffusion immediately prior to the ssNMR measurements.

This strategy led to surprising gains in signal enhancement, allowing the acquisition of multi-dimensional experiments. The DNP-ssNMR approach was first implemented on a eukaryotic system, where we could detect ubiquitin at endogenous levels in human cells. Moreover, the homogeneous distribution of DNP radicals in the nuclear and cytoplasmatic compartments was confirmed using confocal microscopy, which explains the remarkable DNP enhancements. Subsequently, the method was shown to be compatible with bacterial cells. In this case, a targeted labelling approach is crucial to differentiate between the protein of interest and the large amounts of diverse cellular background components.

Additionally, important parameters for optimizing DNP enhancements were investigated, with a particular focus on the reduction of sample protonation by selective deuteration. Deuteration of the cellular background is found to lead to a further increase in signal sensitivity along with an improved selectivity towards the (protonated) target molecule.

The large DNP enhancement factors enabled multi-dimensional NMR experiments which are essential for increasing the spectral resolution. Overall, our approach based on the combination of DNP and cellular solid-state NMR allows for the study of proteins in their natural cellular environment.

In **Chapters 3 and 4**, ssNMR and DNP were applied for the investigation of zeolite-based catalytic systems. The non-invasiveness of NMR Spectroscopy makes it an ideal candidate for examining the reaction mechanisms in heterogeneous catalysis and elucidate molecular structures of the reaction products. A ssNMR approach was developed and used to characterize the materials and identify the species trapped within the post-reacted catalysts. In particular, different multi-dimensional solid-state NMR correlation experiments were adopted, with the aim of spectrally resolving the zeolite-trapped species on the basis of their mobility. By applying through-bond or through-space magnetization transfer schemes, it was possible to distinguish between species with a high or low mobility, respectively. Moreover, the high sensitivity of NMR chemical shifts to the chemical surroundings allowed us to observe moieties in different conformations and/or molecular environments. The results provide important insights into the distinctive host-guest chemistry between the zeolite and trapped organics during catalysis, in terms of their surface mobility, reactivity, and products' distribution. In addition, DNP experiments were performed on these materials. The porous samples were impregnated with a solution containing the DNP agents which induce the polarization of protons of the solvent and neighbouring surface regions. The enhanced polarization is spread by ^1H spin diffusion and then transferred to the target nuclei by cross-polarization. A peculiarity of performing DNP on these systems is the characteristic polarization gradient observed within the material, which can report on

the spatial distribution of the DNP-enhanced species. **Chapter 3** studies the structure-reactivity relationship between the channel topology of the ZSM-5 zeolite and the methanol-to-hydrocarbons (MTH) reaction pathways. Solid-state NMR and DNP experiments revealed that subtle differences in the internal pore structure of a zeolite material can drastically alter the reaction intermediates formed. Sinusoidal channels are found to favour the olefin cycle, while the extended straight channels of zeolite ZSM-5 promote the aromatic cycle with a higher degree of alkylation of aromatics. These findings provide spectroscopic evidence of the effect of zeolite channel geometry on reaction intermediates. In **Chapter 4**, dedicated solid-state NMR techniques in combination with DNP-supported solid-state NMR were used for the molecular elucidation of the ethanol-to-hydrocarbon (ETH) reaction mechanism. The identification of a diverse set of reaction products and intermediates allowed for the determination of the reaction and deactivation pathways of the ETH process. Furthermore, by applying classical diffusion laws, a correlation between the measured DNP enhancements and the distribution of the reaction products inside the zeolite particles could be postulated, which suggests that a specific spatial location is adopted by the zeolite-trapped aromatic species. The knowledge established from reaction mechanism and structure-reactivity relationship studies in the heterogeneous catalysis will not only be useful for the development of superior porous materials but also contribute to the understanding of the zeolite-catalysed hydrocarbon conversion chemistry.

The last two chapters focus on the development of biomolecular DNP-ssNMR at higher magnetic fields from a theoretical and experimental point of view. One major limitation of high-field DNP is the poor performance of currently employed biradicals at increasing magnetic field strength. Therefore, there is a great need to develop new water-soluble biradicals with better DNP performance at high fields so the benefits of strongly enhanced DNP signals can be combined with the increased spectral resolution obtained at high magnetic fields.

