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Volli, e volli sempre, e fortissimamente volli.

-Vittorio Alfieri

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Abstract

Proteins often undergo conformational transitions to fulfill their biological roles. Considering their fundamental importance in physiological processes, an exhaustive characterization of their conformational space is needed. Biomimetic systems, which mimic the features of natural structures and processes, can be employed as models to achieve this goal.

NMR is a powerful method to study structural transitions in highly dynamic biomimetic systems. However, the proteins' conformational space comprises many hardly accessible transient conformations. Additionally, environmental factors, such as protein concentration, temperature, or pH, can influence their structural behavior. These factors limit the suitability of conventional NMR, which demands highly concentrated samples and long acquisition times to obtain informative spectra.

To perform NMR experiments on biomimetic systems under challenging conditions, this thesis focuses on low substrate concentration and short-lived intermediates. I integrated hyperpolarized NMR by dissolution dynamic nuclear polarization (DNP) with established methodologies, such as real-time NMR, ^{13}C - ^{15}N correlated NMR, continuous wave EPR, nanoscale distance measurements, and molecular dynamic (MD) simulations.

In this thesis, I characterized the conformational behavior of four biomimetic models by employing the abovementioned integrated approach:

1. Transcription factors (TFs) feature high conformational plasticity due to the abundant presence of intrinsically disordered regions (IDRs). An example of flexible TF is represented by the MYC-associated factor X (MAX), a partially disordered basic helix-loop-helix leucine zipper (bHLH-LZ) TF involved in cancer. The first part of my thesis focuses mainly on characterizing MAX's conformational balance in the presence of several interaction partners. In solution, MAX forms the homodimer MAX:MAX, which can bind a 6-nucleotides DNA target sequence (EBOX). MAX heterodimerizes in the presence of its main interaction partner MYC, forming the transcriptionally active complex MYC:MAX-DNA. Interestingly, the breast cancer 1 susceptibility protein (BRCA1) also regulates the MYC:MAX-DNA interaction network, directly binding MYC and DNA. However, a detailed residue-resolved description of BRCA1's influence on the trimeric complex is still missing since the dimers provide

low intensity due to strongly anisotropic tumbling. In particular transient states are challenging to characterize. Herein, the modulation expressed by BRCA1 on the conformational transitions involving MYC and MAX during DNA binding and heterodimerization processes is elucidated. BRCA1 competes with MAX homodimer for DNA binding, shifting MAX's structural balance to its monomeric conformation. Additionally, BRCA1 forms a highly dynamic complex with the MAX monomer, hindering the heterodimerization of the latter. Considering the involvement of BRCA1 in breast and ovarian cancer, a deep understanding of BRCA1's modulatory effect on MYC and MAX conformational space is of pivotal importance in finding new possible targets for clinical applications.

2. The protein MAX, introduced in the previous section, features high structural variability, populating different conformations depending on the environmental conditions; therefore, MAX represents the second model of conformational variations presented in this thesis. Particularly, MAX's transition from a monomer to a homodimer, and *vice versa*, is influenced by changes in temperature and pH. Employing dDNP to boost the NMR signals of the protein, MAX's conformational space at low concentrations is explored. Integrating computational techniques to dDNP, it was possible to access MAX at micromolar concentrations and to describe a previously uncharted globular-looking conformational state of MAX. Considering the importance of accessing physiological concentrations in structural dynamics studies, the approach proposed integrates dDNP and MD simulations to provide a detailed characterization of MAX conformational behavior at semi-physiological concentrations.
3. The third part of this thesis supplements the mechanisms for hyperpolarization transfer to biomolecules. The possibility to boost the signals of molecules employing hyperpolarized water has widened the range of substrates compatible with dDNP. However, the modalities of hyperpolarization transfer from the water to the targets of interest are still unclear. To shed light on these mechanisms, dDNP, MD simulations, and cross-relaxation rates predictions are applied to two model systems: an arginine and a polyaspartate solution. In this way, it was possible to characterize how the three main polarization transfer mechanisms, namely direct nuclear Overhauser effect (NOE), exchange-relayed NOE, and chemical exchange contribute to the polarization of biomolecular substrates in dDNP.

4. The last example focuses on characterizing prenucleation species (PNS) in biomimetic solutions to demonstrate the prowess of real-time NMR to describe transient species that are not protein-based but still biologically relevant. Simulated body fluids (SBFs) are metastable solutions mimicking the ionic concentration of human blood plasma, previously used as a model for the study of prenucleation species (PNS). PNS are transient precursors of the final crystalline structures in the recently proposed non-classical crystallization pathway. Employing real-time ^{31}P NMR, calcium potentiometry, x-ray energy dispersive spectra (XEDS), Cryo-TEM, and computer simulations to characterize modified SBF (mSBF, a simulated body fluid with stable pH over time), the evolution of calcium phosphate following the non-classical crystallization pathway is monitored. Calcium phosphate nanoscale inhomogeneities start forming after 5 hours from mSBF preparation and grow into bulkier and more stable structures, up to 200 nm after 24 hours, as confirmed by DOSY NMR and Cryo-TEM. These results indicate that mSBF becomes a stable or metastable solution only after ~24 hours from preparation.

The integration of novel spectroscopic methods into existing structural biology methodologies enables the overcoming of some of the sensitivity constraints of NMR. By means of powerful techniques such as dDNP, NMR, EPR, and MD simulations, the conformational behavior of key transcription factors like MAX or BRCA1 can be determined with atomistic precision even when conventional techniques do not provide enough sensitivity. Delivering novel information on the conformational behavior of medically relevant proteins and peptides could chart novel routes for developing innovative therapeutics, targeting, for example, transient conformational states.

Zusammenfassung

Proteine durchlaufen häufig Konformationsübergänge, um ihre biologischen Funktionen zu erfüllen. In Anbetracht ihrer grundlegenden Bedeutung für physiologische Prozesse ist somit eine umfassende Charakterisierung ihres Konformationsraums erforderlich. Biomimetische Systeme, die die Eigenschaften natürlicher Strukturen und Prozesse nachahmen, können als Modelle eingesetzt werden, um dieses Ziel zu erreichen.

Die NMR ist eine leistungsfähige Methode zur Untersuchung struktureller Übergänge in hochdynamischen biomimetischen Systemen. Der Konformationsraum der Proteine umfasst jedoch viele schwer zugängliche vorübergehende Konformationen. Darüber hinaus können Umweltfaktoren wie Proteinkonzentration, Temperatur oder pH-Wert das strukturelle Verhalten der Proteine beeinflussen. Diese Faktoren schränken die Eignung herkömmlicher NMR Methoden ein, da hochkonzentrierte Proben und lange Aufnahmezeiten erforderlich sind, um aussagekräftige Spektren zu erhalten.

Um NMR-Experimente an biomimetischen Systemen unter herausfordernden Bedingungen durchzuführen, konzentriert sich diese Arbeit auf niedrige Substratkonzentrationen und kurzlebige Zwischenprodukte. Mit diesem Ziel habe ich hyperpolarisierte NMR durch dynamische Kernpolarisation (DNP) mit etablierten Methoden wie Echtzeit-NMR, ^{13}C - ^{15}N Korrelationsspektroskopie, kontinuierlicher Dauerstrich-ESR, Abstandsmessungen im Nanobereich und molekulardynamischen Simulationen (MD) kombiniert.

In dieser Arbeit habe ich das Konformationsverhalten von vier biomimetischen Modellen mit Hilfe des oben erwähnten integrierten Ansatzes charakterisiert:

1. Transkriptionsfaktoren (TFs) zeichnen sich durch eine hohe konformelle Plastizität aus, die auf das häufige Vorkommen von intrinsisch ungeordneten Regionen (IDRs) zurückzuführen ist. Ein Beispiel für einen flexiblen TF ist der MYC-assoziierte Faktor X (MAX), ein teilweise ungeordneter basischer helix-loop-helix lediesecine zipper (bHLH-LZ) TF, der an Krebs beteiligt ist. Der erste Teil meiner Dissertation konzentriert sich auf die Charakterisierung des Konformationsgleichgewichts von MAX in Gegenwart mehrerer Interaktionspartner. In Lösung bildet MAX das Homodimer MAX:MAX, das eine 6-Nukleotide-DNA-Zielsequenz (EBOX) binden kann. In Anwesenheit seines Hauptinteraktionspartners MYC heterodimerisiert MAX und

bildet den transkriptionell aktiven Komplex MYC:MAX-DNA. Interessanterweise reguliert das Brustkrebs-1-Anfälligkeitsprotein (BRCA1) ebenfalls das MYC:MAX-DNA-Interaktionsnetzwerk, indem es MYC und DNA direkt bindet. Eine detaillierte, rückstandsaufgelöste Beschreibung des Einflusses von BRCA1 auf den trimeren Komplex steht jedoch noch aus, da die Dimere aufgrund der stark anisotropen Rotation eine geringe Intensität aufweisen. Insbesondere transiente Zustände sind schwierig zu charakterisieren. In dieser Arbeit wird der Einfluss von BRCA1 auf die Konformationsübergänge von MYC und MAX während der DNA-Bindung und Heterodimerisierung aufgeklärt. BRCA1 konkurriert mit dem MAX-Homodimer um die DNA-Bindung und verschiebt das strukturelle Gleichgewicht von MAX in Richtung seiner monomeren Konformation. Darüber hinaus bildet BRCA1 einen hochdynamischen Komplex mit dem MAX-Monomer, welcher die Heterodimerisierung des letzteren behindert. In Anbetracht der Beteiligung von BRCA1 an Brust- und Eierstockkrebs ist ein tiefes Verständnis der modulierenden Wirkung von BRCA1 auf den MYC- und MAX-Konformationsraum von entscheidender Bedeutung für die Suche nach neuen möglichen Zielen für klinische Anwendungen.

2. Das im vorangegangenen Abschnitt vorgestellte Protein MAX zeichnet sich durch eine hohe strukturelle Variabilität aus und nimmt je nach Umgebungsbedingungen unterschiedliche Konformationen an; daher stellt MAX das zweite in dieser Arbeit vorgestellte Modell für Konformationsvariationen dar. Insbesondere der Übergang von MAX von einem Monomer zu einem Homodimer und umgekehrt wird durch Temperatur- und pH-Änderungen beeinflusst. Unter Verwendung von dDNP zur Verstärkung der NMR-Signale des Proteins wird der Konformationsraum von MAX bei niedrigen Konzentrationen untersucht. Durch die Integration von Computertechniken in dDNP war es möglich, MAX bei mikromolaren Konzentrationen zu untersuchen und einen bisher unbekannten globulären Konformationszustand von MAX zu beschreiben. In Anbetracht der Bedeutung des Zugangs zu physiologischen Konzentrationen in Strukturdynamikstudien integriert der vorgeschlagene Ansatz dDNP und MD-Simulationen, um eine detaillierte Charakterisierung des Konformationsverhaltens von MAX bei semi-physiologischen Konzentrationen zu ermöglichen.

3. Der dritte Teil dieser Arbeit dient zur Ergänzung der Mechanismen der Hyperpolarisationsübertragung auf Biomoleküle. Die Menge der mit dDNP kompatiblen Substrate wurde vergrößert, durch die Möglichkeit, die Signale von Molekülen mit Hilfe von hyperpolarisiertem Wasser zu verstärken. Die Modalitäten der Hyperpolarisationsübertragung vom Wasser auf die Zielmoleküle sind jedoch noch unklar. Um Licht in diese Mechanismen zu bringen, wurden dDNP, MD-Simulationen und Vorhersagen von Kreuzrelaxationsraten auf zwei Modellsysteme angewendet: eine Arginin- und eine Polyaspartatlösung. Auf diese Weise konnte charakterisiert werden, wie die drei wichtigsten Polarisationsübertragungsmechanismen, nämlich der direkte nukleare Overhauser-Effekt (NOE), der austauschvermittelte NOE und der chemische Austausch, zur Polarisation biomolekularer Substrate in dDNP beitragen.
4. Das letzte Beispiel befasst sich mit der Charakterisierung von Pränukleationsspezies (PNS) in biomimetischen Lösungen. Damit soll die Fähigkeit der Echtzeit-NMR zu demonstriert werden, transiente Spezies zu beschreiben, die nicht auf Proteinen basieren, aber dennoch biologisch relevant sind. Simulierte Körperflüssigkeiten (SBF) sind metastabile Lösungen, die die Ionenkonzentration des menschlichen Blutplasmas nachahmen, welches früher als Modell für die Untersuchung von PNS verwendet wurde. PNS sind vorübergehende Vorläufer der endgültigen kristallinen Strukturen auf dem kürzlich vorgeschlagenen nichtklassischen Kristallisationsweg. Mit Hilfe von Echtzeit-³¹P-NMR, Kalziumpotentiometrie, energiedispersiven Röntgenspektren (XEDS), Cryo-TEM und Computersimulationen zur Charakterisierung von modifiziertem SBF (mSBF, einer simulierten Körperflüssigkeit mit zeitlich stabilem pH-Wert) wird die Entwicklung von Kalziumphosphat auf dem nichtklassischen Kristallisationsweg verfolgt. Calciumphosphat-Inhomogenitäten im Nanomaßstab beginnen sich ab 5 Stunden nach der Herstellung von mSBF zu bilden und wachsen zu größeren und stabileren Strukturen heran, die nach 24 Stunden bis zu 200 nm groß sind, wie durch DOSY-NMR und Cryo-TEM bestätigt wird. Diese Ergebnisse zeigen, dass mSBF erst ~24 Stunden nach der Herstellung zu einer stabilen oder metastabilen Lösung wird.

Die Integration neuartiger spektroskopischer Methoden in bestehende strukturbiochemische Verfahren ermöglicht es, einige der Empfindlichkeitsbeschränkungen von NMR Spektroskopie zu überwinden. Mit Hilfe leistungsfähiger Techniken wie dDNP, NMR, EPR und

MD-Simulationen kann das Konformationsverhalten wichtiger Transkriptionsfaktoren wie MAX oder BRCA1 mit atomarer Präzision bestimmt werden, selbst wenn herkömmliche Techniken keine ausreichende Empfindlichkeit bieten. Die Bereitstellung neuer Informationen über das Konformationsverhalten medizinisch relevanter Proteine und Peptide könnte neue Wege für die Entwicklung innovativer Therapeutika aufzeigen, die z. B. auf vorübergehende Konformationszustände abzielen.

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List of abbreviations and acronyms

BRCA1 = breast cancer susceptibility protein 1

CNT = classical nucleation theory

CSA = chemical shift anisotropy

CW = continuous wave

dDNP = dissolution dynamic nuclear polarization

DEER = double electron-electron resonance (also known as PELDOR)

DNP = dynamic nuclear polarization

DQ = double quantum transition

ENDOR = electron-nuclear double resonance

EPR = electron paramagnetic resonance

FT = Fourier transform

HyperW = hyperpolarized water

IDP = intrinsically disordered protein

IDR = intrinsically disordered region

MAS = magic angle spinning

MAX = MYC-associated factor X

MD = molecular dynamic

MRI = magnetic resonance imaging

mSBF = modified simulated body fluid

MW = molecular weight

NCNT = non-classical nucleation theory

NMR = nuclear magnetic resonance

NOE = nuclear Overhauser effect

NOESY = nuclear Overhauser effect spectroscopy

ODNP = Overhauser dynamic nuclear polarization

PAS = principal axis system

PELDOR = pulse electron-electron double resonance (also known as DEER)

PNC = prenucleation cluster

PNS = prenucleation species

RF = radiofrequency

SBF = simulated body fluids

SNR = signal-to-noise ratio

TEM = transmission electron microscopy

TEMPOL = 4-Hydroxy-2,2,6,6-tetramethylpiperidinyloxy

VDI = Virginia Diodes, Inc.

VTI = variable temperature insert

ZQ = zero quantum transition

List of symbols

$\hat{H}_{DD}$ = dipolar coupling Hamiltonian

$\hat{H}_{CS}$ = chemical shift Hamiltonian

$\hat{H}_{jk}$ = J-coupling Hamiltonian

$\hat{I}_j/\hat{I}_k$ = nuclear spin coupling operator

$\hat{J}_{jk}$ = J coupling tensor

$\vec{B}$ = magnetic field vector

B_0 = external magnetic field
 E_{DD} = dipolar coupling energy
 $\hat{I}$ = nuclear spin operator
 I_{tot} = total angular momentum
 $\vec{M}$ = magnetization vector
 M_0 = equilibrium magnetization
 N_α/N_β = number of nuclear spins in the ground state/excited state
 R_1 = nuclear spin-lattice relaxation rate
 R_2 = nuclear spin-spin relaxation rate
 T_1 = nuclear spin-lattice relaxation time
 T_2 = nuclear spin-spin relaxation time
 k_{tot} = exchange rate
 m_e = electron mass
 m_s = spin quantum number
 r_{jk} = distance between coupled nuclear spins
 $\hat{\delta}$ = CSA tensor
 $\hat{\mu}$ = spin magnetic moment
 μ_B = Bohr magneton
 ν_0 = Larmor precession frequency
 $\hat{\sigma}$ = shielding tensor
 σ_L^{NOESY} = cross-relaxation rate
 ω_0 = Larmor frequency
 h = Planck's constant
 $\hbar$ = reduced Planck's constant
 E = energy
 I = spin angular momentum
 $J(\omega)$ = spectral density function
 U = signal amplitude
 W = transition rate
 e = electron elementary charge

g = g factor

Ψ = wavefunction

β = spin temperature coefficient

γ = gyromagnetic ratio

δ = chemical shift

ε = polarization enhancement

η = NOE enhancement

ν = frequency in Hz

ω = frequency in $\frac{rad}{s}$

Chapter 1

Pro and con(formation): tracking conformational transitions by nuclear magnetic resonance

Conformational transitions are necessary for proteins to express their biological roles.¹⁻³ Proteins can change their structural morphology when interacting with a specific target, such as a nucleic acid or another protein, in an induced-fit fashion.⁴⁻⁵ Structural variations are

indeed responsible for proteins' multifunctionality since conformational changes modify their role and relationship with the surrounding environment.⁶⁻⁸

The conformational behavior of proteins in solution was widely studied in the last 60 years⁹, starting from the thermodynamic hypothesis of Christian Anfinsen in 1962¹⁰⁻¹¹ and the subsequent theoretical description of urea-mediated protein unfolding postulated by Charles Tanford in 1965¹². It was shown that proteins often undergo structural transitions, shifting, for example, from a folded to an unfolded state or from a single monomer to a multimeric aggregate.¹³ These structural fluctuations are more frequent in proteins featuring high conformational plasticity, lent by the presence of intrinsically disordered regions (IDRs).¹⁴ Proteins rich in IDRs are denominated intrinsically disordered proteins (IDPs), which play a crucial role in cell homeostasis, actively contributing to cell signaling⁸ and cell cycle regulation¹⁵. Considering their biological relevance, proteins' conformational transitions are of central importance for the understanding of protein structural dynamics.

In order to explore these biologically relevant processes, biomimetic systems can be employed. They mimic biological processes and structures in a controlled laboratory environment. For example, conformational transitions in protein systems can be mimicked by recombinant proteins produced in *E.coli*. Despite the possibility to employ controllable biomimetic systems in vitro, conformational transitions can be challenging to explore with traditional biochemical techniques, such as crystallography or cryo-TEM, due to their highly dynamic nature.

Nuclear magnetic resonance (NMR) spectroscopy represents a valid technique to access dynamic protein substrates. Widely employed as a tool for the identification and the structural elucidation of macromolecules, NMR can be applied to dynamic systems in solution to explore their structural dynamics. Nevertheless, accessing proteins' conformational space can be challenging.

First of all, the conformational behavior of a protein can be influenced by environmental factors, such as concentration, temperature, and pH.¹⁶⁻¹⁷ In eukaryotic cells, proteins reach even nanomolar concentrations.¹⁸⁻²⁰ However, conventional NMR requires high concentrations of the substrate to detect signals with good sensitivity, several orders of magnitudes higher than in physiological conditions. This means that conformational states appearing only in physiological environments are hardly accessible by conventional NMR.

Secondly, the complex conformational space of dynamic proteins can feature short-lived conformational states. Their transient nature hinders the applicability of conventional NMR, which requires long acquisition times to ensure the detection of spectra with good sensitivity, affecting the time resolution of the experiment. Therefore, short-lived states appearing during conformational transitions cannot be detected by NMR.²¹

Finally, the molecular weight of the substrate is not always compatible with the detection limits of NMR. The development of high-field and ultra-high-field spectrometers and their combination with innovative pulse sequences (*e.g.* methyl-TROSY²²) allowed studying protein complexes up to 1 MDa. However, conventional NMR experiences a drop in resolution for large proteins with a slow tumbling time²³⁻²⁵, which broadens the NMR signals and causes spectral crowding.²⁶

In order to address the lack of sensitivity of low concentrated samples and the limited accessibility of transient conformational states, this thesis proposes an innovative strategy combining unconventional techniques, such as dissolution dynamic nuclear polarization (dDNP), with standard approaches, such as NMR, EPR, and MD simulations.

dDNP is a hyperpolarization method first proposed by Jan Henrik Ardenkjær-Larsen and coworkers in 2003, which enhances the NMR sensitivity up to 10,000 folds.²⁷ A dDNP was proven to be compatible for the study of biomacromolecules such as peptides and proteins. However, unstable proteins are often not compatible with the harsh conditions (high temperature, high pressure) necessary for the sample dissolution. Therefore, hyperpolarized water (HyperW) was proposed as a method to access proteins by dDNP without causing denaturation and precipitation of the substrate.²⁸⁻³¹

Employing HyperW dDNP and complementing it with the abovementioned techniques allows to access conformational transitions otherwise incompatible with conventional NMR. Additionally, while HyperW dDNP alone can deliver structural information only for few seconds after dissolution due to the rapid relaxation of the hyperpolarized system, the proposed integrating strategy can provide information on the structural dynamics evolution of the system of interest over longer time.

1.1.1. Aim and structure of the thesis

The main aim of this thesis is to access conformational transitions by integrating dDNP with conventional spectroscopical methods in challenging conditions. For this purpose, four biomimetic systems with a different degree of complexity were chosen as models for conformational transitions (**Figure 1.1**): the interaction complex formed by MYC, MAX (MYC associated factor X), DNA, and BRCA1 (breast cancer susceptibility protein 1); the MAX dimer at low concentrations; a buffered solution of arginine; an SBF (simulated body fluid) solution mimicking the process of calcium phosphate crystallization.

For the study of each biomimetic model, a specific aim was defined:

Describe how, in the MYC:MAX-DNA interaction network, the structural balance of the interacting partners can be modulated by the intrinsically disordered BRCA1;

Elucidate how physiological concentrations can affect the conformation equilibrium of the model MAX:MAX.

Unravel how the polarization can be transferred by HyperW, employing the arginine as a model.

Identify and characterize the intermediates of crystallization in biomimetic conditions provided by SBF.

While **Chapter 1** and **Chapter 2** describe the theoretical background of NMR and DNP, respectively, **Chapter 3** focuses on the MYC:MAX-DNA interaction network and its relationship with the transcription factor (TF) BRCA1. It is demonstrated that the central IDR of BRCA1 actively downregulate the transcriptional activity of MYC by binding to it³² and, therefore, hindering the formation of MYC:MAX. MAX is described as a basic helix-loop-helix leucine zipper (bHLH-LZ) TF, which form DNA-binding homodimers in solution. In this chapter, by integrating real-time NMR, continuous wave (CW) EPR, and MD simulations, two different mechanism of interaction between BRCA1 and the MYC:MAX-DNA complex are elucidated: i) BRCA1 competes with MAX:MAX for the DNA target, destabilizing the MAX:MAX-DNA complex; ii) BRCA1 binds MAX in its monomeric state, hindering the process of heterodimerization. According to the experimental results, the latter mechanism is predominant, leading to a lower transcriptional activity of the interaction complex by binding MYC and MAX; in this way, the heterodimerization of the complex is impeded. This study was submitted in 2023 and published in preprint on Researchsquare.³³

In **Chapter 4**, I focus on the phase behavior of the MAX homodimer. MAX behaves as an IDP, and it was reported in the literature that environmental factors, such as pH or temperature, heavily impact the conformational balance between the dimeric and monomeric states.¹⁶ Employing dDNP and MD simulations as tools for studying MAX at semi-physiological concentrations, it emerged that MAX concentration perturbs its conformational equilibrium, leading to MAX monomerization and folding into a previously undescribed partially disordered semi-globular structure. This study was published on Science Advances in 2022, according to reference ³⁴.

In **Chapter 5**, I explore the polarization transfer mechanisms from hyperpolarized water to a target molecule, employing the amino acid arginine as a model. The main focus of the thesis vests on conformational transitions in biomimetic systems; however, considering the fundamental role of HyperW for protein hyperpolarization in **Chapter 4**, a further understanding of the polarization transfer mechanisms is necessary. **Chapter 5** shows how the three main polarization transfer mechanisms – namely direct nuclear Overhauser effect (NOE), exchange-relay NOE, and chemical exchange – contribute to the polarization transfer. Coupling the dDNP data with NOESY and MD simulations, this study suggests that direct NOE mainly occurs during the first seconds after injection, while exchange-relay NOE and chemical exchange take over only after a few seconds. These results give new insights regarding the importance of NOE in hyperpolarization transfer. The study was published in Chemical Communications in 2022, according to reference ³⁵.

In **Chapter 6**, the SBF is selected as a biomimetic model for calcium phosphate crystallization at semi-physiological conditions since it features the same ionic concentrations of human blood plasma. In particular, the variant mSBF (modified SBF) is employed in this work since its pH remains stable over time.³⁶ **Chapter 6** shows that, after around 5 hours from its preparation, mSBF features nanometric calcium phosphate inhomogeneities in solution. They can be identified as calcium phosphate prenucleation species (PNS), which are formed by smaller units, growing up to 200 nm after 20 hours. By real-time NMR, cryo-TEM, and calcium potentiometry, it was possible to monitor the formation, growth, and aggregation of calcium phosphate in mSBF. Additionally, the stoichiometry of the units forming the bigger nucleation seed were described. By the proposed strategy, this study contributes to the general understanding of crystallization, characterizing the formation and evolution of PNS. This study was published in Analytical Chemistry in 2021, according to reference ³⁷.

Phase transitions in biomimetic systems

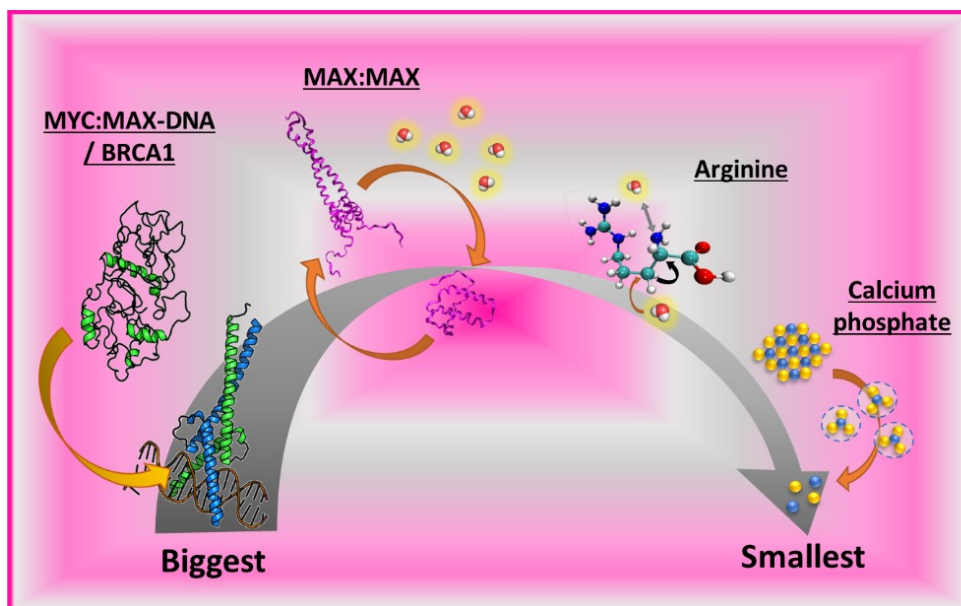


Figure 1.1: Conformational transition studies on different biomimetic systems, from the MYC:MAX-DNA / BRCA1 (the most complex) to the calcium phosphate (the less complex).

1.2 NMR and DNP: brief historical notes

Largely applied in many scientific fields, NMR represented a breakthrough in chemistry and biology. Even if the first NMR spectrum was collected only in 1946 by Bloch *et al.*³⁸, the development of the NMR technique started almost 30 years before.³⁹ The long, intricate history of NMR began at the Johann Wolfgang Goethe University of Frankfurt am Main with the Stern-Gerlach experiment, performed by the physicists Walther Gerlach and Otto Stern

in 1922.⁴⁰ The two scientists proved the existence of spatial quantization by directing a beam of silver atoms through an inhomogeneous magnetic field. Consequent to his discovery, Stern was awarded the Nobel Prize in Physics in 1943.

Another scientist who put the NMR's first milestones was Isidor I. Rabi in 1938. Rabi, a student at Columbia University, shortly worked at the laboratory of Stern in Germany, setting up experiments based on the use of molecular beams passing through a magnetic field. He and his coworkers detected an NMR signal in a beam of LiCl for the first time.⁴¹ The stream of atoms passed through two static magnetic fields. In certain conditions, it was possible to observe a spin transition of the lithium nuclei, corresponding to an intensity fluctuation of the atom beam on the detector. The lithium nuclei were on "resonance" with the magnetic field. Rabi was awarded the Nobel Prize in Physics in 1944. A similar attempt to detect magnetic resonance was performed some years before by the Dutch scientist Cornelis J. Gorter, who tried unsuccessfully to measure the NMR signal from crystals through calorimetric measures of the relaxing system.⁴²

With the advent of the Second World War, scientists of the standing of Rabi, Bloch, Purcell, *etc.*, faced wartime in the labs of MIT, where they largely contributed to the development of radar technologies. Meanwhile, on the other part of the ocean, the Russian physicist Yevgenii K. Zavoiskii laid the foundations for Electron Paramagnetic Resonance (EPR) spectroscopy. Despite his successful attempt to detect NMR signals in 1941, after the invasion of URSS, Zavoiskii's laboratories were moved to Kazan University, where he directed his efforts to build high-frequency radars for the militaries. Only in 1944 he was able to brush up on the academic work of three years before and to detect the electron paramagnetic resonance signal from manganese (II) sulfate heptahydrate.⁴³

In 1946 the Swiss physicist Felix Bloch, with his colleagues William Webster Hansen and Martin Packard, successfully performed the experiment of Rabi *et al.* on a sample of water. In his machinery, the sample was surrounded by a receiver coil and a transmitter coil, shielded from a probe head. While applying an external magnetic field, Bloch excited the ^1H nuclei with radiofrequency energy carried by the transmitter coil. Through the receiver, he was able to detect the electrical signal released by the precession of the proton nuclei to their original position. This phenomenon was called by Bloch "nuclear induction".³⁸⁻³⁹

Contemporarily, similar experiments were performed by Edward M. Purcell, Henry C. Torrey, and Robert V. Pound, who detected radiofrequency (RF) energy absorption in a paraffin

block. A resonant cavity with a frequency of 30 MHz was filled with 850 cm³ of paraffin, and a radiofrequency of 10⁻¹¹ watts was perpendicularly applied to the external magnetic field. Purcell *et al.* observed a reduction of the cavity output, indicating that part of the radiofrequency was absorbed by the paraffin protons on resonance.⁴⁴ The method developed by Bloch on solutions and by Purcell on solids is today known as “continuous-wave NMR” (CW NMR).

Between 1949 and 1950, an alternative method to CW NMR was proposed by the American physicist Erwin Hahn. As anticipated by Bloch, a short RF pulse that fulfills the resonance condition for the nucleus of interest leads to nuclear induction in the absence of an RF field. Starting from this assumption, Hahn *et al.* changed the structure of the Bloch apparatus, using only one RF coil for transmission and reception of the radiofrequency and a fast-responding amplifier to modulate the RF pulse. He carried successfully, for the first time, “pulsed NMR” experiments on Fe(NO₃)₃ solution and glycerol.⁴⁵

At Harvard University, Nicolaas Bloembergen, Purcell, and Pound described for the first time the phenomenon of nuclear relaxation in NMR. They observed the longitudinal relaxation of ¹H, calculating it directly from the saturation recovery of the nuclei or employing the so-called saturation-curve method, in which several saturation curves are obtained, maintaining constant modulation amplitude and varying the output voltage.⁴⁶ In the same years, at the University of Illinois, the term “chemical shift” was used for the first time. The American physicist Herbert S. Gutowsky and Charles J. Hoffman characterized more than 200 compounds by NMR and, based on the previous discoveries of Walter D. Knight and W. C. Dickenson⁴⁷, they defined the resonance frequency difference between the compound and the protons of a water sample.⁴⁸

In 1957, the first ¹³C NMR spectrum of organic compounds was detected by Paul C. Lauterbur.⁴⁹

In 1953, Albert W. Overhauser mathematically theorized the polarization of metallic nuclei through the irradiation of electrons. According to his theory, if the resonance conditions are fulfilled for the electron spins, the polarization will be transferred from the electrons to the protons through paramagnetic relaxation, considering that electrons and protons are hyperfine coupled.⁵⁰ Without being aware of it, Overhauser laid the foundations for what will be known only 10 years later as Overhauser dynamic nuclear polarization (ODNP).

In 1966 the Swiss physicist Richard R. Ernst proposed to substitute the sweep field method, particularly time-consuming, with a combination of short RF pulses and the consequent processing of the data by a Fourier transform (FT). Applying this method on a modified Varian DP60 spectrometer, Ernst *et al.* were able to perform 100 times shorter experiments than with field sweep NMR. The possibility to average more spectra over time led to a 10 times higher intensity in comparison to CW NMR.⁵¹ For his contribution to the field of resonance spectroscopy, Ernst was awarded the Wolf Prize and the Nobel Prize for Chemistry in 1991.

The employment of superconducting magnets, the development of high-field instruments, and the generation of new pulse sequences assessed NMR spectroscopy as one of the most employed techniques in chemistry, physics, and biology after the late '60s. In the latter field, the elucidation of complex macromolecules through NMR earned the Swiss chemist Kurt Wüthrich the Nobel Prize for Chemistry in 2002.⁵²

As already mentioned above, DNP is a “young” technique in comparison to NMR. The concept of DNP was first introduced by Overhauser in 1953, applying it to metals. The possibility to transfer the polarization from the electrons to the nuclei immediately interested the scientific community. In 1958, Abragam postulated the polarization transfer mechanism known today as “solid effect” (see Chapter 2).⁵³⁻⁵⁴ Only five years later, Alexander A. Kessenikh and A. A. Manenkov unraveled another DNP mechanism, namely the “cross effect”.⁵⁵ In 1961, Boris N. Provotorov introduced the concept of different “spin temperatures” associated to the electron Zeeman spin reservoir and the dipolar reservoir in a DNP sample. Based on the Provotorov theory, thermal mixing was postulated in 1964 first by Kozhushner and Provotorov⁵⁶⁻⁵⁷, and later by other physicists, such as Goldman and Borghini⁵⁸. In the last 40 years, DNP experienced a huge development, both from the hardware and the software points of view. The association of magic angle spinning (MAS) NMR with DNP in high magnetic fields embodied one of the main milestones reached in the field of DNP at the end of the last century, and it represents only one of the many applications for which DNP is employed. In the field of solution NMR, dDNP proposed in 2003 by Ardenkjaer-Larsen *et al.*²⁷, became a popular technique. Currently with over 50,000 entries on Google Scholar, dDNP is mainly applied in the field of MRI, biomolecular NMR, material science, and metabolomics.

1.3 How NMR works

1.3.1 The spin

Chapter content:

- What is a spin?
- The bra-ket notation.
- The energy levels.
- The g -factor.

1.3.1.1 What is a spin?

The concept of “spin” was introduced for the first time by Wolfgang Pauli in the 1919⁵⁹, and it is defined as the total angular momentum of charged particles, such as protons and electrons. While in general the total angular momentum would be equal to the sum of the spin angular momentum and the orbital angular momentum, in the case of structureless particles such as electrons, the orbital angular momentum is neglected, and the total angular momentum corresponds to the spin angular momentum. The spin angular momentum, in quantum mechanics, is interpreted as the intrinsic angular momentum of the particle, describing its energy.⁶⁰

Considering the limitations of classical Mechanics applied to quantum particles, a better understanding of spins dynamic was reached only in the 1920s when Niels Bohr and coworkers proposed the so-called “quantum theory”.⁶¹ Spins, considered now as quantizable, could be defined by discrete values describing their quantum states. The spin quantum number (m_s) is the most basic quantum number associated with the spin of a charged particle.

The total angular momentum, named I_{tot} , can be calculated as:

$$I_{tot} = [I(I + 1)]^{1/2} \hbar \quad (1.3.1.1.1)$$

In which $\hbar$ corresponds to the reduced Planck’s constant, equal to 1.054×10^{-34} J·s, and I indicates the spin angular momentum. The total angular momentum identifies all the possible energetic states of a particle, and it can be an integer or a half-integer. The spin resembles the energy of a precessing particle; however, it does not physically precess. The spin can be represented as a vector in the xyz space. In this instance, the interpretation of

the spin angular momentum can be simplified using the $\hat{I}$ operator, combining the three components $\hat{I}_x$, $\hat{I}_y$, and $\hat{I}_z$.

In the absence of a homogeneous magnetic field, named B_0 , the spins of an ensemble of charged particles are degenerate; therefore, the total energy of the system is averaged to zero.

Charged particles can interact with the magnetic field, indeed they have a spin magnetic moment $\hat{\mu}$, defined as:

$$\hat{\mu} = -g\mu_B\hat{I} = -\gamma\hbar\hat{I} \quad (1.3.1.1.2)$$

$$\mu_B = \frac{e\hbar}{2m_e} \quad (1.3.1.1.3)$$

With g corresponding to the g -factor, a proportionality constant equal to ca. 2.002319 for electron spins. The γ identifies the gyromagnetic ratio, strictly depending on the chemical nature of the particle. μ_B is the Bohr magneton, namely the magnetic moment of a precessing electron. The μ_B can be calculated as described in 1.3.1.1.3, with e corresponding to the elementary charge of an electron and m_e as the electron mass.

When a magnetic field is applied to the system, the spins align to B_0 (**Figure 1.2., A. and B.**), and the energy of $\hat{\mu}$ becomes directly proportional to the B_0 , according to the relation:

$$E = |\hat{\mu}|B_0 \quad (1.3.1.1.4)$$

In turn, the energy of $\hat{\mu}$ is related to the relative direction of the vector magnetic field, reaching the highest energy when the vector spin points in the opposite direction of the B_0 . It is important to consider that the single spin is surrounded by many other spins, presenting a magnetic moment; indeed, spins cannot be considered as single entities in space, but as a “bulk”.⁶² This concept is particularly important when the energy of a spin system is taken into account. While in ideal conditions, all the spins align to the external magnetic field, in reality random thermal motions partially disrupt the spins alignment.⁶² Therefore, the single magnetic moments align to B_0 with a certain angle θ , and only few of the spins are perfectly parallel (lowest energy) or antiparallel (highest energy) to the external magnetic field (**Figure 1.2., B.**). By averaging the net aligned and the randomly pointing magnetic moments, it is possible to calculate a, generally low, net magnetization for the whole spin bulk, represented by a *bulk magnetization vector* (**Figure 1.2., C. and D.**).⁶² For the sake of simplicity, in this thesis the concept of spin bulk is assimilated by the definition of “spin”.

Depending on its spin quantum number m_s the spin can populate different energy levels. In the case of ^1H , the simplest example of a diamagnetic spin in a magnetic field, the spin quantum number corresponds to $\pm\frac{1}{2}$. In this case, the spin can populate two different quantum states, the $+\frac{1}{2}$ and the $-\frac{1}{2}$. If the spin quantum number is not equal to $\pm\frac{1}{2}$, more than two states can be observed; for example, this is the case for spin with $m_s = 1$, such as ^{14}N .

The two energy states of a particle with m_s equal to $\pm\frac{1}{2}$ are denoted as α and β , with respectively low and high energy. The separation of the different energy states is known as “Zeeman splitting” or “Zeeman effect”, from the Dutch physicist Pieter Zeeman.⁶³

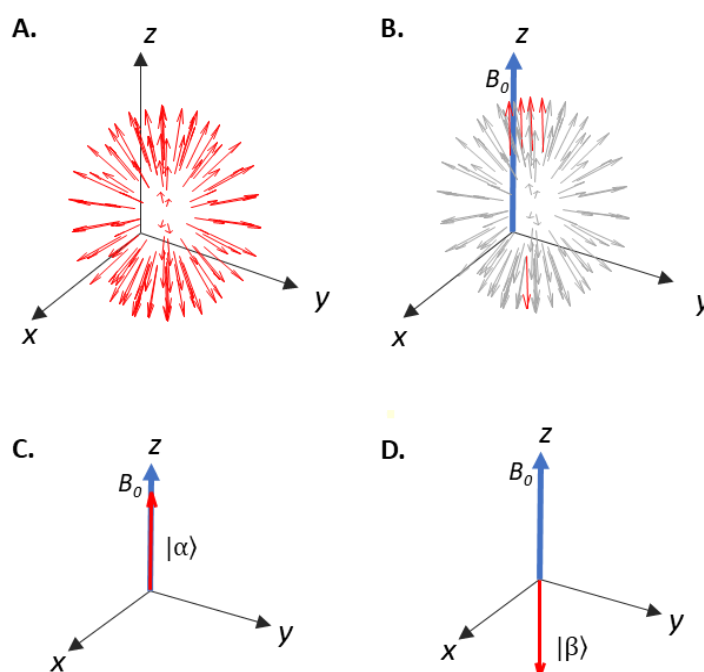


Figure 1.2. Spins in the absence and presence of a magnetic field. **A.)** When no external magnetic field is applied to the spins (red arrows), they degenerate, pointing in all directions. **B.)** When an external magnetic field B_0 is applied to the spins, a small percentage of spins align in parallel or antiparallel to the magnetic field (red arrows). The grey arrows indicate the spins of which alignment is disrupted by random thermal motions. The energy of their magnetic moments depends on the angle between the spin vector and the B_0 . At higher magnetic fields correspond a higher number of aligned spins. **C.)** Bulk magnetization vector of a spin system in its low energy state, pointing in the same direction as the magnetic field. **D.)** Bulk magnetization vector indicating a spin system in its high energy state, pointing in the opposite direction of the magnetic field.

1.3.1.2 The bra-ket notation

In quantum mechanics, the energy states can be defined by the bra-ket notation, also known as Dirac notation. It is written combining angle brackets ($\langle, \rangle$) and vertical bars ($|$), forming the “bra” $\langle$ and the “kets” $|$.

The kets are employed to describe a wavefunction. For example, a quantum mechanical particle can be represented as a one-dimensional wavefunction, $\psi(x)$, with x representing an infinite number of coordinates in space, and it can be written as:

$$\psi = \begin{bmatrix} \psi(x_1) \\ \psi(x_2) \\ \psi(x_3) \\ \vdots \\ \psi(x_n) \end{bmatrix} \quad (1.3.1.2.1)$$

The vector ψ is a column vector denominated “state vector”, living in the so-called Hilbert space. The state vector can be then written in bra-ket notation as:

$$|\psi\rangle = \begin{bmatrix} \psi(x_1) \\ \psi(x_2) \\ \psi(x_3) \\ \vdots \end{bmatrix} \quad (1.3.1.2.2)$$

In this case, the state vector is also called “ket vector”. The “bra vector” $\langle\psi|$ adjoint to the ket vector corresponds then to the transposed, complex-conjugate ket vector, and it can be written as the following row vector:

$$\langle\psi| = [\psi(x_1)^*, \psi(x_2)^*, \psi(x_3)^*, \dots] \quad (1.3.1.2.3)$$

Employing the bra-ket notation is particularly useful to write concisely quantum states and operators, but also to perform algebraic operations, such as the inner product between a bra vector and a ket vector. The inner product is written as $\langle\Phi|\psi\rangle$ and corresponds to the integral of the overlapped area between the two wave functions $\psi(x)$ and $\Phi(x)$.

1.3.1.3 The energy levels

Considering that the magnetic moment is proportional to the spin operator, the energy of a magnetic moment strictly depends on the energy state that the spin populates. Equation 1.3.1.1.4 can be rewritten as:

$$\Delta E = g\mu_B m_s B_0 \quad (1.3.1.3.1)$$

With m_s corresponding to the spin quantum number. Since a spin with m_s of $\pm \frac{1}{2}$ has two different states, the energy difference between high energy and the low energy state, known as “resonance condition”, is equal to:

$$\begin{aligned} \Delta E &= E_\beta - E_\alpha = g\mu_B B_0 \\ &= \gamma \hbar B_0 \end{aligned} \quad (1.3.1.3.2)$$

The energy difference between the two states can be also written as:

$$\Delta E = \gamma \hbar B_0 = \omega_0 \hbar = h\nu_0 \quad (1.3.1.3.3)$$

$$\omega_0 = \gamma B_0 \quad (1.3.1.3.4)$$

Where ω_0 indicates the Larmor frequency, namely the frequency of a rotating object in a magnetic field. The h is the Planck’s constant, and the ν_0 is the Larmor precession frequency expressed in Hz, equal to $\frac{\omega_0}{2\pi}$.

Quantum mechanically, the two energy states can be described also adopting the abovementioned bra-ket notation as:

$$|\alpha\rangle = \left| \frac{1}{2}, +\frac{1}{2} \right\rangle \quad (1.3.1.3.5)$$

$$|\beta\rangle = \left| \frac{1}{2}, -\frac{1}{2} \right\rangle \quad (1.3.1.3.6)$$

The relation between spin and energy state is governed by the time-independent Schrödinger equation:

$$\hat{H}|\psi\rangle = E_\psi |\psi\rangle \quad (1.3.1.3.7)$$

where $|\psi\rangle$ is the eigenfunction of $\hat{H}$, related to the eigenvalue E_ψ . In the case of a spin system $\pm \frac{1}{2}$, it can be rewritten as:

$$\hat{I}_z |\alpha\rangle = +\frac{1}{2} |\alpha\rangle \quad (1.3.1.3.8)$$

$$\hat{I}_z |\beta\rangle = -\frac{1}{2} |\beta\rangle \quad (1.3.1.3.9)$$

The eigenstates correspond to the energy levels that the particle can undertake, illustrated in **Figure 1.2., C.** and **D.**

The Zeeman splitting can be expressed with the Zeeman splitting Hamiltonian $\hat{H}_Z$, proportional to $\hat{I}_z$. It defines the behavior of a spin in a homogeneous magnetic field, contributing to the total Hamiltonian of the spin system.^{60, 62}

$$\hat{H}_Z = -\omega_0 \hat{I}_z \quad (1.3.1.3.10)$$

If the resonance condition is fulfilled, upon the application of an external energetic source to the spin system with $m_s = \pm \frac{1}{2}$, the spins redistribute among the different energy states, populating the high energy level β (**Figure 1.3.**). The spin distribution between high and low energy can be calculated by the Boltzmann distribution, postulated by Maxwell and Boltzmann in 1859⁶⁴:

$$\frac{N_\alpha}{N_\beta} = e^{\left(\frac{\Delta E}{k_B T_S}\right)} = e^{\left(\frac{\gamma \hbar B_0}{k_B T_S}\right)} \quad (1.3.1.3.11)$$

With the proportionality factor k_B as the Boltzmann constant, and the T_S indicating the thermodynamic temperature of the spin, related to its internal energy.⁶⁵

In a spin system with spin quantum number of $\pm \frac{1}{2}$, if an external magnetic field is applied, the spins align to the B_0 . However, the generated net magnetization along the z axes is rather small and almost impossible to detect. What it can be more easily measured is instead the *net transverse magnetization*, perpendicular to B_0 , upon irradiation with an RF in resonance with the spin system. While normally the perpendicular magnetization is close to zero, the RF can rotate the spins, for example of $\frac{\pi}{2}$, generating a precessing net transverse

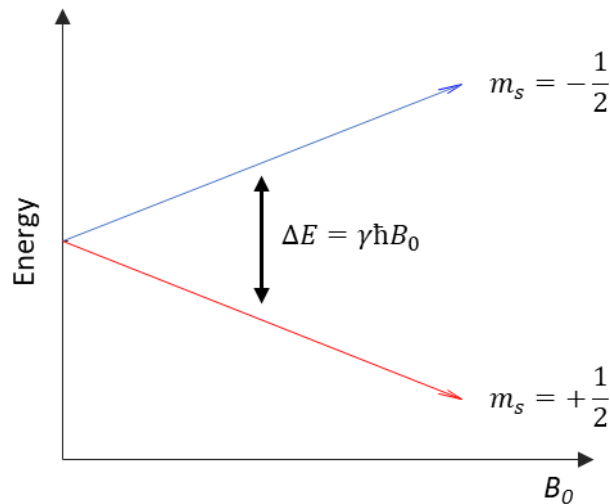


Figure 1.3. Correlation between the spin energy of the system and the magnetic field. The red arrow indicates the low energy state, while the blue arrow indicates the high energy state. The energy of a spin state is directly correlated to the strength of a magnetic field; stronger is a magnetic field, bigger is the energy needed to populate the high energy state.

magnetization perpendicularly to the external field. While precessing, the transverse magnetization also decays over time until it relaxes back to equilibrium. The transverse magnetization is measurable, since a rotating magnetic moment generates a rotating magnetic field, which in turn generates an electric field. The oscillating current is called *free induction decay* (FID) and it is detectable by a wire coil perpendicular to the external magnetic field.⁶⁰

1.3.1.4 The g -factor

When an external magnetic field is applied, electrons align to it. The electron spins can populate a low energy state and a high energy state, and the imbalance between these two populations generate polarization. Note that for the electrons, the high energy state is α , and the low energy state is β .