All DNP experiments require a radical as a source of polarization and the radical properties ultimately determine the efficiency of the DNP mechanism. Particularly under MAS-DNP, the cross-effect (CE) is the prevalent mechanism, which requires the use of biradicals. Recent experimental work and numerical studies have improved our understanding of the phenomenon and resulted in the introduction of new radicals. For example, mixed trityl-nitroxide (TN) radicals are promising candidates for high-field DNP, where the small g -anisotropy of the narrow line trityl species allows for a better saturation of the EPR line. **Chapter 5** presents quantum mechanical simulations for a three-spin system, specifically investigating the CE-DNP performance of trityl-nitroxide biradicals. Our results are consistent with previous work and show that the presence of

the electronic J-coupling can significantly enhance the DNP efficiency, especially at high MAS frequency. Moreover, since Cross Effect DNP relies on both dipolar and J-coupling interactions between the electron spins of the biradical, the effect of both parameters was investigated. The findings indicated that an optimal balance between the electron J-coupling and dipolar interactions must be achieved in order to maximize the DNP efficiency, without interfering with the matching condition required for cross-effect DNP. Additional numerical simulations were employed to explain the measured DNP enhancements for the novel trityl-nitroxide compounds investigated. These MAS-DNP simulations are able to reproduce the relative DNP enhancements observed for the TN radicals under investigation, for which the EPR parameters were known. Combining the experimental results with the simulations suggested that weak J-coupling and dipolar interactions represented the main reason for the comparatively low DNP enhancement measured for the worst performing radical. Next, with the aim to improve the water solubility, a new series of amino acid-linked trityl-nitroxide biradicals (referred to as NATriPols) has been synthesized. **Chapter 6** presents the results obtained for these compounds on model systems and, for the first time, their practical applications to biomolecules. These new TN polarizing agents have almost identical dipolar and exchange interactions but strongly differ in hydrophobicity. The electron-electron magnetic interactions of NATriPols are shown to be independent of the substituents at the α -position of the amino-acid linkers. Nonetheless, a large variation was seen in their experimentally observed DNP performance in a model sample. The results obtained at different biradical concentrations, in combination with EPR data, revealed that DNP enhancements of these biradicals are inversely correlated with their hydrophobicity. Thus, the self-aggregation of NATriPols driven by their hydrophobicity in the DNP matrix is mainly responsible for the difference in their DNP efficiency. Moreover, for the first time, the TN radicals' performance was tested in a biomolecular context. To examine their potential for biological applications, DNP experiments were performed using [^{13}C -, ^{15}N]-labelled amino-acid mixture and [^{13}C -, ^{15}N]-Ubiquitin. In addition, the efficiency of this novel family of radicals is further demonstrated on the membrane-associated peptide Nisin. Our findings confirm that hydrophilic effects strongly dictate the performance of NATriPols and demonstrate their significantly improved DNP efficiency compared to the currently available radicals. The optimized hydrophilic NATriPol-3 exhibited a 10-fold DNP improvement measured on Ubiquitin when compared to the best known TN polarizing agent and showed the highest absolute DNP enhancements thus far obtained at 18.8 T in a biological context. These findings represent the first steps towards a better understanding of this new class of biradicals and pave the way for further optimization of new polarizing agents for biomolecular applications at high magnetic fields.

Outlook

Since the commercialization of MAS DNP instrumentation around 2009, the interdisciplinary DNP community has grown rapidly, continuously pioneering new developments and paving the way for the popularity of MAS DNP as seen today. Due to continuous efforts such as the development of advanced instrumentation, tailored polarizing agents, and optimized sample preparation techniques, DNP has achieved a much broader applicability over the past years. The outstanding gains in sensitivity when using DNP have enabled the investigation of materials that would have otherwise been impossible. At present, DNP enhancement factors of $\epsilon \sim 100$ are readily achieved. This is still far from the theoretical maximum enhancement of $\gamma_e/\gamma_{1H} \sim 660$, but even this “modest” enhancement translates into a reduction of the experimental acquisition time by a factor of 10^4 . Thus, an experiment that would otherwise require ~ 30 years of signal averaging can be obtained in a single day. This has led to a tremendous increase in reported state-of-the-art DNP applications to various problems in material science and biomolecular solid-state NMR.

Still, the theoretical understanding of the underlying quantum-mechanical and chemical processes driving the DNP mechanism is constantly improving and further developments are to be expected in the next few years. As mentioned, further development of new chemical agents to more effectively transfer magnetization of electrons to nuclei at higher fields are ongoing. As resolution and sensitivity in NMR spectroscopy increase with field strength, these advances will open up possibilities to investigate yet larger and more complex chemical structures in life and material science. For instance, in-cell DNP studies will greatly benefit from the increased resolution, allowing the investigation of even larger complexes in their natural setting. Such studies will also improve our understanding of the influence of the local cellular environment on complex cellular processes. Moreover, the ongoing developments in radical synthesis allow the design of targeted DNP polarization agents such as biradical spin-labels, which can enable more efficient transfer of polarization towards the protein of interest^[1, 2]. Molecule-specific DNP enhancement could be obtained in a heterogeneous biological environment by tagging the DNP radical directly to the protein of interest^[2, 3]. Additionally, the use of spin-labels has already been shown in the field of in-cell EPR spectroscopy^[4, 5]. In a similar vein, the use of (caged) DNP radical tagged to the labelled protein under study could be implemented for in-cell DNP-NMR applications. With such advancements, atomic scale investigations of target molecules at endogenous concentrations in the crowded cellular background can be envisaged.

Another aspect of DNP that is likely to drive important changes in the future is the low temperature currently required to perform the DNP experiments. Under the present

conditions necessary for DNP, the lack of motion at such low temperatures often leads to broad ssNMR signals, thereby complicating the spectral analysis. However, efforts are currently underway to develop new instrumentation in DNP that will overcome this problem. Promising developments of high-power pulsed microwave sources at high-frequency are expected to extend the current limitations on the external magnetic fields faced by DNP and several innovative DNP methods could become available that will remove the requirement of low temperatures. Contemporary MAS-DNP exclusively relies on continuous-wave (CW) irradiation of the electron spin resonance and all CW-DNP mechanisms necessitate the correct relaxation times to function properly. This limits the DNP process efficiency, especially at higher magnetic fields and non-cryogenic temperatures. Pulse-shaped microwave irradiation of the electron spins is predicted to overcome these challenges, since these pulsed DNP schemes potentially eliminate the unfavourable field dependence of classical DNP mechanisms.

New approaches for DNP are still in the development phase including time-domain DNP using the NOVEL (nuclear spin orientation via electron spin locking) schemes^[6-10], frequency-swept techniques^[11-13], electron decoupling^[14, 15], or pulse-shaped microwave irradiation using arbitrary-waveform generation (AWG) compatible with MAS-DNP^[16]. As the instrumentation for these novel approaches will, most likely, soon become available, we expect a new wave of exciting DNP research to follow.

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Samenvatting

Nucleaire Magnetische Resonantie (NMR) spectroscopie biedt een unieke kijk op de atomische wereld en onthult informatie over de structuren en dynamica van moleculen zonder hun eigenschappen te veranderen. De intrinsiek lage gevoeligheid van NMR legt de toepassing ervan echter op voor grote uitdagingen. NMR heeft dus enorm geprofiteerd van de opkomst van gevoeligheid verbeterende methoden. Een dergelijke methode is Dynamische Nucleaire Polarisation (DNP), een techniek die specifiek wordt gebruikt om signaal te versterken in vaste stof- en vloeistof NMR evenals MRI. De uitbreiding naar Magic Angle Spinning (MAS)-DNP heeft de aanpak breed toepasbaar gemaakt voor problemen op verschillende gebieden, waaronder structurele biologie, biofysica en scheikunde. In dit proefschrift wilden we bijdragen aan de verdere ontwikkeling van de DNP techniek. Deze bijdragen omvatten methodologische ontwikkelingen en nieuwe toepassingen van DNP-verbeterde vaste stof-NMR (vsNMR). Zeer gevoelige NMR-toepassingen worden op verschillende systemen gebruikt om specifieke vraagstukken te beantwoorden in levens- en materiaalwetenschappen; variërend van in-cel structurele studies, waarbij doelmoleculen rechtstreeks in hun natuurlijke omgeving op atomair niveau worden onderzocht; tot katalytische systemen in zeolieten, waar de onderscheidende gastheer-gast chemie tussen de zeoliet en ingesloten organische stoffen tijdens de katalyse wordt onderzocht. Bovendien worden strategieën beschreven om de gevoeligheid bij hoge magnetische velden verder te verbeteren en worden er nieuwe biradicalaire polariserende stoffen, samen met een onderzoek naar hun potentie voor als biomoleculaire toepassingen, gepresenteerd. De belangrijkste resultaten van het proefschrift worden verder besproken in dit afsluitende hoofdstuk met tevens een blik op de mogelijke spannende toekomstige richtingen van Dynamische Nucleaire Polarisation.

Hoofdstuk 1 introduceert en beoordeelt de belangrijkste aspecten van vaste stof-NMR en geeft een beschrijving van het DNP-mechanisme.

De voordelen van het toepassen van DNP voor de studie van systemen waarin gevoeligheid doorgaans een overheersende rol speelt, worden getoond in **Hoofdstuk 2**. In het bijzonder wordt de succesvolle ontwikkeling van een innovatieve aanpak voor het bestuderen van oplosbare eiwitten in cellen uitgelegd. NMR kan een doorslaggevende rol spelen bij het verschaffen van atomistische details in complexe cellulaire omgevingen. Een van de belangrijkste doelen van cellulaire NMR is het onderzoeken van de structuur en functies van biomoleculen binnen hun natuurlijke omgeving, die hun gedrag sterk kan beïnvloeden. Dergelijke studies worden echter vaak belemmerd door de extreem lage gevoeligheid, vanwege de lage concentratie van het molecuul van belang in de