To redistribute the spins populating the two energy states, the resonance condition must be fulfilled. It is governed by the equation 1.3.1.3.1, and it is written as:

$$\Delta E = \pm \frac{1}{2} g \mu_B B_0 \quad (1.3.1.4.1)$$

With g corresponding to the electron spin g factor, equal to ca. -2.002318.

The electrons, like protons, are charged particles; moving in space, they generate a magnetic field that interacts with the surrounding environment. The two main factors governing the interaction of the electrons with the environment are:

the g -tensor $\rightarrow$ dictates the interaction between the electron and the external magnetic field.

the hyperfine and super-hyperfine coupling $\rightarrow$ describe the interaction with the surrounding nuclear and electron magnetic fields.

Quantum mechanically, the interaction between the electron spin and the external magnetic field can be described by the electron Zeeman Hamiltonian, written as:

$$\hat{H}_{EZ} = \frac{\mu_B}{\hbar} B_0 \cdot \hat{g}_s \cdot \hat{S} \quad (1.3.1.4.2)$$

$\hat{g}_s$ indicates the g -tensor, while $\hat{S}$ identifies the electron spin operator.

The g -tensor is expressed with the following three-dimensional matrix:

$$\hat{g}_S = \begin{bmatrix} g_{xx}^S & g_{xy}^S & g_{xz}^S \\ g_{yx}^S & g_{yy}^S & g_{yz}^S \\ g_{zx}^S & g_{zy}^S & g_{zz}^S \end{bmatrix} \quad (1.3.1.4.3)$$

Considering the symmetry of the tensor, there is a transformation for which the tensor is reduced to a diagonal; this coordinate system is called principal axis system (PAS), and only the diagonal of the matrix is taken into account. Indeed, it can be rewritten as:

$$\hat{g}_S = \begin{bmatrix} g_{xx}^S & 0 & 0 \\ 0 & g_{yy}^S & 0 \\ 0 & 0 & g_{zz}^S \end{bmatrix} \quad (1.3.1.4.4)$$

The three components of the g_S matrix cannot be averaged, since the system is anisotropic. Indeed, the so-called *g-anisotropy* affects the EPR lineshape of asymmetric PA like TEMPOL. In fact, the EPR frequency ω_S depends on the g -tensor length. The spectral density function $g_S(\omega)$ describes the change of frequency depending on the g -tensor and therefore identifying, for each molecular orientation in the sample, the EPR lineshape with the following normalized equation:

$$\int_{-\infty}^{\infty} d\omega g_S(\omega) = 1 \quad (1.3.1.4.5)$$

The presence of multiple paramagnetic centres in solution showing a different total frequency calculated on the orientation of the g -tensor generates a broadening of the EPR lineshape, which varies according to the strength of the external magnetic field, the physicochemical nature of the radical, and the solvent employed.⁶⁶

1.3.2 Scalar, dipolar coupling, and chemical shift

Chapter content:

- The j-coupling.
- The dipolar coupling.
- The chemical shift.

When the FID is Fourier transformed, the final NMR spectrum is obtained. Interestingly a single nucleus can generate multiple peaks, characterized by periodic distance between each other, therefore called *multiplets*.⁶⁷

The presence of *multiplets* in an NMR spectrum is due to the *coupling* between neighbouring spins. The coupling is the interaction between the magnetic fields of two spins close in space.

Different types of couplings, namely *J* and *dipolar coupling* can be described. The former is characterized by the presence of a chemical bond between the interacting nuclei. The latter implicates the coupling of nuclei through space.

1.3.2.1 The J-coupling

The first type of coupling is the J-coupling, also known as scalar coupling. In this case, the interaction between spins is mediated by a shared electron, characteristic of covalent and hydrogen bonds. In the case of two chemically bound nuclei, I_j and I_k , the magnetic field generated by I_j partially polarizes the electron involved in the nuclear bond. The polarization of the electron influences the magnetic field of I_k . Considering that I_j can populate two different spin states, the effective field on I_k changes accordingly, decreasing or increasing. The former generates an upfield shift in the resonance frequency of I_k ; the former shifts the signal downfield. The difference between the two signals is denominated *J* and it is generally expressed in Hz.

The J-coupling is fundamental in protein NMR spectroscopy, where pulse sequences transfer the polarization between covalently bound atoms. The J-coupling manifests in NMR spectra with the presence of multiplets (**Figure 1.4., A.**). The spins couple in several combinations depending on the number of atoms that are coupled and on the spin quantum number of the detected atom.

In the case of a two-spin system with $m_s = \pm \frac{1}{2}$, the states generated by the J-coupling are four, described by the following wavefunctions:

$$\phi_1 = |\alpha_j \alpha_k\rangle \quad (1.3.2.1.1)$$

$$\phi_2 = |\beta_j \alpha_k\rangle \quad (1.3.2.1.2)$$

$$\phi_3 = |\alpha_j \beta_k\rangle \quad (1.3.2.1.3)$$

$$\phi_4 = |\beta_j \beta_k\rangle \quad (1.3.2.1.4)$$

With *j* and *k* referring to the two interacting spins. The coupling between two spins and the influence on the respective magnetic moment is expressed by the following Hamiltonian:

$$\hat{H}_{jk} = 2\pi \hat{I}_j \cdot \hat{J}_{jk} \cdot \hat{I}_k \quad (1.3.2.1.5)$$

In which $\hat{I}_j$ and $\hat{I}_k$ are the spin operators of the two interacting spins, and J_{jk} is the J-coupling tensor. The J-coupling tensor is a vectorial quantity that assumes three different orientations in space, expressed by the matrix:

$$\hat{J}_{jk} = \begin{bmatrix} J_{xx}^{jk} & 0 & 0 \\ 0 & J_{yy}^{jk} & 0 \\ 0 & 0 & J_{zz}^{jk} \end{bmatrix} \quad (1.3.2.1.6)$$

Considering the Principal Axis System (PAS), only the diagonal elements of the matrix are taken into account. The J_{jk} is then calculated as the algebraic average of the three directions x, y, and z:

$$J_{jk} = \frac{[J_{xx}^{jk} + J_{yy}^{jk} + J_{zz}^{jk}]}{3} \quad (1.3.2.1.7)$$

The formula 1.3.2.1.5 is then rewritten as:

$$\hat{H}_{jk} = 2\pi J_{jk} \hat{I}_j \cdot \hat{I}_k \quad (1.3.2.1.8)$$

Considering J_{jk} as a scalar value in case of isotropic systems.

The effect of scalar coupling on a certain spin in resonance is strongly influenced by the nature of the two spins involved. When the coupling is heteronuclear, the difference in frequency between the two spins is greater than the J-coupling ($\Delta\nu > J$). This is the so-called weak coupling case. In this case, the x and y components contribute negligibly to the coupling; therefore, they can be omitted. The equation 1.3.2.1.8 is then rewritten in the final form:

$$\hat{H}_{jk} = 2\pi J_{jk} \hat{I}_z^j \cdot \hat{I}_z^k \quad (1.3.2.1.9)$$

The total Hamiltonian of a coupled, heteronuclear spin system can be then calculated as:

$$\hat{H} = -\omega_j I_z^j - \omega_k I_z^k + 2\pi J_{jk} I_z^j I_z^k = -\nu_j I_z^j - \nu_k I_z^k + 2\pi J_{jk} I_z^j I_z^k \quad (1.3.2.1.10)$$

Employing a time-independent Schrödinger equation (equation 1.3.1.3.7) combined with the wavefunctions defined in 1.3.2.1.1-1.3.2.1.4, it is possible to calculate the energy of each combined state as:

$$\hat{H}|\phi_1\rangle = -\frac{\nu_j}{2} - \frac{\nu_k}{2} + \frac{J}{4}|\alpha\alpha\rangle \quad (1.3.2.1.11)$$

$$\hat{H}|\phi_2\rangle = +\frac{\nu_j}{2} - \frac{\nu_k}{2} - \frac{J}{4}|\beta\alpha\rangle \quad (1.3.2.1.12)$$

$$\hat{H}|\phi_3\rangle = -\frac{\nu_j}{2} + \frac{\nu_k}{2} - \frac{J}{4}|\alpha\beta\rangle \quad (1.3.2.1.13)$$

$$\hat{H}|\phi_4\rangle = +\frac{\nu_j}{2} + \frac{\nu_k}{2} + \frac{J}{4}|\beta\beta\rangle \quad (1.3.2.1.14)$$

The transitions between the different energy levels are *single-quantum transitions* when they imply that only one spin change in population distribution. There are also *double-quantum* and *zero-quantum* transitions, involving a double transition from $\alpha\alpha \rightarrow \beta\beta$ and from $\alpha\beta \rightarrow \beta\alpha$, respectively (**Figure 1.4., B.**). The double-quantum and the zero-quantum transitions do not respect the energy conservation law, since the $\Delta m_s = \pm 2$. They can take place only in specific conditions, for example during DNP. The energy required for the forbidden transitions is calculated as:

$$E_{\alpha\alpha \rightarrow \beta\beta} = \omega_j + \omega_k \quad (1.3.2.1.15)$$

$$E_{\alpha\beta \rightarrow \beta\alpha} = \omega_j - \omega_k \quad (1.3.2.1.16)$$

A more detailed explanation of forbidden transitions can be found in **Chapter 2**.

The second case in which there is scalar coupling is when the difference in Larmor frequency between the two coupled spins is equal or smaller than the J-coupling distance ($\Delta\nu \leq J$). This is the strong coupling case. Generally, this situation is circumscribed to homonuclear systems and it is more frequent at low magnetic fields. The energy calculations for this system cannot be approximated. A further description of J-coupling in homonuclear spin systems go beyond the aim of this thesis.

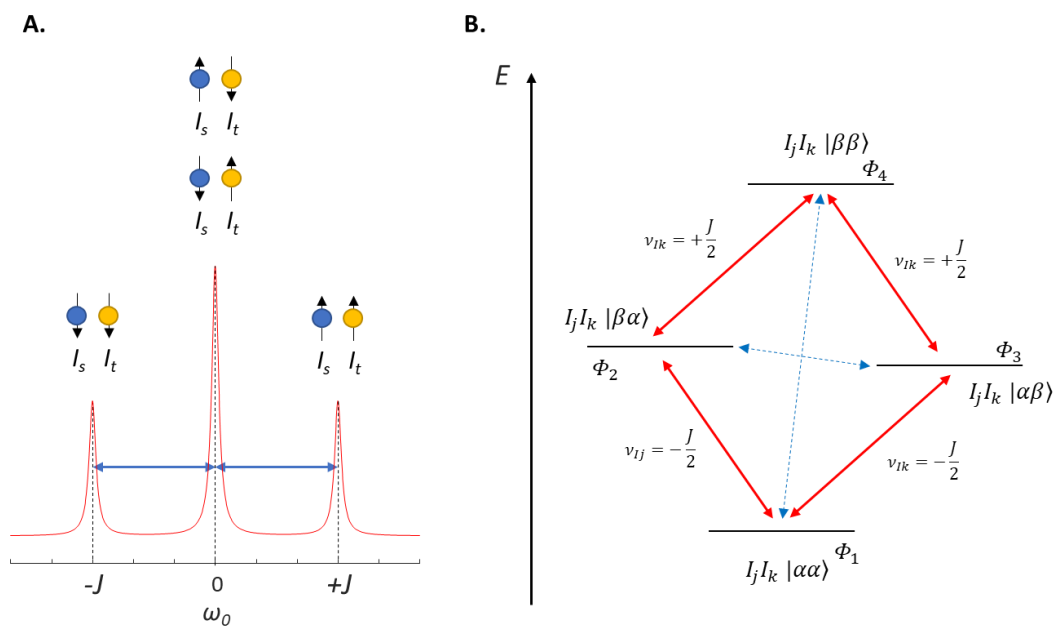


Figure 1.4. Scalar coupling of multiple nuclei. **A.)** Peak multiplets caused by J -coupling in a two-spin system like in the $^{13}\text{CH}_2$ group. The proton spins I_s and I_t populates the energy states as it is shown in the upper part of the figure, affecting the ^{13}C NMR spectrum as illustrated at the bottom. The distance between each peak corresponds to J . Each peak corresponds to a different energy state, populated by the scalar-coupled nuclei. The amplitude of the central peak is double the amplitude of the side peaks since the two states $|\alpha\beta\rangle$ and $|\beta\alpha\rangle$ display almost the same energy, collapsing in a single peak with higher amplitude. **B.)** Energy states diagram of spins I_j and I_k . The red arrows indicate the energy transitions that can happen upon RF irradiation. The blue dashed lines indicate the transitions, generally known as “forbidden”, that do not respect the energy conservation law.

1.3.2.2 The dipolar coupling

The J -coupling is mediated by the presence of a physical bond between the two interacting spins. Nevertheless, spins that are in close proximity to each other interact through their magnetic fields. Importantly, the main factor influencing the dipolar coupling is the distance between the two spins. Because of the possibility to determine inter-spin distances, the dipolar coupling is widely used in NMR spectroscopy for protein structural determination. Moreover, dipolar coupling represents the main mechanism of relaxation in NMR (see **Chapter 1.3.3.3**).

The energy of the dipolar coupling can be expressed as:

$$E_{DD} = -\frac{\mu_0}{4\pi} \left(\frac{3(\vec{\mu}_j \cdot \vec{r}_{jk})(\vec{\mu}_k \cdot \vec{r}_{jk})}{r_{jk}^5} - \frac{\vec{\mu}_j \cdot \vec{\mu}_k}{r_{jk}^3} \right) \quad (1.3.2.2.1)$$

With $\vec{\mu}_j$ and $\vec{\mu}_k$ indicating the magnetic moments of the two coupled spins, and μ_0 stating for the magnetic permeability in free space. The equation can be rewritten as a Hamiltonian, employing the spin operators $\hat{I}_j$ and $\hat{I}_k$.

$$\hat{H}_{DD} = -\frac{\mu_0}{4\pi} \frac{\gamma_j \gamma_k \hbar^2}{r_{jk}^3} \left[\frac{3(\hat{I}_j \cdot \vec{r}_{jk})(\hat{I}_k \cdot \vec{r}_{jk})}{r_{jk}^2} - (\hat{I}_j \cdot \hat{I}_k) \right] \quad (1.3.2.2.2)$$

Where $\vec{r}_{jk}$ indicates the vector of the distance between the two spins (**Figure 1.5.**). Substituting the $\vec{r}$ with the polar coordinates $\vec{r} = (|r|\sin\theta\cos\phi, |r|\sin\theta\sin\phi, |r|\cos\theta)$, it is possible to represent the dipolar Hamiltonian with the so-called “dipolar alphabet” as follows:

$$\hat{H}_{DD} = -\frac{\mu_0}{4\pi} \frac{\gamma_j \gamma_k \hbar^2}{r_{jk}^3} [A + B + C + D + E + F] \quad (1.3.2.2.3)$$

$$A = I_z^j I_z^k (3\cos^2\theta - 1) \quad (1.3.2.2.4)$$

$$B = -\frac{1}{4} (3\cos^2\theta - 1) (I_{j+} I_{k-} + I_{j-} I_{k+}) \quad (1.3.2.2.5)$$

$$C = \frac{3}{2} \sin\theta \cos\theta e^{-i\phi} (I_z^j I_{k+} + I_{j+} I_z^k) \quad (1.3.2.2.6)$$

$$D = \frac{3}{2} \sin\theta \cos\theta e^{-i\phi} (I_z^j I_{k-} + I_{j-} I_z^k) \quad (1.3.2.2.7)$$

$$E = \frac{3}{4} \sin^2\theta \cos\theta e^{-2i\phi} (I_{j+} I_{k+}) \quad (1.3.2.2.8)$$

$$F = \frac{3}{4} \sin^2\theta \cos\theta e^{2i\phi} (I_{j-} I_{k-}) \quad (1.3.2.2.9)$$

In the $\hat{H}_{DD}$ reported above, the two main factors affecting the energy of the spin system are the distance between the two spins r and the angle θ formed between $\vec{r}$ and z .⁶⁷⁻⁶⁸ The ϕ indicates the angle formed between the x axes and the coupled spin (**Figure 1.5.**). The term A of the dipolar coupling Hamiltonian does not describe a state transition of the spins, but the energy difference between the coupled spins.

The energy splitting between the two energy states is calculated applying the term A as follows:

$$\begin{aligned}
E_{\alpha\alpha \rightarrow \beta\alpha} &= E_{\beta\alpha} - E_{\alpha\alpha} \\
&= -\frac{\mu_0}{4\pi} \frac{\gamma_j \gamma_k \hbar^2}{r^3} (3\cos^2\theta - 1) \left[\langle \beta\alpha | I_z^j I_z^k | \beta\alpha \rangle \right. \\
&\quad \left. - \langle \alpha\alpha | I_z^j I_z^k | \alpha\alpha \rangle \right] = -\frac{\mu_0}{4\pi} \frac{\gamma_j \gamma_k \hbar^2}{2r^3} (3\cos^2\theta - 1)
\end{aligned} \tag{1.3.2.2.10}$$

$$\begin{aligned}
E_{\alpha\beta \rightarrow \beta\beta} &= E_{\beta\beta} - E_{\alpha\beta} \\
&= -\frac{\mu_0}{4\pi} \frac{\gamma_j \gamma_k \hbar^2}{r^3} (3\cos^2\theta - 1) \left[\langle \beta\beta | I_z^j I_z^k | \beta\beta \rangle \right. \\
&\quad \left. - \langle \alpha\beta | I_z^j I_z^k | \alpha\beta \rangle \right] = +\frac{\mu_0}{4\pi} \frac{\gamma_j \gamma_k \hbar^2}{2r^3} (3\cos^2\theta - 1)
\end{aligned} \tag{1.3.2.2.11}$$

The other five terms of the “dipolar alphabet” describe the energy of the zero-quantum, double-quantum, and single-quantum transitions. B indicates the probability of a zero-quantum transition to happen, with no effective change on the spin state of the system:

$$(I_{j+} I_{k-} + I_{j-} I_{k+}) |\alpha\beta\rangle \rightarrow |\beta\alpha\rangle \tag{1.3.2.2.12}$$

The C and D describe the single-quantum transitions between different energy states. Considering that, in both cases, only one spin populates a different energy state, C and D are energetically equivalent:

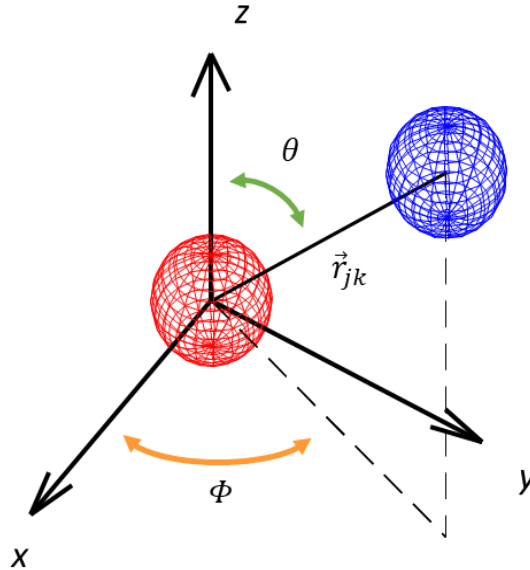


Figure 1.5. Spins I_j and I_k that are dipolar coupled. The distance between the two interacting spins is indicated by the vector $\vec{r}_{jk}$, while θ is the angle formed between the z axes and $\vec{r}_{jk}$. The angle between the x axes and the projection of the distance vector is indicated by Φ .

$$(I_z^j I_{k+} + I_{j+} I_z^k) |\alpha\alpha\rangle \rightarrow |\alpha\beta\rangle + |\beta\alpha\rangle \quad (1.3.2.2.13)$$

$$(I_z^j I_{k-} + I_{j-} I_z^k) |\beta\beta\rangle \rightarrow |\alpha\beta\rangle + |\beta\alpha\rangle \quad (1.3.2.2.14)$$

Finally, E and F describe the two forbidden double-quantum transitions also accessible by DNP, expressed as follows:

$$(I_{j+} I_{k+}) |\alpha\alpha\rangle \rightarrow |\beta\beta\rangle \quad (1.3.2.2.15)$$

$$(I_{j-} I_{k-}) |\beta\beta\rangle \rightarrow |\alpha\alpha\rangle \quad (1.3.2.2.16)$$

It is interesting to mention that, on the one hand, the contribution of the dipolar coupling to the total Hamiltonian of an isotropic system can be negligible if the rotational motion is faster than the $\Delta\omega_{jk}$, namely the difference in frequency between the two coupled signals. In this case, the $\hat{H}_{DD}$ is averaged to zero. On the other hand, the dipolar coupling provides information regarding the intermolecular distance in an anisotropic system, where the interacting molecules are strongly oriented.

1.3.2.3 The chemical shift

While before *heteronuclear* interactions between spins belonging to different atoms were mainly considered, it is necessary to mention that also electrons are moving charges in space and have a magnetic field. The magnetic cloud, generated by the electrons, strongly influences the nuclear spin. The same atoms, if shielded by a different electronic environment, for example a proton in a methyl group and a proton in a carboxylic group, generate small energy perturbations, identified as *chemical shifts*.

The chemical shift is generally represented by numbers on the scale of millionth. To facilitate and standardize the expression of chemical shift, it is calculated in the δ -scale referencing to the resonance frequency of tetramethyl silane (TMS), valid for the ^1H resonance. It is used as a standard and it is expressed as parts per million (*ppm*):

$$\delta = \frac{\nu - \nu_0}{\nu_0} \times 10^6 \quad (1.3.2.3.1)$$

Where ν is the Larmor frequency of the nucleus of interest and the ν_0 is the reference frequency.

Highly isotropic systems are extremely sensitive to nuclear magnetic field perturbations. Even small changes in the total energy of the system cause a substantial signal shift. Since the

surrounding electron cloud affects the magnetic field of the nucleus, the real magnetic field corresponds to the induced $\vec{B}_{ind}$, proportional to the shielding tensor and the external magnetic field:

$$\vec{B}_{ind} = -\hat{\sigma} \cdot \vec{B}_0 \quad (1.3.2.3.2)$$

Where $\hat{\sigma}$ corresponds to the shielding tensor and it is related to the electronic cloud surrounding the nuclei. It strictly depends on the chemical nature of the spin and on its surrounding environment. Adapting the Lamb formula⁶⁷, the isotropic shielding tensor can be calculated as:

$$\hat{\sigma} = \frac{e^2}{3m_e c^2} \int \frac{\rho(r)}{r} dr \quad (1.3.2.3.3)$$

With e corresponding to the energy, m_e to the mass, and the c to the charge. The r indicates the distance between the electron and the nucleus of interest, and ρ is the operator for the density matrix.

While in an anisotropic system the orientation of the chemical shielding is extremely important, in an isotropic system the molecules tumble fast enough to average their orientations. The σ is generally represented by the following matrix, in which all the possible orientations are displayed:

$$\hat{\sigma} = \begin{bmatrix} \sigma_{xx} & \sigma_{xy} & \sigma_{xz} \\ \sigma_{yx} & \sigma_{yy} & \sigma_{yz} \\ \sigma_{zx} & \sigma_{zy} & \sigma_{zz} \end{bmatrix} \quad (1.3.2.3.4)$$

Referring to the PAS, the matrix can be rewritten as follows:

$$\hat{\sigma} = \begin{bmatrix} \sigma_{xx} & 0 & 0 \\ 0 & \sigma_{yy} & 0 \\ 0 & 0 & \sigma_{zz} \end{bmatrix} \quad (1.3.2.3.5)$$

$$\sigma = \frac{[\sigma_{xx} + \sigma_{yy} + \sigma_{zz}]}{3} \quad (1.3.2.3.6)$$

Where the average of the diagonal component represents σ .

Considering all the concepts explained before, the total energy of the system is affected by the electronic perturbation; therefore, the total energy of the perturbed system is described as:

$$E_{CS} = -\vec{\mu} \cdot \vec{B}_{ind} \quad (1.3.2.3.7)$$

And the chemical shift contribution to the total Hamiltonian is:

$$\hat{H}_{cs} = \gamma \hat{I} \cdot \hat{\sigma} \cdot \hat{B}_0 \quad (1.3.2.3.8)$$

At higher electron densities surrounding the nucleus, the resonance frequency of the nuclei of interest shifts downfield. The possibility to discriminate between two nuclei that are very close in space but display different magnetic fields because of the surrounding environment represents one of the biggest advantages of NMR.

1.3.3 Relaxation mechanisms

Chapter content:

- Dipolar coupling and chemical shift anisotropy.
- Spin-lattice relaxation.
- Dipolar coupled spins.
- Spin-spin relaxation.

In a spin system, spins relax when they go back to their low energy state after excitation, due, for example, to the application of a secondary magnetic field. The possibility to relax and to release energy is intrinsic to the spins. The relaxation time of a molecule depends on its size. Therefore, relaxation studies can be employed to characterize spin systems. For example, relaxation experiments can provide structural dynamics information regarding a protein of interest.⁶⁹⁻⁷¹

Two main contributions to spin relaxation can be described:

Spin-lattice relaxation or longitudinal relaxation → the spin system releases its energy to the environment, called “lattice”, which acts as an energy reservoir during a relaxation time T_1 .

Spin-spin relaxation or transverse relaxation → the spin system loses coherent magnetization over a time T_2 .

In the case of spin systems with a spin quantum number of $\pm \frac{1}{2}$, the main mechanisms involved in relaxation are the dipolar coupling, particularly relevant in solution, and chemical shift anisotropy (CSA), caused by molecular electron currents induced by B_0 . This second mechanism becomes particularly relevant at high magnetic fields.

1.3.3.1 Dipolar coupling and chemical shift anisotropy

As it was introduced in the previous section, dipolar coupling is the interaction between two spins through their magnetic fields without chemical bonds connecting them.

The energy of the coupled system, and therefore of the coupled spins, was calculated in equation 1.3.2.17. In the formula, the distance between the spins, r , and the angle formed between them and B_0 , namely θ , are fundamental factors for the energy of the spin systems (**Figure 1.5.**). Higher distances, for example, are related to a weaker coupling, since the energy is inversely proportional to the distance. This relation remains valid also for a rapidly *moving* coupled system: the magnetic field generated by the spin I_j is affected by the time-dependent distance and orientation of the spins.

Regarding the CSA, it corresponds to the chemical shift contribution to the magnetic field under anisotropic conditions. The effect of electron shielding on the energy of a spin system is strictly orientation-dependent. In solution, the shielding tensor can be averaged according to the PAS, since the system is rapidly moving.

To calculate the CSA of a spin system, it is necessary to define the CSA tensor, described as:

$$\hat{\delta} = 1 - \hat{\sigma} = \begin{bmatrix} \delta_{xx} & \delta_{xy} & \delta_{xz} \\ \delta_{yx} & \delta_{yy} & \delta_{yz} \\ \delta_{zx} & \delta_{zy} & \delta_{zz} \end{bmatrix} = \begin{bmatrix} \delta_{xx} & 0 & 0 \\ 0 & \delta_{yy} & 0 \\ 0 & 0 & \delta_{zz} \end{bmatrix} \quad (1.3.3.1.1)$$

Where the $\hat{\sigma}$ represents the shielding tensor. Since the CSA tensor can assume different orientations in space. From the tensor, the CSA is calculated as:

$$\Delta\delta = \delta_{zz} - \frac{\delta_{xx} + \delta_{yy}}{2} \quad (1.3.3.1.2)$$

1.3.3.2 Spin-lattice relaxation

A particle in its high-energy state tends to relax to its ground state. When magnetic field inhomogeneities are present in the surrounding environment, the relaxation process is enhanced. Moving charges around the excited spin behave as energy reservoirs and, therefore, they trigger the return to a ground state with a transition rate W .

While the spin-lattice magnetization I_z corresponds to the difference between the ground state and the excited spin population, the change in spin population in the ground state and in the excited state over time (see equation 1.3.1.3.11) can be calculated as:

$$\frac{dN_\alpha}{dt} = -W(N_\alpha - N_{\alpha 0}) + W(N_\beta - N_{\beta 0}) \quad (1.3.3.2.1)$$

$$\frac{dN_\beta}{dt} = W(N_\alpha - N_{\alpha 0}) - W(N_\beta - N_{\beta 0}) \quad (1.3.3.2.2)$$

Where N_β represents the number of particles populating the excited state and N_α represents the number of particles in the ground state; the $N_{\alpha 0}$ and $N_{\beta 0}$ represent the number of spins at equilibrium before transition. W indicates the transition rate.

A phenomenological description of relaxation was proposed by Felix Bloch in 1946.³⁸ He defined the evolution of magnetization in the presence of an external magnetic field B_0 with the Bloch equation:

$$\frac{d\vec{M}}{dt} = \gamma \vec{M} \times \vec{B} - R\{\vec{M} - M_0\} \quad (1.3.3.2.3)$$

Where $\vec{M}$ indicates the magnetization vector, M_0 is the equilibrium magnetization, and R corresponds to the relaxation rate, which is defined by the following matrix, expressed with PAS:

$$R = \begin{pmatrix} R_2 & 0 & 0 \\ 0 & R_2 & 0 \\ 0 & 0 & R_1 \end{pmatrix} = \begin{pmatrix} 1/T_2 & 0 & 0 \\ 0 & 1/T_2 & 0 \\ 0 & 0 & 1/T_1 \end{pmatrix} \quad (1.3.3.2.4)$$

R_1 indicates the spin-lattice relaxation rate constant, equal to $\frac{1}{T_1}$; while R_2 defines the spin-spin relaxation rate constant, corresponding to $\frac{1}{T_2}$. To describe the longitudinal magnetization returning to equilibrium value with a time T_1 , the z component of the Bloch equation becomes:

$$\frac{dM_z}{dt} = \gamma(M \times B)_z - R_1(M_z - M_0) \quad (1.3.3.2.5)$$

Which in the rotating frame can be rewritten as:

$$\frac{dM_z}{dt} = -R_1(M_z - M_0) \quad (1.3.3.2.6)$$

The differential equation for the calculation of the spin-lattice magnetization is solved by integration, leading to the final form:

$$M_z(t) = [M_z(0) - M_0] \exp(-R_1 t) + M_0 \quad (1.3.3.2.7)$$

The equation 1.3.3.2.7 indicates that the magnetization is driven exponentially from $M_z(0)$ to M_0 depending on the relaxation rate. Generally, nuclei with large magnetic moments, such as ^1H , are characterized by high longitudinal relaxation rates, and, therefore, short relaxation times.⁶⁷

The longitudinal relaxation properties of a nucleus are often explored by inversion recovery (IR) experiments. With a 180°_x pulse, an evolution time τ , and a 90°_x , the R_1 is calculated by employing different τ delays. Considering that the starting magnetization condition $M_z(0) = M_0$ and that the signal amplitude U are proportional to the magnetization in M_z , the equation 1.3.3.2.7 can be rewritten in the following logarithmic form:

$$\ln \left[\frac{U(t) - U}{-2U_0} \right] = -R_1 t \quad (1.3.3.2.8)$$

Where U_0 corresponds to the equilibrium magnetization.

1.3.3.2.1 Dipolar coupled spins

The longitudinal relaxation rate of two coupled spins I_j and I_k with $m_s = \pm \frac{1}{2}$ leads to the transition between the energy states with a rate constant W_1 for the single-quantum transitions, W_2 for the double quantum transition, and W_0 for the zero-quantum one (**Figure 1.6**). The transition rate of each energy state population for coupled spin was described by Solomon in 1955⁷² and then by Redfield in 1957⁷³. While Solomon equations evaluate directly the relaxation rates between two spins I_j and I_k , Redfield describes the relaxation mechanisms with density matrices. The latter can be applied to any non-equilibrium system and to any relaxation mechanism with a semi-classical approach. The relaxation rates can be derived from the Liouville-von Neumann equation, also known as density operator equation of motion:

$$\frac{d\hat{\rho}(t)}{dt} = i[\hat{\rho}(t), \hat{H}_0 + \hat{H}_1(t)] = -i[\hat{H}_0 + \hat{H}_1(t), \hat{\rho}(t)] \quad (1.3.3.2.1.1)$$

Where $\hat{\rho}$ is the density matrix, and the Hamiltonian is the sum of a time-independent part ($\hat{H}_0$) and a time-dependent part ($\hat{H}_1(t)$).

The time-dependent component depends on the interaction with the lattice and it can be defined as:

$$\hat{H}_1(t) = \sum_q F^{(q)}(t) A^{(q)} \quad (1.3.3.2.1.2)$$

Where $A^{(q)}$ indicates the eigenoperators of the spin system. $F^{(q)}$ corresponds to the function of position due to stochastic lattice dynamic processes; they are part of the following autocorrelation function:

$$G^{(q,q')}(\tau) = \overline{F^{(q)}(t) F^{(q')*}(t + \tau)} \quad (1.3.3.2.1.3)$$

The autocorrelation function, if Fourier transformed, generates the spectral density function $J(\omega)$ as follows:

$$J(\omega) = \int_0^\infty G^{(q,q')}(\tau) e^{-i\omega\tau} d\tau \quad (1.3.3.2.1.4)$$

The spectral density function describes the fluctuations of the NMR signal at different frequencies.^{68, 74}

For two like spins dipolar coupled with each other, for example two protons, the relaxation is multiexponential. However, if the two spins are inverted, showing both negative

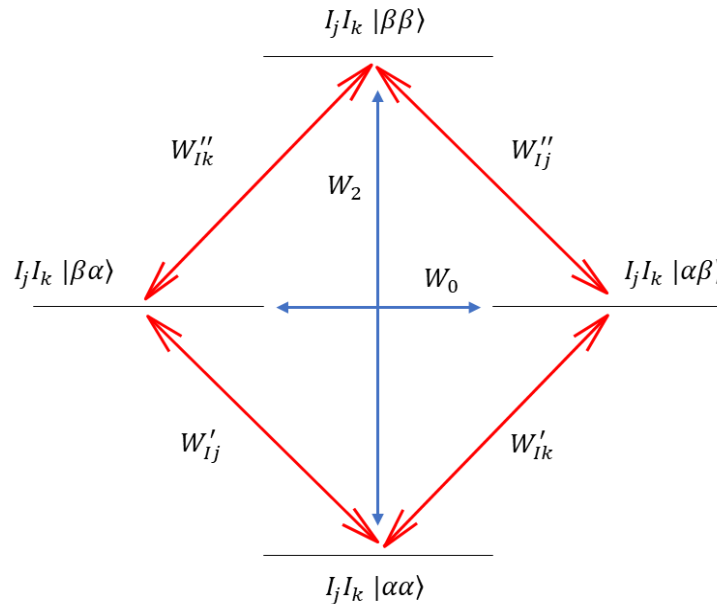


Figure 1.6. Coupled spins populating different energy states. The transition rates are indicated with W . The red arrows indicate the allowed transitions, while the blue arrows correspond to the double quantum (vertical arrow) and zero quantum (horizontal arrow) transitions.

magnetizations, the spin-lattice relaxation rate can be derived from the spectral density function with the following equation:

$$R_1 = \frac{6}{20} d^2 [J(\omega_H) + 4J(2\omega_H)] \quad (1.3.3.2.1.5)$$

$$d^2 = \frac{\hbar^2 \gamma_{I_j}^2 \gamma_{I_k}^2}{r^6} \quad (1.3.3.2.1.6)$$

In which the ω_H indicates the frequency of the ^1H .

In the case of unlike spins, for example a ^1H I_j with a ^{15}N nucleus I_N , the nuclear relaxation is also multiexponential, but a single longitudinal relaxation rate can be calculated upon proton saturation during the magnetization recovery. For bulkier nuclei than protons, the CSA must be taken into account, and the calculation of the longitudinal relaxation rate can be described by:

$$R_1 = \frac{d^2}{10} [J(\omega_{I_j} - \omega_{I_N}) + 3J(\omega_{I_N}) + 6J(\omega_{I_j} + \omega_{I_N})] + \frac{2}{15} \omega_{I_N}^2 \Delta\sigma^2 J(\omega_{I_N}) \quad (1.3.3.2.1.7)$$

Where $\frac{2}{15} \omega_{I_N}^2 \Delta\sigma^2 J(\omega_{I_N})$ indicates the CSA contribution and ω_{I_N} indicates the heteronuclear spin coupled with the ^1H .⁶⁷

1.3.3.3 Spin-spin relaxation

Spin-spin relaxation, also known as transverse relaxation, indicates the loss of transverse magnetization, favouring the longitudinal magnetization. Immediately after applying a $\pi/2$ pulse on the nuclei in resonance, the spins display *coherence* that is lost upon transverse relaxation.

To describe quantum mechanically the spin-spin relaxation of a homonuclear spin system, the Redfield theory is employed. Particularly, the rate of transverse relaxation is derived from the spectral density function $J(\omega)$ in equation 1.3.3.2.1.4 and defined as:

$$R_2 = \frac{3}{20} d^2 [3J(0) + 5J(\omega_H) + 2J(2\omega_H)] + R_{ex} \quad (1.3.3.3.1)$$

Where R_{ex} is the contribution of chemical exchange to the transverse relaxation, often omitted in reference texts⁶⁷, and the $J(0)$ is the spectral density function at zero frequency. The spin-

spin relaxation is influenced negligibly by the CSA, while chemical exchange can strongly influence the R_2 (for an extensive description of chemical exchange, go to **Chapter 1.3.4**). Fast or slow motions characterize the correlation time of the system. In the context of the spectral density function, the correlation time of a molecule consists in the time τ_c of the well-known correlation function.

If the system is in fast motion, the R_1 and R_2 equalize since the spectral density is independent from the frequency ω , as $J(0) = J(\omega) = J(2\omega)$. Therefore, the equation of the spectral density J in 1.3.3.2.1.4 is simplified as:

$$J(\omega) = \int_0^\infty g(\tau_c) e^{-i\omega\tau_c} dt = \lim_{\omega^2\tau_c \rightarrow 0} \left(\frac{2\tau_c}{\omega^2\tau_c^2 + 1} \right) = 2\tau_c \quad (1.3.3.3.2)$$

With τ_c indicates the time that the molecule needs to perform a complete rotation, and it is denominated rotational correlation time. The R_1 and R_2 are then calculated averaging the spectral density function as:

$$R_1 = \frac{6}{20} d^2 [1J + 5J] = \frac{3}{2} d^2 J \quad (1.3.3.3.3)$$

$$R_2 = \frac{3}{20} d^2 [2J + 5J + 2J] = \frac{3}{2} d^2 J \quad (1.3.3.3.4)$$

In the case of a heteronuclear spin system, also the CSA plays a crucial role in the calculation of transverse relaxation rates.⁶⁷ In the case of coupling between a ^1H I_j and a ^{15}N I_N , the transverse relaxation rate is equal to:

$$\begin{aligned} R_2 = \frac{d^2}{20} [& 4J(0) + J(\omega_{I_j} - \omega_{I_N}) + 3J(\omega_{I_N}) + 6J(\omega_{I_j}) \\ & + 6J(\omega_{I_j} + \omega_{I_N})] + \frac{1}{45} \omega_{I_N}^2 \Delta\sigma^2 [4J(0) + 3J(\omega_{I_N})] \\ & + R_{ex} \end{aligned} \quad (1.3.3.3.5)$$

In which both dipolar coupling and CSA contribute. $J(0)$ indicates the loss of coherence of transverse magnetization, while $J(\omega_{I_N})$ and $J(\omega_{I_j} + \omega_{I_N})$ contribute to the relaxation to equilibrium. Finally, $J(\omega_{I_j} - \omega_{I_N})$ affects the dephasing of ^{15}N magnetization, while $J(\omega_{I_j})$ describes the ^1H transition enhancing the dephasing of nitrogen magnetization.

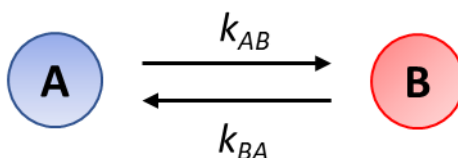
1.3.4 Chemical exchange

Chapter content:

- Slow exchange.
- Intermediate exchange.
- Fast exchange.

The chemical exchange between two different molecular environments influences the relaxation rate. Additionally, chemical exchange affects the lineshape of the NMR spectrum. When a system is in exchange, the chemical environment of the resonant spin is changed; therefore, the NMR spectrum shows a certain number of resonance peaks according to the exchange rate of the system. Chemical exchange has a fundamental role in chemistry, where multiple chemical species are reacting with each other, for example during the process of crystal formation.

A system in exchange can be schematically represented as:



Where “A” and “B” are the two exchanging species and k_{AB} and k_{BA} are the exchange rates. The total exchange rate, indicated as k_{tot} and equal to the sum of k_{AB} and k_{BA} , strongly correlates with the difference in resonance frequency $\Delta\nu$ of the exchanging spin systems. The populations of each single state are expressed as:

$$n_A = \frac{k_{BA}}{k_{AB} + k_{BA}} = \frac{1}{1 + K_{eq}} \quad (1.3.4.1)$$

$$n_B = \frac{k_{AB}}{k_{AB} + k_{BA}} = \frac{K_{eq}}{1 + K_{eq}} \quad (1.3.4.2)$$

$$K_{eq} = \frac{k_{AB}}{k_{BA}} \quad (1.3.4.3)$$

With n_a and n_b indicating the populations of the two states and K_{eq} identifying the reaction equilibrium.

Particularly, three main cases in which the resonance lines are affected by the exchange regime are defined:

$k_{tot} < \Delta\nu \rightarrow$ the system is in *slow exchange* on a millisecond timescale, and two different resonances are identifiable.

$k_{tot} \cong \Delta\nu \rightarrow$ the exchange regime is *intermediate*; therefore, a complex, broadened lineshape can be detected.

$k_{tot} > \Delta\nu \rightarrow$ the exchange is *fast* on a microsecond scale; only a single, generally sharp, resonance can be recorded.

The magnetization of each species is strongly influenced by the exchange rate. The signal of one species decreases when more molecules are exchanged with the other state that, instead, increase in intensity. According to the exchange regime, different experimental approaches can be adopted, as shown in **Figure 1.7**.⁷⁵

The magnetization of the species in exchange is described by the following derivatives:

$$\frac{dM_A}{dt} = -k_{AB}M_A + k_{BA}M_B \quad (1.3.4.4)$$

$$\frac{dM_B}{dt} = +k_{AB}M_A - k_{BA}M_B \quad (1.3.4.5)$$

And the total lineshape of the system is described by the general equation:

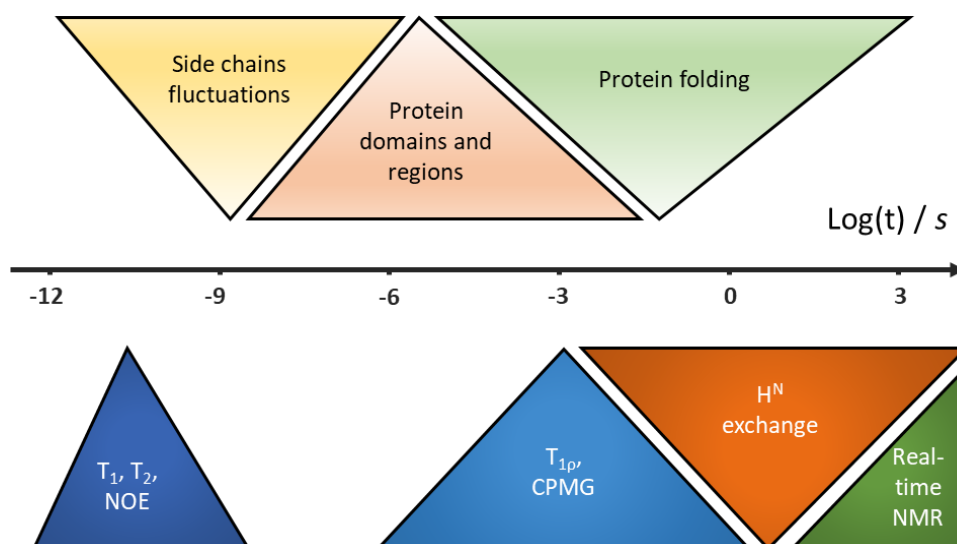


Figure 1.7. Different techniques can be applied to retrieve specific information, according to the speed of the process. While fast phenomena like side chains fluctuations can be explored by T_1 , T_2 , and NOE experiments, the exchange is accessible employing CPMG, H^N exchange, and real-time NMR for fast, intermediate, and slow exchange, respectively.

$$I(\omega) \propto \frac{1}{2} k (\Delta\omega)^2 \frac{1}{(\omega_A - \omega)^2 (\omega_B - \omega)^2 + 4k^2 \omega^2} \quad (1.3.4.6)$$

1.3.4.1 Slow exchange

In slow exchange conditions the exchange rate is lower than the difference in resonance frequencies of the two species. This means that the chemical environment of each species is stable enough to not cause strong perturbations on the surroundings of the resonant spins. In this case, two different peaks are discernible (**Figure 1.8., A.**). Generally, broader linewidths can be associated with the slow exchange since the exchange contributes to the spin-spin relaxation.

The lineshape of a species A in slow exchange is derived from the equation 1.3.4.6 as:

$$I(\omega) \propto \frac{1}{2} k \frac{1}{(\omega_A - \omega)^2 + k^2} \quad (1.3.4.1.1)$$

And the contribution to spin-spin relaxation corresponds to the exchange rate k .

1.3.4.2 Intermediate exchange

A system is in intermediate regime when the exchange rate equalizes the difference in frequency between the resonance frequencies of the two species in exchange. Interestingly, the intermediate exchange causes a broadening of the resonances in the NMR spectrum. The extreme broadening of the peaks, in some cases, can simulate the disappearance of the peak in the spectrum (**Figure 1.8., B.**).

1.3.4.3 Fast exchange

In fast exchange, the two exchanging species result in a single, relatively narrow lineshape. The detected peak is generally located at an equal distance from the resonance frequencies of the species in no exchange (**Figure 1.8., C.**). In this case, through the approximation of ω_A , it is possible to define the lineshape function, starting from the equation 1.3.4.6, as:

$$I(\omega) = \frac{1}{8k} (\Delta\omega)^2 \frac{1}{\frac{1}{k^2} (\Delta\omega)^4 \left(\frac{1}{8}\right)^2 + \omega^2} \quad (1.3.4.3.1)$$

The contribution of the exchange to the spin-spin relaxation is instead defined as:

$$R_2 = \frac{(\Delta\omega)^2}{8k} \quad (1.3.4.3.2)$$

In the case of fast exchange, the frequency contribution to relaxation is not negligible.

Interestingly, if the reaction between the species A and B is imbalanced, tending more to one chemical species than to the other, the single lineshape generated in the spectra does not have a frequency averaged on the single resonance frequency. In the case of an asymmetric reactions, the frequency of the single lineshape is the weighted average of the two lines (see **Chapter 6**). Mathematically, it is described with the formula:

$$\omega_{fin} = \frac{\omega_A + k_{tot}\omega_B}{1 + k_{tot}} \quad (1.3.4.3.3)$$

One of the main problems of the fast exchange is that with conventional NMR pulse sequences it is not possible to discern the single species contributing to the spectrum. However, it is possible to employ relaxation dispersion experiments, such as the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence, to detect the exchange rate when the system is in fast exchange.⁶⁷

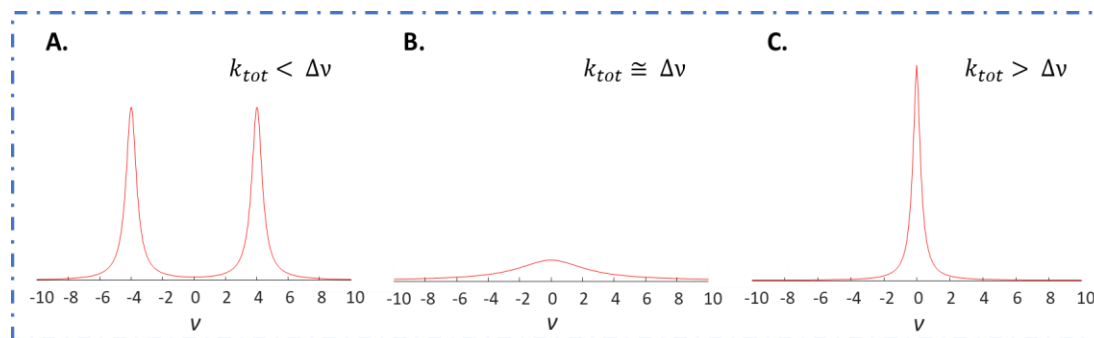


Figure 1.8. Slow (A.), intermediate (B.), and fast exchange (C.). While the two peaks are clearly discernible in the slow exchange regime, they tend to combine in one broad peak when the total exchange rate (k_{tot}) is equal to the difference in frequency of the two signals. In fast exchange, only one narrow peak is visible.

1.3.5 Hyperpolarization techniques

Chapter content:
• SEOP and MEOP.
• PHIP and SABRE.
• DNP.

NMR shows many advantages, but also some disadvantages, when applied to particularly complex, fast relaxing systems. The wide variety of hyperpolarization techniques available have all in common the possibility to polarize thousands of folds the NMR signals.