cellulaire omgeving. We hebben dit probleem aangepakt door DNP te gebruiken en demonstreerden onze methode door het eiwit Ubiquitine in zowel zoogdierlijke- als bacteriële cellen te bestuderen. In beide gevallen werden DNP-middelen onmiddellijk voorafgaan de vsNMR metingen geïntroduceerd door snelle diffusie. Deze strategie leidde tot verrassende verbeteringen in signaalverbetering, waardoor multidimensionale experimenten konden worden verworven. De DNP-vsNMR-aanpak was eerst geïmplementeerd op een eukaryotisch systeem, waar we ubiquitine konden detecteren op endogene niveaus in menselijke cellen. Bovendien werd de homogene verdeling van DNP-radicalen in de nucleaire en cytoplasmatische compartimenten bevestigd met confocale microscopie, wat de opmerkelijke DNP-verbeteringen verklaart. Vervolgens bleek de methode compatibel met bacteriële cellen. In dit geval is een gerichte labelingsaanpak cruciaal om onderscheid te kunnen maken tussen het eiwit van interesse en de grote hoeveelheden diverse cellulaire achtergrondcomponenten. Daarnaast werden belangrijke parameters voor het optimaliseren van DNP-verbeteringen onderzocht, met bijzondere aandacht voor de vermindering van protonatie van monsters door selectieve deuteratie. Gebleken is dat deuteratie van de cellulaire achtergrond leidt tot een verdere toename van de signaalgevoeligheid samen met een verbeterde selectiviteit naar het (geprotoneerde) molecuul van interesse. De grote DNP-verbeteringsfactoren maakten multidimensionale NMR experimenten mogelijk, die essentieel zijn voor het verhogen van de spectrale resolutie. Over het algemeen, maakte onze aanpak, gebaseerd op de combinatie van DNP en cellulaire vaste stof-NMR, de studie van eiwitten in hun natuurlijke cellulaire omgeving mogelijk.

In **Hoofdstukken 3 en 4**, werden vsNMR en DNP toegepast voor het onderzoeken van katalytische systemen in zeolieten. De niet-invasieve aard van NMR spectroscopie maakt het een ideale kandidaat voor het onderzoeken van de reactiemechanismen bij heterogene katalyse en het ophelderen van moleculaire structuren van de reactieproducten. Er werd een vsNMR aanpak ontwikkeld en gebruikt om de materialen te karakteriseren en stoffen te identificeren die opgesloten zaten in de katalysatoren na afloop van de reactie. In het bijzonder werden verschillende multidimensionale vaste stof-NMR-correlatie experimenten gebruikt, met als doel de stoffen die opgesloten zaten in de zeoliet spectraal op te lossen op basis van hun mobiliteit. Door het toepassen van door-binding of door-ruimte magnetisatieoverdrachtschema's was het mogelijk om onderscheid te maken tussen stoffen met een respectievelijk hoge of lage mobiliteit. Bovendien stelde de hoge gevoeligheid van NMR chemische verschuivingen naar de chemische omgeving ons in staat om groepen in verschillende conformaties en/of moleculaire omgevingen te observeren. De resultaten geven belangrijke inzichten in de onderscheidende chemie van gastheer-gast tussen de zeoliet en de ingesloten organische stoffen tijdens katalyse in hun oppervlaktemobiliteit, reactiviteit en distributie van

producten. Daarnaast zijn DNP-experimenten op deze materialen uitgevoerd. De poreuze monsters werden geïmpregneerd met een oplossing die DNP-middelen bevat die de polarisatie van protonen van de oplossing en aangrenzende oppervlaktegebieden induceren. De verbeterde polarisatie wordt verspreid door ^1H -spindiffusie en vervolgens door kruispolarisatie overgebracht naar de doelkernen. Een bijzonderheid bij het uitvoeren van DNP op deze systemen is de karakteristieke polarisatiegradiënt die wordt waargenomen in het materiaal, welke kan rapporteren over de ruimtelijke verdeling van de DNP-verbeterende stoffen. **Hoofdstuk 3** bestudeert de structuur-activiteitsrelatie tussen de kanaaltopologie van de ZSM-5 zeoliet en de methanol-naar-koolwaterstoffen (MTH) reactieroutes. Vaste stof-NMR en DNP experimenten toonden aan dat subtiele verschillen in de interne poriestructuur van een zeolietmateriaal de gevormde reactietussenproducten drastisch kunnen veranderen. Sinusvormige kanalen blijken de olefinacyclus te bevorderen, terwijl de verlengde rechte kanalen van zeoliet ZSM-5 de aromatische cyclus bevorderen met een hogere mate van alkylering van aromaten. Deze bevindingen leveren spectroscopisch bewijs van het effect van de kanaalgeometrie van een zeoliet op reactietussenproducten. In **Hoofdstuk 4** werden specifieke vaste stof-NMR technieken in combinatie met DNP-ondersteunende vaste stof-NMR gebruikt voor de moleculaire opheldering van het ethanol-naar-koolwaterstof (ETH) reactiemechanisme. De identificatie van een diverse set reactieproducten en tussenproducten maakte het mogelijk de reactie- en deactiveringsroutes van het ETH-proces te bepalen. Door klassieke diffusiewetten toe te passen, zou bovendien een correlatie tussen de gemeten DNP-verbeteringen en de distributie van de reactieproducten in de zeolietdeeltjes kunnen worden gepostuleerd, wat suggereert dat een specifieke ruimtelijke locatie wordt aangenomen door de zeoliet-gevangen aromatische stoffen. De kennis die is opgedaan met studies naar reactiemechanismen en structuuractiviteitsrelaties in de heterogene katalyse zal niet alleen nuttig zijn voor de ontwikkeling van superieure poreuze materialen maar zal ook bijdragen aan het begrip van de door zeoliet gekatalyseerde koolwaterstofomzettingschemie.