In hyperpolarization, a higher amount of nuclear spins is polarized, generating a stronger signal during relaxation and, therefore, peaks with higher intensity in the NMR spectrum. The booster effect of hyperpolarization drastically reduces the acquisition time: while in normal NMR the acquisition of intense signals can require hours, the hyperpolarized sample can produce a spectrum with a good sensitivity in couple of scans collected over a minute. The choice for the most suitable hyperpolarization technique is related to the substrates of interest, to the availability of the machinery, and to the economical effort required to set up a hyperpolarization machinery.⁷⁶

The main hyperpolarization techniques are:

Spin exchange optical pumping (SEOP)

Metastability exchange optical pumping (MEOP)

Parahydrogen induces polarization (PHIP)

Signal amplification by reversible exchange (SABRE)

DNP

These technologies are discussed in the next subchapters.

1.3.5.1 SEOP and MEOP

SEOP and MEOP are used to hyperpolarize gases. The SEOP is based on the discoveries of the Nobel Prize for physics Alfred Kastler, and it is employed to hyperpolarize noble gases such as ^3He or ^{129}Xe , the most commonly used gases for this technique.⁷⁷ The machinery for SEOP

is constituted by a borosilicate optical cell, coated with an alkali metal (*e.g.* Rb). After filling the cell with a mixture of noble gas and buffer gas (generally ^2N), the cell is placed into a homogeneous magnetic field and heated up to gasify the alkali metal coating. The cell is then irradiated with polarized light that is absorbed by the alkali metal. Through Fermi-contact interactions, the polarization is transferred from the resonant electrons of the metal to the noble gas.⁷⁸ The SEOP is mostly used for lung MRI, since hyperpolarized noble gases can be safely breathed.⁷⁹⁻⁸⁰

The MEOP differentiates from the SEOP in the stimulus triggering the hyperpolarization of the noble gas. In this case, an electrical discharge excites a population of atoms, generating a metastable species that transfers the polarization to noble gases through metastability-exchange collisions. The applications are similar as for SEOP.⁸¹

1.3.5.2 PHIP and SABRE

The PHIP is a popular hyperpolarization technique in solution, described for the first time in 1987 by Eisenschmidt *et al.*⁸² and based on the addition of parahydrogen as a medium for the hyperpolarization transfer. Parahydrogen is a molecule with energetically symmetrical protons that can hydrogenate other molecules by the polarization transfer schemes known as “parahydrogen and synthesis allow dramatically enhanced nuclear alignment” (PASADENA)⁸²⁻⁸³ and “adiabatic longitudinal transport after dissociation engenders net alignment” (ALTADENA)⁸⁴.

In a H_2 gas, two main isomers are represented: orthohydrogen and parahydrogen. While at room temperature the orthohydrogen is the prevalent form, by cooling to $<50\text{ K}$ in the presence of a catalyst, such as zeolites, the orthohydrogen is converted to parahydrogen. In the latter, the proton spins have an antisymmetric configuration (singlet state), while the orthohydrogen have three possible symmetric configurations (triplet state). The spins in the singlet state relax slowly and remains hyperpolarized for a relatively long time.

The hyperpolarized parahydrogen can transfer its polarization to the molecules of interest through hydrogenation. The parahydrogen, now covalently bound to the target molecule, relaxes to the neighbouring nuclei, such as carbons, which are then polarized. Otherwise, it is possible to directly detect ^1H .

The PHIP is mainly applied to catalytical studies in organic chemistry and in MRI, where the samples hyperpolarized with hydrogenation and used as a medium for imaging are mainly small molecules with a carbon centre, such as succinate or pyruvate.⁸⁵⁻⁸⁶

An alternative to the hydrogenative PHIP is the more recent non-hydrogenated PHIP, better known as Signal Amplification by Reversible Exchange (SABRE).⁸⁷ SABRE employs parahydrogen for polarizing organic substrates. The main advantage of SABRE over PHIP is that the hydrogenation process is not necessary for transferring the polarization. As the extended name of SABRE suggests, the polarization of the substrate takes place through the catalyst-mediated exchange of polarized protons between the parahydrogen and the target molecule. Indeed, the parahydrogen and the target molecule bind simultaneously to the catalyst (generally a metal, e.g. Iridium) and the polarization is transferred by J or spin-spin dipolar coupling.

The SABRE is also employed in biomedical imaging.⁸⁸

1.3.5.3 DNP

Dynamic nuclear polarization (DNP) is a hyperpolarization technique, generically described as the transfer of polarization from an electron to a nucleus. The foundations of DNP were laid in 1953 by the American physicist Albert Overhauser, who postulated for the first time the Overhauser effect in NMR.⁵⁰

DNP further evolved in the second half of the 20th century, branching in different DNP specialities, depending on the technical setup and on the type of substrates used for the experiments. Nowadays, DNP is mainly represented by:

Solid state DNP (ssDNP)

Dissolution DNP (dDNP)

Overhauser DNP (ODNP)

The detailed description of ssDNP and ODNP is beyond the scope of this text; therefore, only dDNP will be treated in detail.

dDNP was proposed for the first time in 2003 by Anderkjaer-Larsen *et al.* as a method to potentially enhance the signal of a liquid sample over 10,000 folds its thermal equilibrium values.²⁷ dDNP is characterized by a unique technical setup, different from the ssDNP. In dDNP, a mixture containing a polarizing agent (PA), such as a nitroxide radical, is inserted in

the DNP apparatus, characterized by a homogeneous magnetic field and a constant temperature below 2 K. The sample is subsequently irradiated with microwaves: the electrons of the PA transfer their polarization to the surrounding nuclei. Finally, the sample is dissolved and transferred from the cryostat to the NMR machine, where the hyperpolarized spectrum is acquired. With proved efficiency and repeatability, dDNP is applied for protein structure and dynamic determination⁸⁹⁻⁹⁰, on nucleic acids⁹¹, and for the study of metabolites⁹²⁻⁹³. DDNP is particularly advantageous for the substantial reduction of the acquisition time and the possibility to access low concentrated liquid samples, impossible to study with conventional NMR.⁹⁴

Despite the great success of DNP, the theory behind it remains cumbersome and not all the phenomena mediating the hyperpolarization transfer are understood in their entirety. In the next Chapter, the basics of DNP theory are discussed, and the dDNP-NMR of the University of Vienna is described as an example of experimental setup for dDNP studies.

1.4 References

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Chapter 2

2.1 What is DNP?

DNP is a technique employed for the hyperpolarization of organic and inorganic substrates and MRI contrast agents.¹⁻⁶ This chapter introduces DNP theory, summarizing the main mechanisms of polarization. The hardware of the dDNP, employed to perform the experiments reported in this thesis, is also presented. Finally, the concept of hyperpolarized water and its application on biomolecules is introduced.

DNP boosts the NMR signal intensity through the polarization transfer from uncoupled electrons to nuclei. The concept of polarization relates to the spin population imbalance between the low and the high energy states, namely α and β , described by the Boltzmann equation (see equation 1.3.1.3.11).⁷

The polarization of a spin with m_s equal to $\pm \frac{1}{2}$ is described as:

$$P_S = \frac{n_\alpha - n_\beta}{n_\beta + n_\alpha} \quad (2.1.1)$$

Where n_α is the number of spins in the low energy state, and n_β is the number of spins in the high energy state. The larger the imbalance, the higher the polarization. The maximum spin polarization is reached when all the spins populate one state, generating a positive or negative polarization. According to the relationship in equation 2.1.1, the polarization is rewritten as:

$$P_S = \tanh \left(\frac{\gamma \beta_s B_0}{2} \right) \quad (2.1.2)$$

$$\beta_s = \frac{\hbar}{k_B T_S} \quad (2.1.3)$$

Where β_s is the inverse spin temperature coefficient, and T_S corresponds to the spin temperature. Spin temperature's real meaning and significance were amply debated in the last century.⁸⁻⁹ While, generally, the spin temperature corresponds to the lattice temperature, this is not always the case, particularly in DNP; for example, when a 180° pulse is applied on a polarized spin, there is no transverse magnetization and the spins populate majorly the high energy state. In this case, the spin temperature is lower than the lattice temperature.¹⁰

In NMR, the degree of spin polarization reached is low. To tackle this problem, higher magnetic fields can be employed, generating a mild increase in the P_s according to equation 2.1.2. Another option consists of exploiting the electrons as polarization reservoirs. Uncoupled electrons are essential in DNP samples; they are generally provided by the addition of a PA to the mixture.

Electrons feature a higher gyromagnetic ratio than nuclei – not considering that conventionally the γ_e is negative. The ratio of the γ of electrons and protons is ca. 660. According to equation 2.1.2, electrons polarize more efficiently than protons. While almost 100% of electrons are polarized at 1 K, nuclei such as ^1H and ^{13}C feature lower polarization. For example, around 70% of the electrons are polarized at a magnetic field of 6.7 T at 5 K; on the contrary, only the 0.13% and 0.034% of protons and carbons, respectively, are polarized (**Figure 2.1.**).

Microwaves in the order of GHz can trigger the polarization transfer from the electrons to the nuclei. In this way, nuclear polarization is boosted.

Three main polarization transfer mechanisms contribute to DNP according to the literature:

Solid effect

Cross effect

Thermal mixing

In the solid effect, the polarization is transferred from the electrons to the nuclei by a flip-flop mechanism, allowing the zero-quantum and the double-quantum transitions to take place. In the case of thermal mixing and cross effect, the polarization is transferred by triple spin flips, involving two coupled electrons and one nucleus.¹¹

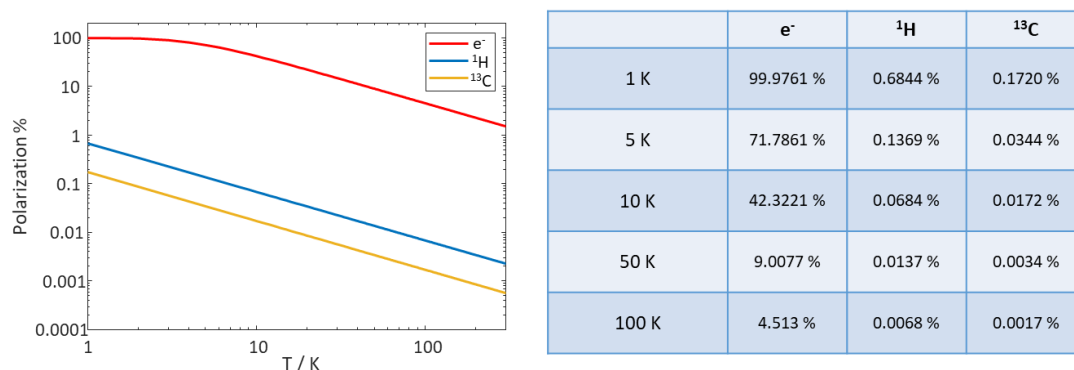


Figure 2.1. Simulation of electrons, protons, and carbons polarization percentage in dependence of temperature with a magnetic field of 6.7 T. The electrons experience a polarization of almost 100 % at 1 K, decreasing dramatically at temperatures above 5 K. The polarization of protons and carbons is overall weaker, as it is reported in the table on the right.

2.2 The EPR spectrum

Chapter content:

- Types of PA employed in DNP.
- The DNP sample preparation.
- Hyperfine and super-hyperfine coupling.
- The hole burning.
- Spectral diffusion.

2.2.1 Types of PA employed in DNP

The choice of a suitable PA for a DNP experiment depends on several factors, such as its radical EPR line, its stability, and its relaxation rate; additionally, the PA must be compatible with the substrate to polarize.¹²

In dDNP, the mostly utilized paramagnetic species belong to two main classes:

Aminoxyl group radicals (commonly known as *nitroxides*)

tri-aryl-methyl radicals

Because of their relative stability and versatility, nitroxides were employed as PA for more than fifty years.¹³⁻¹⁵ Nitroxides feature a broad EPR line, and they are suitable for the

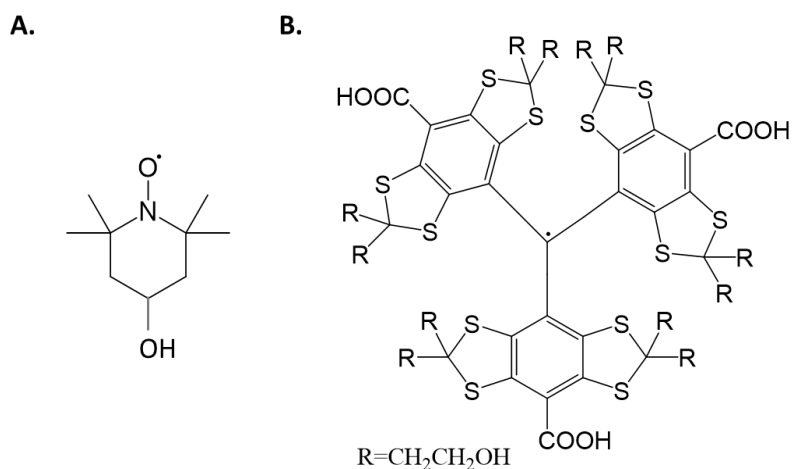


Figure 2.2. Radicals commonly used for DNP. **A.)** 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl, namely TEMPOL. **B.)** OX063 trityl radical.

polarization of high gyromagnetic ratio nuclei, such as ^1H . On the contrary, the polarization of spins with a lower gyromagnetic ratio is less effective.^{12, 16} Generally, the transfer of polarization from the unpaired electrons of nitroxides to the target nuclei mediated by thermal mixing or cross effect is more efficient.¹⁷ One of the most used nitroxide is the 4-hydroxy-TEMPO (TEMPOL) (**Figure 2.2., A.**). From TEMPOL, many other nitroxide derivatives were produced and employed for DNP, such as the biradicals AMUPOL¹⁸ and TOTAPOL¹⁹, mainly utilized in solid-state DNP applications.

The tri-aryl-methyl radicals differs from the nitroxides for the narrow EPR line, ideal for the hyperpolarization of low gyromagnetic ratio nuclei, such as ^{13}C . In this case, the EPR line is relatively slim, and the main polarization transfer mechanism implied is the solid effect. Interestingly, the addition of Gd^{3+} to a DNP sample containing tri-aryl-methyl radicals increases the polarization efficiency.²⁰ One of the most commonly employed radicals belonging to this class is the trytil OX063.

2.2.2 The DNP sample preparation

The PA is not the only element important to formulate an efficient DNP sample. Indeed, another very important factor in the preparation of the DNP sample is the presence of a cryoprotectant, such as glycerol-*d*8 or DMSO.¹² It allows a homogeneous distribution of the PA and substrate in the vitrified sample. The lack of a glassing agent would lead to the crystallization of the DNP sample, and, therefore, to the inhomogeneous compartmentalization of radical and salts. This step is crucial for an efficient DNP experiment, since polarization is heavily influenced by the local concentration of the paramagnetic species that must be tuned according to the substrate and to the radical type.¹⁶

2.2.3 Hyperfine and super-hyperfine coupling

Unpaired electrons not only interact with B_0 , but also with charged particles present in the surrounding environment through two different modalities: the hyperfine and the super-hyperfine coupling. Both of them depends on the distance between the coupled spins; they can be compared to the J- and dipolar coupling occurring between nuclei. In the following excursus, the coupling between an unpaired electron and a nucleus with $m_s = \pm \frac{1}{2}$ is considered.

The hyperfine coupling is expressed by the following Hamiltonian:

$$\hat{H}_{HF} = \hat{S} \cdot \hat{A} \cdot \hat{I} \quad (2.2.3.1)$$

$$\hat{A} = \begin{bmatrix} A_{xx} & A_{xy} & A_{xz} \\ A_{yx} & A_{yy} & A_{yz} \\ A_{zx} & A_{zy} & A_{zz} \end{bmatrix} = \begin{bmatrix} A_{xx} & 0 & 0 \\ 0 & A_{yy} & 0 \\ 0 & 0 & A_{zz} \end{bmatrix} \quad (2.2.3.2)$$

With $\hat{A}$ indicating the hyperfine tensor that, according to the PAS reference system, is described by the matrix in equation 2.2.3.2. If the radical employed belongs to the class of nitroxides, the contributions from A_{xx} and A_{yy} can be very small; hence, they can be neglected. The Hamiltonian in 2.2.3.1 is then rewritten as:

$$\hat{H}_{HF} = A_{zz} \hat{S}_z \hat{I}_z \quad (2.2.3.3)$$

As for the nuclear coupling, the hyperfine tensor depends on the angle formed between the electron and the coupled nucleus. It depends on an isotropic component A_{iso} , known as Fermi-contact interaction²¹⁻²², and an anisotropic component T :

$$A = A_{iso} + T(3\cos^2\theta - 1) \quad (2.2.3.4)$$

$$A_{iso} = \frac{2\mu_0}{3\hbar} g_e \mu_B g_n \mu_n |\psi(0)|^2 \quad (2.2.3.5)$$

$$T = \frac{1}{r^3} \frac{\mu_0}{4\pi\hbar} g_e \mu_B g_n \mu_n \quad (2.2.3.6)$$

On the one hand, the Fermi-contact interaction factor depends on $|\psi(0)|^2$, corresponding to the wavefunction of the electron interacting with the nucleus. On the other hand, the anisotropic component of the hyperfine tensor is influenced by the cube of the distance between the electron and the nucleus.

Interestingly, while the hyperfine tensor affects the EPR profile causing a field shift independently on the magnetic field, the g -anisotropy is strictly related to the external B_0 , affecting the EPR lineshape accordingly.

The interaction between the unpaired electron and many surrounding nuclei, located in different molecules, complicates the calculation of the total electron spin Hamiltonian. For example, the super-hyperfine coupling must be considered.

While the Zeeman component is preponderant, the super-hyperfine contribution, which describes the influence of n -number of nuclei on the energy of one electron, is relatively

small. The exact super-hyperfine contribution is practically impossible to calculate, but it can be evaluated by a statistical approach.

2.2.4 The flip-flop mechanism and spin diffusion

Another factor that influences the EPR lineshape, apart from the g -anisotropy and the super-hyperfine coupling, is the interaction between the nuclear spins with each other.

In coupled spin systems, spins can undergo *flip-flop transitions*. When a spin is populating its high energy state and a proximal nucleus is in its low energy state, a switch of population can occur. The flip-flop mechanism take place between nuclei or between electrons, and it lies at the basics of fundamental DNP mechanisms, such as spectral diffusion (see section 2.2.6), spin diffusion, and solid effect (see section 2.3.2). The inversion of the energy states induced by the flip-flop transition between two nuclei is expressed as:

$$|m_s^{Ik}\rangle|m_s^{Ij}\rangle = \left|+\frac{1}{2}\right\rangle\left|-\frac{1}{2}\right\rangle \leftrightarrow \left|-\frac{1}{2}\right\rangle\left|+\frac{1}{2}\right\rangle \quad (2.2.4.1)$$

The flip-flop mechanism takes place when the difference between the hyperfine tensor energy of the two interacting nuclei with the electron spin is close to zero, meaning that the coupling between nuclei and electrons is very weak. The “flipping” nuclei can be denominated *bulk nuclear spins*.¹¹ The flip-flop mechanism is a time-dependent process happening continuously in the sample, allowing the polarization to flow through the sample. This process is denominated *spin diffusion* and it is fundamental in DNP to homogeneously distribute the polarization among the nuclear spins of the DNP sample.

If the hyperfine tensor is particularly large, the flip-flop transition does not take place. This second case is typical of nuclei surrounding paramagnetic centers, strongly coupled to them; indeed, the anisotropic component of A is inverse proportional to the distance between the two particles. They form the so-called *local nuclear spin* environment. In this second case, the strong coupling between nuclei and electrons prevents any flip-flop transition, due to the energy difference between them. The local nuclear spins and the bulk nuclear spins interact with each other through the *diffusion barrier*. The concept of diffusion barrier was introduced for the first time by Blumberg *et al.* in 1960²³ and the physical characteristics of it are still debated. It was recently published that the nuclear spin diffusion barrier for nuclei surrounding a vanadium paramagnetic center lies between 4 and 6.6 Å.²⁴ These results were

confirmed later on by DNP, showing that the diffusion barrier surrounding an electron has a radius $<6 \text{ \AA}$ (**Figure 2.3., A.**).²⁵

Considering the different characteristics of local nuclear spins and bulk nuclear spins, they can affect the EPR spectrum in different ways. Indeed, while the local nuclear spins correlate with the static *inhomogeneous broadening*, the bulk nuclear spins are linked to a time-dependent *homogeneous broadening* of the EPR lineshape. The several “contributions” to the EPR spectrum are called *spin packets*, graphically represented as narrow homogeneous lines “covered” by an inhomogeneous profile (**Figure 2.3., red curves in B.**). The inhomogeneous broadening, indeed, influences the total EPR profile consistently by the static nuclear spin populations located close to the electrons (**Figure 2.3., blue profile in B.**).

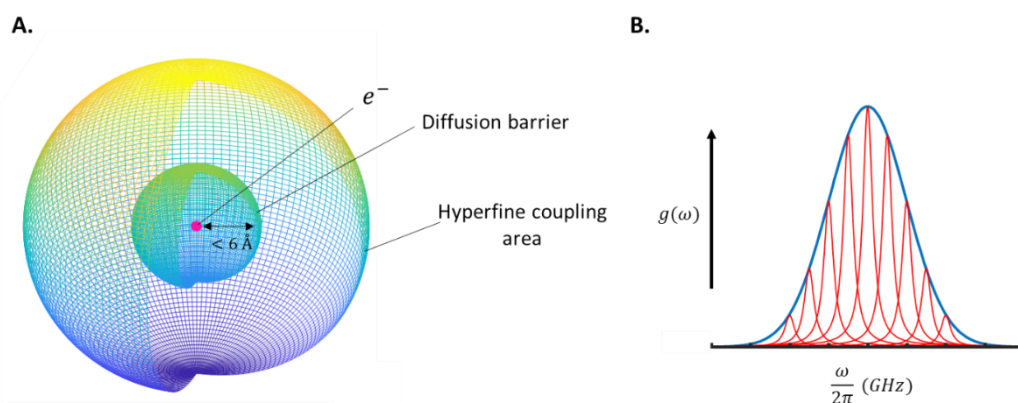


Figure 2.3. The flip-flop mechanism takes place between the spins of the bulk, generating homogeneous and inhomogeneous broadening of the EPR spectrum. **A.)** Simplified schematic representation of the surrounding of a paramagnetic center. The local nuclear spins are localized in the diffusion barrier, generally with a distance from the electron of less than $6 \text{ \AA}$. The bulk nuclear spins localize between the diffusion barrier and the hyperfine coupling area. **B.)** The EPR line is characterized by homogeneous (red curves) and inhomogeneous (blue profile) broadening. The spin packets contribute to the broadening through single narrow resonances derived from the strong coupling between nuclei and electron.

2.2.5 The hole burning

The electron spins absorb energy when irradiated with microwaves on resonance. At certain frequencies, the electrons can absorb the amount of energy necessary to equalize the “up” and “down” populations, leading to the cancellation of the EPR signal at that specific ω ; this phenomenon is called *saturation*. In DNP, when the electron signal of a specific spin packet is saturated, the homogeneously broadened line features an absorption dip, defined as a *hole*. The DNP sample undergoes the effect of an external B_0 , influenced by the many magnetic fields present in the surrounding of the electron, leading to a specific local field.

Upon the microwave irradiation that generates the magnetic field B_1 , only certain spin packets are saturated. Therefore, the change in polarization of the saturated electron spins “burns a hole” as in **Figure 2.4**.¹¹

The broadening of the hole burnt in the EPR spectrum depends on the *spectral diffusion*. The mechanism of spectral diffusion consists of the transfer of spin polarization between electrons (see section 2.2.6). The width of the hole is directly proportional to the spectral diffusion speed since the spectral diffusion affects the homogeneous broadening of the EPR spectrum. Faster the polarization flows from one electron to the other, wider will be the hole generated by the saturation of a spin packet.

The hole burning involves only the homogeneous broadening of the EPR line, which is affected by time-dependent changes in the bulk nuclear spins. Therefore, when a hole is burnt, the local environment of a non-saturated electron can change to the point that some spin packets shift on the inhomogeneously broadened EPR line. This particular phenomenon is known as packet-shifting and it can be observed in ^{29}Si ²⁶⁻²⁷ and ^{31}P ²⁶ electron-nuclear double resonance (ENDOR) experiments.²⁸

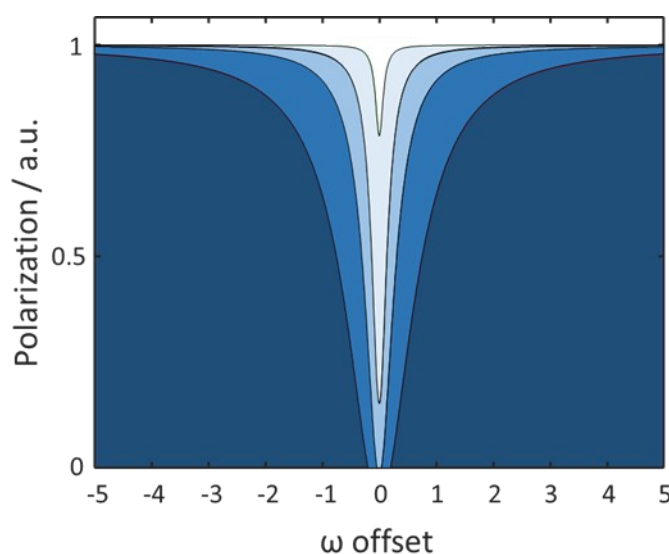


Figure 2.4. Hole burning effect at a certain frequency caused by signal saturation. The size of the hole burnt depends on the frequency used and the Larmor frequency of the electron in its own local environment. The different colors indicate burnt holes can have different width according to the spectral diffusion speed.

2.2.6 Spectral diffusion

When electrons mutually interact through hyperfine and super-hyperfine coupling, they can transfer the polarization to the neighboring electrons with flip-flop transitions. This mechanism is the abovementioned spectral diffusion.

In a sample containing a PA, the resonance frequency of each electron spin packet is different. When the sample is irradiated with a frequency ω_m , only few electrons are saturated. The excitation of the resonant electrons induces a flip-flop “cascade” mechanism, diffusing the polarization to the hyperfine coupled electrons. The initially saturated spins relax to their low energy state; at the same time, the proximal electron spins not in resonance are polarized. This phenomenon allows the transport of polarization homogeneously in the sample, despite the frequency employed for the irradiation resonating only with a few electron spins (**Figure 2.5.**).^{11, 29}

While spectral diffusion causes homogeneous broadening on the EPR lineshape, inhomogeneous broadening derived from electron-electron transient interactions can be predicted by applying the Provotorov’s theory of magnetic resonance saturation. Postulated in 1961 by the Russian physicist Boris N. Provotorov, it describes the change in polarization that two coupled spins undergo in high temperature approximation when a pulse is applied.³⁰⁻³² Despite the initial application to homogeneous broadening in solid samples, later it was adapted for studies of inhomogeneous broadening at low temperatures.

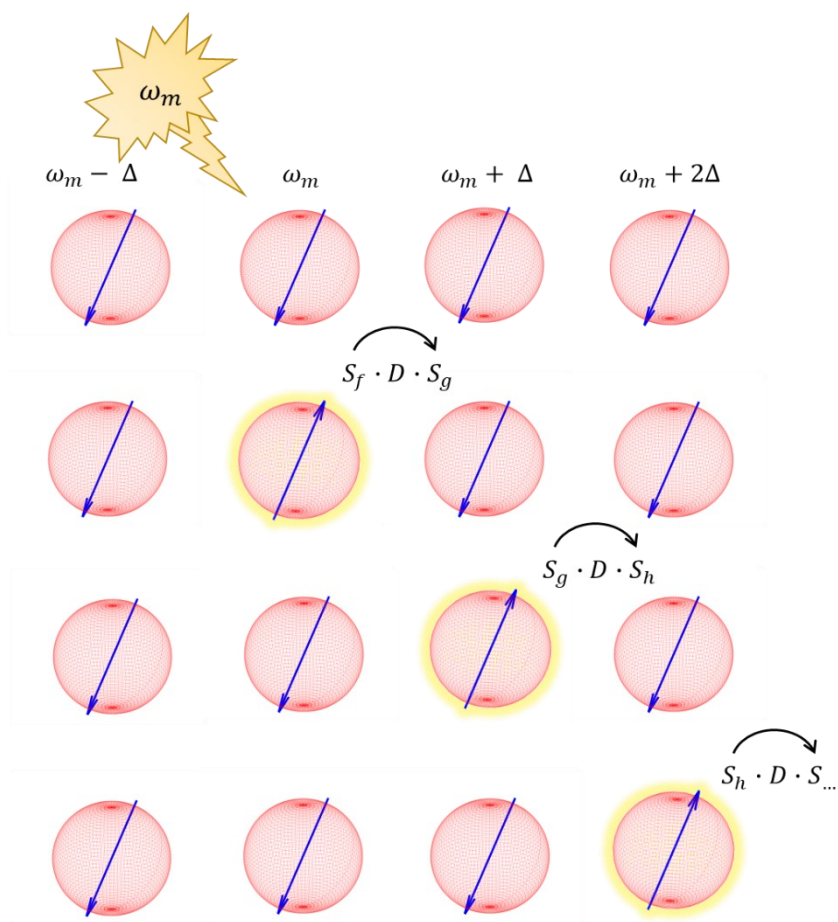


Figure 2.5. Mechanism of spectral diffusion in a DNP sample. The spin packets, surrounded by their unique environment, show different resonance frequencies. When a microwave frequency ω_m is applied to the system, only certain spins resonate. The polarized spins, now in their high energy state, is dipolar coupled with the neighbouring electrons. The coupling allows a flip-flop transition between the two spins, leading to the relaxation of the resonant spin and the polarization of the coupled spin. This mechanism occurs then for the other electrons until reaching a homogeneous electron polarization and a broadening of the EPR spectrum.

2.3 Hyperpolarization mechanisms

Chapter content:

- The nuclear Overhauser DNP.
- The solid effect.
- The thermal mixing.
- The cross effect.

2.3.1 The nuclear Overhauser DNP

After the first description of the nuclear Overhauser effect provided by Albert Overhauser in 1953³³, the French physicist Ionel Solomon experimentally measured the NOE on samples of hydrofluoric acid.³⁴

A theoretical explanation of NOEs is provided in **Chapter 5**. NOEs are at the basics of the so-called Overhauser DNP (ODNP).³⁵ ODNP consists of the microwave irradiation of the electrons hyperfine coupled with the surrounding nuclei and the consequent increase in nuclear signal in a liquid sample; it is generally more efficient at low magnetic fields. At high B_0 the electron Larmor frequency is higher than the modulatory effect of the dipolar interaction between the spins. However, ODNP was recently adapted to magnetic fields over 400 MHz³⁵⁻³⁸, keeping a good efficiency. This hyperpolarization technique is employed in solution to characterize the hydration shell of macromolecules and proteins.³⁹ The possibility to hyperpolarize the substrates of interest without employing high fields represents an advantage for applications in MRI, where water hyperpolarized by ODNP can be employed.⁴⁰⁻

⁴¹ Recently, ODNP was also proposed for portable DNP NMR setups.⁴²

2.3.2 The solid effect

First described by Jeffries in 1957⁴³ and lately by the group of Ubersfeld and by Abragam and Proctor⁸, the solid effect consists in the microwave-dependent one-step transfer of polarization from the electrons to the nuclei upon irradiation, mediated by a flip-flop or a flip-flip mechanism.⁴⁴ The polarization then diffuses from one nucleus to the other by spin diffusion.

In a system with a nucleus coupled to an electron, the transition $\alpha\beta \leftrightarrow \beta\beta$ and $\alpha\alpha \leftrightarrow \beta\alpha$, involving the nuclear spin, can be stimulated by RF at the right frequency (**Figure 2.6.**, green arrows). The transitions of the electron spin $\alpha\beta \leftrightarrow \alpha\alpha$ and $\beta\beta \leftrightarrow \beta\alpha$ can instead be aroused by microwaves (**Figure 2.6.**, red arrows). The double quantum (DQ) and the zero quantum (ZQ) transitions (**Figure 2.6.**, blue and pink arrow, respectively), involving a two-spin transition, can be achieved, for example, by DNP. A microwave frequency equal to the difference or the sum of the resonance frequency of the electron spins and the nearby nuclear spins excites the electron spins; through hyperfine coupling, electrons flip the coupled nuclear spin. These double transitions are generally forbidden, because they

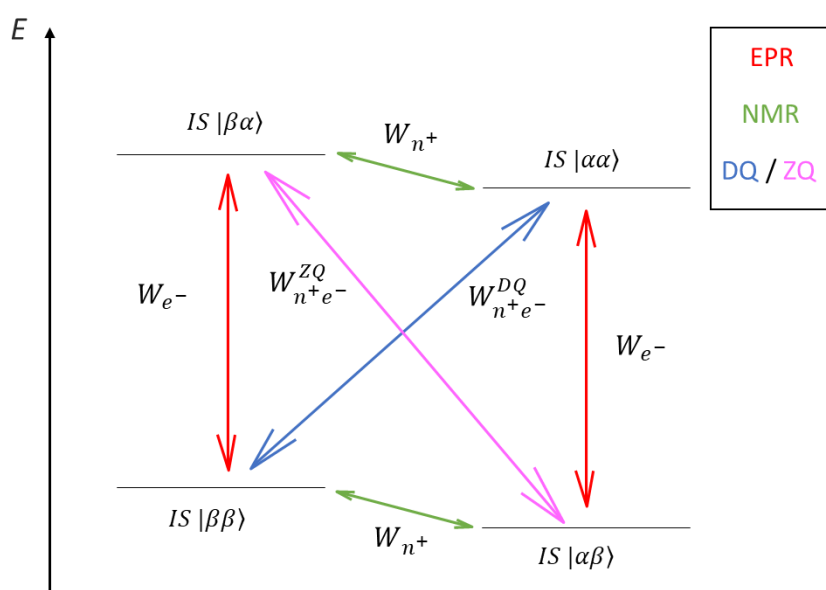


Figure 2.6. Energy transition diagram of an electron-nucleus coupled spin system IS . The transitions involving the nuclear spin are triggered by RF irradiation; the transitions rate is indicated by W_{n+} . The high energy state of the electron spin state is populated upon irradiation with microwaves at the rate W_{e-} . Finally, the transitions achieved by DNP are double quantum (W_{n+e-}^{DQ}) and zero quantum (W_{n+e-}^{ZQ}).

implicate the simultaneous redistribution of nuclear and electron spins, not respecting the energy conservation law. The possibility to induce a double-quantum or a zero-quantum transition depends on the microwave frequency employed.

According to the classification provided by Wenckebach in reference ¹¹, three different types of solid effect can be distinguished: the *well-resolved solid effect*, the *differential solid effect*, and the *microwave-induced thermal mixing*. The first is observed when low amounts of radical are used and when the type of radical employed shows a *narrow EPR profile* in comparison to the nuclear resonance frequencies (**Figure 2.7.**). In this case, the polarization can be positive or negative; these two conditions can coexist in the case of the integrated solid effect, in which strong microwave pulses are employed and there is slow spectral diffusion. One example of “narrow-line” radical is the Trytil (OX063).

The second mechanism is achieved in a slow spectral diffusion regime employing a radical with a broader EPR linewidth, on the same order of magnitude of the nuclear Larmor frequency. While a hole can still be burnt at a certain frequency ω_s , the resonances at $\omega_s \pm \omega_{0,I}$ can coexist, generating a hybrid NMR hyperpolarized spectrum. Considering the EPR lineshape as composed of more spin packets “contained” in an inhomogeneous line, at a certain microwave frequency few spin packets are excited. The electron spins at frequency $\omega_s \pm \omega_{0,I}$ then flips with a nucleus in the vicinity. Since the spectral diffusion is slow, it can

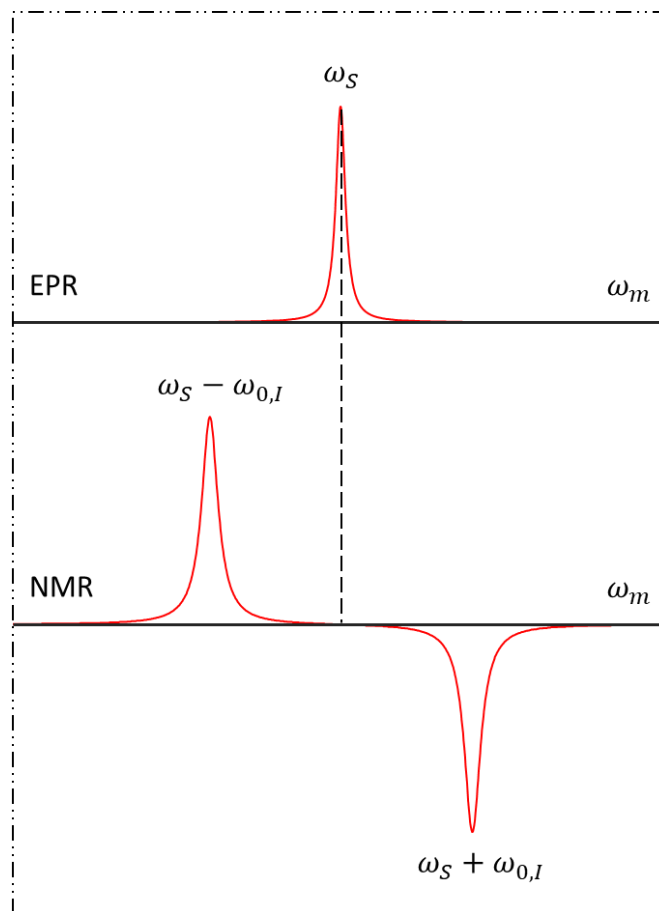


Figure 2.7. Schematic representation of the EPR lineshape of a radical compatible with solid effect and the NMR signals of the boosted nuclei. The narrow EPR profile of the paramagnetic center does not cover the resonance of the coupled nucleus. If there is solid effect, the resonance of the nuclear signal is equal to $\omega_S - \omega_{0,I}$, generated by a ZQ transition, and it shows a positive signal in the spectrum. If there is DQ transition, the NMR signal has a center equal to $\omega_S + \omega_{0,I}$ and it has a negative intensity.

be assumed that the electrons display the same polarization shown at thermal equilibrium. The polarization of the nucleus is then governed by the flow of polarization from electrons to protons, both in ZQ and DQ transition. The net polarization is considered as the difference between the positive polarization and the negative polarization.

The third case is characterized by PA with an EPR spectrum of the same order of magnitude of the NMR lineshape and by a fast spin diffusion process. This last case was theoretically described by Provotorov.¹¹

Later in this chapter, the well-resolved solid effect will be the main effect discussed and it will be addressed just as “solid effect”.

The solid effect can enhance the polarization of the bulk in the DNP sample. The total rate of nuclear polarization is described by the following equation:

$$\frac{\partial P_I}{\partial t} = -\frac{2N_S A^2}{N_I \omega_{0,I}^2} [Tr_g^0(\omega_m + \omega_{0,I})[P_I - P_S] + Tr_g^0(\omega_m - \omega_{0,I})[P_I + P_S] + R_{1,S}[1 - P_S^2]P_I] \quad (2.3.2.1)$$

$$Tr_g^0(\omega) = \frac{1}{2} \pi \omega_S^2 g(\omega) \quad (2.3.2.2)$$

Where Tr_g^0 is the electron polarization transfer rate, known as *spin-pumping rate*, and the $g(\omega)$ is the EPR lineshape; the $R_{1,S}$ is the relaxation rate of the electrons. The polarization rate of the nuclear spins is directly proportional to the number of electron spins present in the sample and on the anisotropic component of the dipolar coupling between the electrons and the nuclei; stronger is the coupling, stronger is the solid effect. This mechanism is particularly suitable for the hyperpolarization of slowly-relaxing nuclei with a low gyromagnetic ratio. The equation 2.3.2.1 takes into account both the contribution of the negative polarization, mediated by the double-quantum transition, and the positive polarization, mediated by the zero-quantum transition (**Figure 2.7.**). The spin-pumping rate is the balance between the relaxing nuclei and the polarized electron spins. The relaxation rate of the electron spins decreases exponentially when their polarization increases.

2.3.3 Thermal mixing

With the introduction of the concept of spin temperature by Alfred G. Redfield⁴⁵ and the later attribution of different temperatures to spin reservoirs by Provotorov^{32, 46}, the understanding of the DNP mechanisms widened. In 1964, several scientists, such as Goldman and Abragam, described a polarization transfer mechanism, previously uncharted, denominated *thermal mixing*.

The thermal mixing is particularly efficient for fast relaxing nuclei. The model of thermal mixing consists in *triple spin flip* transitions between two free electrons and one nucleus (**Figure 2.8., A.**). This polarization mechanism is particularly efficient when the PA has a broad EPR lineshape, covering the resonance frequency of the nucleus involved in the flip-flop-flip transition (**Figure 2.8., B.**). Broad EPR spectra are typical of nitroxide radicals, such as TEMPOL. Considering that the thermal mixing involves many electrons interacting with nuclei, the concentration of the radical is necessarily higher than for the solid effect, in order to have an excess of unpaired electrons to flip with protons.¹¹

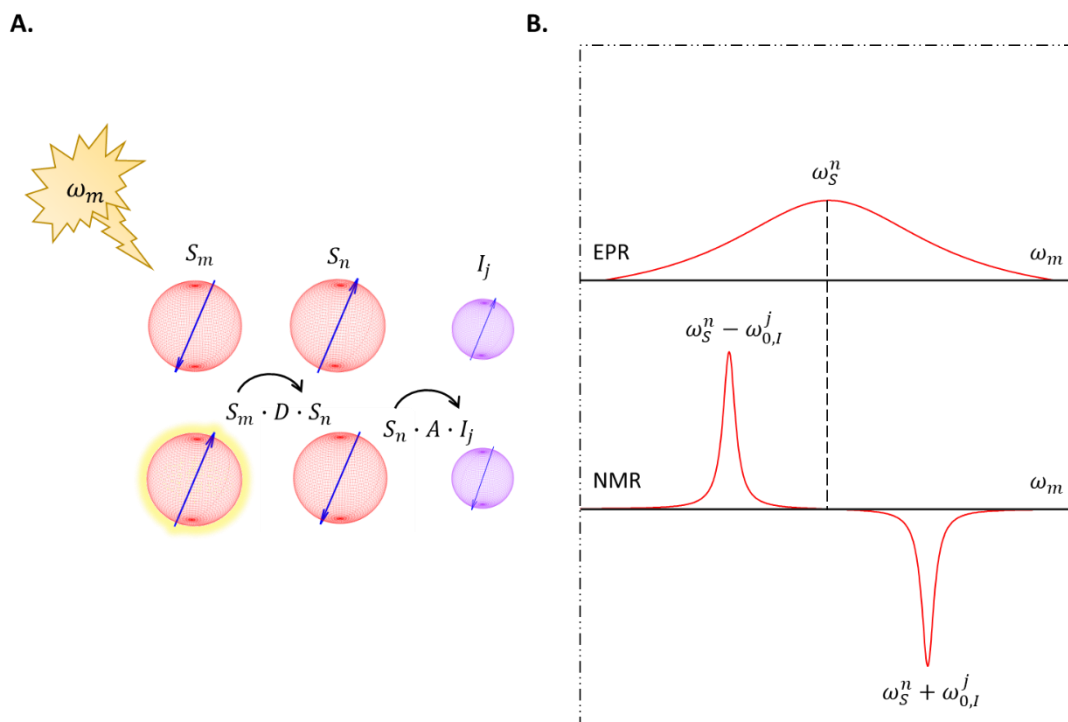


Figure 2.8. Schematic representation of the triple spin flip transition and the characteristic EPR line. **A.)** Upon microwave irradiation, the nucleus is polarized through spin flips between two electron spins coupled with each other and, in turn, coupled with a nucleus. The different sizes of electrons and nuclei indicate the different degrees of polarization. **B.)** The radicals compatible with thermal mixing are characterized by a broad EPR line that “cover” the resonance of the coupled nucleus.

In the triple spin flip model, three main parameters contribute to the total energy of the spin system: the *electron Zeeman interaction* of the spins with the external magnetic field, the *dipole-dipole interaction* between the electrons, and the *nuclear Zeeman interaction* between the nuclei and the B_0 . Because of super-hyperfine interactions, the electron spin fluctuations between the low energy state and the high energy state “mixes” with the nuclei; therefore, the nuclei are polarized and the nuclear spin temperatures are equalized, independently on their gyromagnetic ratio.⁴⁷

The electron spins populating the energy states are called “Zeeman reservoirs”. According to the polarization of the electron spins, an inverse spin temperature coefficient, denoted as $\beta = \frac{1}{k_B T_S}$, is attributed to each reservoir. When the two energy levels are equally populated, $\beta = 0$. In case of uneven spin populations, the inverse spin temperature can assume positive or negative values, indicating respectively a higher population of the low or the high energy state.

The dipolar coupling between the electron spins causes the further division of the Zeeman reservoirs in several sublevels, known as “dipolar reservoirs” (**Figure 2.9.**). The spins populate

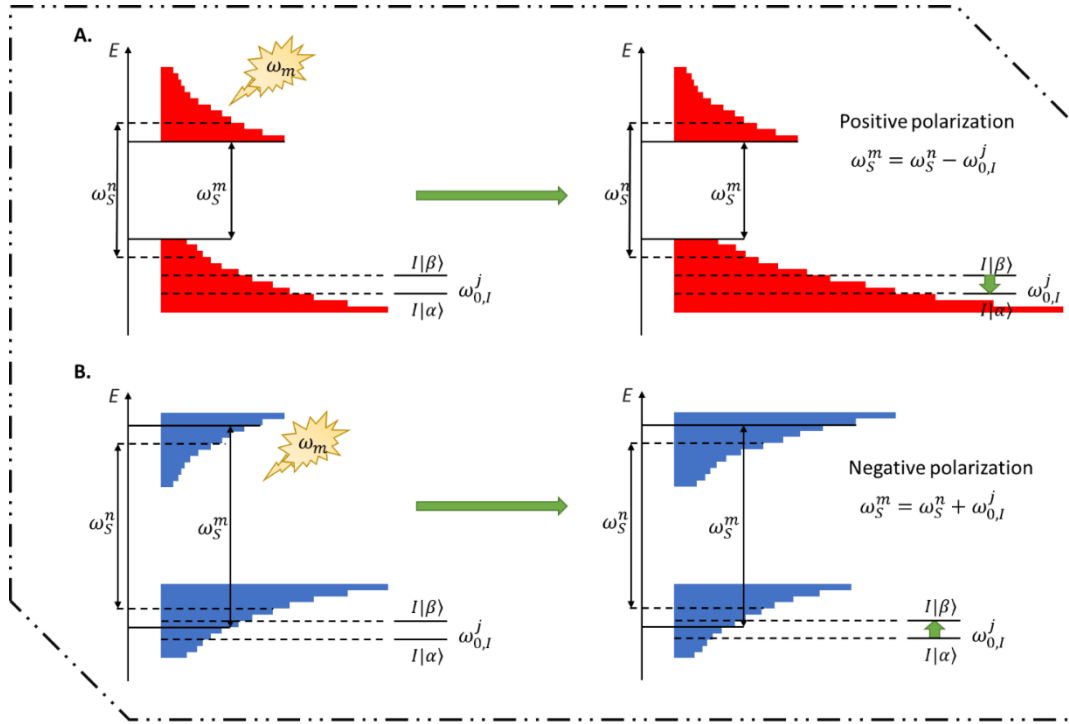


Figure 2.9. Simplified representation of energy levels in thermal mixing. The electron population is split by the electron Zeeman and dipole-dipole interactions in many energy levels. **A.)** The frequency applied is equal to the difference $\omega_S^n - \omega_{0,I}^j$, where the first term corresponds to the energy gap between the high and low energy states of the electron spin reservoirs and the second term indicates the energy needed for the polarization of the nucleus coupled to the electrons. The microwaves trigger the redistribution of the spins majorly in the low energy state, therefore generating a positive polarization in the nuclei, which also populates mainly the low energy state. **B.)** When the frequency applied is equal to $\omega_S^n + \omega_{0,I}^j$ the electron spins populate mainly the high energy states, triggering the nuclear spins population redistribution, generating a negative polarization in the NMR profile.

the energy sublevels unevenly; therefore, some spin packages give a stronger EPR signal in comparison to others, contributing to the inhomogeneous broadening of the EPR line.

The third “flip” involves the nuclei. The energy nuclear transition features a smaller bandwidth than the electrons’; therefore, the polarization can flow from the electrons to the nuclei. Depending on the difference in Larmor frequency between the coupled electrons, three different cases of transitions can be described:

Pure electron flip-flop transition $\rightarrow$ if the Larmor frequency of the two electrons is equal, $\omega_S^m \approx \omega_S^n$, the irradiation with this microwave frequency burns a hole in the EPR spectrum.

Triple spin flips with $\omega_S^m \approx \omega_S^n + \omega_{0,I}^j \rightarrow$ it generates negative polarization of the nuclear spins.

Triple spin flips with $\omega_S^m \approx \omega_S^n - \omega_{0,I}^j \rightarrow$ it generates positive polarization of the nuclear spins.

An efficient model of thermal mixing at low temperature was proposed by Michel Borghini in 1968, deriving it from the Redfield theory.⁴⁸⁻⁵⁰ According to Borghini, the EPR lineshape can be calculated as follows:

$$\sum_i g(\omega) \tanh\left(\frac{1}{2}\beta(\omega)\right) \cong -\omega_m P_L + \frac{N_S}{N_I} \omega_{0,I} \frac{T_{1,S}}{T_{1,I}} P_I \quad (2.3.3.1)$$

Where the left part of the equation defines the EPR lineshape of the electrons, taking into account the polarization of a spin and its spectral density function, as in the equation 1.3.1.4.5. The ω_m corresponds to the resonance frequency of a specific spin reservoir, while the P_L is the lattice polarization of the electron spin. The $-\omega_m P_L$ indicates the polarization of the system in the absence of microwave irradiation. $\omega_{0,I}$ and P_I indicates the nuclear Larmor frequency and nuclear spin polarization, respectively.