The laatste twee hoofdstukken zijn gericht op de ontwikkeling van biomoleculaire DNP-vsNMR bij hogere magnetische velden vanuit een theoretisch en experimenteel perspectief. Een belangrijke beperking van hoog-veld DNP is de slechte prestatie van momenteel gebruikte biradicalen wanneer de magnetische veldsterkte toeneemt. Daarom is er een grote behoefte om nieuwe wateroplosbare biradicalen te ontwikkelen met betere DNP-prestaties bij hoge velden. Hiermee kunnen de voordelen van sterk verbeterde DNP-signalen worden gecombineerd met verhoogde spectrale resolutie die wordt verkregen bij hoge magnetische velden. Alle DNP-experimenten vereisen een radicaal als polarisatiebron en de radicaaleigenschappen bepalen uiteindelijk de efficiëntie van het DNP-mechanisme. Vooral onder MAS-DNP is het cross-effect (CE)

het overheersende mechanisme dat het gebruik van radicalen vereist. Recent experimenteel werk en numerieke studies hebben ons begrip van het fenomeen verbeterd wat leidde tot de introductie van nieuwe radicalen. Gemengde trityl-nitroxide (TN) radicalen zijn bijvoorbeeld veelbelovende kandidaten voor hoge-veld DNP, waar de kleine g-anisotropie van tritylsoorten met een smalle lijn een betere verzadiging van de EPR-lijn mogelijk maakt. **Hoofdstuk 5** presenteert kwantummechanische simulaties voor een systeem met drie spins, waarbij specifiek de CE-DNP prestatie van trityl-nitroxide biradicalen wordt onderzocht. Onze resultaten komen overeen met eerder werk en laten zien dat de aanwezigheid van elektronische J-koppeling de DNP-efficiëntie significant kan verbeteren, vooral bij een hoge MAS frequentie. Omdat bovendien Cross Effect DNP afhankelijk is van zowel dipolaire als J-koppelingsinteracties tussen de elektronspins van de biradicalen, werd het effect van beide parameters onderzocht. De bevindingen gaven aan dat een optimale balans tussen de elektronen J-koppeling en dipolaire interacties moet worden bereikt om de DNP-efficiëntie te maximaliseren, zonder de overeenkomende voorwaarden die nodig is voor cross-effect DNP te verstoren. Aanvullende numerieke simulaties werden gebruikt om de gemeten DNP-verbeteringen voor de onderzochte nieuwe trityl-nitroxide stoffen te verklaren. Deze MAS-DNP simulaties kunnen de relatieve DNP-verbeteringen reproduceren die zijn waargenomen voor de onderzochte TN radicalen, waarvan de EPR parameters bekend waren. Het combineren van de experimentele resultaten met de simulaties suggereerde dat zwakke J-koppelingen en dipolaire interacties de belangrijkste redenen waren voor de relatieve lage DNP-verbetering gemeten voor de slechtst presterende radicaal. Vervolgens, is een nieuwe reeks van aminozuur-gekoppelde trityl-nitroxide biradicalen (aangeduid als NATriPols) gesynthetiseerd met als doel de wateroplosbaarheid te verbeteren. **Hoofdstuk 6** presenteert de resultaten die voor deze radicalen zijn verkregen op modelsystemen en, voor de eerste keer, hun praktische toepassingen op biomoleculen. Deze nieuwe TN-polarisatoren hebben bijna identieke dipolaire- en uitwisselingsinteracties, maar verschillen sterk in hydrofobiciteit. De elektron-elektron magnetische interacties van NATriPols blijken onafhankelijk te zijn van de substituenten op de α -positie van de aminozuurlinkers. Desalniettemin werd er een grote variatie gezien in hun experimenteel waargenomen DNP-prestatie in een modelmonster. De resultaten verkregen bij verschillende biradical concentraties in combinatie met EPR-gegevens toonden dat DNP-verbeteringen van deze radicalen omgekeerd gecorreleerd zijn met hun hydrofobiciteit. Het samenbundelen van NATriPols, gedreven door hun hydrofobiciteit in de DNP-matrix, is dus voornamelijk verantwoordelijk voor het verschil in hun DNP-efficiëntie. Bovendien werden de prestaties van de TN radicalen voor het eerst in een biomoleculaire context getest. Om hun potentie voor biologische toepassingen te onderzoeken, werden DNP-experimenten uitgevoerd met $[^{13}\text{C}, ^{15}\text{N}]$ -gelabeld aminozuurmengsel en $[^{13}\text{C}, ^{15}\text{N}]$ -Ubiquitine.