Other important factors impacting the final EPR spectrum of a radical in thermal mixing are the total number of electron spins in comparison to nuclear spins and the relaxation time of nuclei and electrons to the lattice. The former, denominated in the formula as $\frac{N_S}{N_I}$, suggests that an excess of electrons leads to better polarizations; according to a recent study¹⁷, the polarization efficiency correlates directly to the radical concentration. The latter indicates that fast thermal mixing polarizes particularly well fast relaxing nuclei, such as ^1H . Interestingly, all the nuclei in solution hypothetically share the same spin temperature when they are in pure thermal mixing.

The thermal mixing was described for the first time by Cox, Bouffar, and Goldman⁵¹, who proved the equalization of thermally mixed spin temperatures on a sample containing ^7Li and ^{19}F ions. After the dynamic polarization of the abovementioned nuclei, the lithium signal was saturated for a short period of time. Surprisingly, during the mixing period, the polarization of the ^{19}F diminished, finally equalizing to the one of ^7Li .⁵¹

2.3.4 The cross effect

The cross effect is a polarization mechanism, proposed for the first time in 1967 by Atsarkin, Mefed, and Rodak⁵²⁻⁵³, who described this effect on Cr^{3+} -ions doped ruby crystals (Al_2O_3).

The cross effect relies on triple spin flips between two electrons and one nucleus. This polarization transfer mechanism takes place when the Larmor frequency of the nuclei $\omega_{0,I}^j$ satisfy the following condition:

$$\Delta > \omega_{0,I}^j > \delta \quad 2.3.4.1$$

Where Δ and δ corresponds to inhomogeneous and homogeneous linewidth, respectively.⁵⁴ The maximum of polarization transfer is reached when the nuclear Larmor frequency is equal to the difference of the two hyperfine coupled electrons' Larmor frequencies in the equation:

$$|\omega_S^m - \omega_S^n| = \omega_{0,I}^j \quad 2.3.4.2$$

When the energy levels $| - + + \rangle$ and $| + - - \rangle$ (blue rectangles in **Figure 2.10.**) are degenerate, the microwave frequency saturates the electron energy levels and the polarization flows to the coupled nucleus. The cross effect is exploited in magic angle spinning (MAS) DNP applications.⁵⁴ Its polarization transfer efficiency is boosted when biradicals are employed, such as bis-TEMPO-n-ethylene glycol (BTnE)⁵⁵ and TOTAPOL¹⁹. In this case, the distance between the paramagnetic centers located on the biradical is short (ca. 12 Å for the abovementioned biradicals). This represents an advantage since the short interelectronic distance is independent on the concentration, allowing 4-fold higher enhancement at 4-fold lower concentrations.⁵⁴

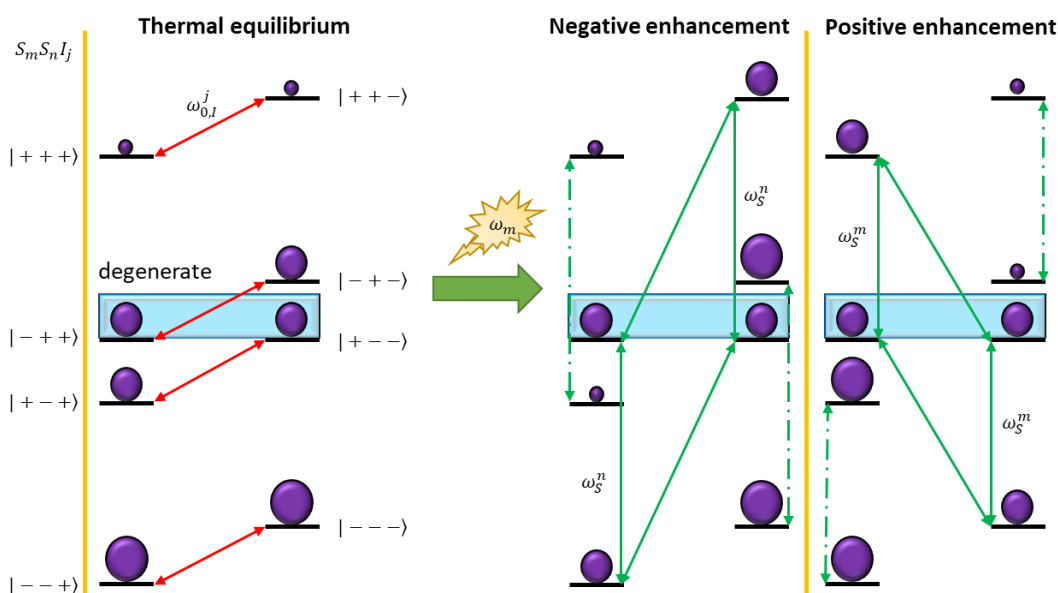


Figure 2.10. The polarization mechanism of cross effect on a coupled electron-electron-nucleus system. The central energy levels are degenerate (indicated by the blue box). When microwaves irradiate the spins, the spin population redistributes since the electrons are saturated, allowing two-spin transitions. According to the microwave frequency employed for irradiation, the polarization can be positive or negative.

2.4 dDNP: from the cryostat to the magnet

Chapter content:

- The cryostat.
- The probe and the microwave source.
- The fluid path.
- The dissolution and the transfer.
- The sample injector and the NMR setup.

The experimental hyperpolarization setup can differ according to the type of DNP employed. For example, in MAS DNP the apparatus comprises a gyrotron oscillator, a low temperature NMR probe, and a transmission line. On the contrary, the dDNP setup is characterized by:

A cryostat

A microwave source

A dissolution system

An NMR spectrometer

Often, some of the main dDNP components can be customized according to the needs of the research group, developing and then improving new prototypes.⁵⁶⁻⁵⁸ A commercial version of dDNP, the SPINlab system, was proposed by GE Healthcare and launched in 2011, indicating a growing interest in hyperpolarization techniques for clinical applications.

In this chapter, the dDNP setup of the University of Vienna, employed in several studies presented in this thesis, is described.

2.4.1 The cryostat

The magnet-cryostat combined system employed for the hyperpolarization experiments was purchased from Cryogen Ltd. Characterized by a magnetic field of 6.7 T, it reaches a stable temperature between 1.3-1.6 K.

The system is “cryogen free”, meaning that the machine does not have to be continuously supplied by an external He source to maintain a temperature of few Kelvin; instead, it is equipped with a liquefier, which recondenses He in a closed cycle. The He recovery system represents a big advantage, both from the economical and the practical point of view. The DNP is composed by three main gas circuits (**Figure 2.11.**):

A He compressor is connected to the cold head in a closed circuit to maintain the temperature of the magnet stably below the threshold of 4 K.

The He is pumped into the He pot from a He tank, in which is present a constant pressure of 1.125 kPa. A charcoal filter avoids the introduction of any pollutant into the cryostat. The volume of He necessary for cooling the probe and the variable temperature insert (VTI) can be manually (by a needle valve) or automatically (by a heater) regulated. The temperature of the needle valve ranges from 38 to 42 K, indicating, respectively, the complete opening and the complete closing of the valve. The evaporated He is then dry pumped from the cryostat back to the tank where it is reliquefied.

Both the temperature of the He pot and the VTI are controlled by two different temperature sensors. The use of superconducting magnets cooled by the He closed cycle is compatible with the magnetic field homogeneity required for dynamic nuclear polarization ($\frac{\Delta B_0}{B_0} <$

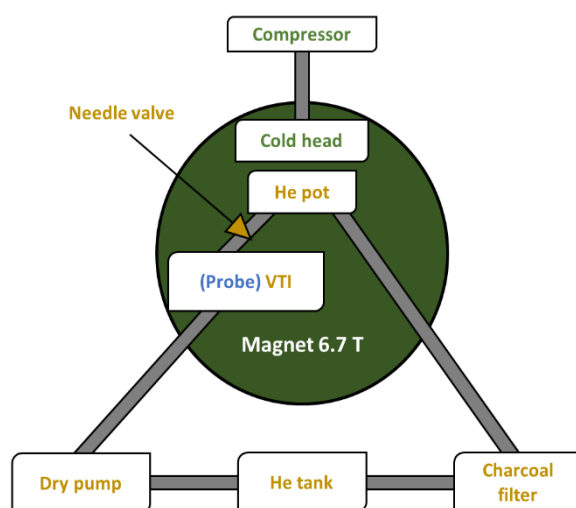


Figure 2.11. Schematic representation of the DNP system. The green elements of the DNP identify the closed cycle for the cooling of the magnet. The yellow components belong to the refrigeration system of the VTI. The liquid He is evaporated and then pumped to a He tank where it is compressed; the reliquefied He is then filtered to avoid the presence of possible pollutants and pumped back into the system. The amount of He to regulate the temperature and pressure of the VTI is regulated manually by a needle valve. The blue elements indicate the probe.

10 ppm). The DNP machine was generally built and employed on the model of reference ⁵⁹, and then adapted according to ⁵⁶ (**Figure 2.12.**).

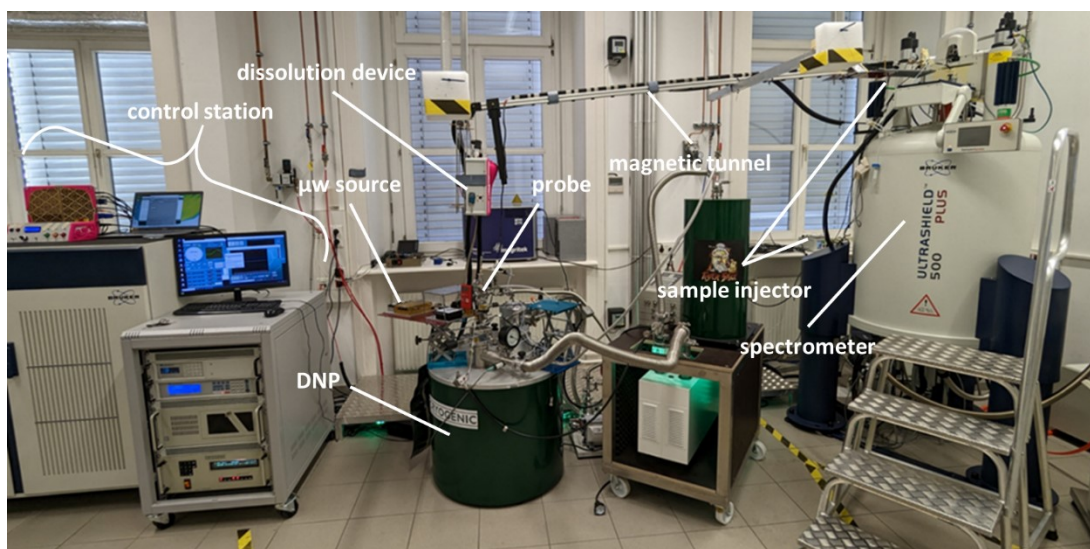


Figure 2.12. The dDNP system of the NMR core facility at the University of Vienna.

2.4.1.1 The probe and the microwave source

The cryostat is equipped with a stainless-steel probe of 24 mm external diameter and 725 mm height. (**Figure 2.13.**) The probe head is connected to the microwave source by a customized microwave guide. From the top of the probe head, a sample holder with maximum 18 mm of diameter can be inserted. It presents three ports: one connected to a He source, the second linked to a turbo pump, and the third to an airlock. The isolation of the inner probe from the air moisture, which could form nitrogen crystals at temperatures <80 K, is guaranteed by the presence of the airlock. On the bottom part of the probe, where the sample should accommodate, the two receiver coils are placed with 90° angle between each other and tuned, respectively, on ^1H and on a second nucleus, such as ^{31}P or ^{13}C . The coils can also be adapted for the detection of other nuclei upon the manipulation of two external tuning and matching boxes, one for each channel. The bottom part of the probe is covered by a leak-tight copper head, sealed to the probe by bonding assembly silicon and secured with Wood's metal. To allow the maximum irradiation of the probe, the microwaves are steered to a brass mirror with an inclination of 45°. The temperature, monitored by a dedicated sensor, is maintained at 1.4 K; during microwave irradiation, it can reach 1.6 K.

The Virginia Diodes, Inc. (VDI) microwave source employed in the DNP system is characterized by the emission of microwaves ranging from 187.36 to 188.8 GHz, upon coupling with a x16 multiplier. The final microwave output power is 50 mW. The microwave source is regulated by the specific software provided by Virginia Diodes, Inc. An adjustable microwave suppression is applied on the irradiated microwaves. During the experiments, the suppression represented the 3.9% of the total output.

When irradiated, the signal of the solid state DNP sample is monitored by a 400 MHz Bruker Avance III console, equipped with a broad-band preamplifiers for the ^1H and ^{13}C channels, resonating at 285.3 MHz and 71.72 MHz, respectively.⁵⁶

In a standard DNP sample with 50% Glycerol, 10% ddH₂O, 40% D₂O, and 40 mM TEMPOL, the polarization plateau is reached after approximately 1.5 hour with a continuous wave irradiation at 188 GHz.

2.4.2 The fluid path

The fluid path employed for the experiments was custom made on the model proposed in reference ⁵⁷ (**Figure 2.14.**). It is characterized by a 0.158 cm inner capillary and a 0.317 cm

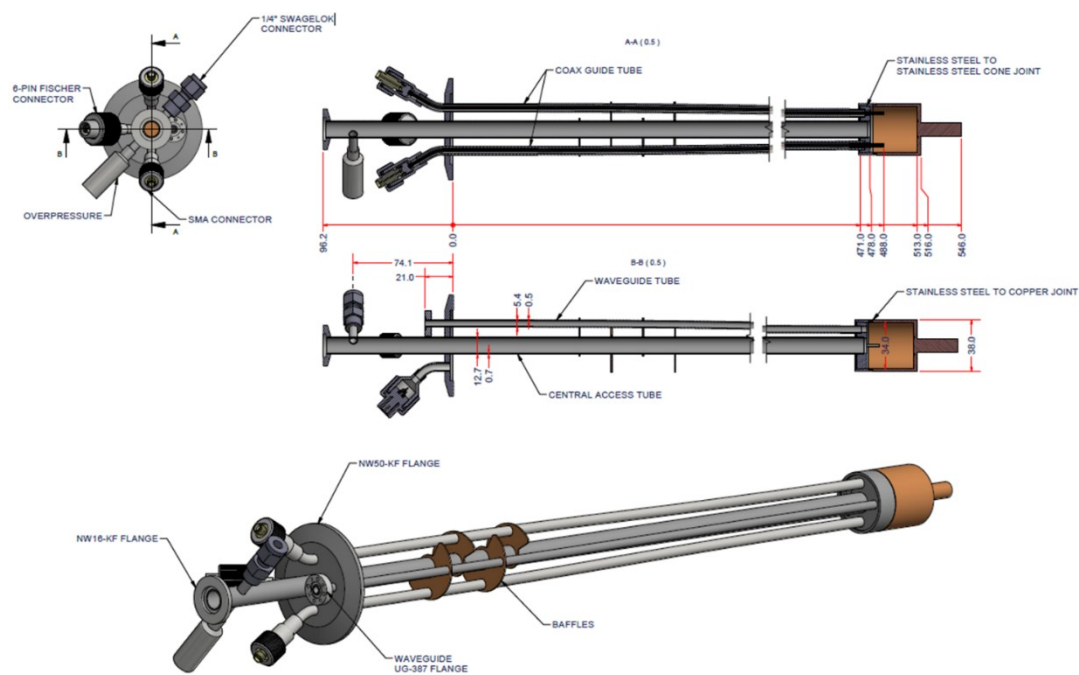


Figure 2.13. Schematic representation of the DNP probe.

outer capillary made out of PEEK. The capillaries are connected to a stainless steel “flow separator” (**Figure 2.14.**, indicated as **1.**) that, in turn, has two different outputs: from one side it is connected to the dissolution device (**Figure 2.14.**, indicated as **2.**); on the other side it is characterized by a “one-way valve” (**Figure 2.14.**, indicated as **3.**), to avoid leaks of air in the polarizer when linked to the magnetic tunnel.

On the bottom, the capillary is attached to a PEEK sample cap (**Figure 2.14.**, indicated as **4.**), composed by a top that can be screwed and tightened by PTFE tape inside the detachable bottom part. The system needs to be air-tight, in order to avoid nitrogen blockages during the experiments; therefore, a “dynamic sealing”⁵ made out of PEEK allows to move the capillary downward during the insertion and upward during the dissolution process without compromising the sample low pressure.

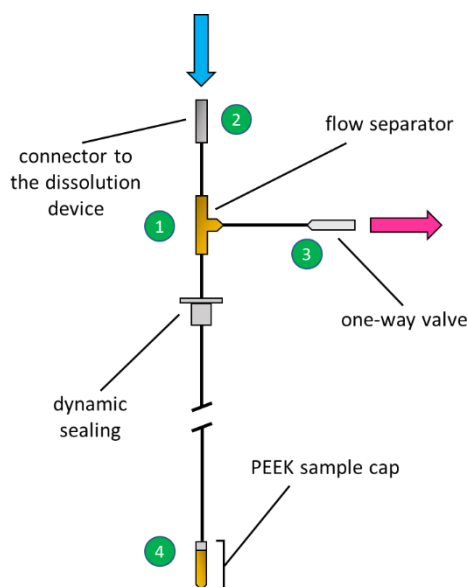


Figure 2.14. Illustrative scheme of the fluid path. The blue arrow indicates where the heated D₂O enters. It then passes through the inner capillary to the sample cap, where the sample is dissolved. The hyperpolarized buffer flows between the inner and outer capillary and it goes out from the fluid path through the one-way valve, as the pink arrow indicates. The numbers indicate the different parts of the fluid path described in the main text.

2.4.3 The dissolution and the transfer

As soon as the DNP sample is polarized, it is ready for dissolution. In order to dissolve and transfer the sample from the DNP machine to the NMR spectrometer, 5 mL of D₂O are pressurized and heated up to 15 bar and ~220° C; subsequently, the sample is dissolved and transferred.

In order to avoid a complete relaxation of the polarized sample during the transfer, the dDNP system is equipped with a magnetic tunnel, which provides a constant magnetic field of ~ 0.5 T.⁶⁰ The magnetic tunnel is a home-built Halbach array, composed by four magnets surrounding a PTFE tubing. The magnetic tunnel is positioned on a hanging aluminium scaffolding that allows to reach the borehole of the 500 MHz NMR spectrometer employed in the experiments.

The sudden change of magnetic field strength between the tunnel and the tubing outside impacts the relaxation rate of the polarized sample. Therefore, the PTFE tubing outside the magnetic tunnel is coated with a copper solenoid, providing a magnetic field of 37 mT.

2.4.4 The sample injector and the NMR setup

When the sample reaches the sample injector, a pre-set volume is selected and transferred in the NMR spectrometer, avoiding the introduction of gas bubbles.⁶¹ The sample injector is composed by a complex system of valves that open in series (**Figure 2.15.**). The hyperpolarized sample is detected by a photodiode positioned before a three-ports valve. To avoid a fast relaxation of the hyperpolarized sample, a magnetic plate generates a magnetic field below the valves. The three-port valve and the degassing valve 1 then opens; the liquid is transferred into a collection chamber. While the three-port valve closes, the degassing valve 2 opens, generating a flux of air that allows the sample to degas. The pinch valve then opens and the hyperpolarized buffer is injected with pressurized He at 3 bar into the NMR tube. The pinch valve closes and the three-ports valve switches to the waste for the subsequent cleaning procedure. As soon as the buffer is settled inside the NMR machine, the acquisition starts automatically. The whole process of transfer and injection takes few seconds. The degassing delay and the pinch valve opening time can be manually regulated through a controller box. The technology behind the pinch valve of the home-built sample injector is based on the design proposed by Kouřil *et al.*⁶¹ and adapted to classical dDNP.

The DNP system at the University of Vienna can be operated together with a low-field Magritek SpinSolve Phosphorous at room temperature and 1 T or with a Bruker NEO 500 MHz NMR spectrometer. The latter is also equipped with a cryoprobe Prodigy BBFO. According to the experimental needs, one or the other spectrometers are used, generally performing real-time NMR acquisitions to monitor the evolution and the decay of the sample polarization. The NMR experimental temperatures that can be employed range between 4 and 50° C. For the protein dDNP studies of this thesis, hyperpolarized water was employed.

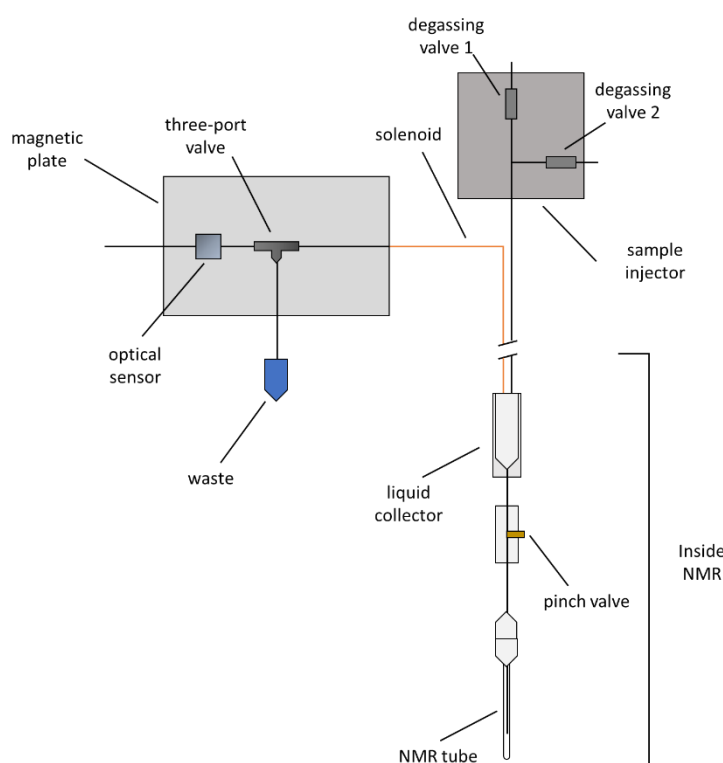


Figure 2.15. Schematic representation of the injection system. The sample arrives from the magnetic tunnel, activating an optical sensor and triggering the opening of a three-port valve for a settable number of seconds. The hyperpolarized buffer is then accumulated into a liquid collector. The latter is degasified, and a controllable amount of buffer is injected into the NMR machine. A solenoid covers the tube connecting the injector and the liquid collector to provide a magnetic field until the sample is inside the spectrometer.

2.5 A bit of “magic”: hyperpolarized water

dDNP is a versatile method to boost the signal of substrates of interest. However, in case of particularly sensitive molecules, such as peptides or proteins, the rapid cooling and the dissolution process could damage the sample. Additionally, relatively large proteins would experience short T_1 , leading to the almost complete loss of polarization during the transfer.

To tackle these problems, the natural tendency of water protons to exchange with labile chains can be exploited.

Water represents a versatile polarization reservoir that delivers the polarization from the DNP to the NMR spectrometer, where the target molecule is waiting. Upon mixing, the polarization is transferred from the protons of the water to the substrate, which gains signal intensity. The main mechanisms implicated in the polarization transfer are: NOE, exchange-relay NOE, and chemical exchange (see **Chapter 5**).⁶²

In the last years, hyperpolarized water represented the elective strategy to improve the signal of biomacromolecules otherwise incompatible with dDNP. This application was pioneered by Lucio Frydman and coworkers in 2014, where they hyperpolarized arginine and other small biomolecules through the injection of HyperW water.⁶³ They also suggest to improve this method with 2D pulse sequences; for example, ^1H - ^{15}N correlation experiments could provide information regarding the aminic groups of a protein backbone.

From its first usage, hyperpolarized water was applied to bigger proteins, such as trypsin⁶², ubiquitin⁶⁴, and on IDPs like MAX⁶⁵, α -sinuclein⁶⁶, and osteopontin⁶⁷.

It was recently shown that hyperpolarized water can be employed for the polarization of nucleic acids, such as RNA⁶⁸, or in ligand-binding⁶⁹⁻⁷¹, protein folding^{65, 72-73}, and membrane interaction⁷⁴⁻⁷⁵ studies.

In dDNP, hyperpolarized water applications are not limited to biomacromolecules. For instance, recently it was shown the suitability of this techniques for MRI.⁷⁶⁻⁷⁸ Unsurprisingly, hyperpolarized water can be employed as a perfectly biologically compatible contrast agent; nevertheless, prior the injection into a potential patient, the radical must be removed from the sample. Subsequent to dissolution, the radical (usually TEMPOL) in the hyperpolarized water can be reduced by ascorbic acid to diamagnetic piperidine⁷⁹ or filtered⁸⁰ from the hyperpolarized solution. It was demonstrated that hyperpolarized water gives high contrast images in angiographic applications and in perfusion studies. A third method to remove the PA from the dissolved sample consists in its binding to an organic phase after dissolution.^{63,}

⁸¹ Particularly in some applications, such as MRI, the removal of the radical is of fundamental importance for the experimental success; and radical reduction, capture, and filtration allows an efficient elimination of the radical from the hyperpolarized buffer. However, these methods are relatively time consuming, leading to a significant loss of hyperpolarization during the process. To tackle this issue, it was recently proposed to employ labile UV-

generated radicals to hyperpolarize dDNP samples.⁸² The instability of these radicals, produced from biologically compatible precursors such as pyruvic acid, allows to hyperpolarize the DNP sample without a subsequent radical neutralization.

In this thesis, hyperpolarized water was employed to boost the signal of IDPs (**Chapter 4**) and small molecules (**Chapter 5**), and, respectively, to study the folding behaviour and the modalities of polarization transfer from the water protons to the target.

2.6 References

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Chapter 3

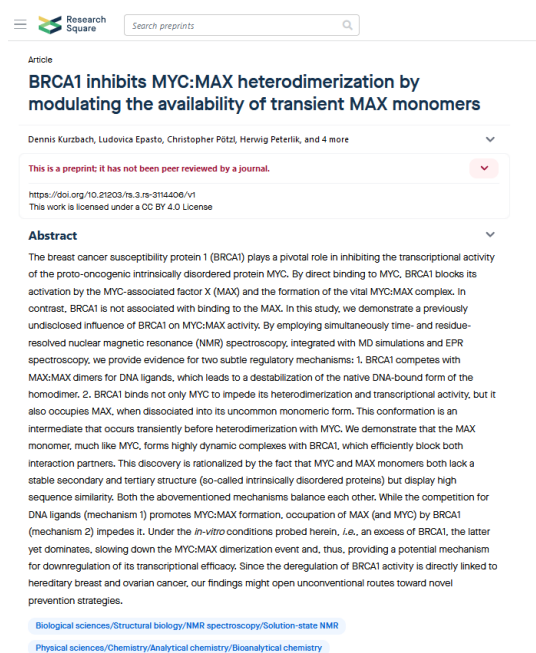
BRCA1 inhibits MYC:MAX heterodimerization by modulating the availability of transient MAX monomers

Manuscript 1

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BRCA1 inhibits MYC:MAX heterodimerization by modulating the availability of transient MAX monomers

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Abstract

The breast cancer susceptibility protein 1 (BRCA1) plays a pivotal role in inhibiting the transcriptional activity of the proto-oncogenic intrinsically disordered protein MYC. By direct binding to MYC, BRCA1 blocks its activation by the MYC-associated factor X (MAX) and the formation of the vital MYC:MAX complex. In contrast, BRCA1 is not associated with binding to the MAX. In this study, we demonstrate a previously undisclosed influence of BRCA1 on MYC:MAX activity. By employing simultaneously time- and residue-resolved nuclear magnetic resonance (NMR) spectroscopy, integrated with MD simulations and EPR spectroscopy, we provide evidence for two subtle regulatory mechanisms: 1. BRCA1 competes with MAX:MAX dimers for DNA ligands, which leads to a destabilization of the native DNA-bound form of the homodimer. 2. BRCA1 binds not only MYC to impede its heterodimerization and transcriptional activity, but it also occupies MAX, when dissociated into its uncommon monomeric form. This conformation is an intermediate that occurs transiently before heterodimerization with MYC. We demonstrate that the MAX monomer, much like MYC, forms highly dynamic complexes with BRCA1, which efficiently block both interaction partners. This discovery is rationalized by the fact that MYC and MAX monomers both lack a stable secondary and tertiary structure (so-called intrinsically disordered proteins) but display high sequence similarity. Both the abovementioned mechanisms balance each other. While the competition for DNA ligands (mechanism 1) promotes MYC:MAX formation, occupation of MAX (and MYC) by BRCA1 (mechanism 2) impedes it. Under the *in-vitro* conditions probed herein, *i.e.*, an excess of BRCA1, the latter yet dominates, slowing down the MYC:MAX dimerization event and, thus, providing a potential mechanism for downregulation of its transcriptional efficacy. Since the deregulation of BRCA1 activity is directly linked to hereditary breast and ovarian cancer, our findings might open unconventional routes toward novel prevention strategies.

Introduction

Transcription factors (TFs) play essential roles in cellular development and replication, exhibiting high specificity in recognizing defined DNA consensus sequences, and, thus, mediating diverse biological functions.^{1–3} Often, the structural flexibility of TFs, driven by intrinsically disordered regions (IDRs)^{4–6}, endows TFs with their versatile yet precise functionality. Intrinsic disorder particularly characterizes cancer-associated TFs overexpressed in tumor cells.⁷ However, this inherent conformational variability poses challenges for studying TF dynamics using conventional biochemical and biophysical techniques. Thus, despite their medicinal relevance, the molecular details of TF structure-function relationships remain often unexplored.

One vital TF example is the MYC-associated factor X (MAX), a partially disordered basic helix-loop-helix leucine zipper (b-HLH-LZ) TF.⁸ In solution, MAX predominantly forms a transcriptionally inactive homodimer, MAX:MAX, specifically binding to the well-known 6-nucleotide consensus enhancer box (EBOX) sequence found in gene promoters.^{8–9} Interaction with partners like MYC¹⁰, Mad¹¹, and Mxi1¹² requires homodimer dissociation and subsequent heterodimerization, forming transcriptionally active

complexes. Herein, we focus on MYC, another intrinsically disordered DNA-binding bHLH-LZ TF, that folds upon binding to MAX, forming the ubiquitous MYC:MAX complex.¹³

Although MAX primarily exists as a folded homodimer in cells, environmental factors like the abundance of co-solutes, temperature¹⁴ or concentration¹⁵ can shift its conformational equilibrium towards dissociated monomers. Given the prominent role of the MYC:MAX-DNA interaction network in tumorigenesis^{16–19}, precise modulation of this transcriptional complex is vital for maintaining cellular homeostasis^{20–22}.

BRCA1, a large partially disordered protein containing 1863 amino acids, is an established key player in regulating the MYC:MAX-DNA network.^{23–25} While its function in double-strand break repair mechanisms and role in hereditary breast and ovarian cancer (HBOC) is well-studied^{26–28}, BRCA1 also exerts regulatory control over gene expression. Specifically, the intrinsically disordered region of BRCA1, spanning amino acids ~ 200 to ~ 500, interacts with both MYC and its consensus DNA sequence housing the CACGTG EBOX motif.²⁹ The modulation of MYC by BRCA1 is furthermore linked to MYC overexpression in BRCA1-associated HBOC.²⁵

In contrast, BRCA1 is not associated with direct MAX binding, despite the latter's high sequence similarity with MYC.^{24, 30}

Herein, we expand this longstanding fundamental aspect of the human transcriptional machinery. We show that BRCA1 impacts the activity of MAX in MYC:MAX heterodimerization events. We identified two counteractive mechanisms: (i) BRCA1 destabilizes MAX:MAX-DNA complexes by competing with the DNA-ligands that bind both MAX monomers together, and (ii) BRCA1 interacts with transient, intrinsically disordered MAX monomers that result from dimer dissociation. While the first route favors MYC:MAX heterodimerization by enhanced exposure of dissociated MAX monomers to MYC, the second route hinders this process by masking the monomers. Under our experimental conditions, route 2 dominated *in-vitro* and slowed down the formation of the transcriptionally active MYC:MAX complex. Thus, we provide an unconventional perspective on BRCA1 within the MYC/MAX transcriptional machinery. We elucidate the molecular mechanism responsible for the regulation of MYC by BRCA1 and demonstrate its indirect influence on MAX activity.

Results and Discussion

The bHLH-LZ transcription factor MAX features a characteristic homodimer structure. Each unit of the dimer consists of two helices separated by a loop (helix-loop-helix, HLH domain), flanked by the intrinsically disordered basic N-terminus and a C-terminal leucine zipper (LZ) domain. The MAX conformational space is quite heterogeneous: The homodimer exists in a conformational equilibrium with the dissociated partially disordered monomers (Fig. 1A). The latter refold upon heterodimerization with MYC into MYC:MAX.^{31–32} Both, MAX:MAX and MYC:MAX dimers display high affinity to EBOX DNA motifs, in contrast to the respective monomers.

Employing a combination of residue-resolved real-time spectroscopy³³, ¹³C-¹⁵N-correlated (¹H^N-CON) NMR experiments³⁴, continuous wave (CW) and double electron-electron resonance (DEER) EPR spectroscopy³⁵, as well as molecular dynamic (MD) simulations, we illustrate the structural and dynamic features of the MYC:MAX-DNA interaction network and how BRCA1 impacts the relevant conformational space. The impact of BRCA1 on the MAX:MAX-DNA + MYC → MYC:MAX-DNA + MAX heterodimerization kinetics will be described first, followed by a mechanistic explanation of the BRCA1 activity.

Residue-resolved real-time NMR reveals the impact of BRCA1 on MYC:MAX heterodimerization kinetics.

To investigate the impact of BRCA1 on MYC:MAX heterodimerization, we injected the MAX:MAX complex in its typical EBOX DNA-bound conformation (using a 5'-CACGTG-3' double-stranded DNA hexamer as ligand) into a MYC solution, either in the presence or absence of BRCA1 (Fig. 1B). Fast mixing by injecting the MAX:MAX-DNA solution under mild overpressure proceeded *in-situ* in an NMR tube, waiting in an NMR spectrometer. We employed a setup similar to that of Brutscher and co-workers³⁶ (Fig. 1C). Immediately after mixing, we monitored the conformational changes upon heterodimerization from the perspective of MYC by a time-resolved ¹³C/¹⁵N-edited series of ¹H-detected 1D spectra (*i.e.*, the first increment of a BEST-HNCO experiment). We recorded one spectrum every 8 s (corresponding to 16 averaged free induction decays, FID). Indeed, MAX:MAX dissociation is a slow process, particularly when bound to DNA, enabling real-time monitoring by NMR.

To avoid signal overlap in the 1D spectra, we selectively ¹³CO labeled MYC's Leu and Ile residues using the α-keto-isocaproate as amino acid precursor.³⁷ The resulting four signals were unambiguously assigned (see Fig. SI 1 for details): D13, E27, Q71, and E96 (corresponding to *i* + 1 resonances relative to the ¹³CO labeled residues; Fig. 1D).

To trace the spectral evolution after injection of the MAX:MAX-DNA complex, each of the four resonances was fitted to a Lorentzian function for each time step. Then, the changes in line integral were evaluated over time. Figure 1E shows the resulting time traces. As MYC folds upon binding to MAX, the signals of the free IDP decay to naught within ca. 70 s (recorded on a 600 MHz NMR spectrometer equipped with a cryogenically cooled probe; c(MYC) = c(MAX:MAX-DNA) = 0.1 mM in 25 mM NaCl, 25 mM MES, and 100 mM ArgHCl buffer at pH 5.5; *T* = 5.5° C). The resulting strongly anisotropic MYC:MAX complex leads to prohibitive signal broadening¹⁴, hence resulting in an overall signal amplitude reduction. In our experiments, the weak signals of the MYC:MAX heterodimer even remained below the detection threshold upon completion of the reaction, as our rapid sampling scheme did not allow for signal averaging for longer periods. Table 1 lists the rates of signal decay for the detected residues. Within the error, the decay rates were similar for all observed residues. Assuming the absence of long-lived intermediates, these rates, thus, correspond to the heterodimerization rate constant.

Repeating the same experiment, but in the presence of a BRCA1 fragment housing the intrinsically disordered DNA- and MYC-binding domain (aa 219–504; henceforth denoted BRCA1^{219–504}) the decay

rates decreased by roughly one order of magnitude for all four resonances. All signals needed > 250 s to decay to naught (Fig. 1F). Again, within the experimental errors all residues displayed similar decay rates.

Table 1. Signal decay rates for sMYC upon heterodimerization (cf. Fig. 1E-F).

Decay rate (s ⁻¹)				
	D13	E27	Q71	E96
MYC:MAX-DNA	0.0248 (±0.0325)	0.0370 (±0.0158)	0.0321 (±0.0126)	0.0412 (±0.0091)
MYC:MAX-DNA / BRCA1	0.0062 (±0.0092)	0.0098 (±0.0027)	0.0119 (±0.0040)	0.0060 (±0.0013)

Hence, the real-time data unambiguously showed that the employed BRCA1^{219–504} fragment kinetically impedes the formation of the MYC:MAX heterodimer.

Two possible scenarios could be conceived to explain these findings: either BRCA1 binds MYC and/or MAX monomers to slow down their mutual dimerization or it stabilizes the MAX:MAX-DNA trimer.

In the following sections, we elucidate both putative candidates of action mechanisms.

BRCA1 destabilizes MAX:MAX-DNA complexes.

To unravel the inhibiting effect of BRCA1^{219–504} on the MYC:MAX-DNA complex formation, we performed a temperature series on homogeneously ¹³C/¹⁵N labeled MAX:MAX in the absence and presence of BRCA1. We found that, competition of BRCA1^{219–504} with MAX:MAX for DNA ligands destabilizes the MAX:MAX-DNA complex. Hence, this competition promotes the heterodimerization of MYC:MAX, since this process necessitates the presence of both partners in their monomeric states. Therefore, this mechanism alone could not account for the observed inhibition of MYC heterodimerization with MAX.

It is well established that the MAX dimer dissociates at higher temperatures^{14,32}. The dissociation temperature depends on the complex stability, which in turn is concentration- and pH-dependent. In addition, DNA-binding further stabilizes the complex.¹⁴ Melting, *i.e.*, dissociation and unfolding of MAX can be readily identified by pronounced non-homogeneous chemical shift perturbations (CSP).

However, to trace the CSP upon dissociation, even ¹H-¹⁵N TROSY (transverse relaxation optimized spectroscopy) did not provide high-quality spectra under our conditions (*c* = 0.1 mM; *B*₀ = 14.1 T; *T* = 15°-40° C; Fig. 2A) due to the strongly anisotropic tumbling of the MAX:MAX dimer. In contrast, we found that ¹³C-detected ¹H^N-CON experiments led to intense and well-resolved spectra thanks to improved

chemical shift dispersion and reduced impact of slow anisotropic tumbling on transverse relaxation (Fig. 2B and Fig. SI 2 to SI 5)³⁴.

The spectral changes for amino acids Ser29 and Arg70 upon melting are exemplarily shown in Fig. 2C. These are located in helix H1 close to the DNA-binding site and the LZ (see Fig. 1A), distant from the DNA-binding site. Figure 2D and E displays the respective melting curves, obtained by plotting the CSP against the sample temperature ($15^{\circ}\text{C} < T < 40^{\circ}\text{C}$). For both residues, the chemical shift changes upon unfolding leading to sigmoidal melting profiles. Temperature changes alone did not lead to similarly significant changes over ranges of up to 20°C . The dissociation temperatures were determined *via* the inflection point of the sigmoidal curves. We then tested the influence of EBOX-DNA and/or BRCA1^{219–504} on the structural stability of the MAX dimer (Fig. 2D and E). Binding to EBOX DNA raises the melting temperature significantly, as expected. It stabilizes the homodimer by binding the N-terminal domains of the two subunits together. At the same time, BRCA1^{219–504} did not significantly influence the melting of the MAX:MAX dimer (in accordance with earlier reports showing no interaction between MAX:MAX and BRCA1²⁴) (Fig. SI 6).

In contrast, and most importantly, BRCA1 reduced the melting temperature for Ser29 of the MAX:MAX-DNA complex substantially, hence, destabilizing the HLH domain. The conformational equilibrium of MAX is, thus, shifted toward its monomeric state (Fig. 2D and E). In our earlier work, we found that the employed BRCA1^{219–504} fragment binds to the EBOX DNA hexamer.²⁹ Hence, competition for the DNA with MAX is very likely the underlying reason for the observed effect.

A competition of MAX:MAX and BRCA1 for EBOX DNA ligands

To further corroborate this hypothesis, we conducted EPR experiments of the melting event. To this end, we spin-labeled MAX with the nitroxide MTSL at three mutation sites: R5C, G35C, and R55C, *i.e.*, at the N-terminus, the loop, and the LZ (Fig. 3A). These mutations do not hinder MAX dimerization or the binding to its interaction partners.^{38–39}

Temperature series of continuous wave (CW) EPR spectra of the three mutants reproduced the melting temperatures found in the NMR experiments within the precision of the experiments. To derive the melting curves, we fitted the EPR spectra at each temperature to a superposition of one slowly and one fast tumbling species (Fig. SI 7 to SI 18). We associated the slow contribution with the folded dimer, and the fast one to unfolded MAX monomers present after dimer dissociation. The two contributions' weights were plotted versus the sample temperature (Fig. 3B and Fig. SI 19 to SI 22) and the dimer dissociation temperatures were again extracted based on the inflection point of sigmoidal fits to the data (Fig. 3C).

We found that the destabilizing effect of BRCA1 is most pronounced for the R5C mutant located in the DNA-binding N-terminal domain (NTD) of MAX. While the EBOX DNA increased the dissociation temperature by ca. 8 K in the absence of BRCA1^{219–504}, it only rose by 4 K in the presence of BRCA1^{219–}

⁵⁰⁴ (Table 2). A similar, yet somewhat less pronounced behavior was found for mutant G35C in the HLH region. In contrast, for mutant R55C in the LZ domain, the melting temperature was independent of the presence of DNA and/or BRCA1^{219–504}. Hence, the EPR data suggest that the stabilizing effect of the DNA ligand is most pronounced in the DNA-binding NTD and HLH, while the LZ domain can dissociate independently of the presence of a ligand. Addition of BRCA1^{219–504} reduces the stabilizing effect of the DNA on the NTD and HLH by competing for the ligand, again without interfering with the dissociation of the LZ (Fig. 3C).

Note that we observed a stronger effect of the EBOX DNA and BRCA1^{219–504} on Ser29 than on Arg70 in the NMR temperature series (see Fig. 2), too. Also in these experiments the stabilizing effect was found more pronounced close to the DNA-binding than for the distant LZ.

Table 2. Melting temperatures of MAX in the presence of different ligands at pH 5.5 for the three different spin-labeled mutants.

Melting temperatures (K)			
Mutation	MAX:MAX	MAX:MAX-DNA	MAX:MAX-DNA / BRCA1
R5C	305.1	322.5	313.6
G35C	312.1	323.1	321
R55C	321.7	321.3	323.5

A two-step dissociation mechanism rationalizes the MAX:MAX melting behavior

To rationalize the EPR-based observations, we performed coarse-grained MD simulations (Fig. 3D, all details can be found in the Experimental section) of the MAX:MAX dissociation event at elevated temperatures (60° C). These showed a two-step process: The dissociation of MAX starts with an unwinding of the LZ domain followed by a dissociation of the HLH and NTD domains. The observation of varying impact of DNA binding on the stability of the different MAX segments can therefore be explained as follows: DNA-binding stabilizes only the NTD by connecting the two MAX subunits. Unwinding of the LZ can proceed independently while NTD and, to some degree, the HLH domain remain intact. This leads to changes in local dynamics and chemical environment, to which NMR and EPR are both intrinsically sensitive, within the LZ, independently of the presence of a ligand. Since LZ melting takes place at always similar temperatures as observed in both, NMR and EPR experiments, the ligand-independent unwinding process provides a straightforward rational. The second step, *i.e.*, the dissociation of the NTD and HLH domains, is yet impeded by the presence of the DNA. Hence, DNA binding will

increase their melting temperatures and competition for the DNA ligand by BRCA1^{219–504} will reduce the impact of the nucleic acid (again as observed in the EPR and NMR experiments).

Note that the MD simulation only show that two distinct processes take place on the single molecule level but they do not allow for any statement about the kinetics or the temperature dependence of the two dissociation process on the level of the ensemble average observed by NMR and EPR.

Nanoscale distance measurements confirm the competition between BRCA1 and MAX:MAX

Finally, we confirmed the competition between MAX and BRCA1 by nanoscale distance measurements, *i.e.*, DEER (double electron-electron resonance) EPR (Fig. 4). To this end, we doubly labeled a 12-nucleotide DNA sequence (5'-CACGTGCACGTG-3'), *i.e.*, two times the 6-nucleotide EBOX, with a nitroxide derivative at the 5' and 3' ends. The DEER experiments revealed a bimodal distance distribution with two peaks centered at ca. 4 and 5 nm (Fig. 4B and Fig. SI 23 and 24) in the absence and presence of EBOX DNA and/or BRCA1^{219–504}. An MD simulation of the labeled EBOX showed that the spin labels populate two different conformations (Fig. 4C and D). A histogram of the NO-NO distances sampled in the simulations showed that the two major peaks found in the experimental distribution $P(r)$ (Fig. 4E and F), can be traced back to these two conformations.

The population weights of these two conformations shift upon binding of the DNA to BRCA1^{219–504} towards the more compact state (Fig. 4B). Binding to MAX:MAX yet led to only insignificant population shifts. Upon addition of MAX:MAX to the BRCA1^{219–504}-DNA complex, the distribution of the MAX:MAX-bound DNA is restated. This observation corroborates our above interpretation that BRCA1^{219–504} competes with MAX:MAX for the DNA. In addition, it can be seen that the affinity of MAX:MAX to its cognate EBOX DNA is higher than that of BRCA1^{219–504}.

In sum, competition for the DNA by BRCA1^{219–504} destabilizes the NTD of MAX:MAX complex, yet, leaves the LZ untouched. However, this finding cannot account for the inhibition of the MYC:MAX heterodimerization observed in the real-time NMR experiments. Hence, another explanation needs to be found.

BRCA1 interacts not only with MYC, but also with MAX monomers.

The interaction between MYC and BRCA1 is well established,²⁴ in contrast to binding of BRCA1 to MAX. Herein, we complement the BRCA1 interaction network by showing that the BRCA1 does bind MAX, too, yet only when in its monomeric state. The occupation of either MYC or MAX by BRCA1 can straightforwardly explain the inhibition of the heterocomplex formation.

To probe both, the MYC- and MAX-BRCA1^{219–504} complexes, we recorded residue-resolved NMR signal amplitudes changes, relaxation times and heteronuclear Overhauser spectroscopy (Fig. 5; CSP data can be found in the Supporting Information).

For the already documented MYC-BRCA1 interaction, we found that the binding of our BRCA1^{219–504} construct to MYC significantly reduces the signal amplitudes S relative to those of free MYC S_0 along the entire primary sequence. This observation indicates a complex binding mechanism between the two IDPs that involved a large part of MYC and leads to reduced tumbling of the entire IDP. Interestingly, no strong CSP was observed (Fig. 4A Supporting Information Fig. SI 25) pointing towards the formation of a “fuzzy” complex⁴⁰ that retains a high degree of intrinsic disorder and does not lead to strong changes in secondary and tertiary structures. Heterogeneous changes in transverse relaxation rates R_2 and $^1\text{H}\{-^{15}\text{N}\}$ NOE η upon complex formation along the entire primary sequence, furthermore, corroborated the “fuzzy” binding mode (Fig. 5A-C). Particularly positive differential relaxation rates ΔR_2 and NOE $\Delta\eta$ were observed between aa 350 and 400 of MYC pointing towards effectively reduced dynamics on the nano- and picoseconds timescale in this region due to the proximity of BRCA1^{219–504}, in agreement with earlier reports.²⁴

Notably, we observed a comparable “fuzzy” binding mode for the MAX monomer, too. We conducted our measurements at a temperature of 35°C and pH 5.5 to populate the monomeric state by > 90%.¹⁴ Fig. 5D shows that the signal amplitudes are again reduced along the entire primary sequence upon binding to BRCA1^{219–504}. Relaxation rates R_2 and $^1\text{H}\{-^{15}\text{N}\}$ NOE η were also found to again increase along the entire primary sequence indicating slower conformational dynamics upon complex formation (Fig. 5E-F). The most pronounced changes were observed for residues 30–50, 75–80, and the C-terminal region. This observation again points to a complex binding mode involving large shares of the MAX monomer.

To further confirm this formerly undocumented interaction, we conducted and isothermal titration calorimetry (ITC) and small angle X-ray scattering (SAXS) experiments on the MAX- BRCA1^{219–504} system (Fig. 5G-H). ITC showed a clear titration profile yielding a K_D of $3.9 \pm 1.1 \mu\text{M}$ and a stoichiometry of $\approx 1:1$ (see the Supporting Information for all fit parameters.)

In agreement, SAXS led to clear increase in atom-to-atom distances upon addition of BRCA1 to MAX again corroborating the interaction of the MAX monomer with BRCA1. The pair distance distribution functions (PDDF), extracted from SAXS intensity curves, show an asymmetric profile, typical for elongated proteins, with only one maximum for BRCA1^{219–504}, but two maxima for mixtures with MAX indicating the formation of joint complex.

Overall, the combined NMR, ITC and SAXS data clearly indicate that BRCA1 interacts with both, MYC and MAX monomers in a similar “fuzzy” manner. On the one hand, this finding is unexpected as the MAX:MAX dimer does not bind to BRCA1²⁴ - a fact that can be considered as a paradigm of TF activity. On the other hand, the high sequence similarity between the two fully disordered proteins MYC and MAX in their monomeric states readily rationalizes the observed interaction. Hence, not only masking of MYC by BRCA1^{219–504} impedes MYC:MAX complex formation, but also binding of BRCA1^{219–504} to the small population of MAX monomers coexisting with the MAX:MAX dimer^{14,32} can slow down this event.

Conclusions

BRCA1 can impact MYC:MAX heterodimerization *via* two routes: by competing with MAX:MAX for bound DNA ligands, leading to a destabilization of the complex, and by occupying MYC and MAX monomers to inhibit their mutual dimerization. Considering that our real-time NMR assays show that the presence of BRCA1 slows down MYC:MAX heterodimerization, the first route (destabilization) cannot be the dominant mode of interaction under our *in-vitro* conditions. Instead, it is very likely that binding to MYC and MAX monomers – the latter being necessary intermediates of the $\text{MAX:MAX} + \text{MYC} \rightarrow \text{MYC:MAX} + \text{MAX}$ reaction – slows down the complex formation by masking the respective monomers.

Previous studies showed that BRCA1 plays a regulatory role in the MYC:MAX heterodimer transcription activity by making MYC. However, no interaction was reported between BRCA1 and active MAX monomer species.²⁴ A perspective on the effect of BRCA1 on the entire MYC:MAX-DNA network including the lowly populated monomer species was still lacking. Notwithstanding our experimental conditions are not identical to the cellular environment, we yet provide such a comprehensive description under physiological solution-state conditions of the effect of BRCA1 on the structural dynamics of the MYC:MAX-DNA interaction network.

In conclusion, our study provides new perspectives to approach the vital MYC:MAX-DNA interaction network, considered as an important target of cancer therapeutics for decades.^{41–44}

Materials and methods

Protein expression and sample preparation

The his-tagged MYC, MAX, and BRCA1 were expressed in Rosetta 2 *E. coli* cells, transformed with pET21(a) + vectors subcloned with the codifying DNA sequences of interest. The bacterial cultures were grown at 37° C until reaching an absorbance of 0.6 at 600 nm. The cells were resuspended in M9 (with homogeneously ¹³C labeled glucose 1 g/L and ¹⁵N ammonium chloride 1 g/L) before induction through IPTG. The expression was carried out overnight at 30° C. While for BRCA1 and MAX we followed the procedures reported in ²⁹ and ³¹, respectively, MYC was produced employing a protocol for insoluble proteins. The cells were disrupted, and the insoluble fraction was resuspended in denaturing buffer (25 mM Tris, 100 mM NaCl, 6 M GuCl, pH 8.0) overnight. MYC was then purified with affinity chromatography and dialyzed step-wise to a GuCl-free buffer. The his-tag was removed by TEV cut; MYC was finally transferred into the MES buffer (25 mM NaCl, 25 mM MES, 100 mM ArgHCl, pH 5.5) with centricones (cutoff 10,000 kDa). For the MYC selective labeling on the leucine and isoleucine, the ¹³C glucose of the M9 was substituted with unlabeled glucose and 105 mg/L of α-isocaproate, purchased from Eurisotope.

For the EPR experiments, we purchased three different pET21(a) + vectors subcloned with the sequence codifying the three variants R5C, G35C, and R55C. The expressed proteins were MTSL-tagged according

to the reference⁴⁵. MTSL-tagged MAX mutants were buffer exchanged against MES buffer. We checked the successful MTSL labeling by CW EPR.

We purchased the CACGTG DNA filaments forming the EBOX double-strand from Eurofins Austria.

The synthesis of TEMPO-labeled oligonucleotides (EBOX, formed by 12 nucleotides) has been performed in two steps as previously described^{46–48}. Thus, the solid phase assembly process was performed first by using the phosphoramidite chemistry (to insert site-specifically the alkyne function), followed by a post functionalization of the DNA strands (based on the CuAAC click chemistry) with the azido-TEMPO residue. In Supporting Information from Fig. SI 26 to SI 31, the characterization experiments (MALDI-TOF MS and LC-ESI MS) have been reported.

NMR

The $^1\text{H}^{15}\text{N}$ HSQC, $^{13}\text{C}^{15}\text{N}$ CON, real-time HNCOC experiments were performed on a 600 MHz Avance Neo Bruker spectrometer equipped with a Bruker Prodigy TCI cryo-probe. The TROSYs were performed on a 700 MHz Bruker spectrometer equipped with a helium cooled Bruker QCI cryo-probe.

All samples were prepared in 25 mM MES buffer at pH 5.5. 10% D_2O was added as lock solvent.

The injection system for the real-time MYC:MAX-DNA / BRCA1 experiments were performed using a homemade injection system, composed by a plastic tubing connected on one side to a syringe and on the other side to the NMR tube.³⁶ We filled the tube with water as shuttling solvent and then loaded the protein, separated by an air cushion. The protein sample was injected directly into the NMR tube containing MYC, while acquiring a time resolved $^{13}\text{C}/^{15}\text{N}$ -edited ^1H NMR series (first increment of an HNCOC) with a rate of 1 scan per second. To calculate the decay rate, the MYC NMR signals were baseline corrected and fitted in MATLAB using a Lorentzian function to calculate the final area of the peak.

For the H^{N} -CON selective ^1H excitation and refocusing were achieved using PC9⁴⁹ (bandwidth 6.25 ppm, duration 2001 μs), and REBURP⁵⁰ (bandwidths 6.0-6.05 ppm, durations 1600–1614 μs) pulses, respectively. Selective proton pulses were centered at 8.3 ppm.

Selective ^{13}C excitation and refocusing were achieved using Q5 (bandwidth 136 ppm, duration 300 μs), and Q3 (bandwidth 115 ppm, duration 199 μs) pulses.⁵¹ Selective ^{13}C pulses were centered at 173 and 54 ppm for CO and Ca, respectively. IPAP acquisition was used to suppress CO-Ca couplings during acquisition. Non-selective ^{15}N 90 pulse durations were 32 μs . the number of scans was 32, the recycle delay was increased to 1 s, and the number of t_1 increments collected was 256.

^1H , ^{13}C , ^{15}N BEST HNCOC spectra were recorded using the Bruker library pulse sequence 'b_hncogp3d' (modified to generate shaped ^1H pulses directly from input values of offsets, flip-angles, and bandwidths).⁵² Selective ^1H pulses were PC9 (bandwidth 4.17 ppm, duration 2251 μs), REBURP

(bandwidth 4.85 ppm, duration 1498 μ s), EBURP2 and EBURP2tr (bandwidths 4.3 ppm, durations 1439 μ s), and BIP720, 50, 20.1 (150 μ s).⁵³ Except for the BIP pulse (set on resonance at 4.7 ppm), all ^1H selective pulses were centered at 8.3 ppm.

Selective ^{13}C pulses were G4 and G4tr (bandwidth 125 ppm, duration 308 μ s), and Q3 (bandwidth 82 ppm, duration 210 μ s).⁵¹ Selective ^{13}C pulses were centered at 173 ppm for CO, or 53.2 ppm for Ca. Non-selective ^{15}N 90 pulse durations were 39.75 μ s.

All further NMR parameters, i.e., spectral width, pulse lengths and powers, delays, phase cycles etc. can be found in the data depository specified in the data availability section.

All the NMR analysis were performed using Topspin 4.0.7, CCPNMR and MATLAB R2019a. Again, all processing scripts can be found in the code depository (see below).

EPR

The Continuous Wave (CW) X-Band measurements have been recorded using a Bruker ElexSys E500 instrument (9.34 GHz) equipped with a super-high Q resonator (ER4122SHQE-W1). Samples (20 μ L) with a concentration of 100 μ M were loaded into a microcapillary tube. All measurements were recorded with 100-kHz field modulation. The temperature-stabilized measurements were performed using a temperature controller (ER 4131 VT, Bruker). The spectra were recorded with 0.25 G modulation amplitude for measurements in the range 290 K – 335 K.

Pulsed ELection DOuble Resonance (PELDOR), known also as Double Electron-Electron Resonance (DEER) Q-band EPR measurements (34 GHz) were recorded at 50 K with a Bruker Elexsys E580 spectrometer equipped with an ER5106QT-2w resonator and an arbitrary waveform generator (AWG) pulse channel using Xepr. Cryogenic temperatures (50 K) were achieved by an Oxford flow cryostat. A total of 60 μ L of 100 μ M TEMPO-labeled Ebox in buffer (10% v/v of glycerol- d_8) have been filled in 2 mm o.d. quartz capillaries, flash frozen and stored in liquid nitrogen between measurements. The field-swept EPR spectra were recorded by electron spin echo (ESE) detection, carried out with the pulse sequence: $\pi/2 - \tau - \pi - \tau - \text{echo}$. The microwave pulse lengths used were $t_{\pi/2} = t_{\pi} = 10/12$ ns and an interpulse delay of $\tau = 248$ ns. A two-step phase-cycle was applied to remove all unwanted echoes. PELDOR / DEER EPR data were acquired with the 4-pulse DEER (4pDEER) sequence $\pi/2_{\text{obs}} - \tau_1 - \pi_{\text{obs}} - (\tau_1 + t) - \pi_{\text{pump}} - (\tau_2 - t) - \pi_{\text{obs}} - \tau_2$. 4pDEER measurements have been recorded with rectangular pulses of length $t_{\pi, \text{pump}} = 12$ ns, applied at the maximum of the nitroxide Q-band spectrum and observer pulses with $t_{\pi/2, \text{obs}} = t_{\pi, \text{obs}} = 10$ ns, offset 56 MHz; a 16-step phase cycle has been applied. The evaluation of the DEER data was performed using DeerAnalysis2022.⁵⁴ Parameter-free and Gaussian distance distributions $P(r)$ were computed via Tikhonov regularization, using generalized cross-validation (GCV) to select the optimal regularization parameter.^{55–57}

The home-written MatLAB routine (script) for fitting of the room temperature CW EPR spectra can be found in the Supporting Information.

MD simulations

The MD simulation of MAX₂ was performed using GROMACS 2022.2^{58–65} and the MARTINI3.0.0 force field⁶⁶. The 1r05.pdb file from the RCSB PDB⁸ was coarse-grained using the MARTINI maker on the CHARMM-GUI web server^{67–68}. MAX₂ was placed into a cubic box with an edge length of 16.4 nm³ and dissolved in 35742 coarse-grained water molecules, 395 Na⁺-ions and 403 Cl⁻-ions. The solution system was minimized using the steepest descent algorithm until the maximum force was 10.0 kJ·mol⁻¹·nm⁻¹. The minimized system was equilibrated in a canonical ensemble with the V-rescale thermostat at 310.15 K for 1000 ps. Then a second equilibration was performed in a microcanonical ensemble with the Berendsen barostat at 1 bar was performed for 1000 ps. The production run was performed with the Parrinello-Rahman barostat instead of the Berendsen barostat at 310.15K for 50 μs.

The MD simulations of the spin labeled EBOX were performed using Yasara 22.9.24.L.64 and the AMBER14 force field⁶⁹. The EBOX with the labeled cytosine was placed in a cubic box with an edge length of 6.39 nm³ and dissolved in 8552 water molecules, 36 Na⁺-ions and 15 Cl⁻-ions. The simulation was performed with the V-rescale thermostat and pressure control. The overall duration was 607 ns. The EBOX with the labeled guanosine was placed in a cubic box with an edge length of 6.39 nm³ and dissolved in 8473 water molecules, 38 Na⁺-ions and 15 Cl⁻-ions. The simulation was carried out with the V-rescale thermostat and pressure control. The overall duration was 734 ns.

ITC

Titration calorimetric measurements were carried out using a Microcal iTC200 calorimeter. The product of the initial ligand concentration and the association constant $c = K_b \cdot [\text{Max}]$ was in the range of $2 < c < 200$. The starting conditions were 130–150 μM BRCA1 in the measurement cell (200 μL) and 0.3 mM of MAX in the injection needle (40 μL at pH 5.5 where the monomer state dominates the conformational space). Titration of ligand proteins into buffer gave only minor heat release. Nevertheless, these titrations were subtracted from runs performed with BRCA1. Measurements were carried out with a stirring speed of 1000 rpm. The time delay between the injections was set to 180 s to allow peak recovery to baseline. Nonlinear least-squares fits (one binding site) were employed to interpret the experimental data.

SAXS

SAXS scattering patterns were collected using X-rays either from a microfocus source (Incoatec IμS High Brilliance) or a liquid metal jet technology (Excillum) and a 2D position sensitive detector (Vantec 2000, Bruker AXS). The sample-to-detector distance was chosen to cover a range of scattering vector q from 0.1 to 2.8 nm⁻¹. All SAXS patterns were radially averaged to obtain scattering intensities depending on the scattering vector $q = 4\pi(\sin\theta)/\lambda$ where 2θ is the scattering angle and $\lambda = 0.1542$ nm is the X-ray wavelength in the case of the microfocus source and $\lambda = 0.13414$ nm for the metal jet source. The SAXS

data are analyzed by the indirect Fourier transformation (IFT) method using the GIFT software.⁷⁰ The resulting pair distance distribution function (PDDF), extracted model-free from the scattering pattern, provided information on the structure of the scattering objects. The MAX and BRCA1 concentrations were 0.1 mM, again at pH 5.5 to overpopulate the MAX monomer.

Declarations

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Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

All data are available under DOI [tba upon publication].

Code Availability

All codes are available under DOI [tba upon publication].

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Figures

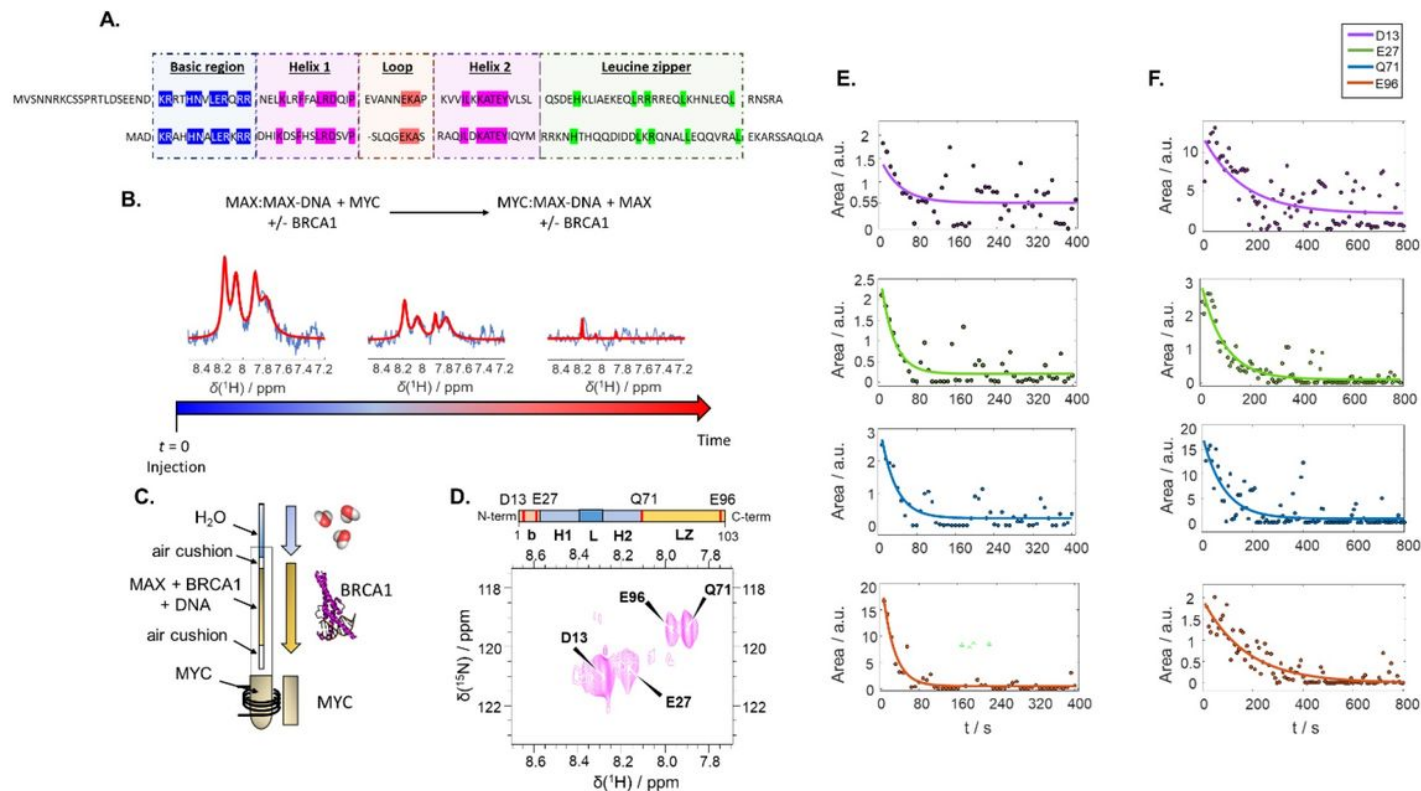


Figure 1

MYC:MAX heterodimerization assays. A) Sequence alignment of the MYC and MAX constructs used herein. The different domains of the basic-HLH-LZ structures are indicated. B) Schematic of the injection experiments. At $t = 0$ a MAX:MAX-DNA solution is mixed with sIMYC in the absence or presence of BRCA1²¹⁹⁻⁵⁰⁴. As the heterodimerization proceeds, the four sIMYC resonances decay (spectra blue, Lorentzian fits red). C.) Schematic representation of the injection apparatus. The MAX:MAX-DNA or MAX:MAX-DNA / BRCA1 solution is loaded inside a tubing, filled with water as a driving liquid, separated from the sample by an air cushion. The solution is injected by applying pressure on the driving liquid into an NMR tube, located directly inside the NMR spectrometer, where sIMYC is waiting. Mixing was complete within 1 s due to the force of the injection. D.) HNCO spectrum of sIMYC. The assigned amino acids assignment is indicated in the spectrum and in the MYC architecture scheme (top). E.) Decay of the four sIMYC signals upon mixing with a MAX:MAX-DNA solution at 5.5° C. The dots indicate the experimental data points fitted with an exponential function (solid line). All the signals decay to naught within 80 sec. F.) Decay of the sIMYC signals upon mixing with MAX:MAX-DNA in the presence of BRCA1²¹⁹⁻⁵⁰⁴. BRCA1 prolongs the signal decay by hindering the MYC:MAX-DNA complex formation.

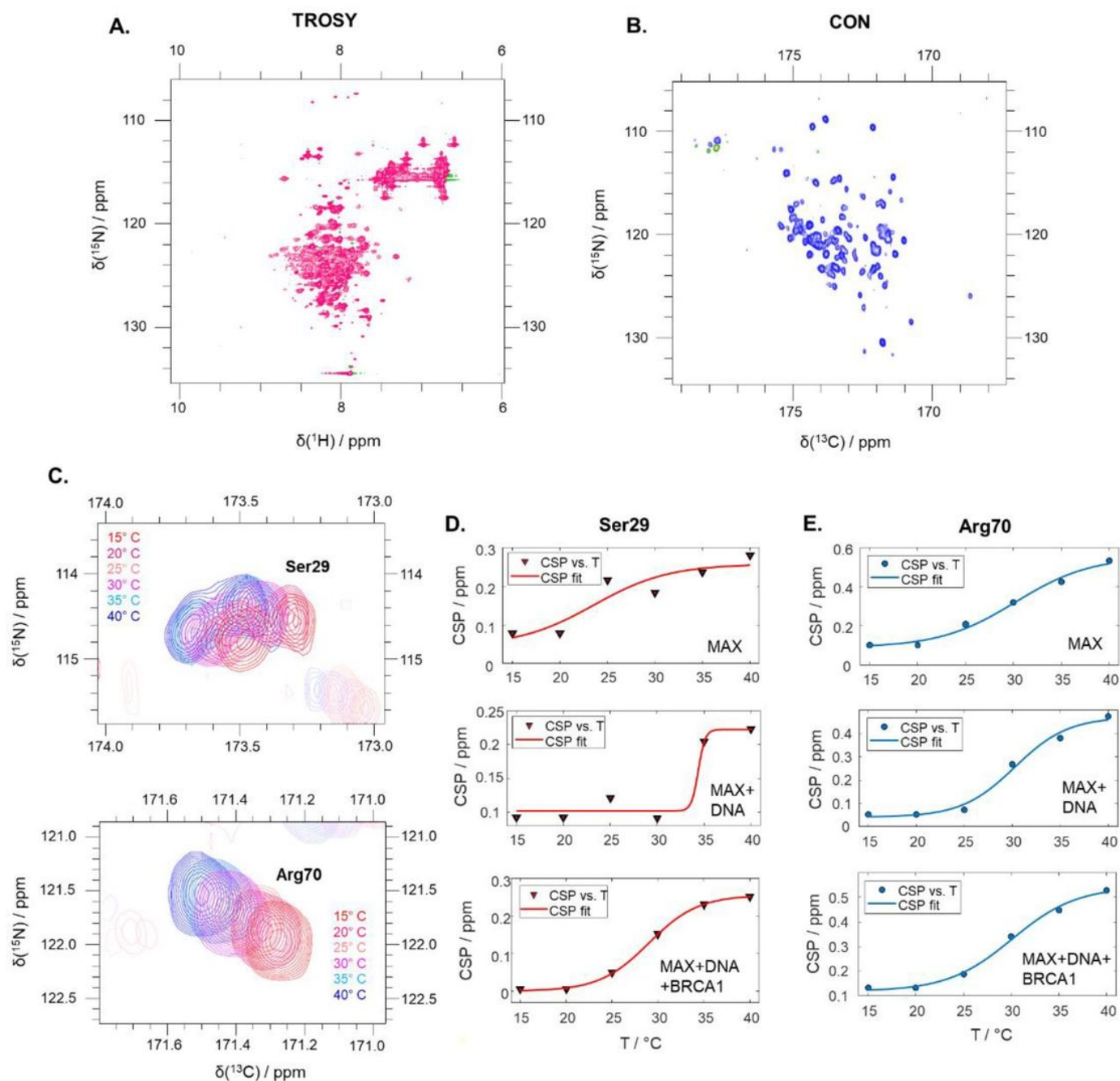


Figure 2

CON NMR spectra and chemical shift perturbation plots of MAX in combination with BRCA1 and DNA. A.) ^1H - ^{15}N TROSY spectrum of ^{15}N labeled MAX. $c = 0.1$ mM in MES buffer at 20°C . Strong signal broadening and overlap is observed. B.) ^{13}C - ^{15}N correlated NMR spectrum of homogeneously ^{13}C - ^{15}N labeled MAX under the same conditions as in panel A. The signals appear well-defined. C.) Chemical shift of Ser29 and Arg70 between 15°C (red) and 40°C (blue). One resonance per residue was observed at all temperatures. Hence, the system was always in fast exchange. D) CSP vs. T profiles for MAX Ser29 in the absence or presence of EBOX DNA and/or BRCA1. The CSP was calculated from the ^{13}C - ^{15}N experiments. Experimental CSP: red triangles sigmoidal fits: red line. The addition of BRCA1 to MAX:MAX-DNA complex reduces the Ser29 melting temperature significantly. E.) The same as in D, but for MAX Arg70.

The effects of the DNA and/or BRCA1²¹⁹⁻⁵⁰⁴ ligands are less pronounced due to the distance of residue Arg70 from the DNA-binding site.

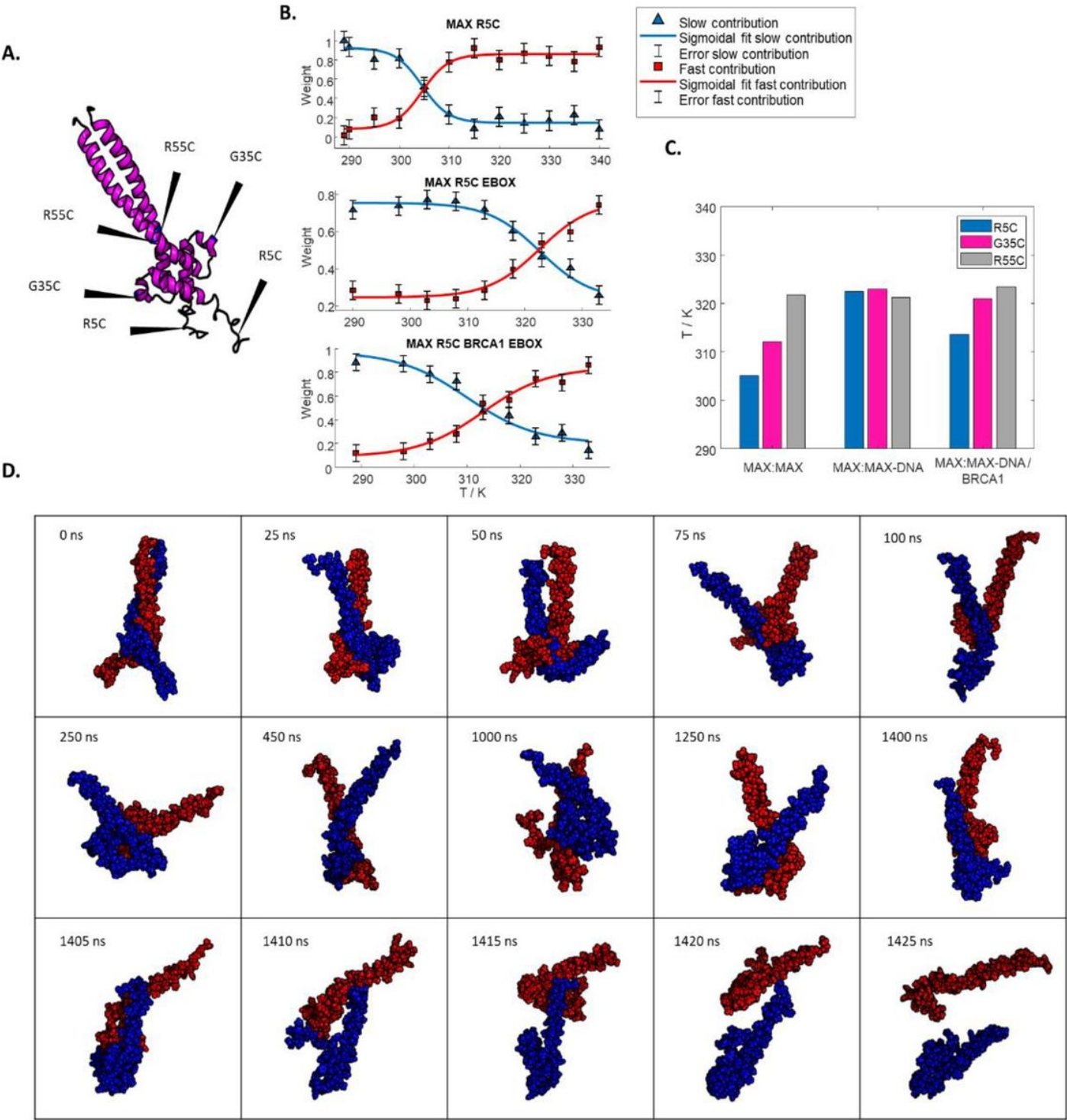


Figure 3

EPR and MD simulations of MAX:MAX, EBOX DNA, and BRCA1²¹⁹⁻⁵⁰⁴. A.) Localization of the MTSL labeling sites of MAX at positions R5C, G35C, and R55C. B.) EPR melting curves of an R5C mutated MAX with and without BRCA1 and/or the EBOX DNA. The blue triangles and the red squares indicate the

contributions of the folded (dimer) and unfolded (monomer) species in CW EPR spectra at different temperatures; the simulated data were fitted by sigmoidal functions (blue and red lines). The addition of both DNA and/or BRCA1²¹⁹⁻⁵⁰⁴ strongly impacts residues in (R5C) or close (G35C) to the basic DNA-binding site. C.) Comparison of the dissociation temperatures of MAX R5C (blue), G35C (pink), and R55C (grey) upon addition of the different interaction partners. The addition of DNA to the MAX:MAX complex increases the stability of the folded conformations, for R5C and G35C. Adding BRCA1 to the MAX:MAX-DNA trimers reduce the melting temperatures again. The melting temperature difference is less pronounced for R55C. D.) Coarse-grained MD simulation of the dissociation of MAX:MAX at 60° C. The dissociation starts with an unwinding of the LZ domain followed by dissociation of the HLH domain.

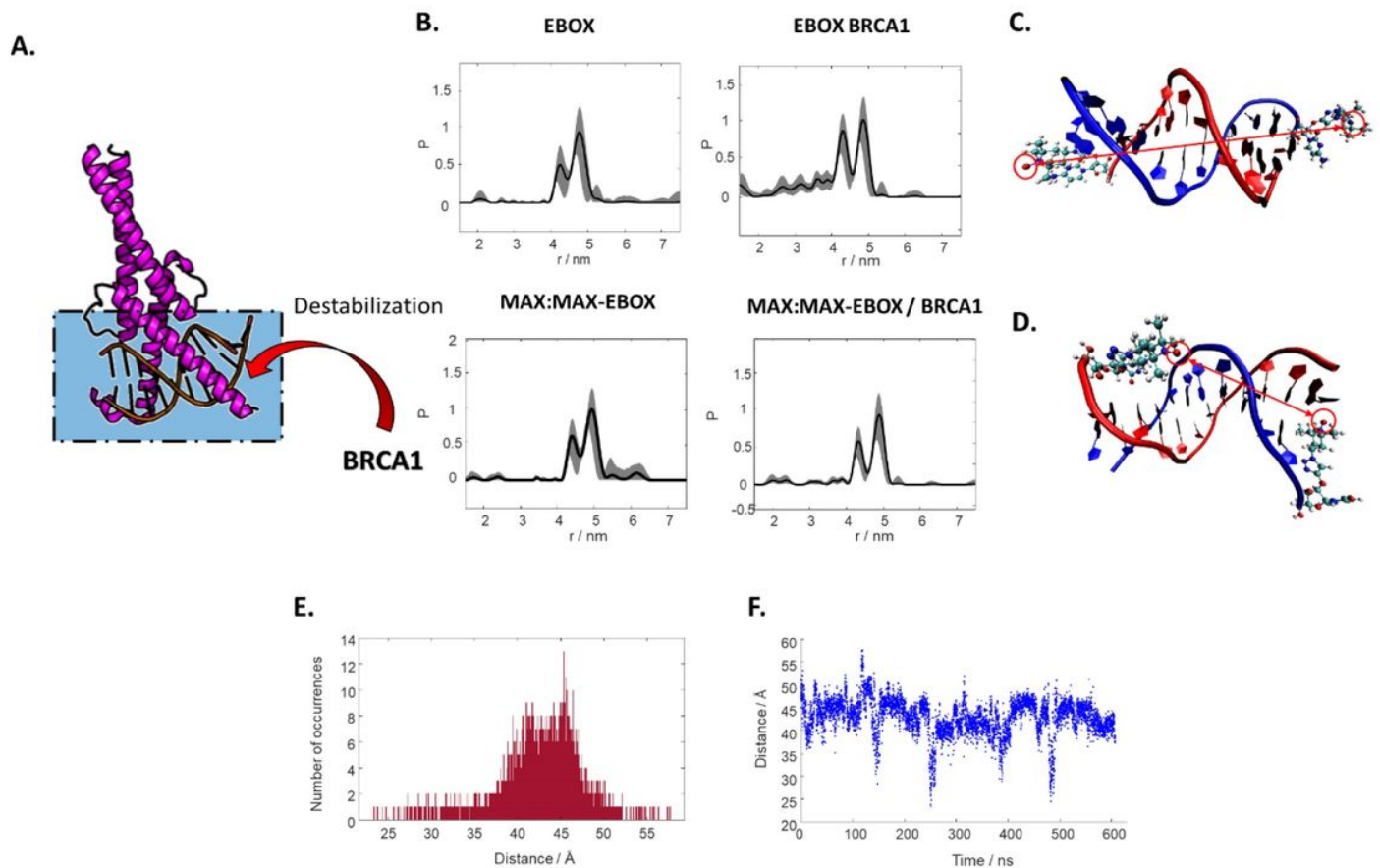


Figure 4

Nanoscale distance measurements confirm MAX-BRCA1 competition. A) Sketch of the interaction triangle. BRCA1 competes with MAX:MAX for a bound EBOX DNA ligand. B) Distance distributions $P(r)$ between spin labels attached to the two terminal cytosines of the DNA ligand in the absence and/or presence of BRCA1²¹⁹⁻⁵⁰⁴. C,D) MD-derived snapshots of the extended and compacted conformations, leading to the two main distances found in the DEER experiments. The red circles indicate the NO groups of the nitroxide labels; the red arrows highlight the distance between the labels. E) NO-NO distance distribution histogram derived from MD simulations. The profile resembles the experimental distance distribution. F) Inter-label distance vs. simulation time underlying the histogram in panel E.

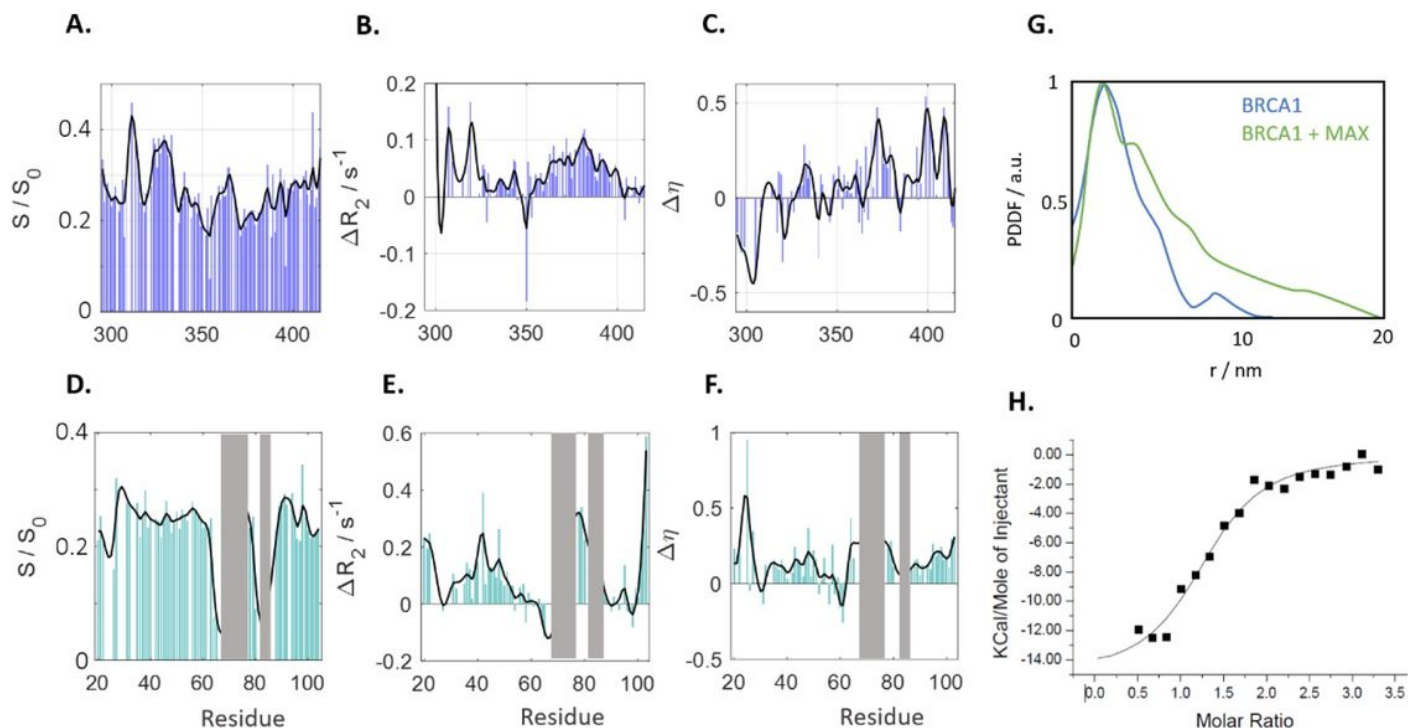


Figure 5

Fingerprint of BRCA1 binding to MAX monomers. A-C) Signal intensity ratios S/S_0 , differential transverse relaxation rates ΔR_2 , heteronuclear NOE $\Delta\eta$ of MYC upon binding to BRCA1. The black lines serve as a guide to the eye. D-F) Same as in panels A-C, but for the MAX monomer instead of MYC. Significant changes point towards a binding mode comparable to that of MYC. The grey bars indicate stretches that could not be assigned unambiguously in our experiments. G.) SAXS PDDF of BRCA1 in the absence (blue) and presence of MAX (green). H) ITC profile upon titration of MAX into a BRCA1 solution.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Epastoetal.ElucidationofMYCMAXBRCA1interactionnetworkbehaviorthroughNMREPRandMDSI.docx](#)

Chapter 4

Towards Protein NMR at Physiological Concentrations by Hyperpolarized Water – Finding and mapping uncharted conformational spaces

Manuscript 2

Ludovica Martina Epasto, Kateryna Che, Fanny Kozak, Albina Selimovic, Pavel Kadeřávek,
Dennis Kurzbach

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BIOCHEMISTRY

Toward protein NMR at physiological concentrations by hyperpolarized water—Finding and mapping uncharted conformational spaces

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Pavel Kadeřávek², Dennis Kurzbach^{1*}

Nuclear magnetic resonance (NMR) spectroscopy is a key method for determining the structural dynamics of proteins in their native solution state. However, the low sensitivity of NMR typically necessitates nonphysiologically high sample concentrations, which often limit the relevance of the recorded data. We show how to use hyperpolarized water by dissolution dynamic nuclear polarization (DDNP) to acquire protein spectra at concentrations of 1 μ M within seconds and with a high signal-to-noise ratio. The importance of approaching physiological concentrations is demonstrated for the vital MYC-associated factor X, which we show to switch conformations when diluted. While in vitro conditions lead to a population of the well-documented dimer, concentrations lowered by more than two orders of magnitude entail dimer dissociation and formation of a globularly folded monomer. We identified this structure by integrating DDNP with computational techniques to overcome the often-encountered constraint of DDNP of limited structural information provided by the typically detected one-dimensional spectra.

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Statement of contribution: *I published, together with my coworkers, this work in Science Advances in 2022, according to the DOI: 10.1126/sciadv.abq5179. I performed the DDNP and NMR experiments, and the MD simulations. I took care of the data analysis and methodology. The experiment planning and the writing were performed by myself under the supervision of Assoc. Prof. Dr. Dennis Kurzbach.*

BIOCHEMISTRY

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Nuclear magnetic resonance (NMR) spectroscopy is a key method for determining the structural dynamics of proteins in their native solution state. However, the low sensitivity of NMR typically necessitates nonphysiologically high sample concentrations, which often limit the relevance of the recorded data. We show how to use hyperpolarized water by dissolution dynamic nuclear polarization (DDNP) to acquire protein spectra at concentrations of 1 μ M within seconds and with a high signal-to-noise ratio. The importance of approaching physiological concentrations is demonstrated for the vital MYC-associated factor X, which we show to switch conformations when diluted. While in vitro conditions lead to a population of the well-documented dimer, concentrations lowered by more than two orders of magnitude entail dimer dissociation and formation of a globularly folded monomer. We identified this structure by integrating DDNP with computational techniques to overcome the often-encountered constraint of DDNP of limited structural information provided by the typically detected one-dimensional spectra.

INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy is a key method for determining the structures and dynamics of proteins and nucleic acids (1–3). Currently, NMR is the only method that enables complete structural characterization of biomacromolecules with atomistic resolution in their native solution environment. However, because of the intrinsically low sensitivity of the method, the target molecule's concentration needs to be much higher than under typical physiological conditions. This is crucial because interaction kinetics, complex structures, and accessible conformational spaces are often concentration dependent (4–7). As a result, typical in vitro conditions limit the relevance of NMR data. In contrast, developing NMR methods that enable access to the low micromolar physiological concentration regime can help to improve the medicinal relevance of NMR and thus widen the scope of its applications. Such developments remain challenging, even despite extensive and continuous efforts, such as developing costly ultrahigh-field magnets and cryogenically cooled probes (8).

Here, we demonstrate an unconventional route toward overcoming the NMR concentration limitation. We show how to record NMR spectra of proteins within seconds at micromolar concentration and a signal-to-noise ratio (SNR) > 15 by dissolving them in “hyperpolarized water” (9–17). This means that the NMR signals of the water protons are enhanced in comparison to conventional NMR by pretreating it in a dedicated DNP (dynamic nuclear polarization) apparatus before being used for dissolving the target protein. Other applications of hyperpolarized water can, for example, be found in magnetic resonance imaging (18, 19) or low-field (triplet or Overhauser DNP) NMR (20–26). The hyperpolarization boosts the water NMR signal intensities by more than two orders of magnitude. Through exchange interactions between the water and the target

biomolecule, the signals of the latter are similarly boosted by orders of magnitude, hence enabling access to equivalently >100-fold reduced concentration regimes (9–17).

The value of this methodological advance is demonstrated using the ubiquitous transcription factor MAX (MYC-associated factor X; M_w = 10.9 kDa per monomer) (27–32). We show that MAX adopts an undocumented conformation when concentrations approach physiological levels (33) in contrast to the high-concentration regime typically encountered in in vitro assays (32–34). By combining the hyperpolarization methodology with molecular dynamic (MD) simulations, chemical shift prediction, and established structural biology methods, we characterized the newly found MAX conformation in atomistic detail.

This is relevant because the vital dimerization of MAX with its partner molecule MYC inevitably depends on MAX's conformation (5, 35). The importance of this process can be assessed considering that the MYC:MAX heterodimer plays a crucial role in cell proliferation and apoptosis (31) and that its dysregulation is associated with a plethora of cancers (31).

MAX is typically described as a stably folded homodimer in solution. To bind to MYC, the homodimer yet needs to dissociate. Despite its significance, the question of how the process of MAX homodimer destabilization proceeds remains unanswered, not least because of a strong dimerization tendency and nanomolar homomolecular affinities (33). To tackle this dilemma, we map a formerly uncharted part of MAX's conformational space, i.e., the regime at physiological concentrations, and characterize the structures found therein with DNP-boosted NMR. This might help shed light on the MYC:MAX dimerization enigma from the viewpoint of the MAX monomer.

RESULTS

To obtain NMR spectra of MAX at physiological concentrations in hyperpolarized water, we used dissolution DNP (DDNP) (36–38). We followed the protocol detailed in (39, 40). In brief, hyperpolarized water was produced in a dedicated DNP apparatus under cryogenic

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conditions ($T_{\text{DNP}} = 1.3$ K) by microwave irradiation of dissolved stable radicals [15 mM TEMPOL (4-Hydroxy-2,2,6,6-tetramethylpiperidinyloxy)]. To this end, we used the home-built prototype described by Kress *et al.* (41). After polarization buildup, the hyperpolarized water was rapidly heated to ambient temperatures and pneumatically transferred to an NMR spectrometer where a MAX solution was waiting “in situ” in the NMR tube. The hyperpolarized water was then mixed with the MAX solution (0.3 mM), diluting it 300-fold before the NMR acquisition (Fig. 1A). Upon detection, the final concentration was 1 μM . The entire dissolution and mixing process took ca. 2 s.

Upon mixing, the proton spin hyperpolarization of the water is spontaneously transferred to the target protein through chemical exchange and nuclear Overhauser effects, boosting the signal amplitudes of the target (17). A series of ^{15}N -edited ^1H one-dimensional (1D) NMR spectra was then recorded for 120 s with a sampling rate of 2 s^{-1} [the pulse sequence was similar to the first increment of a

BEST-HMQC (band-selective short transient heteronuclear multi-quantum correlation) (42); see fig. S1].

The resulting signal intensity as a function of time is shown in Fig. 1B. An intense spectrum can be observed directly after mixing. It decays to thermal equilibrium within ~ 10 s. The initial SNR was found to be >15 . The typical comparison to thermal equilibrium data was complicated because not all peaks were visible in the nonhyperpolarized spectra after decay to thermal equilibrium. The enhancement of the bulk signal was ca. 120 (see fig. S2 for the spectrum in thermal equilibrium). It should be noted that the data were recorded on a 500-MHz spectrometer equipped with a broadband probe, which is designed for the detection of heteronuclei. Much better SNR could be obtained with a probe dedicated to ^1H detection and higher magnetic fields. Other targets, particularly intrinsically disordered proteins, that typically feature sharper NMR signals (11) might even further push the detection limit.

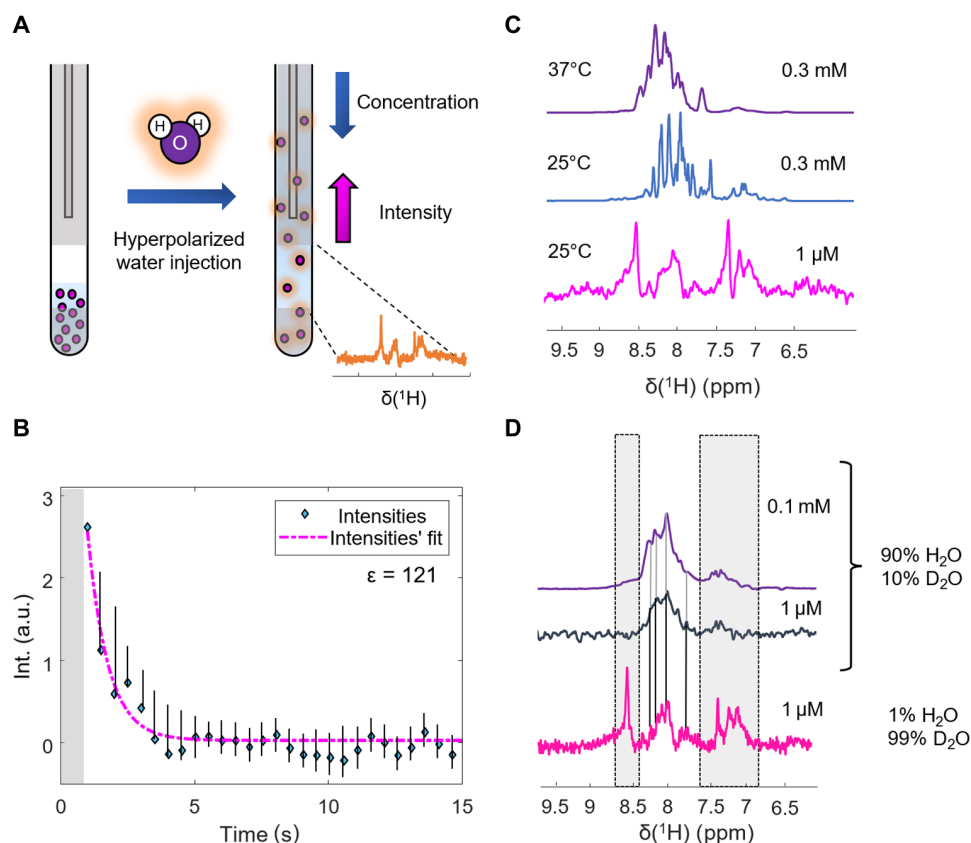


Fig. 1. NMR and DDNP of MAX. (A) Sketch of the experimental concept. Upon injection of hyperpolarized water, the target protein is diluted, while at the same time, the signal loss is (over)compensated by exchange between the water and the target, which introduces hyperpolarized protons into the latter. (B) Signal decay (bulk integrals) of the ^1H resonance of MAX after dilution. Directly after injection, turbulences obscure the spectra (gray bar). After 1 s, the turbulences settle, and signal-amplified spectra become detectable. After 10 s, the signal amplitude has decayed below the noise level. The experiment was repeated two times. The error bars indicate the maximum deviation between the repetitions. (C) Comparison of skyline projections on the ^1H dimension of an HSQC (in purple) and a TROSY (in blue) experiment detecting ^{15}N -labeled MAX (0.3 mM) with a hyperpolarized MAX spectrum (first detected spectrum) at near-physiological concentration (1 μM). The latter was recorded using an adapted BEST-HMQC pulse sequence. The spectra at higher concentrations can be considered fingerprints of the folded dimer (25°C) and the unfolded state (37°C). The spectrum at low concentrations matches neither of these cases, indicating the presence of a third conformational state. (D) ^{15}N -edited ^1H spectra of ^{15}N -labeled MAX at different dilutions detected on an 11.7-T NMR spectrometer in thermal equilibrium (purple and blue) compared to the hyperpolarized ^{15}N -edited ^1H spectrum (magenta; average over the first 20 detected spectra). The black straight lines trace the position of significant peaks from the thermal equilibrium to the hyperpolarized spectrum. The dashed boxes indicate significantly boosted signals in the NMR experiment that remained undetected in the thermal equilibrium spectrum. The DDNP experiment highlights almost invisible signals in the spectral envelope obtained in the standard experiments. a.u., arbitrary units.

Concerning the experimental times, one must consider that the buildup of water hyperpolarization takes ca. 2 hours (16), while the detection only takes a few minutes. Taking the preparation of the dissolution experiments into account, too, the time needed is comparable to that of a conventional NMR experiment recorded via signal averaging. However, as the gain in signal amplitude is >120 for the case at hand, >14,000 signal averages would be required to reach a similar SNR.