Bovendien wordt de efficiëntie van deze nieuwe familie van radicalen verder gedemonstreerd met de membraan-geassocieerde peptide Nisin. Onze bevindingen bevestigen dat hydrofiele effecten de prestaties van NATriPols sterk dicteren en laten de aanzienlijk verbeterde DNP-prestaties, in vergelijking met momenteel gebruikte radicalen, zien. De geoptimaliseerde hydrofiele NATriPol-3 vertoonde een 10-voudige DNP-verbetering gemeten op Ubiquitine in vergelijking met de best bekende TN-polarisator en vertoonde de hoogste absolute DNP-verbetering tot nu toe verkregen bij een 18.8 T in een biologische context. Deze bevindingen vormen de eerste stappen naar een beter begrip van deze nieuwe klasse van biradicalen en maken de weg vrij voor verdere optimalisatie voor nieuwe polarisatiemiddelen voor biomoleculaire toepassingen bij hoge magnetische velden.

Perspectieven

Sinds de commercialisering van MAS DNP-instrumentatie rond 2009 is de interdisciplinaire DNP-gemeenschap snel gegroeid, die voortdurend pionierende nieuwe ontwikkelingen onderging en de weg vrij maakte voor de populariteit van MAS DNP zoals we die vandaag de dag kennen. Door voortdurende inspanningen zoals de ontwikkeling van geavanceerde instrumentatie, op maat gemaakte polarisatiemiddelen en geoptimaliseerde monstervoorbereidingstechnieken, heeft DNP de afgelopen jaren een veel bredere toepasbaarheid bereikt. De opmerkelijke winst in gevoeligheid bij het gebruik van DNP heeft het mogelijk gemaakt om materialen te onderzoeken die anders onmogelijk waren geweest. Momenteel worden DNP-verbeteringsfactoren van $\epsilon \sim 100$ gemakkelijk bereikt. Dit is nog ver verwijderd van de theoretische maximale verbetering van $\frac{\gamma_e}{\gamma_{1H}} \sim 660$, maar zelfs deze “bescheiden” verbetering vertaalt zich in een vermindering van de experimentele acquisitietijd met een factor van 10^4 . Dus een experiment dat anders ~ 30 jaar aan gemiddeld signaal zou vereisen, kan in één dag worden verkregen. Dit heeft geleid tot een enorme toename van gerapporteerde state-of-the-art DNP-toepassingen voor verschillende problemen in materiaalwetenschappen en biomoleculaire vaste stof-NMR. Vooralsnog verbetert het theoretische begrip van de onderliggende kwantummechanische en chemische processen die het DNP-mechanisme aandrijven voortdurend en zijn verdere ontwikkelingen in de komende jaren te verwachten. Zoals gezegd vindt de verdere ontwikkeling van nieuwe chemische middelen die de magnetisatie efficiënter kunnen overdragen van elektronen naar kernen bij hogere velden nog altijd plaats. Naarmate de resolutie en gevoeligheid in NMR spectroscopie toenemen met veldsterkte, zullen deze vooruitgangen mogelijkheden openen om nog grotere en complexere chemische structuren in de levens- en materiaalwetenschappen te onderzoeken. In-cel DNP-studies zullen bijvoorbeeld veel

baat hebben bij verhoogde resolutie, waardoor zelfs grotere complexen in hun natuurlijke omgeving kunnen worden bestudeerd. Dergelijke studies zullen ook ons begrip van de invloed van de lokale cellulaire omgeving op complexe cellulaire processen verbeteren. Bovendien maken de voortdurende ontwikkelingen in de synthese van radicalen het ontwerp van gerichte DNP-polarisatiemiddelen mogelijk zoals biradicalen spinlabels die een efficiëntere polarisatieoverdracht naar het eiwit van interesse mogelijk maken^[1, 2]. Molecuul-specifieke DNP-verbetering kan worden verkregen in een heterogene biologische omgeving door het DNP-radicaal direct te koppelen aan het eiwit van interesse^[2, 3]. Bovendien is het gebruik van spinlabels al aangetoond op het gebied van in-cel EPR spectroscopie^[4, 5]. Op een vergelijkbare manier zou het gebruik van (gekooide) DNP-radicalen die gekoppeld zijn aan het onderzochte gelabelde eiwit kunnen worden geïmplementeerd voor in-cel DNP-NMR-toepassingen. Met dergelijke verbeteringen kunnen onderzoeken op atomaire schaal van doelwitmoleculen op endogene concentraties in de overvolle cellulaire achtergrond worden overwogen.