Figure 1C shows a comparison of the spectrum obtained in hyperpolarized water with spectra recorded by conventional NMR. Notably, the hyperpolarized spectrum obtained at physiological concentrations differs from the other spectra recorded under typical *in vitro* NMR conditions. In particular, we compared the DDNP-derived spectrum obtained at a concentration of 1 μM with HSQC (heteronuclear single-quantum coherence) and TROSY (transverse relaxation-optimized spectroscopy) spectra, obtained at a concentration of 0.3 mM at 37° and 25°C, respectively. Under these conditions, MAX populates one of two well-documented conformations, a stably folded helical homodimer (43) at 25°C and a monomeric random-coil state (29) at 37°C (the elongated dimer tumbles slowly around one axis, necessitating TROSY). Evidently, the low-concentration spectrum matches neither of these two cases. Hence, a third conformation in an undocumented conformational subspace seems to be populated when concentrations approach physiological levels, independent of the environmental temperature.

In the next step, we confirmed this conclusion by comparing the hyperpolarized spectra with references in thermal equilibrium recorded with the same real-time ^{15}N -edited ^1H 1D BEST-HMQC detection sequence. The result is shown in Fig. 1D. Two experiments were carried out at 0.1 mM and 1 μM . It is evident that the conformation populated at low concentration leads to a spectrum significantly different from the one recorded at higher concentration. Only upon reducing the concentration to 1 μM , the features of the reference spectrum can be observed in the hyperpolarized spectrum, too (see fig. S3 for a more detailed representation). It should be noted that with hyperpolarization, some signals can be observed that remained below the detection threshold in the thermal equilibrium case, particularly in the regions around 8.5 and 7.5 parts per million (ppm) (Fig. 1D, gray boxes).

These resonances are significantly stronger enhanced (compared to thermal equilibrium) than other parts of the spectrum. An analysis of the predicted chemical shifts (cf. table S1) showed that these resonances belong exclusively to Q-, D-, N-, and H-type residues. These amino acids all feature labile side-chain protons, and it was recently shown (16, 40) that amino acids with such moieties display stronger signal enhancements in hyperpolarized water than amino acids void of exchangeable side-chain protons. It was concluded (16, 40) that exchange-relayed nuclear Overhauser magnetization transfers from the side chains to the detected amides additionally boost these resonances. A similar effect likely underlies the data presented in Fig. 1D, too. Similar reasoning may account for the substantial enhancements of the side-chain resonances between 7 and 7.5 ppm of N and Q residues.

Hence, we conclude from the data in Fig. 1 that the DDNP results show a spectral fingerprint of MAX in a region of its conformational space that is dominated neither by the MAX homodimer (high concentrations, low temperature) nor by its random-coil state (high concentrations, high temperatures). HSQC experiments in thermal equilibrium further support this (see figs. S4 and S5), showing, despite low SNR, that spectra obtained at near-physiological concentrations match neither that of the folded nor that of the intrinsically disordered state.

We want to stress that data can be recorded at concentrations of 1 μM in thermal equilibrium (in particular when using cryogenically cooled probes), but important features/peaks of the spectra might be overlooked as these remain below the sensitivity threshold. Contrary, with DDNP, weak signals can be recovered that vividly exchange with the solvent, such as those of the Q-, D-, N-, and H-type residues.

Note that damage of MAX due to the injection of hyperpolarized water is unlikely. Control DDNP experiments at higher concentration (fig. S6) and thermal equilibrium spectra of reconcentrated MAX after a DDNP experiment in a radical-free buffer all led to NMR spectra of the intact MAX dimer (see fig. S7).

An intuitive explanation for the conformational switch is a concentration-dependent monomer-to-dimer ratio and that the monomer dominates the conformational space at low concentrations. However, neither conventional NMR nor crystallography can be used to resolve the structural dynamics of monomeric MAX at such high dilution. We, therefore, used MD simulations to identify a possible monomeric MAX species. Because of the lack of a known monomer structure, we selected one chain from the NMR solution structure of the homodimer reported in the literature (32) as starting point [this choice was supported by structure prediction through the PHYRE2 software (44), which yielded a similar conformation for the MAX monomer; see fig. S8]. We then ran MD simulations of the monomer in explicit solvent for >500 ns. The result is shown in Fig. 2 (A to C) (for details, see the Materials and Methods). The initially elongated monomer rapidly switches its conformation to a globular shape with stable secondary and tertiary structural elements (Fig. 2A). The major structural elements of MAX, i.e., the helix-loop-helix motif, are retained, yet the N-terminal helix that typically forms the leucine zipper (LZ) domain folds back toward the N terminus. Considering that the N and C termini feature opposite charges (MAX is one of the strongest dipoles found in nature) (45), such a configuration is likely energetically favorable in the absence of a second unit to form a dimer and constitute the LZ. In our simulations, this transition was evidenced by a sudden reduction in hydrodynamic radius (Fig. 2B), which drops from 2.5 to 1.5 nm upon transition from the elongated to the globular state. Once globularly folded, MAX did not return to the initial state in our simulation. The result was reproduced in three independent runs (see figs. S9 to S13).

To integrate the DDNP and MD data, we then used the Sparta+ software package (46) to calculate the ^1H chemical shifts of the newly found globularly folded structure. We randomly chose 16 conformations from the last 20 ns (Fig. 2C) of the MD trajectory and predicted the amide $^1\text{H}^{\text{N}}$ chemical shift (see table S1). Computing histograms of the chemical shift distribution (combining all 16 data-sets) then yielded predictions of the ^{15}N -edited ^1H spectra. The results for the three independent simulations are shown in Fig. 2 (D to F). The measured hyperpolarized spectra and predicted ^{15}N -edited ^1H spectra match well within the precision of the approach. The resemblance of experimental and predicted data strongly supports our conclusion that a formerly undocumented conformation of a MAX monomer exists in lowly concentrated solutions. The match suggests that the globularly folded MAX monomer found in the MD simulations represents the conformation detected by DDNP-boosted NMR at physiological concentrations.

Note that the simulations do not consider differential line broadening of individual residues or the dependence of the individual residue signal intensities on the site-specific effectiveness of solvent exchange in the DDNP experiments. This could lead to mismatches between

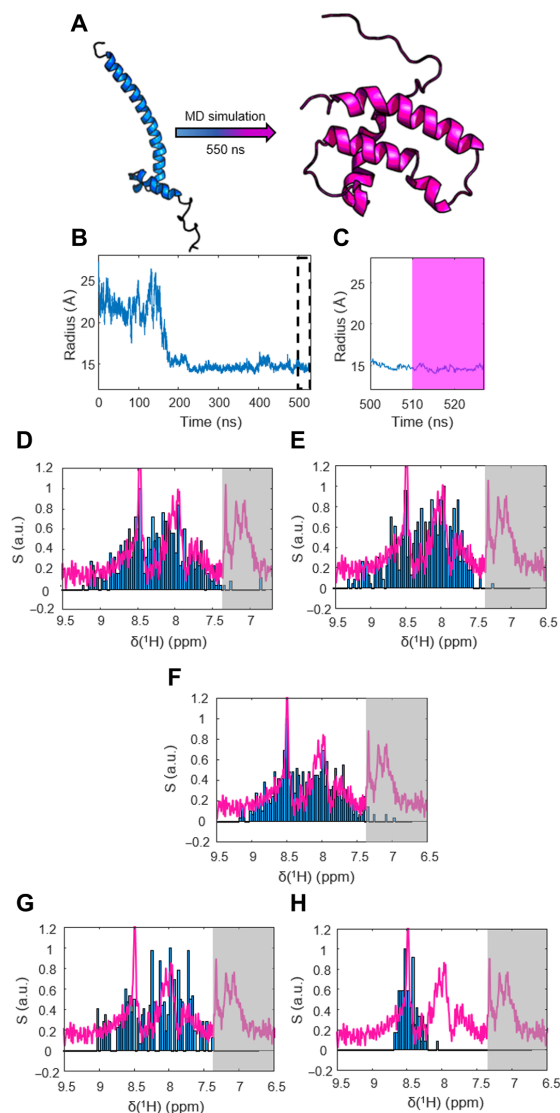


Fig. 2. Integration of MD simulations with the DDNP spectra. (A) Structural transition observed for the MAX monomer in a 550-ns MD simulation in a 15 nm³ large box. Stripping the well-documented dimer of one subunit provided the starting structure. After 150 ns, a transition into a globular, tightly folded conformation could be observed. (B) Development of the hydration radius R_h of MAX in the MD simulations over time. After ~150 ns, MAX converts from the initial elongated shape into a compact conformation. The dashed box indicates the part of the simulation that we considered equilibrated and used for chemical shift predictions. (C) Zoom onto the R_h trajectory and the last 30 ns of the simulation. All conformations used for chemical shift prediction stem from the last 20 ns of simulation, highlighted in pink. (D to F) Comparison of the hyperpolarized spectrum (S) (pink line; average over the first 20 detected spectra) and the spectrum predicted on the basis of the MD simulations (blue bars) of the MAX monomer for three independent runs. The gray shade indicates side-chain resonances not included in the chemical shift calculations. The good match between the experimental and the calculated spectrum supports our conclusion that the monomer dominates the conformational ensemble at low concentrations. (G) “Negative control” comparing the experimental hyperpolarized spectrum with a spectrum predicted from the elongated MAX dimer structure. The mismatch shows that the experiment does not reflect this structure. (H) Negative control comparing the experimental hyperpolarized spectrum with a spectrum predicted for a random-coil state. The mismatch shows that the experiment does not reflect this structure either.

experimental and simulated results and data misinterpretation even for similar protein conformations. Therefore, it is important to perform negative control experiments and compare the DDNP-derived spectra to known cases. For MAX, the unfolded state, as well as the coiled-coil heterodimer, can be chosen as control structures.

We predicted the ¹⁵N-edited ¹H spectra for the MAX dimer, using the available NMR solution structure as input for Sparta+ and a random-coil state of MAX, using neighborhood-corrected chemical shift prediction (Fig. 2, G and H) (47). In both cases, the experimental spectrum was not reproduced, supporting our conclusion that the hyperpolarized water–boosted NMR experiments detected a globularly folded MAX monomer.

Both control cases do not suffice to explain our results, although these two conformations are the primary constituents of the (so far documented) MAX conformational space [see, e.g., the work of Fieber *et al.* (33)]. Hence, necessarily, the DDNP experiments show a third, so far unknown, conformation.

The number and type of required control experiments depend on the detail of information that one wants to extract from the DDNP-enhanced spectra. Here, we compare our simulations to the overall experimental line shape, i.e., we refrain from residue-resolved data analysis. For this type of analysis, the two reported controls are sufficient as both clearly do not match the experimental line shape and width.

DISCUSSION

In conclusion, we find that hyperpolarized water can boost the sensitivity of protein NMR spectra to access the low–micromolar concentration regime at high SNR and within short experimental times (acquisition in less than 1 min). This is possible even for a challenging target such as MAX, which is typically studied by NMR at concentrations of >1 mM (32). This enabled the identification and characterization of globularly folded monomeric MAX.

The predominance of a globular conformation at low concentrations is independently supported by fluorescence resonance energy transfer data reported by Vancraenenbroeck and Hofmann (48). They showed that at very low concentrations, monomeric globular conformations dominate the conformational space of MAX. In contrast, the coiled-coil conformation of the MAX dimer features a strongly anisotropic shape, which could only be found at higher concentrations. However, this study focused on kinetic analyses and did not report a resolved conformational model at low concentrations. Our combined DDNP/MD approach yet can provide atomistic protein structures to map the conformational spaces at high detail. In the broader context of cellular transcription, the monomer concentration of unbound MAX in the cell nucleus is of particular interest as it might be directly involved in the dimerization rate with the MYC transcription factor.

It should be noted, however, that the presence of DNA in the cell nucleus, where MAX resides *in vivo*, favors the population of the homodimer because of the high stability of the MAX:MAX-DNA complex (33). Under *in vivo* conditions, MAX remains mainly in this form. The concentration of unbound MAX might therefore be much lower than 1 μM.

MATERIALS AND METHODS

Protein production

His-tagged MAX was subcloned into a pET-21a(+) expression vector and transformed into *Escherichia coli* Rosetta2 cells. The bacteria were grown at 37°C in M9 [for ¹⁵N labeling, ¹⁵N ammonium chloride

(1 g/liter) was added] and induced at an optical density corresponding to A_{600} (absorbance at 600 nm) = 0.6 with 1 mM isopropyl- β -D-thiogalactopyranoside before incubation at 30°C overnight. Cell pellets were homogenized in 25 mM Tris, 100 mM NaCl, and 2 mM β -mercaptoethanol (pH 8.00). After cell disruption, the supernatant was purified through Ni^{2+} affinity chromatography. The his-tag was cleaved by Tobacco Etch Virus (TEV) protease in a ratio of 1:15 to MAX at 4°C overnight. The cut was confirmed through mass spectrometry, reporting a mass of 11.035 kDa, consistent with the expected ^{15}N -enriched protein mass (see fig. S14).

The sample was buffer-exchanged using ultracentrifugal filters with a cutoff of 3 kDa into the buffer used for the NMR experiments [25 mM MES, 25 mM NaCl, and 100 mM ArgHCl (pH 5.5)]. Last, MAX was concentrated up to 0.3 mM and aliquoted.

Dissolution dynamic nuclear polarization

For DNP, 150 μl of a solution of 15 mM TEMPOL in a mixture of 50% glycerol- d_8 , 40% D_2O , and 10% H_2O was hyperpolarized at 1.4 K in a magnetic field of 6.7 T for 2500 s using continuous-wave microwave irradiation at 188.08 GHz. DNP samples were always freshly prepared to avoid ripening effects (49). A Virginia Diodes Inc. (VDI) microwave source was used together with a 16 $\times$ frequency multiplier that provided an output power for the microwave of ca. 50 mW. The magnet-cryostat combination was purchased from Cryogenic Ltd. and operated as described in (41).

For detection of the solid-state polarization, a 400-MHz Bruker Avance III system was adapted to a ^1H resonance frequency of 285.3 MHz and a ^{13}C frequency of 71.72 MHz by using a broadband preamplifier for both channels. The detection circuit and the external tune-and-match system were home-built, as described in (50). To monitor the buildup, detection pulses with a flip angle of 1° were applied every 5 s.

After DNP, the sample was dissolved with a burst of 5 ml of D_2O at 1.5 MPa as described in (41). The hyperpolarized liquid was then pushed with helium gas at 0.7 MPa to the detection spectrometer. The dissolution process used a home-built pressure heater actuated with an Arduino microcontroller. A home-written MATLAB-based user interface controls the dissolution and injection steps.

Detection was carried out using a 500 MHz Bruker NEO spectrometer equipped with a Prodigy BBFO cryogenic probe. The pulse sequence for detection corresponded to a series of detections based on the first increment of a BEST-HMQC (see fig. S1) (42). We used PC9 and RSNOB (51) selective 90° and 180° pulses covering a bandwidth of 4 ppm centered around a carrier frequency of 9.5 ppm to excite and invert the protons. The pulse lengths were 3495 and 1165 μs , respectively. The 90° pulse for the ^{15}N channel was 15 μs long. The ^{15}N carrier frequency was adjusted to 118 ppm. Heteronuclear decoupling was achieved using the GARP (52) scheme as preinstalled in Bruker TOPSPIN 4. The delay d_0 was fixed to 0.1 μs . Note that varying d_0 between 0 and 0.2 μs does not significantly affect the recorded spectra.

For sample injection, a “syringe” device similar to the one by Kouril *et al.* (53), but adapted to liquids, was used that injects a controlled volume into the NMR tube (see fig. S15 for a sketch of the setup). It should be noted that protein samples are often showing temperature-dependent conformational changes. For the dissolution system used here, the hyperpolarized water arrived with a temperature of 24° to 26°C in the NMR tube waiting in the spectrometer at 25°C (as controlled by the built-in temperature control system of the Bruker NEO

spectrometer), such that no strong temperature gradients biased the protein conformation. However, other DNP systems that use other dissolution systems might lead to different sample temperatures.

To perform DDNP experiments with MAX at higher concentrations, the same procedure was followed, but MAX was only diluted by a factor of 3 during the injection, resulting in a final concentration of 0.1 mM upon detection (see fig. S6 for the results). For reproduction experiments, see fig. S16.

Nuclear magnetic resonance

TROSY and HSQC spectra were recorded using a Bruker NEO 600-, 700-, or 850-MHz spectrometer equipped with cryogenically cooled probe heads optimized for ^1H detection. Spectra were recorded in the States-TPPI/PFG mode for quadrature detection with carrier frequencies for ^1H and ^{15}N of 4.73 and 120.0 ppm, respectively. The samples contained 1 μM , 0.1 mM, or 0.3 mM MAX; 25 mM MES; 100 mM ArgHCl; and 25 mM NaCl (pH 5.5) in a 90% H_2O /10% D_2O mixture.

All NMR spectra were processed and analyzed using TOPSPIN 4.0.7 and MATLAB R2019a. NMRPipe and SPARKY were used to process and analyze the recorded TROSY and HSQC data (54, 55). A squared and 60° phase-shifted sine bell window function was applied in all dimensions for apodization. Time-domain data were zero-filled to twice the dataset size, before Fourier transformation.

In addition, we considered the possibility that a shift in pH could influence MAX's configuration. Therefore, we performed a TROSY experiment on MAX in a 30 times diluted buffer (fig. S5). Despite the significant variation of pH (from 5.5 to 7.15) in the lowly concentrated buffer, the detected spectrum does not show any similarities with the spectra obtained from a lowly concentrated protein sample (fig. S4C).

MD simulations and chemical shift prediction

MD simulations were performed using the YASARA software package (56, 57). The AMBER14 force field was used with periodic boundary conditions (58). Explicit water and a NaCl concentration of 0.9% were used for filling and neutralizing the simulation cell. Nonbonded interactions were cut off at 1.05 nm. Long-range Coulombic interactions were treated by a smoothed particle-mesh Ewald method (59). MD trajectories of >500-ns length were then accumulated. In total, three trajectories were computed. Intermolecular forces were recalculated at every second simulation substep. Temperature rescaling was used with a set temperature of 25°C. The box dimensions (cubic of >150-Å side length) were controlled to yield a solvent pressure of 1 bar. Snapshots of the simulations were taken every 4000 fs.

The chemical shifts were predicted from the MD simulations or the MAX dimer NMR structure (Protein Data Bank code 1R05) using the Sparta+ webserver (46). To this end, a set of 16 random snapshots from the last 20 ns of each MD trajectory was submitted for chemical shift prediction. The spectra were then calculated by computing averages over histograms (with 100 bins each) that represent the distribution of resonances over the $\delta(^1\text{H})$ dimension for each single snapshot. These tasks were performed using the MATLAB software package.

Neighborhood-corrected chemical shift calculations were carried out using the ncIDP web server (<https://st-protein02.chem.au.dk/ncIDP/>). These calculations predict chemical shifts (47) of unfolded proteins and peptides on the basis of their primary amino acid sequence. For calculating the chemical shift, a random-coil conformation is

assumed for the protein or peptide. Database values for documented chemical shifts of all 20 amino acids in random-coil type structures are combined with sequence-dependent left- and right-neighbor (in terms of the primary sequence) correction factors. This results in an accurate prediction of the chemical shift for the protein when existing unfolded. Hence, this type of prediction can be used for the negative control in Fig. 2H (benchmark against unfolded MAX).

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <https://science.org/doi/10.1126/sciadv.abq5179>

[View/request a protocol for this paper from Bio-protocol.](#)

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Toward protein NMR at physiological concentrations by hyperpolarized water— Finding and mapping uncharted conformational spaces

Ludovica M. Epasto, Kateryna Che, Fanny Kozak, Albina Selimovic, Pavel Kadeřávek, and Dennis Kurzbach

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Chapter 5

Nuclear Overhauser spectroscopy in hyperpolarized water - chemical vs. magnetic exchange

Manuscript 3

Ludovica Martina Epasto, Philipp Honegger, Kateryna Che, Fanny Kozak, Florian Jörg, Christian Schröder, Dennis Kurzbach

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Nuclear Overhauser spectroscopy in hyperpolarized water – chemical vs. magnetic exchange†

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Abstract

Dissolution dynamic nuclear polarization (dDNP) is a versatile hyperpolarization technique to boost signal intensities in nuclear magnetic resonance (NMR) spectroscopy. The possibility to dissolve biomolecules in a hyperpolarized aqueous buffer under mild conditions has recently widened the scope of NMR by dDNP. The water-to-target hyperpolarization transfer mechanisms remain yet unclear, not least due to an often-encountered dilemma of dDNP experiments: The strongly enhanced signal intensities are accompanied by limited structural information as data acquisition is restricted to short time series of only one-dimensional spectra or a single correlation spectrum. Tackling this challenge, we combine dDNP with molecular dynamics (MD) simulations and predictions of cross-relaxation rates to unravel the spin dynamics of magnetization flow in hyperpolarized solutions.

Statement of contribution: I published, together with my coworkers, this work in *Chemical Communications* in 2022, according to the DOI: 10.1039/D2CC03735A. I performed the experiments and the MD simulations. Additionally, I conceived the study and wrote the paper under the supervision of Assoc. Prof. Dr. Dennis Kurzbach.



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Dissolution dynamic nuclear polarization (dDNP) is a versatile hyperpolarization technique to boost signal intensities in nuclear magnetic resonance (NMR) spectroscopy. The possibility to dissolve biomolecules in a hyperpolarized aqueous buffer under mild conditions has recently widened the scope of NMR by dDNP. The water-to-target hyperpolarization transfer mechanisms remain yet unclear, not least due to an often-encountered dilemma of dDNP experiments: The strongly enhanced signal intensities are accompanied by limited structural information as data acquisition is restricted to short time series of only one-dimensional spectra or a single correlation spectrum. Tackling this challenge, we combine dDNP with molecular dynamics (MD) simulations and predictions of cross-relaxation rates to unravel the spin dynamics of magnetization flow in hyperpolarized solutions.

Spin hyperpolarization denotes a population distribution between nuclear spin levels far from thermal equilibrium. Such a state invokes strongly improved signal amplitudes, often over 10 000-fold enhanced, compared to conventional nuclear magnetic resonance (NMR) spectra. Various methods, from para hydrogenation to dynamic nuclear polarization (DNP) to optical pumping,¹ enable the creation of hyperpolarized spin states for a wide array of applications. The use of hyperpolarized water to boost signal intensities in biomolecular NMR spectra has recently received ample attention^{2–5} as dissolving a target biomolecule in a hyperpolarized buffer enables new approaches at substantially enhanced sensitivity: residue- and time-resolved protein NMR,⁶

2D and 3D correlation spectra,⁷ protein folding and folding monitoring,⁸ membrane interactions,⁹ structural dynamics,¹⁰ and exchange processes.¹¹ However, the understanding of the hyperpolarization transfer mechanism¹² from the buffer to the targets remained incomplete; not least as data acquisition in the, most often used, dissolution DNP (dDNP) experiments is typically limited to short periods of 1–2 min that do not allow for determining the exchange pathways *via* complex pulse sequences. Important insights into hyperpolarization transfer by nuclear Overhauser effects (NOE) between water protons and molecules dissolved therein have, *e.g.*, been achieved by Marco-Rius *et al.* (¹³C to ¹H)¹³ or Hu *et al.* (¹H to ¹H or ¹⁹F).¹⁴ To this understanding, we here add a description of the interplay between direct solvent-solute NOE and exchange-relayed NOE in hyperpolarized water; an interesting phenomenon which invokes shifted weights of the different contributions to the magnetization flow as NOE and chemical exchange are differently affected by spin-hyperpolarization. Herein, we provide an understanding of these effects and, hence, also of the transient signal enhancements in biomolecular dDNP. In a dDNP experiment, the sample is *ex situ* pretreated to boost the spin resonances before transfer to an NMR spectrometer for detection. As the resulting spin-hyperpolarization after the transfer constitutes a non-equilibrium state, its lifetime is necessarily limited. We here demonstrate how to unravel the active transfer mechanisms despite a short time window by integrating dDNP experiments^{1,15,16} with cross-relaxation rates^{17,18} obtained from molecular dynamics (MD) trajectories; providing formerly inaccessible details.

Experimentally, we employed the dDNP protocol detailed in ref. 19 (see the ESI†). In brief, we hyperpolarized a water-glycerol mixture²⁰ using a prototype system²¹ operating at a temperature of $T_{\text{DNP}} = 1.4$ K and a magnetic field of $B_{0,\text{DNP}} = 6.7$ T. After build-up of the ¹H spin hyperpolarization, the sample was dissolved with a burst of D₂O and transferred to a conventional 11.7 T NMR spectrometer for detection at 298 K. The hyperpolarized sample was then mixed in the NMR tube waiting in the spectrometer with a solution of the target molecules under study. After mixing, the

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† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d2cc03735a>



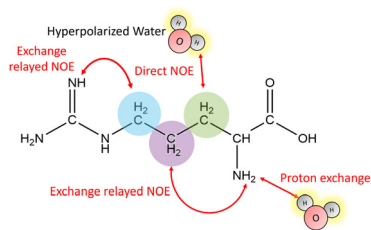


Fig. 1 Hyperpolarization transfer pathways between water and arginine: direct nuclear NOE between the solvent and the target, chemical proton exchange of labile moieties, or exchange-relayed NOE. The β -, γ and δ -hydrogens are indicated by the green, violet and blue spheres, respectively.

hyperpolarization is readily transferred from the water to the target. As a model target to study the transfer mechanisms, we chose the ubiquitous amino acid arginine due to its widespread use and its variety of labile as well as non-exchanging protons.

Fig. 1 outlines the potential polarization transfer pathways: chemical proton exchange, direct water-to-target NOE, and exchange-relayed NOE. Phenomenologically, the magnetization transfer upon dissolution in hyperpolarized water results in a transient change of the arginine ^1H NMR intensities. Fig. 2a and b show the resulting data. Detection started 2 s after mixing to allow the solution to settle before acquisition. The resonances of H_2O , and Arg- H^β , H^γ , as well as H^δ can be discerned. However, the strong water resonance covered H^α . As hyperpolarization is a non-equilibrium state, the water signal decays exponentially to its thermal equilibrium value within *ca.* 40 s (Fig. 2c). During this period, the non-equilibrium polarization is transferred to the target. We observed that for $t < 5$ s, all arginine signal intensities are reduced compared to thermal equilibrium Fig. 2b. Then, the signals rise over their equilibrium values during a time of *ca.* 40 s before reaching the thermal equilibrium. Fig. 2d and e show the negative signal enhancement computed as $1/\varepsilon$ where ε corresponds to the ratio of the signal amplitude $S(t)$ at time t and the thermal equilibrium amplitude S_{TE} . All observed arginine protons, Arg- H^β , H^γ , and H^δ do not chemically exchange with the solvent and yet show substantial signal amplitude changes during this period. Note that the water signal decay is strictly monotone, which outrules biases in signal intensities due to incomplete mixing or convection. The ESI† contain similar data for poly-aspartate, as a proof-of-concept that the reported method can be applied to other amino acids and macromolecular systems, too.

The observed behavior correlates with the well-documented^{22,23} superposition of a negative direct NOE between the solvent and the target that dominates at the beginning of the time trace and reduces the arginine signal amplitudes and the effects of chemical exchange and exchange-relayed NOE that dominates during the later stages of the trajectory and increases the signal amplitudes. The initial negative enhancements ε_i are listed in Table 1.

The differences in build-up and decay rates in Fig. 2b–e result from proton position-dependent efficiencies of hyperpolarization transfer, either *via* direct or exchange-relayed NOE.

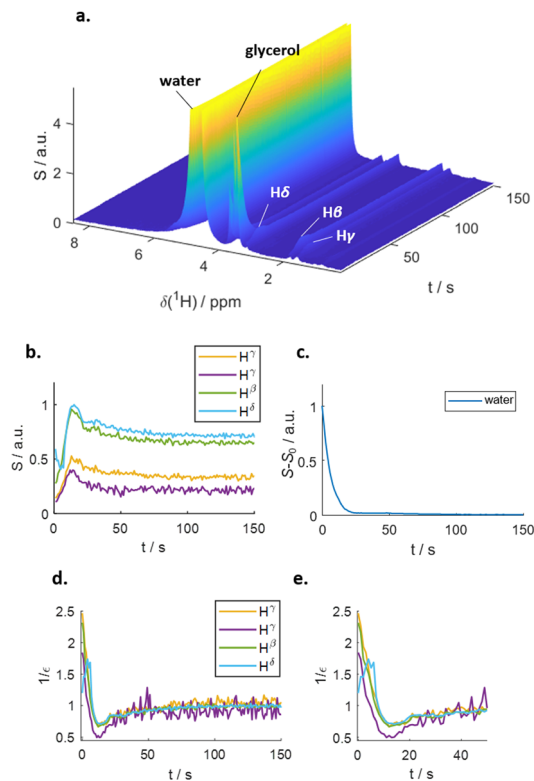


Fig. 2 (a) Time series of proton NMR spectra of arginine in hyperpolarized water. $t = 0$ corresponds to the start of the acquisition period. Next to the aliphatic protons, the water and glycerol lines are visible. (b) Intensities of the aliphatic arginine protons. An initial negative enhancement for 5 s can be discerned, prior to a positive enhancement at $t > 5$ s. (c) Intensity of the water signal. The decay is strictly monoexponential. (d) Negative signal enhancements $1/\varepsilon$ vs. time. (e) The first 50 s of panel d.

Table 1 Experimental initial signal enhancements $1/\varepsilon_i$ and computational values for the arginine hydrogens. For the coordination number and the residence time τ only water molecules closer than six Å to an arginine hydrogen were considered

Atom	$1/\varepsilon_i$ [a.u.]	σ_L [a.u.]	Distance < 6 Å	
			# H_2O	τ [ps]
H^α	—	4.98	24	8.3
H^β	2.30	4.95	24	7.6
H^γ	2.22	4.70	23	7.2
H^δ	1.24	4.55	23	6.5

To corroborate this interpretation as well as that of the negative signal enhancement at $t < 5$ s as direct NOE between solvent and target, we employed MD simulations (see ESI† for details) to calculate the direct water-arginine polarization exchange using Redfield relaxation theory.²⁴

The first 20 ns of each trajectory were discarded to avoid the inclusion of possible slow equilibration artifacts. To compute cross-relaxation rates, five hundred starting points were evenly spaced along the trajectory for each correlation function. At each starting point, the distance of the spin pairs was minimized by centering the reference amino acid spin in the



simulation box. Subsequently, the coordinates of all molecules were unfolded to undo the coordinate jumps caused by periodic boundary conditions, restoring the natural diffusive motion of the spin pairs. The resulting correlation functions were Fourier transformed using NumPy, and the cross-relaxation rates were averaged over all arginines.

For the computation of the NOE, we capitalized on the fact that the magnetization transfer between two interacting spins I and S with a nuclear spin of 1/2 (e.g. an arginine hydrogen atom and a water hydrogen atom) takes place strictly *via* a dipole-coupled mechanism. The corresponding time correlation function

$$G_{IS}(t) = \left\langle \left(\frac{1}{r_{IS}(0)^3} \frac{1}{r_{IS}(t)^3} \right) \left(\frac{3}{2} \cos^2(\theta_{IS}(t)) - \frac{1}{2} \right) \right\rangle \quad (1)$$

depends on two terms: The first is a function of the vector $\vec{r}_{IS}$ joining the two interacting spins I and S; its randomization rate is determined by the translational diffusion of these two spins towards or away from each other. Typically, this is the numerically dominant term in intermolecular NOEs.²⁵ The second term depends on the angle θ_{IS} swept by the spin-joining vector during time t . This term represents the gyration of the two spins around a common center. The spectral density $J_{IS}(\nu)$ can be obtained by real-part Fourier transformation

$$J_{IS}(\nu) = \text{Re} \left[\int_0^\infty e^{i2\pi\nu t} G_{IS}(t) dt \right] = \int_0^\infty \cos(2\pi\nu t) G_{IS}(t) dt \quad (2)$$

and yields the laboratory-frame cross relaxation rate σ_L^{NOESY} via^{26,27}

$$\sigma_L^{\text{NOESY}}(\nu) = 0.6J_{IS}(\nu_I + \nu_S) - 0.1J_{IS}(|\nu_I - \nu_S|) \quad (3)$$

as a function of the Larmor frequencies ν_I and ν_S of the interacting nuclei I and S. Choosing the field used in our experiments for detection (11.7 T corresponding to a Larmor frequency of 500 MHz for protons; see ESI† Fig. S2), we could extract the relative efficiencies of the solvent NOE for Arg-H α , H β , H γ , as well as H δ .

The spectral density function in eqn (2) is a function of the distance of the interacting species. The distance therefore also affects σ_L^{NOESY} .²⁸ The most important contributions to σ_L^{NOESY} should be covered within a distance of 6 Å.²⁸ Using this limit, we counted approximately 24 water molecules in the vicinity of the H α , H β , H γ and H δ protons. The corresponding residence times (see Table 1) correlate well with the NOE relaxation rate as shown in the radio plot in Fig. 3.

More importantly, the measured initial negative signal enhancements also correlate with the computed values (Fig. 3) corroborating our interpretation of the signal reduction in the dDNP experiments as direct solvent-to-target NOE. The H β (green dots) atoms showing the strongest initial negative enhancement $1/\epsilon_i$ lead to the highest rates σ_L . In contrast, water molecules near the arginine H δ (blue dots) led to a much reduced $1/\epsilon_i$ as accompanied by slower NOE transfer and shorter residence times. H γ (purple dots) consistently laid between H β and H δ for all three parameters.

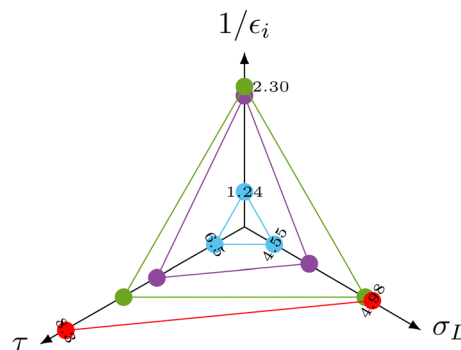


Fig. 3 Radio plot of the correlation between the experimental negative signal enhancement immediately after start of the detection $1/\epsilon_i$, the NOE relaxation rate σ_L and the residence time τ of the interaction water molecules. H α (red) was masked in the experiments by the broadened water line.

However, the correlation of computed and experimental data suffers from the drawback that chemical exchange is neglected in our simulations. Hence, we further complemented our conclusions with conventional NOESY (Nuclear Overhauser Enhancement Spectroscopy). We found that both, direct NOE between the water and the different arginine protons as well as exchange processes, are effectively transferring nuclear spin polarization (Fig. 4; see the ESI† for other mixing times). For all mixing times, negative cross-peaks (relative to negative diagonal peaks) between the labile H N and H n resonances of arginine and the water indicate chemical proton exchange. In addition, we observed cross-peaks between the water and H β , H γ , as well as H δ indicating direct NOE between arginine and solvent. Intramolecular NOE between all involved arginine protons could be observed, as expected (Fig. 4).

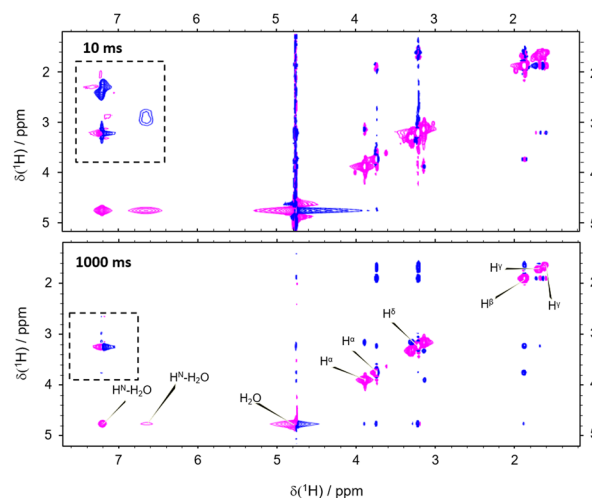


Fig. 4 Thermal equilibrium NOESY with a mixing time of 10 ms (top) and 1 s (bottom). Positive cross-peaks between the water and the non-exchangeable protons evidence intermolecular NOE. Cross-peaks between the water and the labile protons indicate proton exchange. The dashed box highlights magnetization transfer between exchanging and aliphatic protons, i.e., exchange-relayed NOE.



Importantly, the NOESY show the simultaneous action of chemical proton exchange and intramolecular NOE leading to exchange-relayed NOE. This is crucial for understanding the hyperpolarization transfer. Since the solvent NOE features an opposed sign relative to the diagonal peaks, an exchange-relayed NOE is necessarily responsible for the positive signal enhancements of the non-exchangeable protons at $t > 5$ s. The observation of cross-peaks between the signals of the labile H^N protons and H^β , H^γ , as well as H^δ confirms the flow of magnetization from exchanging to non-exchanging moieties (Fig. 4 dashed rectangle).

Summarizing, all three pathways (direct solvent NOE, chemical exchange, as well as exchange relayed NOE) depicted in Fig. 1 can transfer hyperpolarization from the water to a target molecule to boost NMR spectra of biomolecules in a hyperpolarized buffer. For arginine at $B_0 = 11.7$ T, we find negative signal enhancements for all non-labile protons by direct NOE for $t < 5$ s after dissolution in hyperpolarized water and positive enhancements for $t > 5$ s by exchange-relayed NOE. As water is a ubiquitous solvent, the reported mechanisms can be readily extended and exploited for other target molecules. The combination of dDNP and computational approaches can thereby be a powerful asset to enlighten both, structural as well as spin dynamics of hyperpolarized solutions. It should be noted that in experiments that detect vividly exchanging protons, as typical for 1H - ^{15}N cross-peaks or ^{15}N -edited 1H signals of peptides, nucleic acids, and proteins, the contribution of the negative, direct solvent NOE is canceled by the much more effective proton exchange pathway reaching enhancements of $\epsilon > 100$. However, for non-labile protons, this effect cannot be neglected and competes with the exchange-relayed pathway. The importance of exchange-relayed effects is further supported by earlier work⁶ that indicated that backbone 1H - ^{15}N signal enhancements are strongest for residues with protic side chains. This observation can readily be explained with exchange-relayed NOE between the side chain and backbone protons.

All raw data are available under <https://doi.org/10.5281/zenodo.7113098>.

LME, KC and FK performed the experiments. PH developed the software to analyze the MD trajectories. LME, PH and FJ performed simulations. LME, CS and DK analyzed data and conceived the study.

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Conflicts of interest

There are no conflicts to declare.

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Chapter 6

Formation and evolution of nanoscale calcium phosphate precursors under biomimetic conditions

Manuscript 4

Ludovica Martina Epasto, Tristan Georges, Albina Selimović, Jean-Michel Guigner, Thierry Azaïs, Dennis Kurzbach

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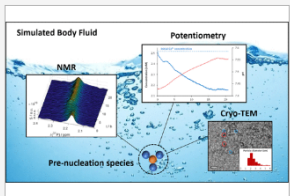
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SUBJECTS: Anatomy, Cluster chemistry, Crystallization, Ions, Phosphates

Abstract

Simulated body fluids (SBFs) that mimic human blood plasma are widely used media for *in vitro* studies in an extensive array of research fields, from biomineralization to surface and corrosion sciences. We show that these solutions undergo dynamic nanoscopic conformational rearrangements on the timescale of minutes to hours, even though they are commonly considered stable or metastable. In particular, we find and characterize nanoscale inhomogeneities made of calcium phosphate (CaP) aggregates that emerge from homogeneous SBFs within a few hours and evolve into prenucleation species (PNS) that act as precursors in CaP crystallization processes. These ionic clusters consist of ~2 nm large spherical building units that can aggregate into superstructures with sizes of over 200 nm. We show that the residence times of phosphate ions in the PNS depend critically on the total PNS surface. These findings are particularly relevant for understanding nonclassical crystallization phenomena, in which PNS are assumed to act as building blocks for the final crystal structure.

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Statement of contribution: I published, together with my coworkers, this work in *Analytical Chemistry* in 2021, according to the DOI: 10.1021/acs.analchem.1c01561. I performed all the NMR experiments and the simulations presented in the paper. Additionally, I conceived the study and wrote the paper under the supervision of Assoc. Prof. Dr. Dennis Kurzbach with the equal contribution of Tristan Georges.

Formation and Evolution of Nanoscale Calcium Phosphate Precursors under Biomimetic Conditions

Ludovica M. Epasto,^{||} Tristan Georges,^{||} Albina Selimović, Jean-Michel Guigner, Thierry Azaïs,^{*} and Dennis Kurzbauch^{*}



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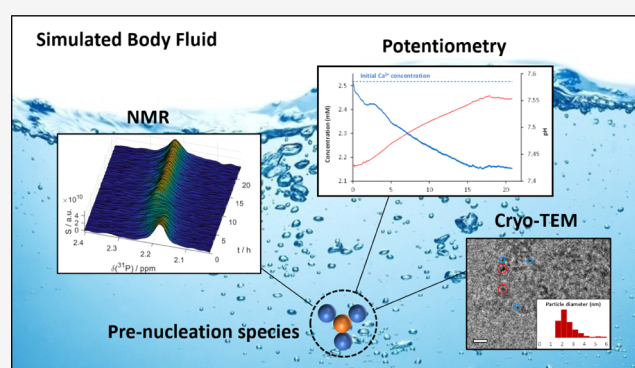


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ABSTRACT: Simulated body fluids (SBFs) that mimic human blood plasma are widely used media for *in vitro* studies in an extensive array of research fields, from biomineralization to surface and corrosion sciences. We show that these solutions undergo dynamic nanoscopic conformational rearrangements on the timescale of minutes to hours, even though they are commonly considered stable or metastable. In particular, we find and characterize nanoscale inhomogeneities made of calcium phosphate (CaP) aggregates that emerge from homogeneous SBFs within a few hours and evolve into prenucleation species (PNS) that act as precursors in CaP crystallization processes. These ionic clusters consist of ~2 nm large spherical building units that can aggregate into suprastructures with sizes of over 200 nm. We show that the residence times of phosphate ions in the PNS depend critically on the total PNS surface. These findings are particularly relevant for understanding nonclassical crystallization phenomena, in which PNS are assumed to act as building blocks for the final crystal structure.



INTRODUCTION

Biomineralization is defined as the ability of living organisms to produce mineral phases embedded mostly in calcified tissues such as bone and teeth of vertebrates.^{1–3} Beyond its fundamental aspect, its understanding is also of imminent interest for developing nature-inspired materials with tailored properties such as improved bone implants⁴ or functional materials.^{5,6} In this context, simulated body fluids (SBFs) that mimic the ionic composition of human blood plasma^{7,8} are ubiquitous and used in a wide array of research fields, from the design of bone graft materials^{9,10} and tissue engineering^{11,12} to corrosion and biodegradation studies^{13–15} to bioinspired material design.^{16,17} In particular, SBF plays a crucial role as it is the most widespread biomimetic medium used to assess the bioactivity of materials.^{18–20} Notwithstanding its frequent use, the “nanostructure” of SBF remains unsolved to large degrees. A deeper understanding of the structural dynamics is yet essential due to the influence of solvent properties on mineralization and crystallization phenomena.^{21,22} Indeed, environmental conditions often determine the morphology of solid phases precipitating from solution.

In this regard, SBFs have gained particular attention as a result of the recent observation of so-called prenucleation species (PNS) and their involvement in the early onset of biomimetic calcium phosphate (CaP) precipitation (e.g., hydroxyapatite).^{23,24} These PNS are highly dynamic nano-

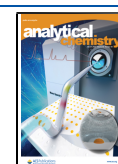
metric ionic clusters that spontaneously form in solution preceding the precipitation of solid crystalline phases.²⁵ Today, PNS have been documented for CaP^{21,23,26–29} and carbonates,^{30–33} as well as for iron oxides.³⁴ Interestingly, their involvement in the formation of biominerals challenges the long-standing nucleation-and-growth paradigm,^{35,36} which has stimulated an ongoing update of the existing crystallization theories.^{26,37,38}

In addition, it has been suggested that the PNS structure and dynamics determine the crystallization path a material takes.²² Hence, a better understanding of PNS behavior in SBFs promises new avenues for the rational design of solid biomimetic ceramics via control over the initial reaction conditions that regulate the molecular architecture of the final material. However, the structure and dynamic behavior of PNS (in particular in SBF) remain poorly understood, and the factors that control the associated nucleation events remain unclear, leaving significant gaps in the current understanding of crystallization.^{39,40}

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Indeed, the highly dynamic behavior of SBFs and its ionic constituents is challenging to characterize experimentally from both, structural and dynamical, viewpoints. To overcome this bottleneck, we here present an integrative approach, including the first observation of PNS by real-time nuclear magnetic resonance (NMR) spectroscopy in solution combined with cryoelectron microscopy and calcium potentiometric measurements. We shed light on the constitution of SBFs at the nanoscale, revealing dynamical and structural inhomogeneities therein and thus providing a significant advancement in their understanding.

In particular, we show how SBF undergoes defined and irreversible molecular rearrangements with time, challenging the long-standing notion of its (meta)stability.^{23,41,42} We find that nanometric CaP clusters with diameters of ca. 2 nm form within 5 h after preparing the fresh SBF. We identify these clusters as previously described CaP-based PNS.²³ Subsequently, these PNS can aggregate to form soluble suprastructures of several hundreds of nanometers in size that might precede the precipitation of solid CaP. We find calcium triphosphate units to be the basic units of the PNS. Furthermore, we characterize the exchange kinetics of phosphates between PNS-bound and free states, demonstrating that the frequency of exchange depends critically on the quantity of PNS in solution.

■ EXPERIMENTAL SECTION

Sample Preparation. Modified SBFs (mSBFs) were prepared following the protocol by Oyane et al.^{43,44} In brief, neat water was stirred at 37 °C, and the salts constituting mSBF were added successively to achieve concentrations comparable to those found in human blood plasma and in particular, a P_i concentration of 1 mM. After the addition of all the salts, the pH was adjusted to 7.4 using 1.0 M NaOH. After preparation, the mSBF was immediately transferred to an NMR tube, and 10% v/v D_2O was added as the lock solvent.

NMR Spectroscopy. All NMR data were acquired at 37 °C on a Bruker NEO NMR spectrometer operating at 11.7 T (500 MHz proton Larmor frequency) equipped with a 5 mm Bruker Prodigy BBO cryoprobe. For real-time detection, 1D ^{31}P NMR spectra were acquired every 7.5 min by averaging 64 flame-ionization detectors (FIDs). For detection, 90° flip-angle pulses were applied with a length of 12 μ s every 5 s. Prior to Fourier transformation, all data were zero filled and apodized using a Gaussian window function. Subsequent to Fourier transformation, all data were baseline-corrected. To extract signal intensities, the NMR signals were fitted to two Lorentzian functions using home-written scripts embedded in the MATLAB software package using the “fitNlorentzian” function.

For diffusion ordered spectroscopy (DOSY), the spectra were acquired using the *dsteppgp3s* pulse sequence⁴⁵ embedded in the Bruker TopSpin 4 pulse sequence library. The spectra were acquired over 5 h 15 by averaging 150 FIDs. For detection, 90° flip-angle pulses were applied with a length of 12 μ s every 10 s. A six-step *z*-gradient ramp was used with 2, 14, 25, 37, 48, and 60% of the maximum gradient strength.

Data were analyzed using the GNAT software package⁴⁶ for MATLAB. Prior to Fourier transformation, all data were zero-filled and apodized using a Lorentzian window function. Subsequent to Fourier transformation, all data were baseline-corrected. Diffusion coefficients were extracted using mono-exponential fits to the Stejskal–Tanner equation.⁴⁷

Calcium Ion Potentiometry. Calcium concentration and pH were monitored using a calcium-sensitive electrode (Ca-ISE, Metrohm) and a pH glass electrode (Unitrode, Metrohm) connected to a Titrando titration device (Metrohm) and analyzed with Tiamo software (Metrohm). The Ca-ISE was calibrated at room temperature using three modified SBF-like solutions with respective calcium concentrations of 0.1, 1, and 3 mM. These m-SBF-like solutions were synthesized by following the procedure of Oyane et al.^{43,44} omitting phosphate salts. For each solution, the potential (in millivolts) was measured and automatically fitted to the Nernst equation with a least-square algorithm. The pH electrode was calibrated using three buffered solutions at pH 4, pH 7, and pH 9 (Metrohm). Sample evolution was monitored for 24 h recording potential every 10 s under gentle stirring.

Cryoelectron Microscopy and XEDS. m-SBF solutions were vitrified at specific time points after preparation (1, 5.5, and 23 h). The morphology and the size of nanoparticles were determined from cryotransmission electron microscopy (cryo-TEM) images. A drop (3 μ L) of solution was deposited on “quantifoil” (Quantifoil Micro Tools GmbH, Germany) carbon membrane grids. The excess of the liquid on the grid was absorbed with a filter paper, and the grid was quenched quickly in liquid ethane to form a thin vitreous ice film. The samples were transferred in the microscope and observed at low temperature (−180 °C). Cryo-TEM images were recorded on ultrascan 1000, 2k × 2k CCD camera (Gatan, USA), using a LaB₆ JEOL JEM2100 (JEOL, Japan) cryomicroscope operating at 200 kV with a JEOL low-dose system (Minimum Dose System, MDS) to protect the thin ice film from any irradiation before imaging and reduce the irradiation during the image capture.

X-ray energy-dispersive spectra (XEDS) characterizing the elemental composition of the samples have been recorded with a JEOL (Japan) XEDS detector with 140 eV resolution using a JEOL (Japan) 2100F, field-emission gun instrument operating at 200 kV under cryo-conditions. Images were recorded on an UltraScan 4000 Gatan (USA) camera with a 4096 × 4096 charge-coupled device.

■ RESULTS AND DISCUSSION

In the following, we will first describe the formation and internal dynamics of PNS in SBFs by employing real-time NMR. Second, we describe the PNS constitution by means of transmission electron microscopy (TEM) and potentiometric experiments.

We want to stress that we herein collectively term all CaP aggregates that occur in solution during the early onset of CaP precipitation, for the sake of generality, prenucleation species or “PNS”. This notion should here be understood as neutral, that is, as not referring to any nucleation or crystallization theory.^{26,37–39}

Real-Time NMR of the Evolving SBF. We initially observed the formation and evolution of PNS by real-time NMR of the modified SBF^{43,44} (mSBF which provides improved pH stability over neat SBF). Capitalizing on the strong dependence of the chemical shift of phosphate ions on their local environment, these experiments could distinguish the signals of phosphates bound in a PNS from free phosphates in solution. They revealed that PNS form within 5 h after preparing fresh mSBF and undergo continuous changes throughout a 15 h period.

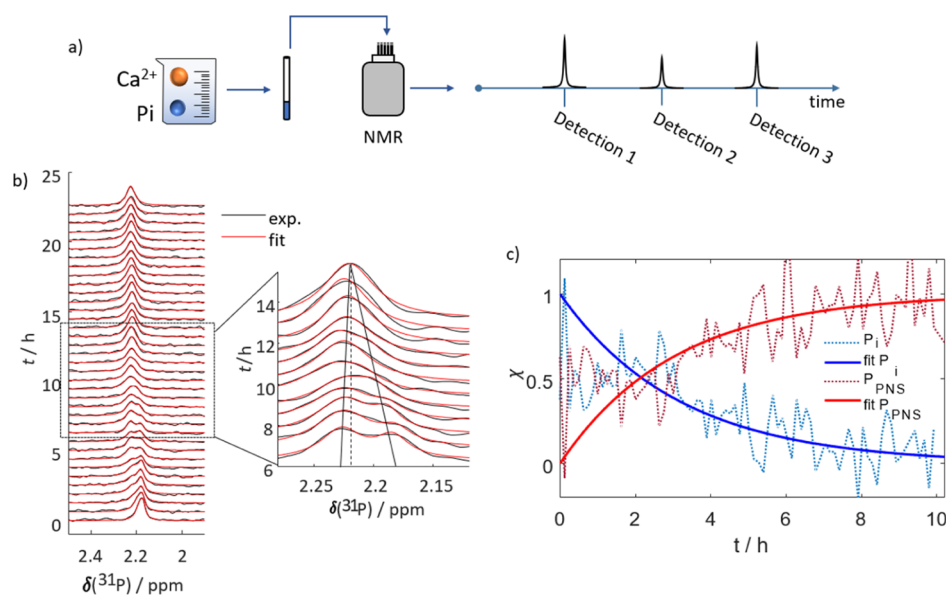


Figure 1. Monitoring of PNS formation in mSBF through real-time NMR. (a) Scheme of experimental workflow. The evolution of PNS at 37 °C in mSBF is traced by consecutive detections of ^{31}P spectra. (b) ^{31}P NMR signals detected for mSBF over a period of 24 h (black) and corresponding fits to two Lorentzian functions (red). Initially, only a single resonance can be observed. After ~ 5 h, a second peak appears. Between 8 and 10 h, the first and second peaks merge, forming a single signal. The insert highlights the transition period during which the two signals merge. The resulting single line resonates at a frequency between those of the precursor signals. At $t > 15$ h, again, only a single line is visible. Note that the spectra are normalized. (c) Time-dependence of the fractional contribution χ of the two lines to the overall spectrum. The contribution of free phosphate is shown in blue and that of PNS-bound phosphate in red. The dotted lines indicate the experimental data, while the solid lines represent fits of the experimental data to monoexponential functions. After 10 h, the signals merged and the two contributions could not be separated anymore.

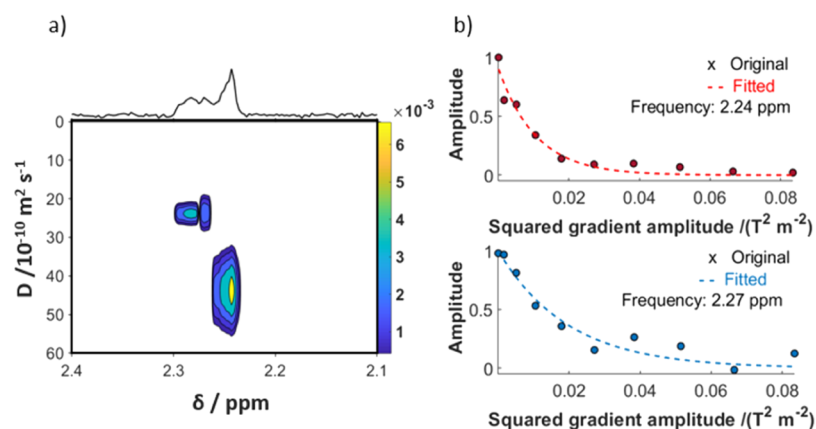


Figure 2. DOSY analysis of mSBF. (a) Mapped diffusion coefficients in dependence of the chemical shift. When both the P_i and the P_{PNS} signals, appear well-separated in the ^{31}P NMR spectra, DOSY of the system allows one to discern two phosphate species via their diffusion coefficients. A slower diffusion coefficient is observed for the species underlying P_{PNS} (left) compared to the signal underlying P_i (right). The diffusion coefficients were $\sim 24 \times 10^{-10}$ vs $\sim 43 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$, respectively. The data were acquired at 37 °C for 5 h at a phosphate concentration of 5 mM. (b) Monoexponential fits of the two DOSY profiles underlying the data in panel (a). The P_{PNS} signal decays slower with increasing gradient amplitude than the P_i signal.

We recorded a series of ^{31}P NMR spectra, as outlined in Figure 1a, starting immediately after the sample preparation. Figure 1b shows the resulting spectra (black lines) and superimposed fits (red lines) to two Lorentzian functions that model the NMR line shape (see the Experimental Section for details). At $t = 0$, a single resonance at 2.18 ppm is detectable, corresponding to free inorganic phosphate (P_i) dissolved in mSBF similar in position and linewidth to the control experiment (Figure S1). After 3–5 h, a second broader peak appears at 2.23 ppm. We assign this signal to emerging PNS (*vide infra*) and denote the involved phosphates henceforth as P_{PNS} . During this period, our fitting routine can quantitatively

reproduce the spectra with only two lines. From 8 to 10 h after preparation, both signals are broadened compared to their initial line width and slowly approach each other.

After 10 h, the signals merge, and only a single line remains at a chemical shift of 2.21 ppm (insert Figure 1b). Notably, the total signal intensity is constant throughout the detection period (up to 30 h), indicating that CaP does not precipitate from mSBF under our conditions ($T = 37$ °C; $\text{pH} = 7.4$) throughout the experiment.

Figure 1c shows the relative fractions χ of the two spectral components of P_i and P_{PNS} of the spectra during the initial

period of evolution. The figure highlights how P_i is consumed to form P_{PNS} .

Diffusion-Ordered Spectroscopy Provides Evidence of PNS. To characterize the two different phosphate species further, we employed DOSY. It showed that the hydrodynamic radius of the PNS is ca. 1.7-fold larger than that of free P_i .

Due to the low signal intensities, these experiments had to be acquired at a P_i concentration of 5 mM (a fivefold increase compared to neat mSBF). However, the results can be safely qualitatively interpreted as, similar to mSBF, two distinct resonances appear after 10 to 15 h of maturation (Figure S2). Figure 2 displays the DOSY analysis of modified mSBF acquired over a 5 h period (10–15 h after sample preparation). Notably, two distinct diffusion coefficients are evidenced. A coefficient of $D = \sim 24 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ could be observed for the single P_{PNS} peak. The P_i signal yielded to a faster coefficient of $\sim 42 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$. Solving the Stokes–Einstein relation assuming spherical particles, we find that the average hydrodynamic radius R_h of P_{PNS} is ~ 1.7 -fold larger than that of P_i , amounting to ~ 1.0 and ~ 0.6 nm, respectively.

Therefore, the volume of the detected particles is ca. fourfold larger than that of free P_i . Hence, we deduce that the second signal appearing in the ^{31}P spectra in Figure 1 indeed stems from phosphate units embedded in larger ionic clusters that, despite their size, remain in solution, that is, in a PNS.

Theoretical Modeling of the Phosphate Exchange. To better understand the R_h variations and the real-time NMR data, we theoretically modeled our data assuming two-site chemical exchange. We found that the mSBF system passes from a slow to an intermediate to a fast exchange regime with growing PNS concentration. This circumstance causes the apparent spectral changes in the NMR time series.

To form PNS, it is necessary that the phosphates exchanges between the pool of free P_i in solution and the pool of newly bound species P_{PNS} . This process influences the shape of the NMR spectra in Figures 3a (similar to 1b). The time series starts with a single P_i signal, followed by the emergence of a well-distinguishable P_{PNS} signal. Later, both signals first broaden and then merge into a single, again sharp, resonance. This behavior is typical for exchange-driven spectral coalescence.⁴⁸ Figure 3c illustrates how both signals are broadened and reduced in amplitude during the period of intermediate exchange.

We could model this behavior using the Norris equation⁴⁹ for two-site exchange, which can be considered as a simplified version of the Carver–Richards equation⁵⁰

$$\rho_T = \left[-iC \sum_l F_l \right] \left[1 + \left(\frac{1}{\tau} \right) \sum_l F_l \right]^{-1} \quad (1a)$$

$$F_l = n_l \left[i(\omega - \omega_k) - \frac{1}{(T_2)_l} - \frac{1}{\tau} \right]^{-1} \quad (1b)$$

ρ_T is the resulting spectrum, τ stands for the average inverse exchange rate k_{ex} and n_l is the fractional population of the l th site. ω is the resonance frequency and $1/(T_2)_l$ is the line width of the l th species in the absence of exchange. C is an overall proportionality constant assumed here to be 1.

The exchange rate constant $1/\tau$ was modeled by combining the classical collision theory Arrhenius law with a first-order kinetic equation

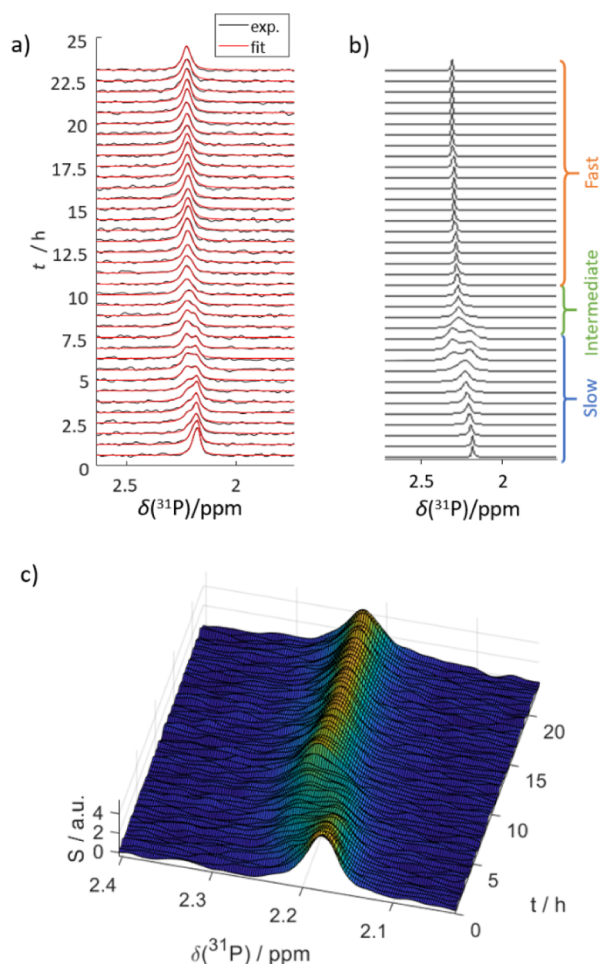


Figure 3. Comparison of (a) experimental results of mSBF evolution at 37 °C with (b) simulated data. The prediction was performed with a dynamic two-site exchange model, as described in the main text. In both, the second species appears at ca. 3–5 h, following the exchange path of the NMR-detected peak. The brackets indicate the different exchange regimes. (c) Non-normalized surface plot of the ^{31}P NMR signal versus time. The representation illustrates that the signals are broadened and reduced in amplitude during the transition from slow to fast exchange.

$$Z = n_{P_i} n_{PNS} R_{h,PNS} \sqrt{\frac{8k_b T}{\pi m_{red}}} \quad (2a)$$

$$1/\tau = Z \exp\left(-\frac{E_A}{RT}\right) \quad (2b)$$

$$\frac{\partial[n_{PNS}]}{\partial t} = -k[n_{P_i}] \quad (3)$$

where n_{P_i} and n_{PNS} indicate the concentrations of free and PNS-bound phosphates, respectively. $R_{h,PNS}$ is the estimated radius of hydration of a PNS (assumed to be 1 nm, in line with the DOSY experiments). k_b is the Boltzmann constant, T is the temperature of the system, and m_{red} is the reduced PNS mass. E_A is the activation energy, and R is the gas constant. In eq 3, k is the kinetic constant of the conversion $P_i \rightarrow P_{PNS}$.

Combining eqs 1a–2a, we could reproduce the experimental data, as shown in Figure 3a,b. At this stage, the following understanding emerges of the exchange processes in mSBF:

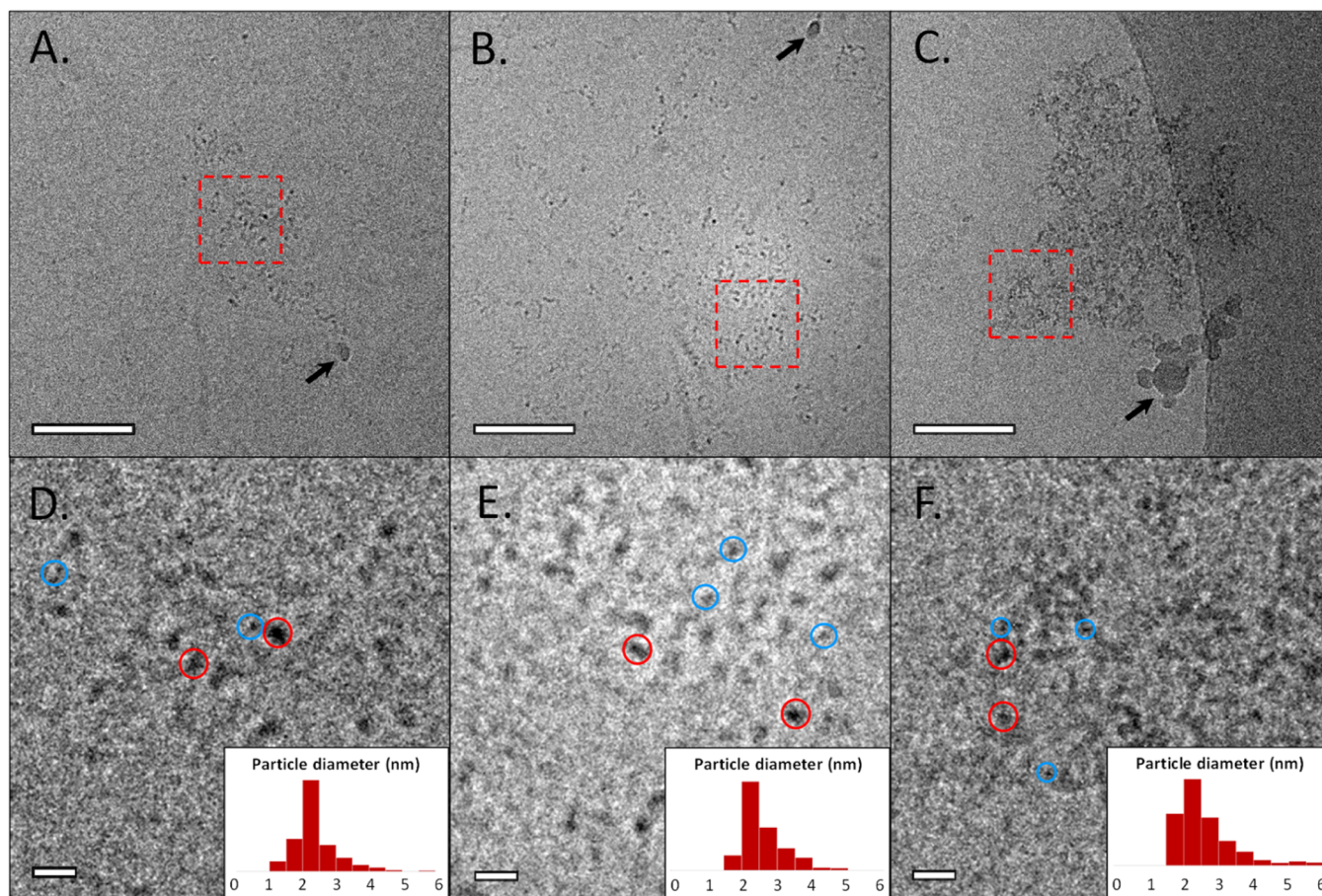


Figure 4. Cryo-TEM observations of mSBF solution at different time points—(A) 1 h, (B) 5.5 h, and (C) 23 h after mSBF preparation, respectively. (D–F) Zoom of the corresponding zones delimited by the red squares in (A–C). Insert displays size evaluation of individual PNS. Blue and red circles give an example of smaller and bigger PNS, respectively. The black arrows indicate the presence of polluting agents, due to the freezing process. Scale bar (A–C) = 50 nm. Scale bar (D–F) = 10 nm.

Initially, aggregation of P_i and Ca^{2+} ions leads to CaP-PNS formation and the emergence of a corresponding second signal. At this time, the system is in slow exchange as the number of PNS is still small, compared to free P_i , such that only a small “exchange surface” is available and a small number of encounters (cf. eqs 2a and 2b; the prefactor n_{PNS} is small, leading to slow exchange rates $1/\tau$). After 5 h, the two peaks broaden as the system undergoes a transition into an intermediate exchange regime, where $\Delta\delta(^{31}P) = 1/\tau = k_{ex}$. In other words, the exchange surface grows together with the number of PNS, n_{PNS} . Finally, the two peaks merge after ca. 15 h, with a chemical shift averaged between the two initial lines (cf. Figure 1b). For this case, the model calculations indicate that the phosphates experience a fast exchange between both states, that is, $\Delta\delta(^{31}P) < 1/\tau$.

The real-time NMR data, diffusion measurements, and theoretical predictions thus suggest a process in which (i) PNS forms in mSBF spontaneously after its preparation and continues to emerge over a time of >15 h, (ii) the diameter of the PNS detected by solution-state NMR is on the order of 2 nm, and (iii) the exchange between freely dissolved and PNS-bound phosphates accelerates when the concentration of PNS grows.

During the slow exchange phase, the chemical shift difference between the two species amounts to 0.05 ppm, which corresponds to an exchange rate of $k_{ex} < 10$ Hz.

Interestingly, earlier studies on CaP precipitation (dicalcium phosphate dehydrate)⁵¹ under nonbiomimetic conditions (pH 8 and ion concentrations >10 mM) reported much higher limits of $k_{ex} < 250$ to 350 Hz, highlighting the importance of environmental conditions for crystallization studies.

Cryo-TEM Analysis Reveals PNS Suprastructures. To further analyze the PNS structure, we performed cryo-TEM observations. Data were acquired 1, 5.5, and 23 h after sample preparation, following the exchange regimes derived by NMR (Figures 4 and S3). After 1 h, we observed the appearance of the first PNS (Figure 4a). The number of PNS visibly increased after 5.5 h (Figure 4b,c), supporting the time-dependent emergence of PNS in the mSBF. After 23 h of maturation, some PNS are found to aggregate into higher-order structures of several hundreds of nanometers including typically ~100 to ~400 PNS (Figure 4c). In addition, free PNS were also observed to coexist in solution (Figure S3).

Higher magnification allows the evaluation of PNS size and distribution (Figure 4d–f). After 1 and 5.5 h, individual PNS with sizes ranging from 1.5 to 3.5 nm were observed in good agreement with the DOSY NMR data. After 23 h, PNS aggregates were found constituted by individual spherical units again with diameters of 1.5–3.5 nm. In addition, bigger units of 4–6 nm in size were found to coexist at this time of maturation (red circles vs blue circles in Figure 4d–f).

The observed aggregates and clusters remain in solution in the mSBF for an extended period before CaP precipitation takes place. Hence, these structures likely define the starting point of the pathway CaP takes toward its solid crystalline state.^{23,24,27}

Note that solution-state NMR is limited to aggregates of a few nanometers due to line-broadening effects for bigger molecular assemblies. It is very likely that the detected P_{PNS} resonance through ^{31}P NMR species do not reflect all PNS observed by cryo-TEM. Larger species might not be detected. The determined R_h of ~ 1 nm through DOSY NMR corroborates this assumption.

EDXS Analysis Reveals Phosphate-Rich PNS. We employed energy-dispersive X-ray spectroscopy (EDXS) of mSBF 23 h after preparation to determine the PNS composition after complete equilibration. In the electron micrograph, we selected a region of $\sim 1 \mu\text{m}^2$ containing a large PNS aggregate (Figure 5a) and another region devoid of any PNS (Figure 5b) as a negative control.

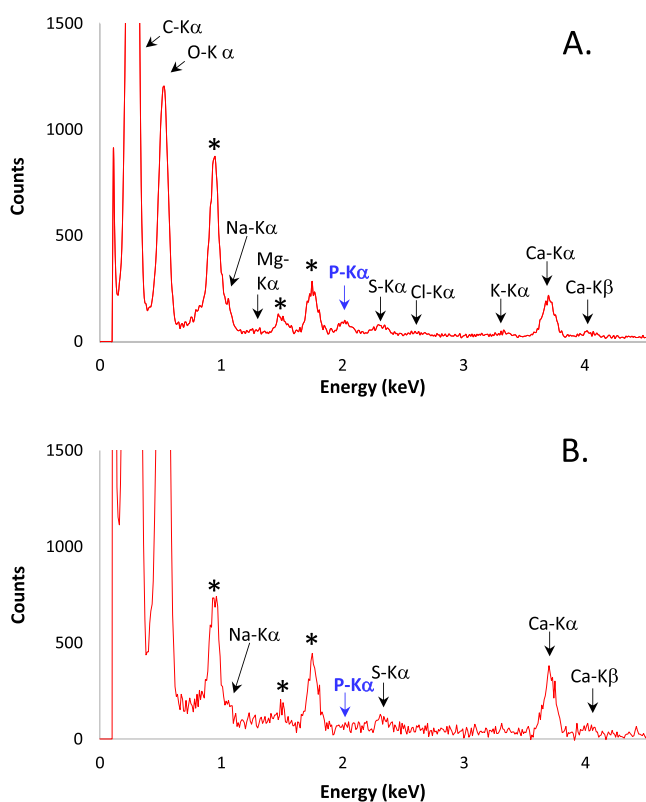


Figure 5. EDXS analysis of the mSBF performed 23 h after preparation. (a) Selected zone contains a large amount of PNS. (b) Selected zone does not contain a significant amount of PNS (Figure S4). The electron beam area is $\sim 1 \mu\text{m}^2$. Black stars at 0.93, 1.49, and 1.74 keV indicate Cu-L α , Al-K α , and Si-K α peaks, respectively, corresponding to the grid, its plasma treatment, or pollution.

The Ca K α and Ca K β peaks are both visible in the absence and presence of PNS. In opposition, the P K α peak is only visible in the presence of PNS, indicating the abundance of phosphorous atoms within the ionic clusters. Interestingly, S K α and Na L α signals (markers of sulfate and Na^+), of comparable intensity to the P K α peak, are present in both cases (Figure 5a,b).

Thus, the EDXS results suggest that the mSBF, once equilibrated, is depleted in free P_i , while most phosphate ions are localized within the PNS units. These data are consistent with the ^{31}P NMR data, in which the detected fraction of free P_i is very small at $t > 15$ h.

Ca^{2+} Potentiometry Reveals the PNS Ca/P Ratio. To further corroborate the NMR-derived kinetics and determine the protonation state of the phosphate units, we potentiometrically determined the calcium ion concentration over a period of 21 h following mSBF preparation.

Figure 6 displays the time dependence of the free Ca^{2+} ion concentration throughout our experiments. After mSBF

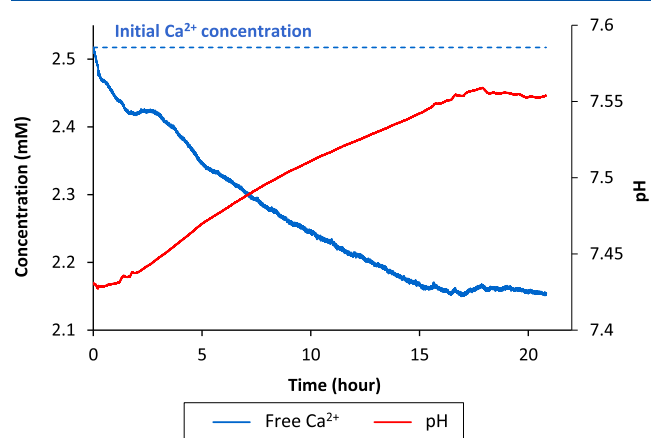


Figure 6. Evolution of free Ca^{2+} concentration (blue curve) and pH (red curve) of mSBF over time. Concentration curves were temperature-corrected to mitigate biases in apparent concentrations.

preparation, the concentration continuously decreases until it reaches a steady state at $t > 16$ h. This decrease results from the uptake of Ca^{2+} ions by the PNS (in line with the NMR and TEM data). The proportion of bound calcium ions after ca. 16 h amounts to 14% of the initial Ca^{2+} concentration, that is, the free ion concentration drops from 2.5 to 2.15 mM (Figure 6). In return, the amount of bound Ca^{2+} is ca. 0.35 mM (Figure S5). Moreover, from the NMR experiments and EDXS analysis, we can estimate the amount of P_{PNS} after 21 h to ca. 1 mM considering that the majority of phosphate ions are embedded in PNS. Thus, we determine that one Ca^{2+} ion interacts with 2.8 (~ 3) P_i , forming PNS as “calcium triphosphate units”.

Interestingly, we also observed a small pH variation strongly correlated with the free Ca^{2+} species (Figure 6). The pH increases from 7.42 to 7.56 ($\Delta\text{pH} = 0.14$), reaching a plateau after 16 h similar to the concentration of free Ca^{2+} .

This observation is important as the pH influences the phosphate speciation. The pH values suggest that PNS tend to accommodate both HPO_4^{2-} and H_2PO_4^- ions but with a higher proportion of the latter ($[\text{H}_2\text{PO}_4^-]/[\text{HPO}_4^{2-}] = 0.36$) (Figure S5).

Considering relative contributions of bound H_2PO_4^- and HPO_4^{2-} of 36% and 64%, respectively, the PNS basic unit amounts to average nominal composition of $[\text{Ca}(\text{H}_2\text{PO}_4)_{1.04}(\text{HPO}_4)_{1.76}]_{n}^{2.56-}$ (not considering coordinated water molecules), very similar to the calcium triphosphate units proposed by Habraken et al.²⁷

CONCLUSIONS

Our data show that the mSBF is a highly dynamic system that undergoes a continuous transformation after its preparation. It can be considered as stable or metastable only after an extended equilibration period of >24 h. Priorly, the dynamic emergence of CaP PNS continuously changes the nanoscopic constitution of the solution.

Figure 7 summarizes the picture we developed of PNS in the mSBF from individual ions to small clusters to larger

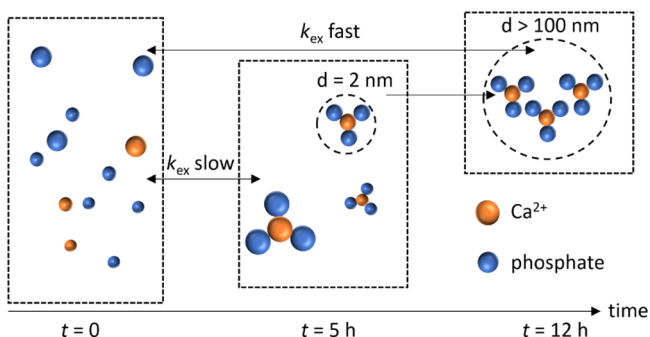


Figure 7. Scheme summarizing the PNS evolution in mSBF. After preparation, the ions are freely dispersed in solution. After 5 h, they aggregate to form CaP species with a diameter of ca. 2–3 nm. The exchange rate of phosphates between free and bound states is slow (on the NMR timescale) at this stage. After 12 h, the CaP clusters can aggregate to form suprastructures of more than 100 nm in size, where individual PNS are still present. The phosphate exchange is fast at this stage. The PNS are constituted by three phosphate units per Ca^{2+} ion.

suprastructures. Initially, PNS solute species of ~ 2 nm in diameter form right after mSBF preparation and experience accelerating phosphate exchange with maturation time. After equilibration, mSBF is found to be depleted of free phosphate as PNS form with an estimated Ca/P molar ratio of 1/3.

Given its widespread use, the understanding of SBF and PNS dispersed therein might be useful in a wide array of research fields. Among other examples, the investigation of nonclassical crystallization pathways in the field of biomineralization or the development of novel bone-graft replacements relies heavily on SBF use.

The combination of NMR, Cryo-TEM, and Ca^{2+} titration thereby provides the possibility of tracing the transformation of P_i into P_{PNS} in real time and quantify the size and composition of PNS. Therefore, the presented integrative methodology might open new avenues for spatiotemporally resolved data sets that enable characterization of nanoscale inhomogeneities in solution and associated crystallization events at an improved level of detail.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.analchem.1c01561>.

Reference NMR experiments, supplementary cryo-TEM images, and supplementary potentiometry experiments (PDF)

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Notes

The authors declare no competing financial interest.

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General conclusions and outlook

After the structural determination of ribonuclease A in 1957 by Saunders and coworkers¹, a plethora of biomacromolecules, such as peptides, proteins, and nucleic acids, were structurally characterized by NMR. However, despite its potential, NMR has limited access to low-concentrated, high molecular-weight substrates, and short-lived conformational intermediates.

Sample concentration is a central factor affecting the application range of NMR. To obtain intense NMR signals, employing a protein sample with a concentration of hundreds of micromolar is a primary requirement. However, high protein concentrations are incompatible with physiological conditions, which are often impossible to reproduce and analyze with good sensitivity by NMR. Indeed, naturally occurring protein concentrations are usually estimated to reach even nanomolar concentrations.²⁻⁴

The size of the substrate of interest also limits the application of conventional NMR. Molecules of hundreds of kilodaltons are generally hardly accessible by conventional NMR. Macromolecules with a high molecular weight are characterized by slow tumbling, leading to a fast transverse relaxation; therefore, they produce NMR spectra with low-intensity signals and broad lines. In 2003, Key and coworkers proposed the methyl-TROSY experiment to study bulkier molecular complexes up to 1 MDa and high molecular weight DNA.⁵⁻⁶ However, to apply this technique to the substrate of interest, it is necessary to have a high degree of deuteration and ¹³CH₃-labelled methyl groups.⁷ Adopting a complex labeling scheme represents a limitation of the method's applicability since it could be unsuitable for some protein substrates.

Another challenge for conventional NMR is detecting highly dynamic proteins since they often feature transient conformational states. While conventional NMR can easily detect the protein ground state, the folding intermediates are hardly accessible due to their short lifetime. To overcome this problem, real-time NMR monitoring can be employed to access temporary conformations. Brutscher and coworkers proposed an example of real-time NMR for transient protein folding elucidation, monitoring the transient conformation of the amyloidogenic protein β -2 microglobulin by HNCO experiments.⁸ However, the low SNR due

to the short life of the conformational intermediate limits the possibility of employing real-time NMR as the only method for structural dynamics elucidation.

This thesis proposes an unconventional route to address protein conformational spaces and low-concentrated samples. The approach proposed herein integrates hyperpolarized water by dDNP, a technique employed in the literature for protein hyperpolarization in solution⁹⁻¹¹, with real-time NMR, CW EPR, pulsed EPR, and MD simulations. The proposed strategy can be a versatile option to overcome the challenging conditions posed by low concentrations and short-lived conformations' accessibility. First, the integrated method herein reported does not require a special labeling scheme. In principle, dDNP can also be performed on unlabelled samples, observing only the evolution of the proton signals. For example, in **Chapter 5** the decay of the hyperpolarized proton signals is observed. Already a standard labeling strategy, such as homogeneously ¹⁵N or ¹³C, is sufficient to obtain good quality spectra. Secondly, the proposed approach can detect high intensity spectra of transient conformations. The SNR of the protein spectrum is significantly boosted by the hyperpolarized water, therefore providing more atomistic information in comparison to the thermal equilibrium state. Even if the substrate of interest has a short lifetime, it is possible to access it with high sensitivity, tackling the long acquisition times often needed by NMR. Additionally, by employing HyperW, low concentrated samples are accessible. While hundreds of micromolar are necessary to obtain a good SNR in an NMR experiment, dDNP can detect even 1 μ M of protein substrate, as suggested in **Chapter 4**.

Combining several methodologies increases the versatility of the proposed strategy, providing detailed information on the structural dynamics of the systems under study. In this thesis, four biomimetic models are employed to test the proposed combined approach. In summary:

Chapter 3 showed that the IDP BRCA1 modulates the MYC:MAX-DNA interaction networks by forming highly dynamic complexes. Previous studies suggested that BRCA1 actively binds MYC, but no direct interaction between BRCA1 and MAX was demonstrated. By means of real-time NMR, it was possible to monitor MYC:MAX-DNA heterodimerization, showing that the presence of BRCA1 slows down the complex formation. Complementing the real-time NMR experiments with ¹³C-¹⁵N NMR, CW EPR, and nanoscale distance measurements, it was possible to observe that BRCA1 modulates MYC:MAX-DNA by interacting with MYC and MAX monomer; therefore, the presence of BRCA1 hinders the heterodimerization process. Highly

dynamic multimeric complexes can feature slow tumbling due to their size, therefore generating broad NMR signals difficult to interpret. Additionally, the transition between the MAX homodimer to the MAX heterodimer features short-lived transient states, hardly detectable by NMR. The proposed method successfully overcomes these difficulties. Additionally, it allows the tracing of intermediate conformational states prior to binding, exploring them from the perspective of MYC (monitored by real-time NMR), MAX (studied by ^{13}C - ^{15}N NMR and CW EPR), and DNA (explored by DEER EPR). This work suggests a new perspective on how the interaction between BRCA1 and the MYC:MAX-DNA complex occurs.

In **Chapter 4**, dDNP was employed as the method to characterize the structural dynamic features of the model protein MAX, accessing conformational states represented only at micromolar concentrations. By corroborating the dDNP results with conventional NMR experiments and MD simulations, the semi-globular monomeric state of MAX was described at low concentrations. In contrast, at higher concentrations, MAX populated its dimeric form. Concentration is a cardinal factor in regulating the conformational balance of a dynamic protein, such as MAX. This study shows that the proposed integrated approach can be employed to explore the structural behavior of proteins at low concentration and characterize their structural dynamic features. This is particularly important since the natural concentration of proteins in eukaryotic cells is low, therefore leading to possible concentration induced modifications.

Chapter 5 discusses the mechanisms of polarization transfer from hyperpolarized water to an arginine model. By hyperpolarizing the arginine with HyperW and calculating the NOEs from MD simulations, it was possible to show how the NOEs, exchange-relay NOE, and chemical exchange contribute to polarization transfer. Considering that HyperW is employed in **Chapter 4** to hyperpolarize a protein substrate, this chapter provides information on the polarization transfer modalities in biomolecular samples. It is demonstrated that the proposed integrated method can also be employed to characterize the polarization transfer to the protein substrate and could be employed ad-hoc to calculate and compare experimental and simulated NOEs, providing information on the water shell surrounding a macromolecule in solution.

Finally, **Chapter 6** introduces the concept of PNS and their presence in biomimetic solutions such as mSBF. The study focuses on the structural characterization of nanometric inhomogeneities by real-time NMR, monitoring their time-dependent growth. The

understanding of the transition from a homogeneous ionic solution to the development of amorphous structures of hundreds of nanometers is particularly relevant for the development of novel bioactive materials.¹² The strategy combined herein can be employed to characterize the crystallization process, providing molecular details on the conformational evolution of PNS in solution.

This thesis shows that it is possible to access transient and low-concentration-driven conformations by integrating different methods. Additionally, fundamental mechanisms of polarization transfer are experimentally and computationally explored.

dDNP could also provide interesting insights on the conformational behaviour of the MYC:MAX-DNA / BRCA1 interaction network and on CaP evolution, on which it was not yet employed. For example, the application of dDNP on MYC:MAX-DNA / BRCA1 could provide residue-resolved information on the transition of MAX from a homodimer to a heterodimer, and additionally trace the specific interaction of BRCA1 with the forming complex. dDNP could also characterize the behaviour of the semi-globular monomeric MAX at low concentrations in the presence of an interaction partner, such as MYC. A further understanding of the interaction process between low-concentrated MAX and MYC could offer a valuable overlook on protein heterodimerization processes at semi-physiological concentrations, considering the naturally low concentrations of proteins in the cells.

While PNS formation in mSBF cannot be directly explored by dDNP because of the PNS slow evolution, dDNP was already applied on highly concentrated calcium phosphate solutions for their characterization since the evolution in this case is faster than in mSBF.¹³

Overall, the elucidation of conformational transitions in biologically relevant biomacromolecules represents a step forward in the understanding of protein structural behavior at semi-physiological conditions. A detailed atomistic characterization of the structural balance of medically relevant biomolecules is fundamental in pharmacological research. The urge for new, more efficient drugs first claims the identifications of previously undescribed targets. Proteins involved in life-threatening diseases like cancer are often intrinsically disordered, featuring a highly dynamic conformational space to fulfill their biological roles.¹⁴⁻¹⁶ In the presence of mutations, highly dynamic proteins can determine the transition of a cell from its physiological to its pathological state. Focusing on specific conformations involved in pathological processes represents an ideal route to develop potentially novel therapeutics.

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Appendix A

BRCA1 inhibits MYC:MAX heterodimerization by modulating the availability of transient MAX monomers

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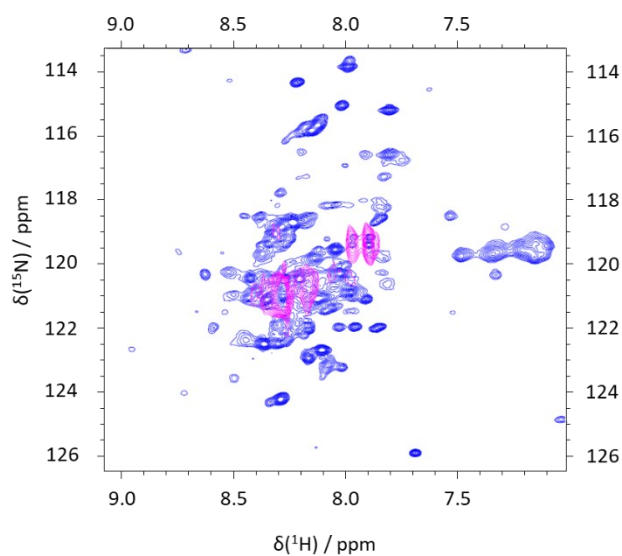


Figure SI 1. MYC spectra. Blue: ^1H - ^{15}N TROSY spectrum of a 0.1 mM sIMYC sample, after mixing 1:1:1 with MAX:MAX and EBOX DNA; the experiment was recorded at 25° C, in MES buffer at pH 5.5. Pink: HNC0 projection on the ^1H - ^{15}N plane, showing only the signals of the selectively labeled residues. Thus, the peaks of the HNC0 could be identified by comparing them to the reference spectrum.

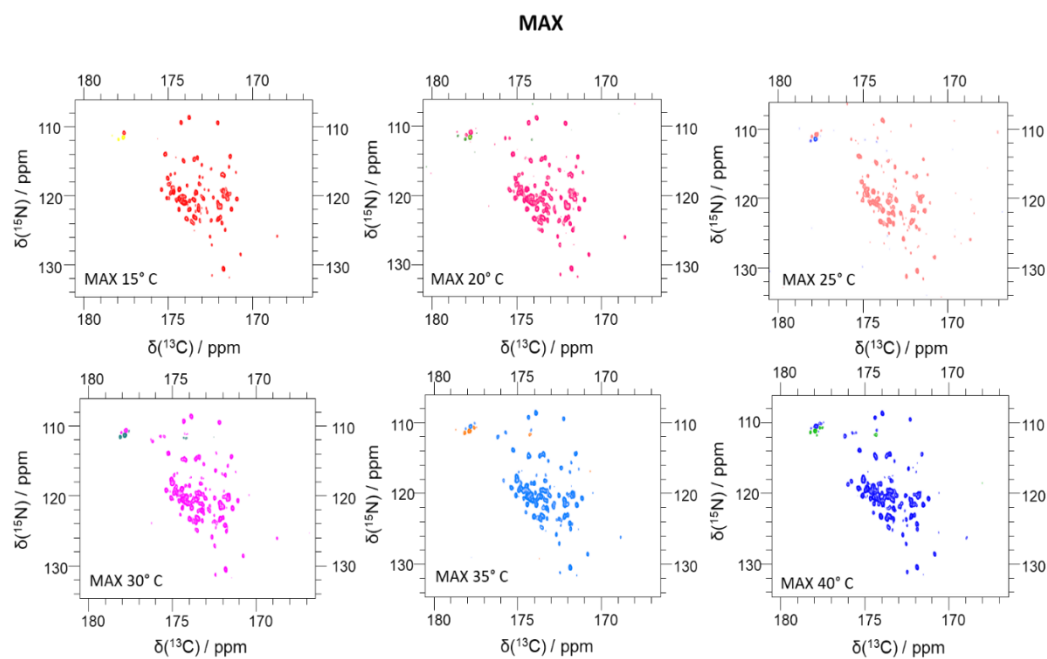


Figure SI 2. ^{13}C - ^{15}N correlated NMR spectra of 0.1 mM ^{13}C - ^{15}N labeled MAX in at pH 5.5 at temperatures ranging from 15 to 40° C.

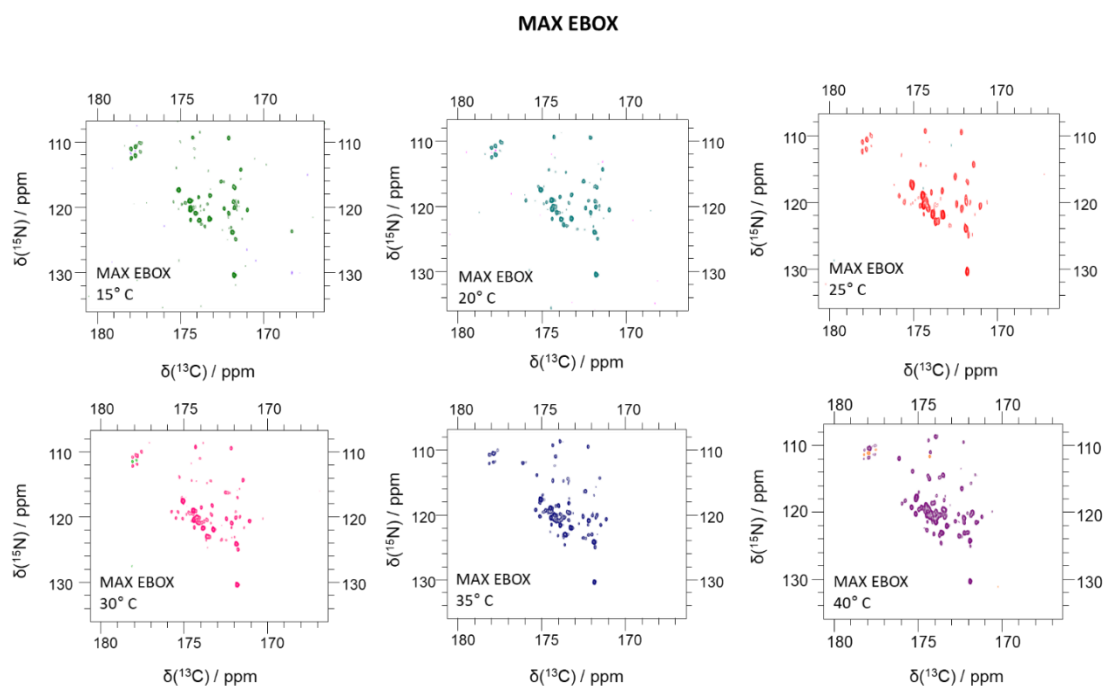


Figure SI 3. ^{13}C - ^{15}N correlated NMR spectra of 0.1 mM ^{13}C - ^{15}N labeled MAX in the presence of 1 eq. EBOX DNA at pH 5.5 at temperatures ranging from 15 to 40° C.

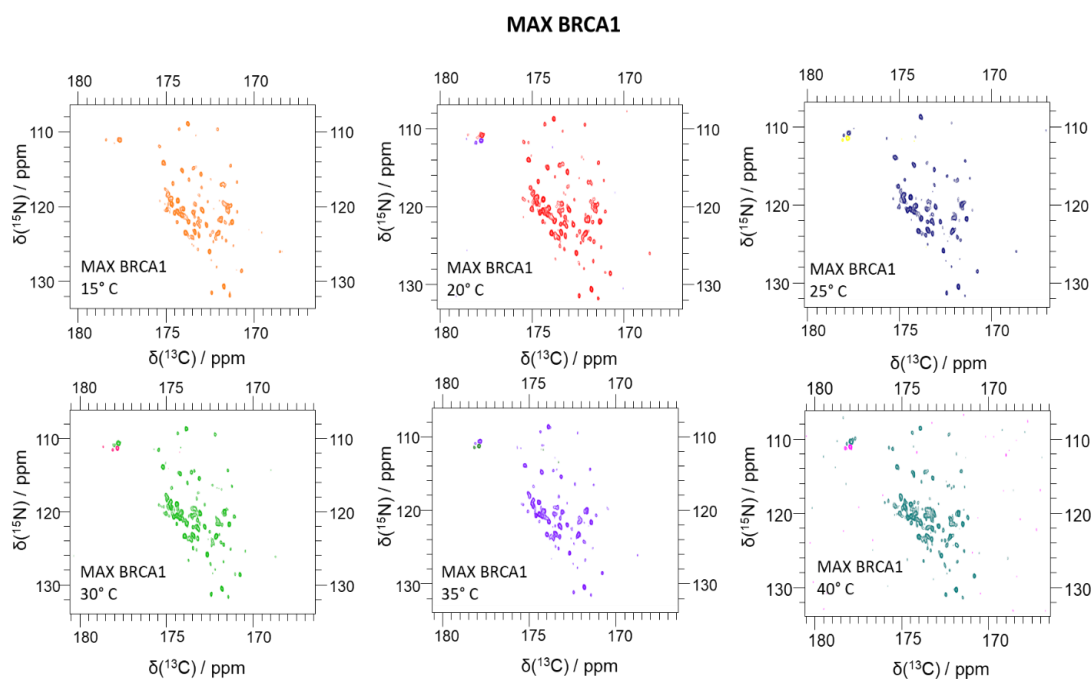


Figure SI 4. ^{13}C - ^{15}N correlated NMR spectra of 0.1 mM ^{13}C - ^{15}N labeled MAX in the presence of 1 eq. BRCA1 at pH 5.5 and temperatures between 15 and 40° C.

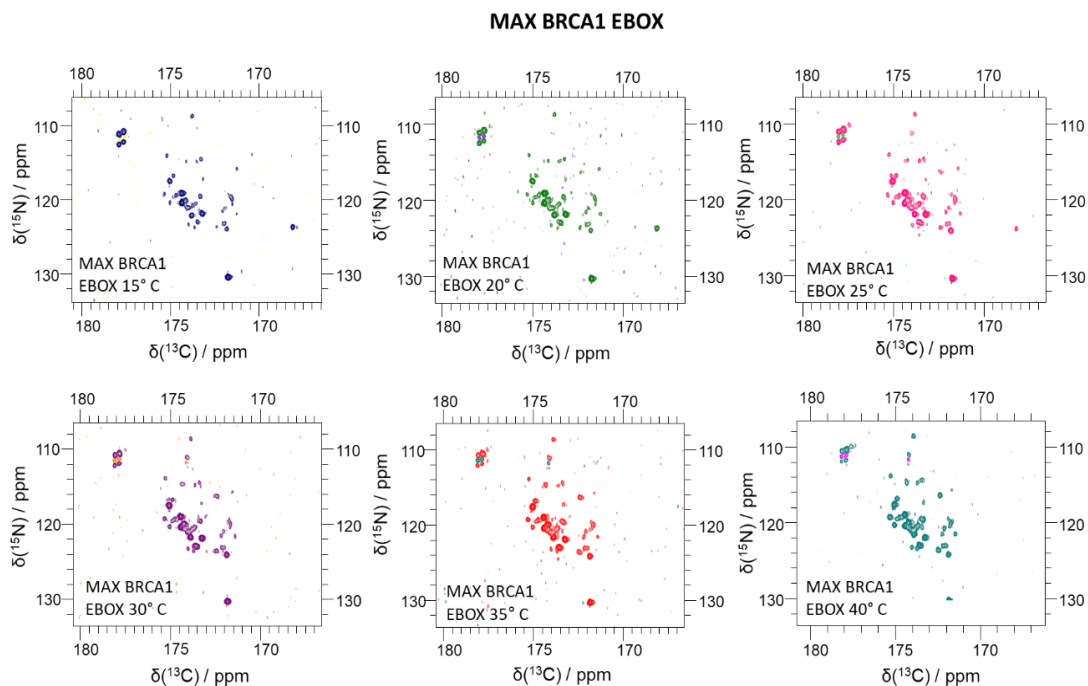


Figure SI 5. ^{13}C - ^{15}N correlated NMR spectra of 0.1 mM ^{13}C - ^{15}N labeled MAX in the presence of 1 eq. BRCA1 and EBOX DNA at pH 5.5 and temperatures between 15 and 40° C.