Een ander aspect van DNP dat in de toekomst waarschijnlijk belangrijke veranderingen zal veroorzaken, is de lage temperatuur dat momenteel is vereist om de DNP-experimenten uit te voeren. Onder de huidige omstandigheden die noodzakelijk zijn voor DNP, leidt het gebrek aan beweging bij zulke lage temperaturen vaak tot brede vsNMR signalen, wat de spectrale analyse compliceert. Echter worden er momenteel inspanningen gedaan om nieuwe instrumenten in DNP te ontwikkelen die dit probleem zullen overbruggen. Veelbelovende ontwikkelingen van gepulste microgolffbronnen met hoog vermogen bij hoge frequentie zullen naar verwachting de huidige beperkingen op de externe magnetische velden, waarmee DNP wordt geconfronteerd, uitbreiden. Ook zouden verschillende innovatieve DNP-methoden beschikbaar kunnen worden die de behoefte aan lage temperaturen zullen wegnemen. Hedendaagse MAS-DNP vertrouwt exclusief op continue golf (CW)-straling van de elektronenspinresonantie en alle CW-DNP-mechanismen vereisen de juiste relaxatietijden om goed te functioneren. Dit beperkt de efficiëntie van het DNP-proces bij hogere magnetische velden en niet-cryogene temperaturen. Verwacht wordt dat pulsvormige microgolffstraling van de elektronenspin deze uitdagingen zal overwinnen, aangezien deze gepulseerde DNP-schema's mogelijk de ongunstige veldafhankelijkheid van klassieke DNP-mechanismen elimineren. Nieuwe benaderingen voor DNP zijn nog steeds in de ontwikkelingsfase, waaronder tijdsdomein DNP die de NOVEL (nucleaire spinoriëntatie via elektronspinvergrendeling) schema's gebruikt^[6-10], frequentie-swept-technieken^[11-13], elektronenontkoppeling^[14, 15] of pulsvormige microgolffstraling met behulp van willekeurige golfvormgeneratie (AWG) gebruikt die compatibel is met MAS-DNP^[16]. Zodra de instrumentatie beschikbaar is voor deze nieuwe benaderingen, verwachten we dat er een nieuwe golf van spannende DNP-onderzoek zal volgen.

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List of publications

- 1) **A. Lucini Paioni**, S. Narasimhan, J. van der Zwan, and M. Baldus. “DNP preparations for in-cell solid-state NMR spectroscopy” – *in preparation*
- 2) W. Zhai*, **A. Lucini Paioni***, X. Cai, S. Narasimhan, J. Medeiros-Silva, W. Zhang, A. Rockenbauer, M. Weingarth, Y. Song, M. Baldus and Y. Liu. “Postmodification via Thiol-click Chemistry Yields Hydrophilic Trityl-nitroxide Biradicals for Biomolecular High-Field Dynamic Nuclear Polarization” – *in press, J. Phys. Chem. B* (2020) DOI: 10.1021/acs.jpcc.0c08321 [** these authors contributed equally*]
- 3) D. Fu*, **A. Lucini Paioni***, C. Lian, O. van der Heijden, M. Baldus and B.M. Weckhuysen. “Elucidating Zeolite Channel Geometry–Reaction Intermediate Relationships for the Methanol-to-Hydrocarbon Process” – *Angew. Chem. Int. Ed.* 59, 2–9 (2020) [**these authors contributed equally*]
- 4) M. Çağlayan, **A. Lucini Paioni**, E. Abou-Hamad, G. Shterk, A. Pustovarenko, M. Baldus, A. Dutta Chowdhury and J. Gascon. “Initial Carbon-Carbon Bond Formation during the Early Stages of Methane Dehydroaromatization” - *Angew. Chem. Int. Ed.* 59, 16741 – 16746 (2020)
- 5) R. Shukla, J. Medeiros-Silva, A. Parmar, B.J.A. Vermeulen, S. Das, **A. Lucini Paioni**, S. Jekhmane, J. Lorent, A. M.J.J. Bonvin, M. Baldus, M. Lelli, E.J.A. Veldhuizen, E. Breukink, I. Singh, M. Weingarth. “Mode of action of teixobactins in cellular conditions” - *Nat Commun* 11, 2848 (2020)
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- 7) S. Chordia, S. Narasimhan, **A. Lucini Paioni**, M. Baldus & G. Roelfes. “In Vivo Assembly of Artificial Metalloenzymes and Application in Whole-Cell Biocatalysis” – *submitted, ChemRxiv*.
<https://doi.org/10.26434/chemrxiv.12485993.v1>

- 8) S. Narasimhan, C. Pinto, **A. Lucini Paioni**, J. van der Zwan, G. E. Folkers, and M. Baldus. "Characterizing proteins in a native bacterial environment using solid-state NMR spectroscopy". – *submitted for an invited contribution to Nature Protocols*
- 9) M. Velthoen, **A. Lucini Paioni**, I. Teune, M. Baldus, B. M. Weckhuysen. "Matrix Effects in a Fluid Catalytic Cracking Catalyst Particle: Influence on Structure, Acidity, and Accessibility". *Chem. Eur. J.* 26,11995–12009, (2020)
- 10) J.A.W. Jong, Y. Guo, C. Veenhoven, M.E. Moret, J. van der Zwan, **A. Lucini Paioni**, M. Baldus, K.C. Scheiner, R. Dalebout, M.J. van Steenberg, M.C. Verhaar, R. Smakman, W.E. Hennink, K.G.F. Gerritsen & C.F. van Nostrum. "Phenylglyoxaldehyde-Functionalized Polymeric Sorbents for Urea Removal from Aqueous Solutions". *ACS Applied Polymer Materials*, 2 (2), 515-527, (2020)
- 11) Y. Luo, S. Xiang, **A. Lucini Paioni**, A. Adler, and M. Baldus. "Solid-state NMR Spectroscopy for Studying microtubules and microtubule-associated proteins" - *Methods in Molecular Biology*, accepted
- 12) B. Luna-Murillo*, M. Pala*, **A. Lucini Paioni**, M. Baldus, F. Ronsse, W. Prins, P. C. A. Bruijninx, and B. M. Weckhuysen. "Catalytic fast pyrolysis of biomass: Catalyst characterization reveals the feed-dependent deactivation of a technical ZSM-5-based catalyst" – *submitted*
- 13) Helena Ehren, Felicitas Kolbe, **A. Lucini Paioni**, Eike Brunner, Marc Baldus. "DNP-supported solid-state NMR studies of ^{13}C , ^{15}N , ^{29}Si -enriched biosilica of *Cyclotella cryptica* and *Thalassiosira pseudonana*" – *under review* DOI:10.21203/rs.3.rs-44217/v1
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- 16) R.M. Danisi, J. E. Schmidt, **A. Lucini Paioni**, K. Houben, J.D. Poplawsky, M. Baldus, B.M. Weckhuysen & E.T.C. Vogt. "Revealing Long- and Short-range Structural Modifications within Phosphorus-treated HZSM-5 Zeolites by Atom Probe Tomography, Nuclear Magnetic Resonance and Powder X-Ray Diffraction". *Physical Chemistry Chemical Physics*, 20 (44), 27766-27777, (2018)
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- 18) E. Heracleous, E. Pachatouridou, A.M. Hernández-Giménez, H. Hernando, T. Fakin, **A. Lucini Paioni**, M. Baldus, D.P. Serrano, P.C.A. Bruijninx, B.M. Weckhuysen & A.A. Lappas. "Characterization of deactivated and regenerated zeolite ZSM-5-based catalyst extrudates used in catalytic pyrolysis of biomass". *Journal of Catalysis*, 380, 108-122 (2018)
- 19) F. Caracciolo, **A. Lucini Paioni**, M. Filibian, L. Melone, P. Carretta. "Proton and Carbon-13 Dynamic Nuclear Polarization of Methylated β -Cyclodextrins". *J Phys Chem B*. 122(6):1836-1845 (2018)
- 20) J. Medeiros-Silva, S. Jekhmane, **A. Lucini Paioni**, K. Gawarecka, M. Baldus, E. Swiezewska, E. Breukink & M. Weingarth. "High-resolution NMR studies of antibiotics in cellular membranes". *Nature Communications* 9, 3963 (2018)
- 21) A. Dutta Chowdhury, **A. Lucini Paioni**, K. Houben, G. T. Whiting, M. Baldus, B. M. Weckhuysen. "Bridging the Gap between the Direct and Hydrocarbon Pool Mechanisms of the Methanol-to-Hydrocarbons Process". *Angew. Chem. Int. Ed.* 57, 8095 –8099 (2018)
- 22) **A. Lucini Paioni**, M. A.M. Renault, M. Baldus. "DNP and Cellular Solid-State NMR", *eMagRes*, Volume 7, Issue 3 (2018)

Curriculum Vitae

Alessandra Lucini Paioni was born on the 10th of February 1992 in Crema, Italy. After obtaining her high school diploma in classical studies in 2011, she enrolled at the University of Pavia (Italy) where she completed her Bachelor of Science in Physics in 2014. She then continued her studies in Physical Sciences and was awarded a Master of Science (*cum laude*) in 2016. For her master's research project she studied the ¹³C nuclear spin-lattice relaxation and the Dynamic Nuclear Polarization (DNP) process in ¹³C-labelled β-cyclodextrins, focusing on the application of DNP for in vivo real-time metabolic magnetic resonance imaging. Her master thesis was completed under the supervision of Prof. Pietro Carretta. In October 2016, she moved to Utrecht to start her PhD in the solid-state NMR group of Prof. Dr. Marc Baldus at the Bijvoet Centre for Biomolecular Research (Utrecht University). In the NMR Spectroscopy Research Group she mainly focused on the development of novel applications of DNP-enhanced solid-state NMR to study complex molecular systems in life and material science.