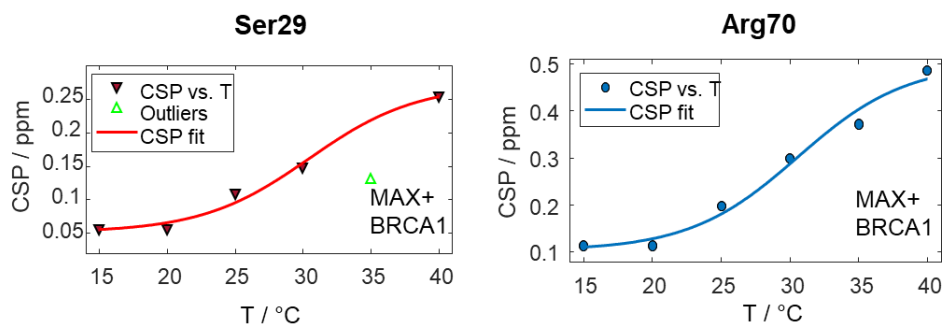


Figure SI 6. Effect of BRCA1 on the melting behavior of MAX for residues Ser29 (left) and Arg70 (right). The reverse triangles and the blue dots indicate the experimental CSPs data. The data were fitted with a sigmoidal function (red and blue lines).

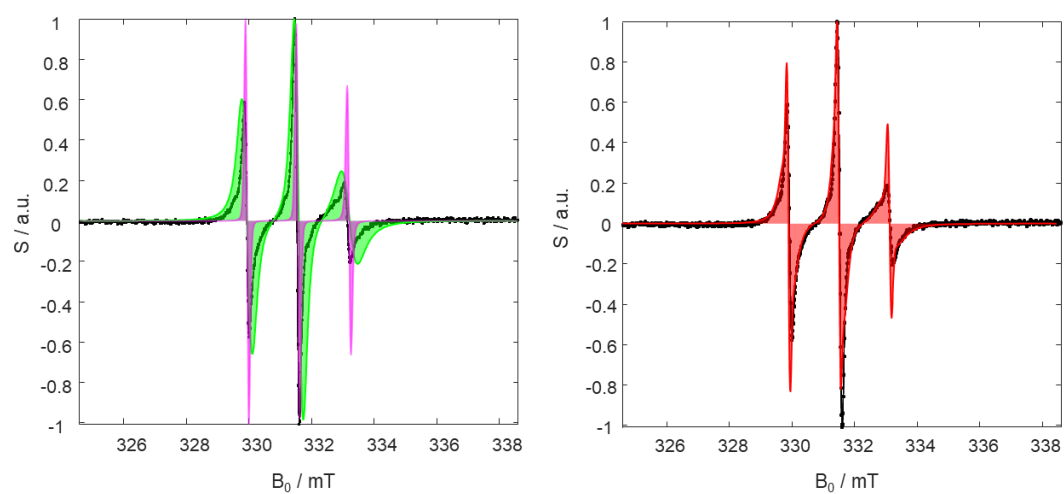


Figure SI 7. Schematic representation of a CW EPR spectrum (black) characterized by two main simulated contributions, one slow (in green) and one fast (in pink), shown on the left. The sum of the two contributions is depicted in red on the right spectrum.

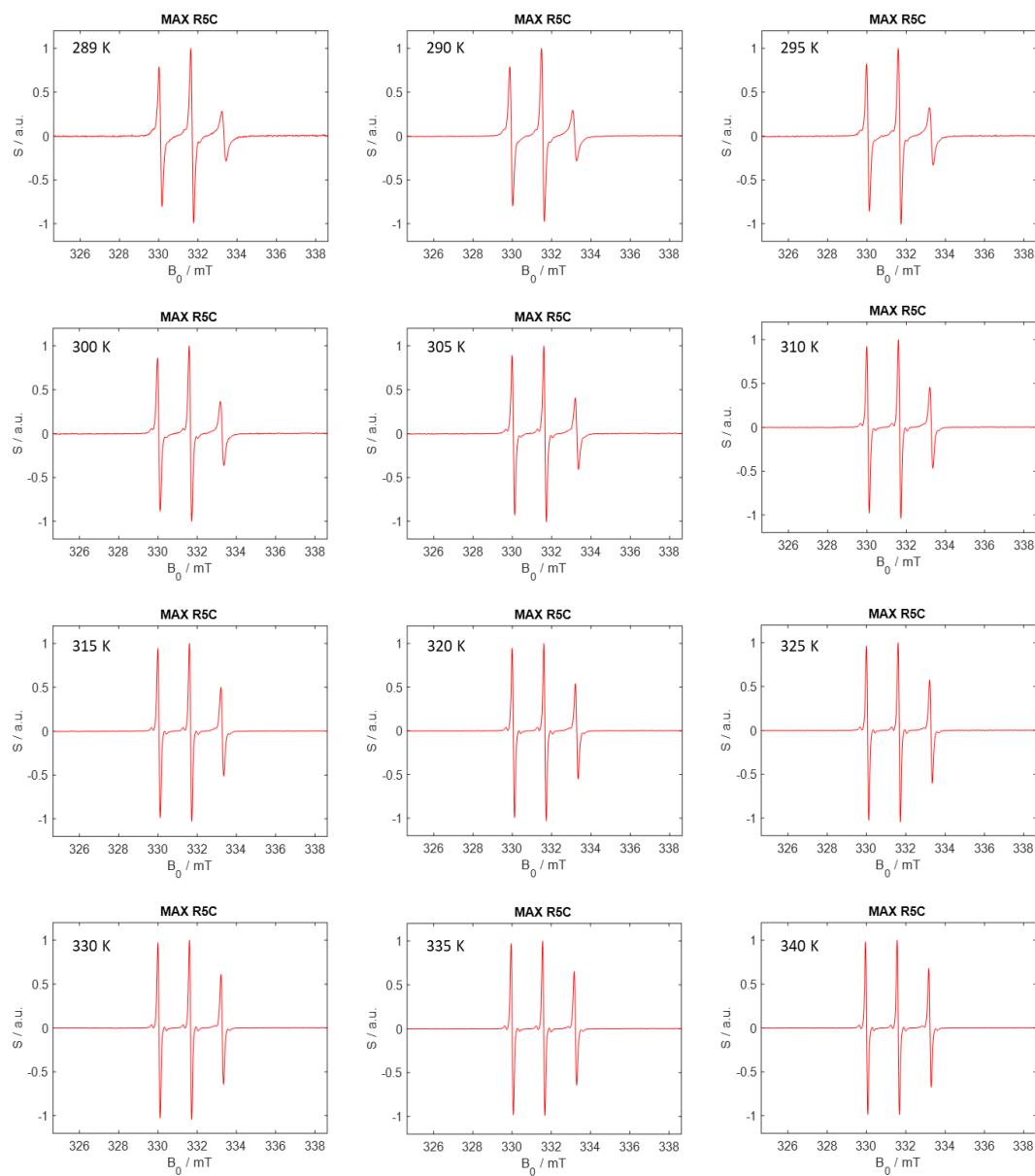


Figure SI 8. CW EPR spectra of MAX R5C. The experimental temperatures are indicated.

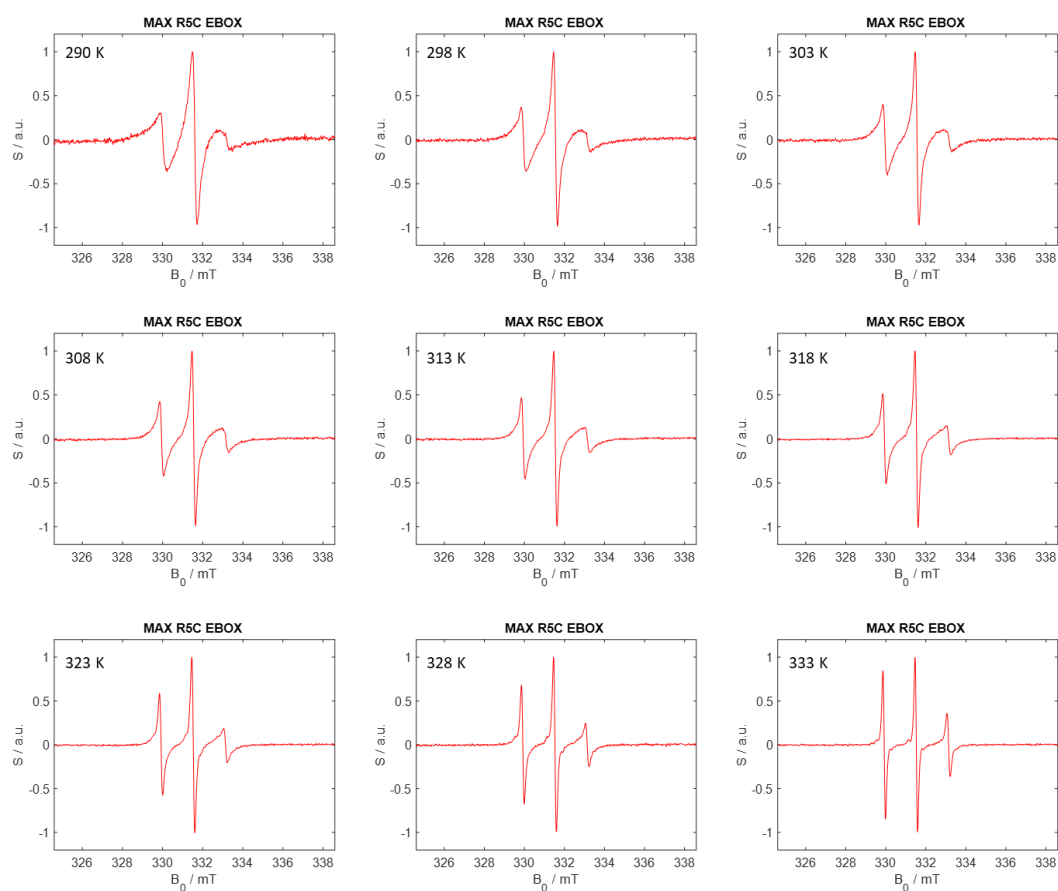


Figure SI 9. CW EPR spectra of MAX R5C + EBOX DNA. The experimental temperatures are indicated.

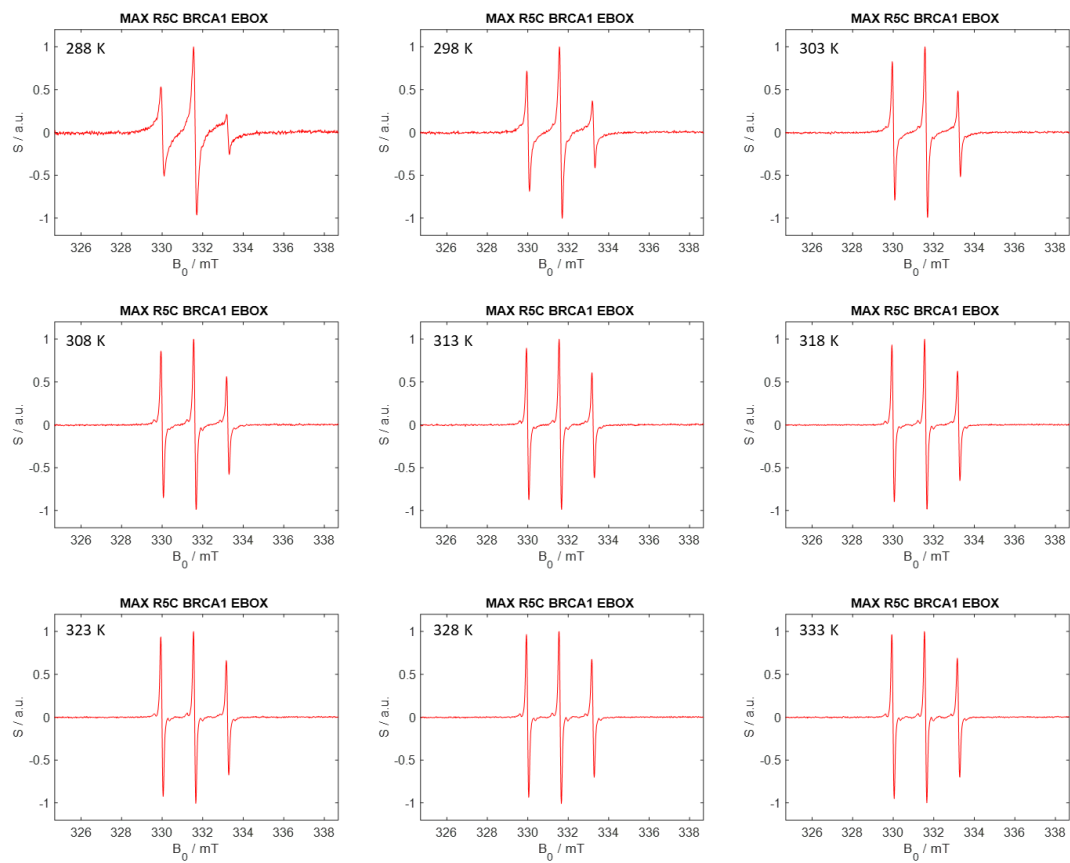


Figure SI 10. CW EPR spectra of MAX R5C + EBOX DNA + BRCA1. The experimental temperatures are indicated.

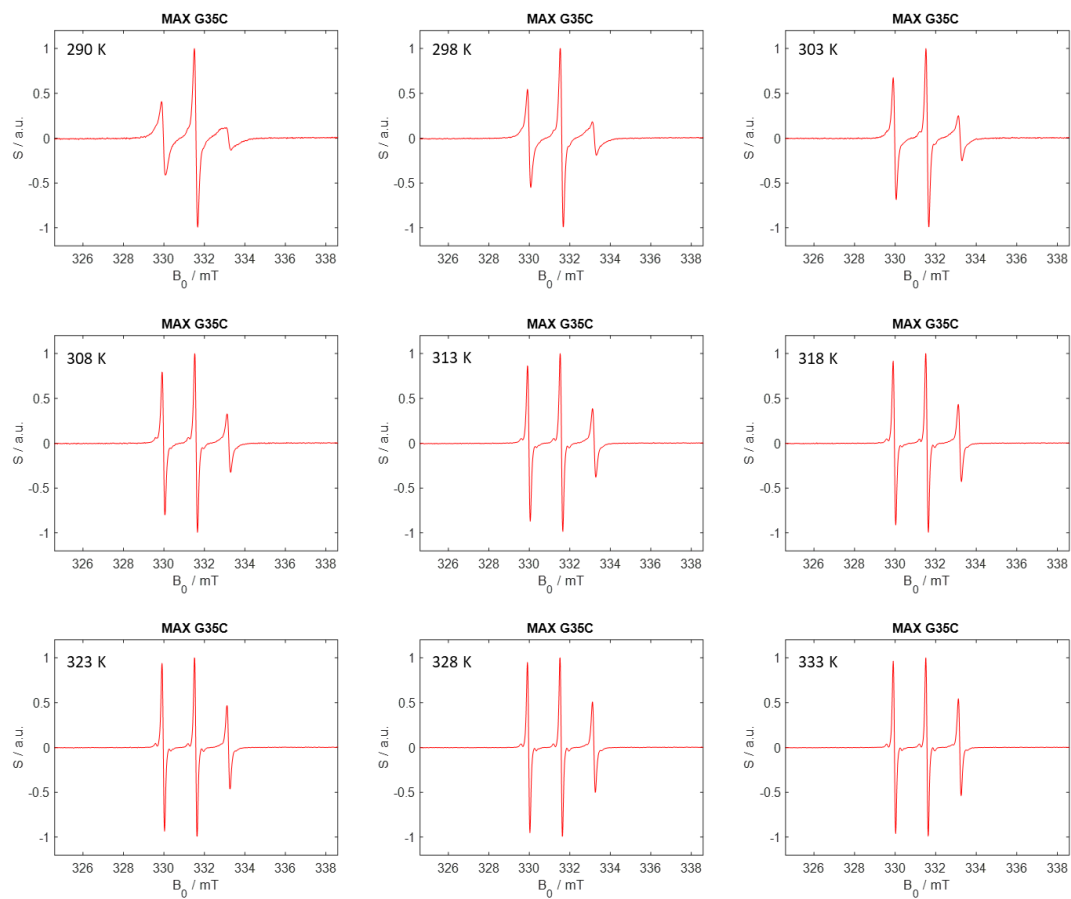


Figure SI 11. CW EPR spectra of MAX G35C. The experimental temperatures are indicated.

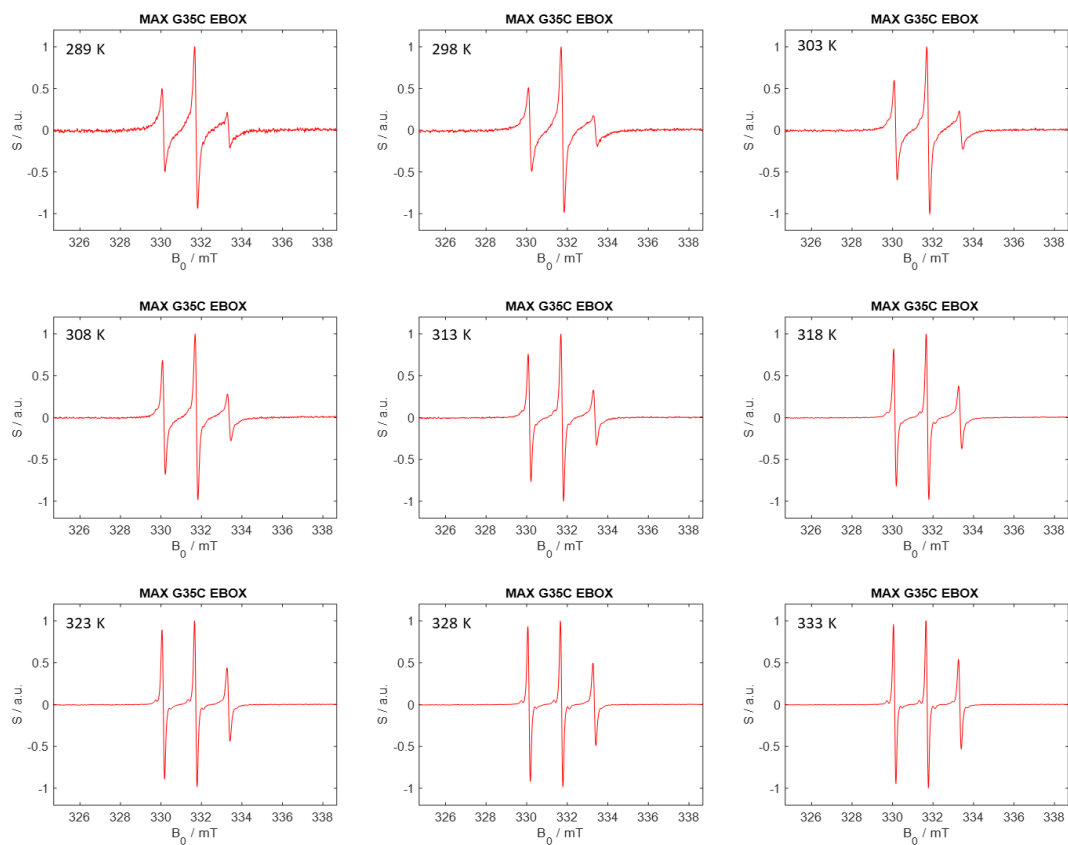


Figure SI 12. CW EPR spectra of MAX G35C + EBOX DNA. The experimental temperatures are indicated.

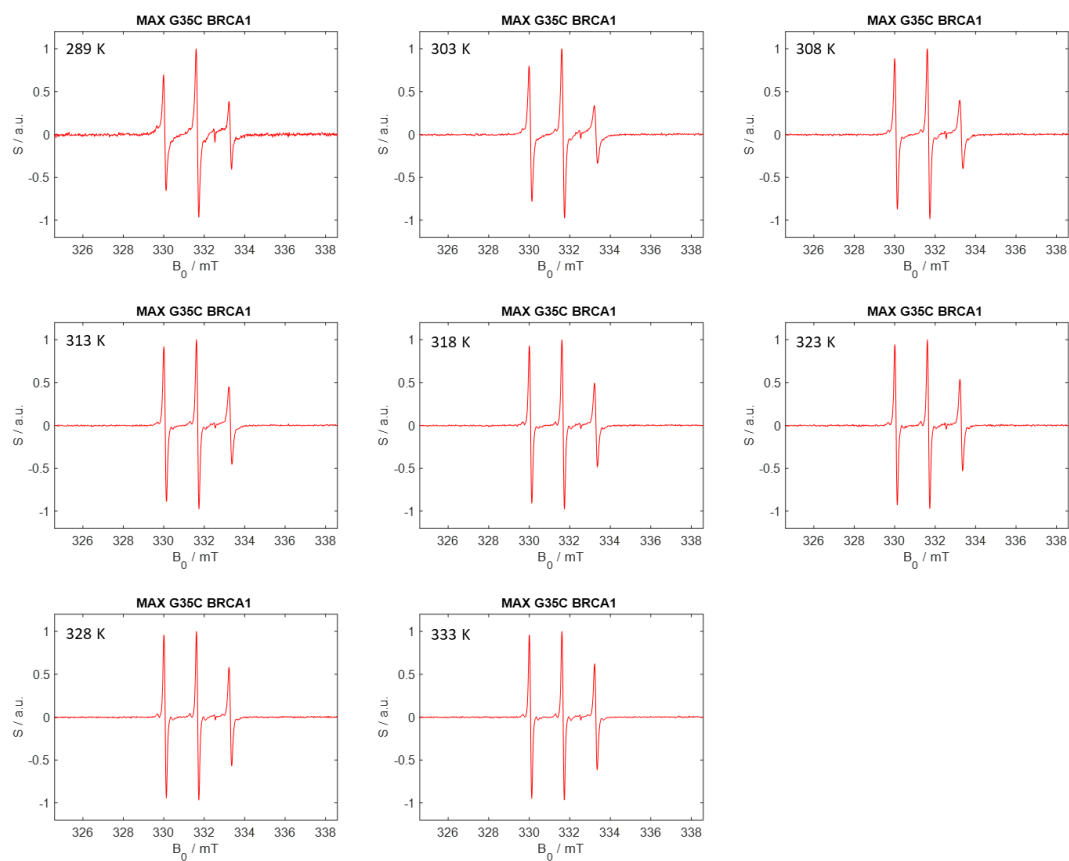


Figure SI 13. CW EPR spectra of MAX G35C + BRCA1. The experimental temperatures are indicated.

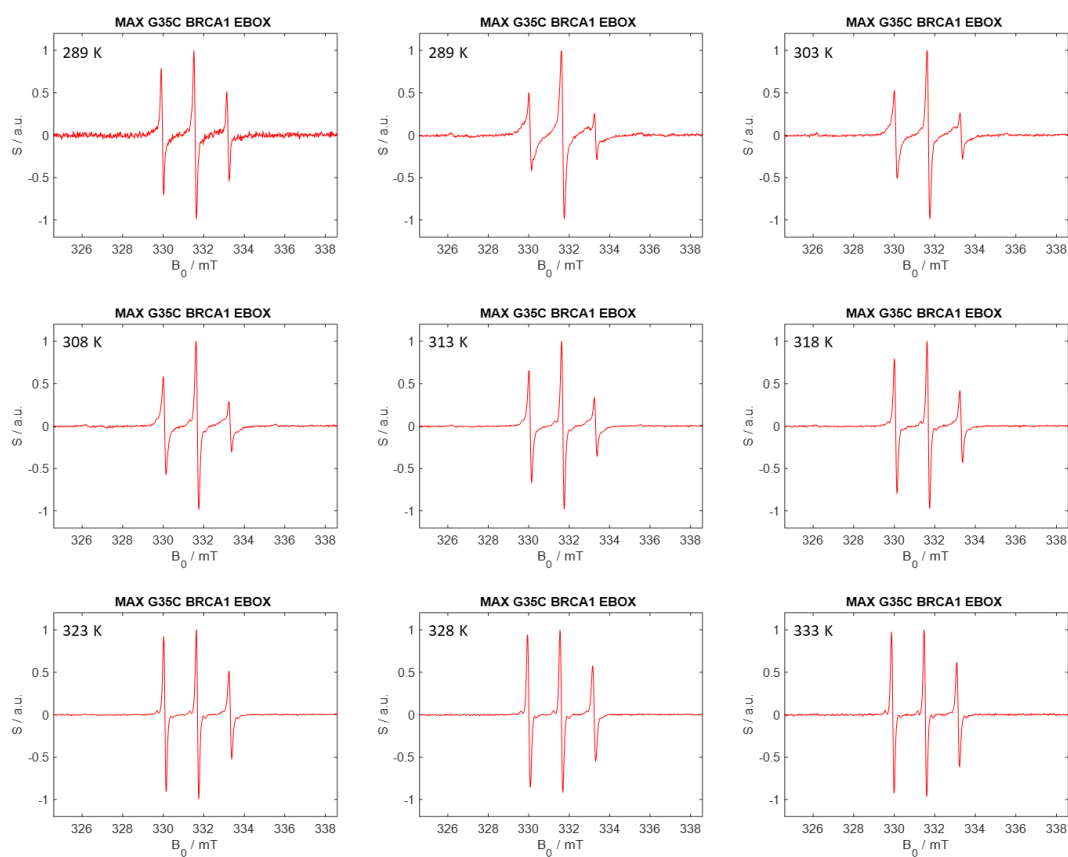


Figure SI 14. CW EPR spectra of MAX G35C + EBOX DNA + BRCA1. The experimental temperatures are indicated.

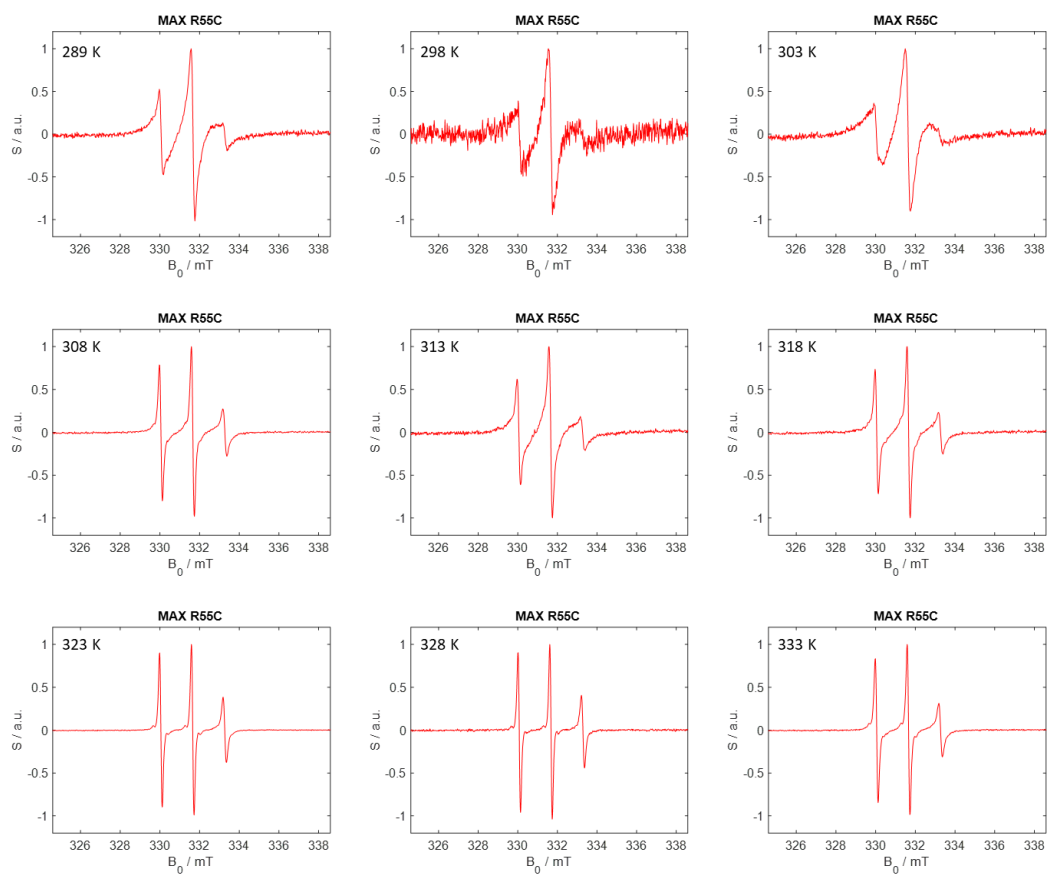


Figure SI 15. CW EPR spectra of MAX R55C. The experimental temperatures are indicated.

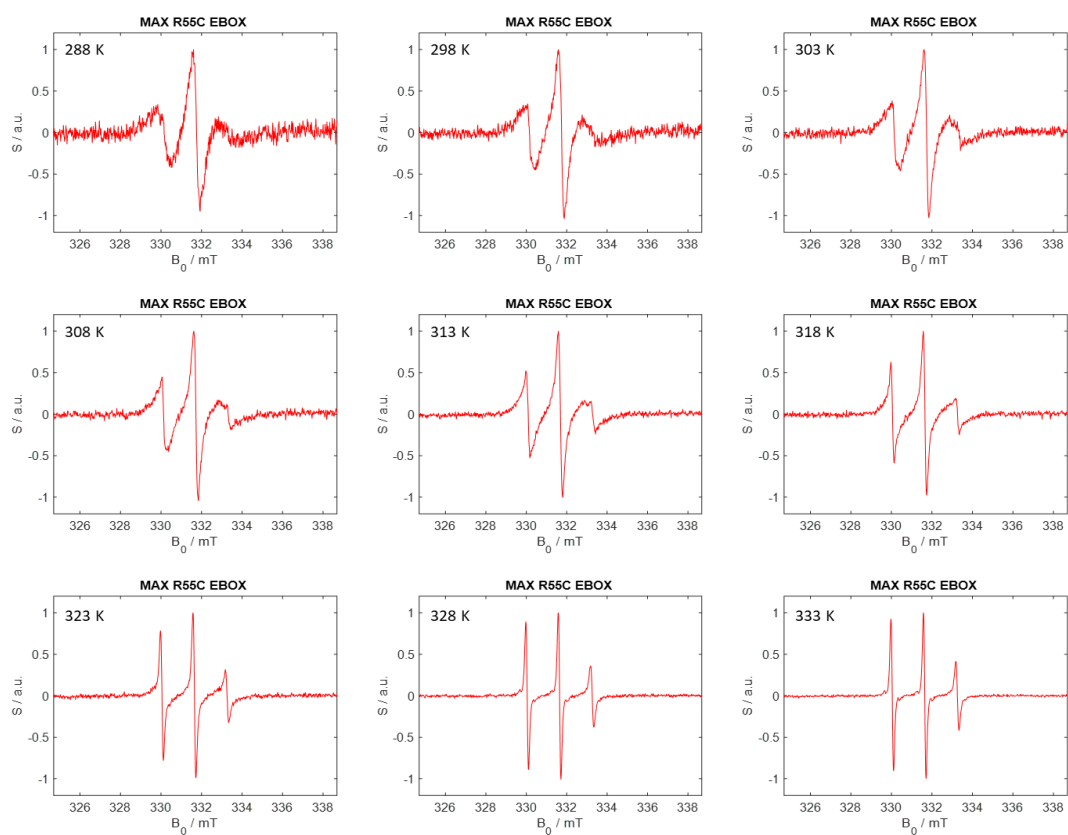


Figure SI 16. CW EPR spectra of MAX R55C + EBOX DNA. The experimental temperatures are indicated.

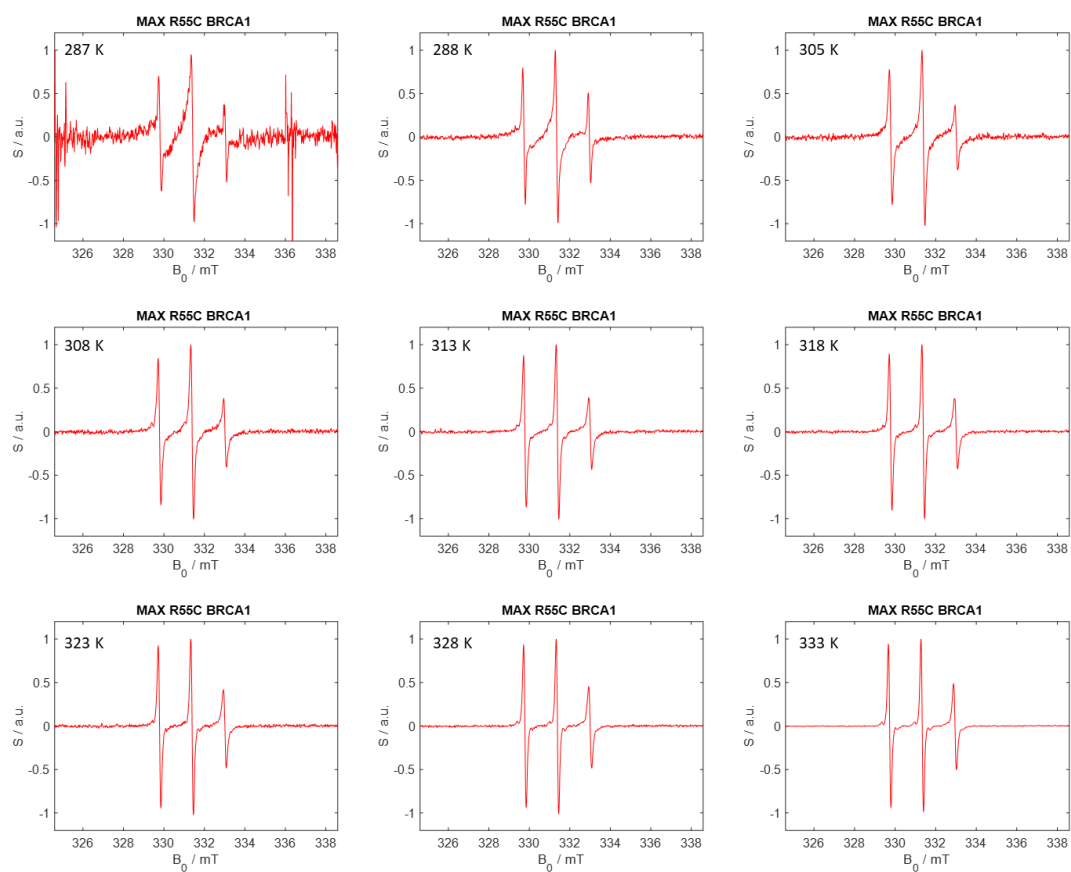


Figure SI 17. CW EPR spectra of MAX R55C + BRCA1. The experimental temperatures are indicated.

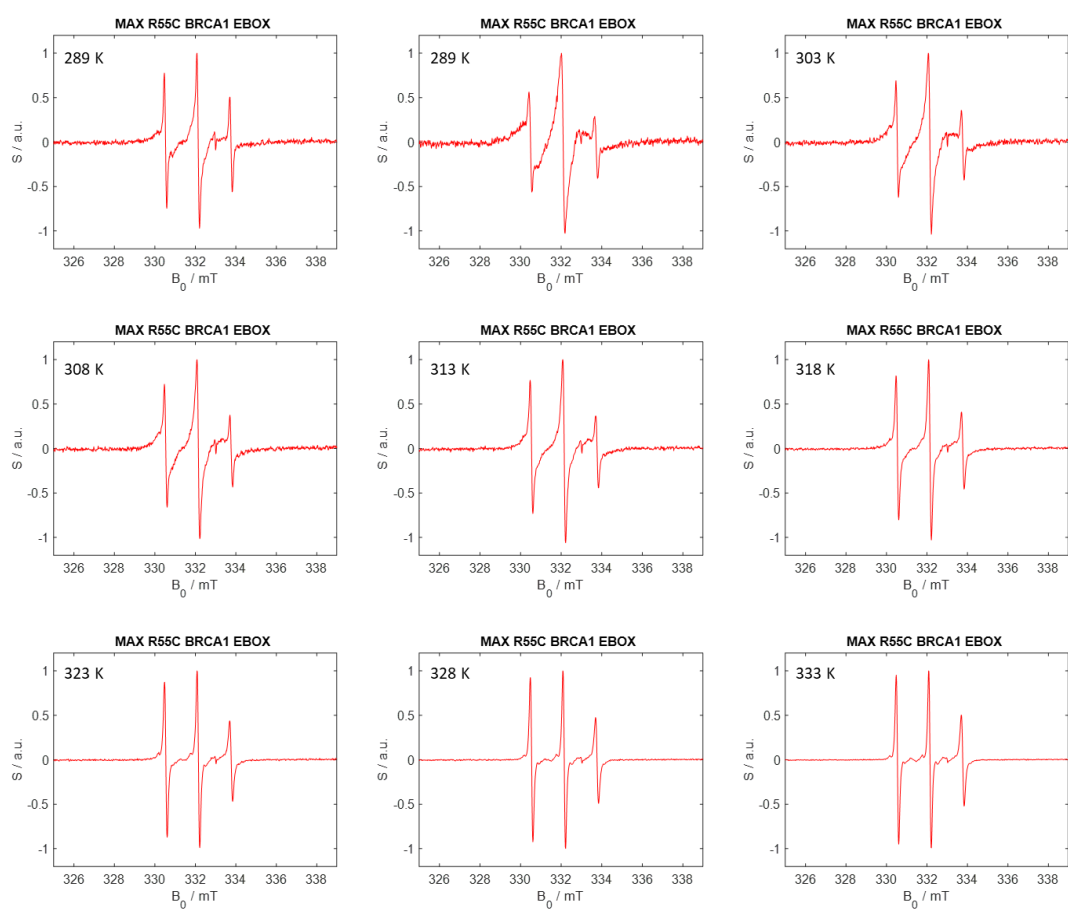


Figure SI 18. CW EPR spectra of MAX R55C + EBOX DNA + BRCA1. The experimental temperatures are indicated.

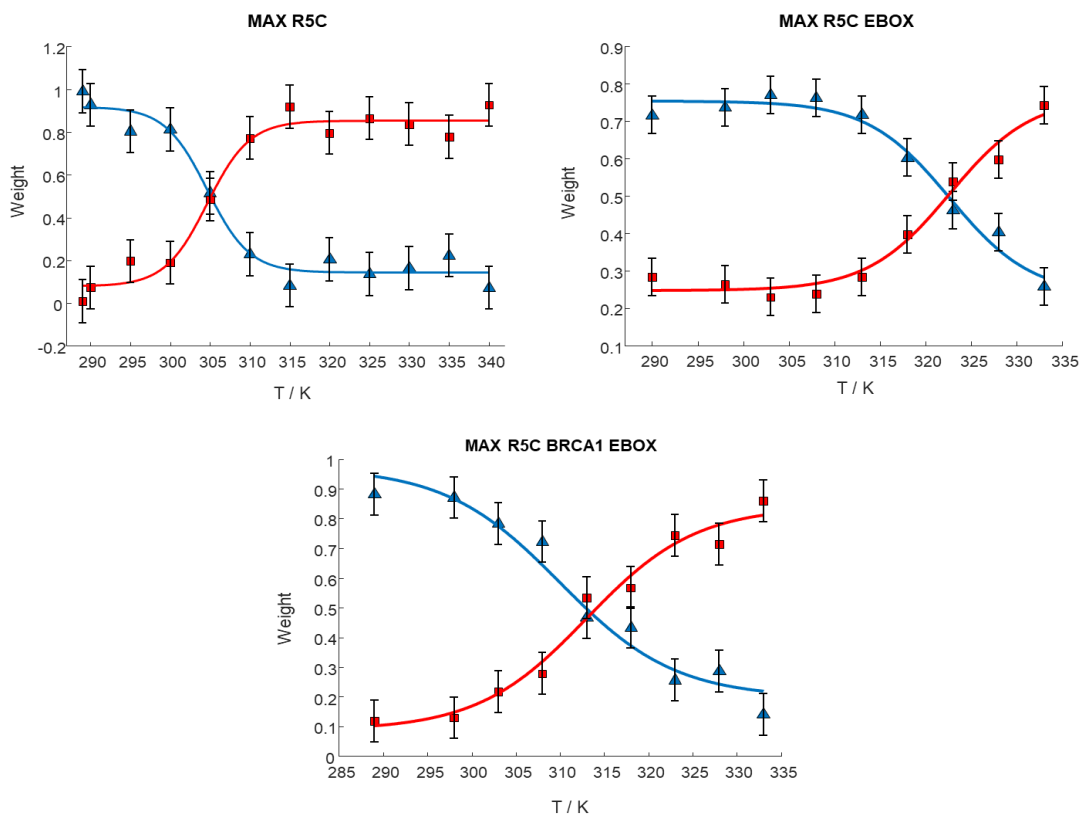


Figure SI 19. Weights of the slow contribution (blue triangles) and fast contribution (red squares) obtained from simulating the CW EPR spectra of MAX R5C at different temperatures. The experimental data were fitted with a sigmoidal function (blue and red lines, respectively). The melting temperature is identified as the crossing point between the fits. The experiments were performed in the absence or the presence of EBOX DNA and/or BRCA1.

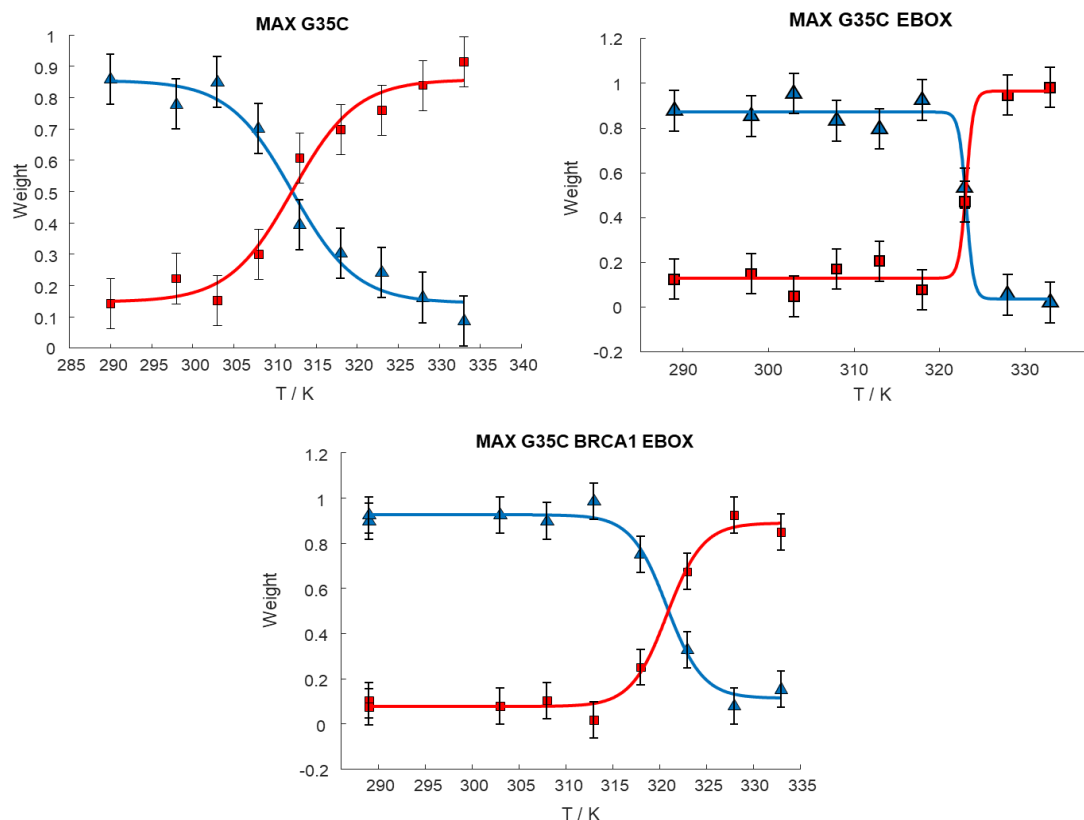


Figure SI 20. Weights of the slow contribution (blue triangles) and fast contribution (red squares) for MAX G35C at different temperatures. The experimental data were fitted with a sigmoidal function (blue and red lines, respectively). The melting temperature is identified as the crossing point between the fits. The experiments were performed in the absence or the presence of EBOX DNA and/or BRCA1.

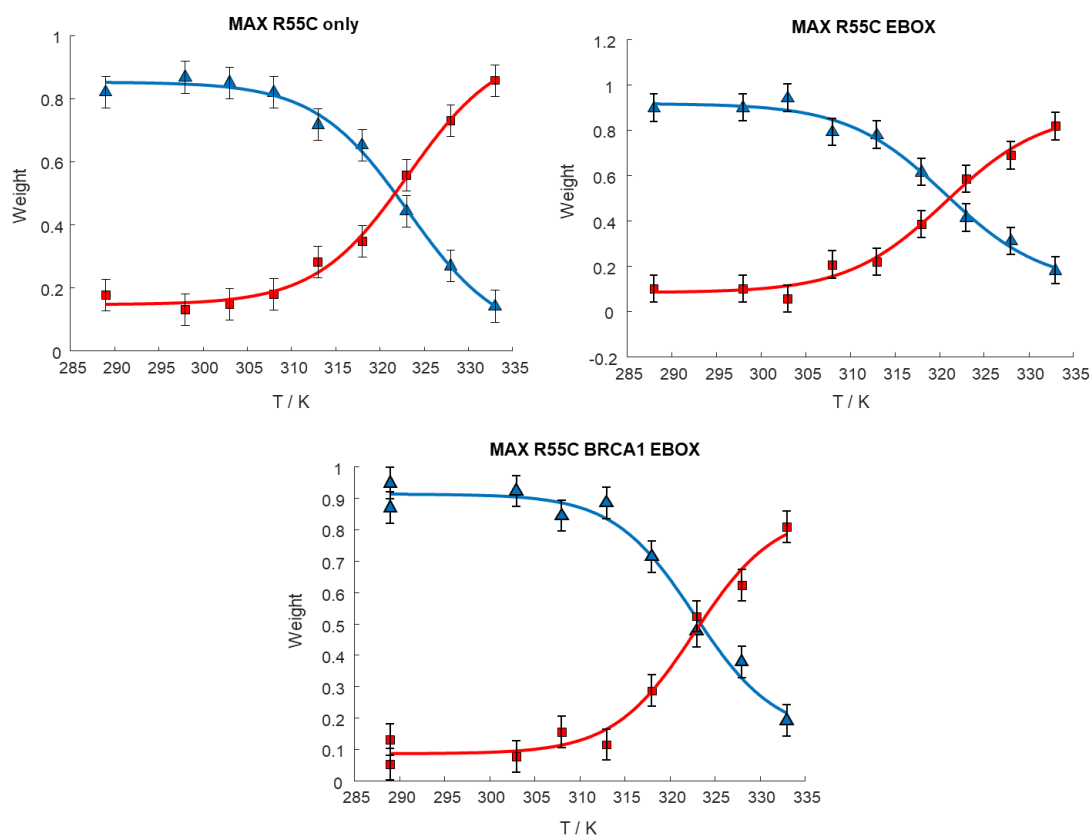


Figure SI 21. Weights of the slow contribution (blue triangles) and fast contribution (red squares) for MAX R55C at different temperatures. The experimental data were fitted with a sigmoidal function (blue and red lines, respectively). The melting temperature is identified as the crossing point between the fits. The experiments were performed in the absence or the presence of EBOX DNA and/or BRCA1.

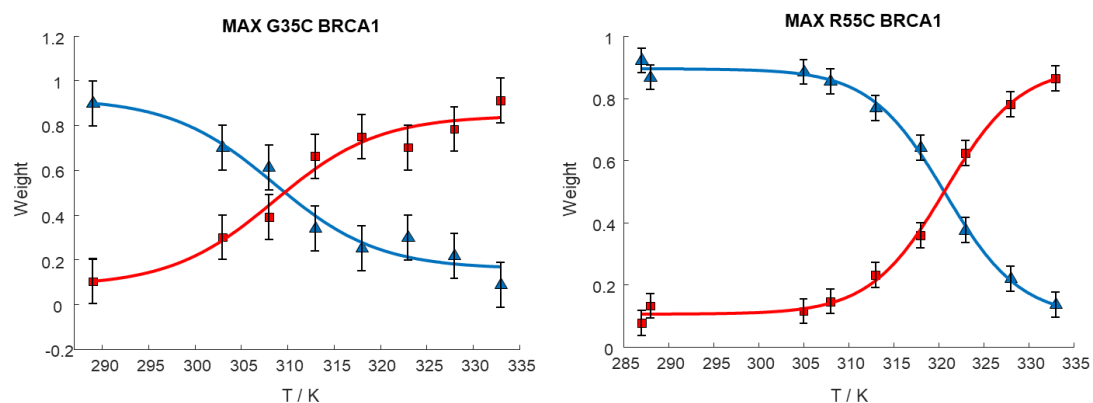


Figure SI 22. Weights of the slow contribution (blue triangles) and fast contribution (red squares) for MAX G35C and R55C at different temperatures. The experimental data were fitted with a sigmoidal function (blue and red lines, respectively). The melting temperature is identified as the crossing point between the fits. The experiments were performed in the presence of BRCA1.

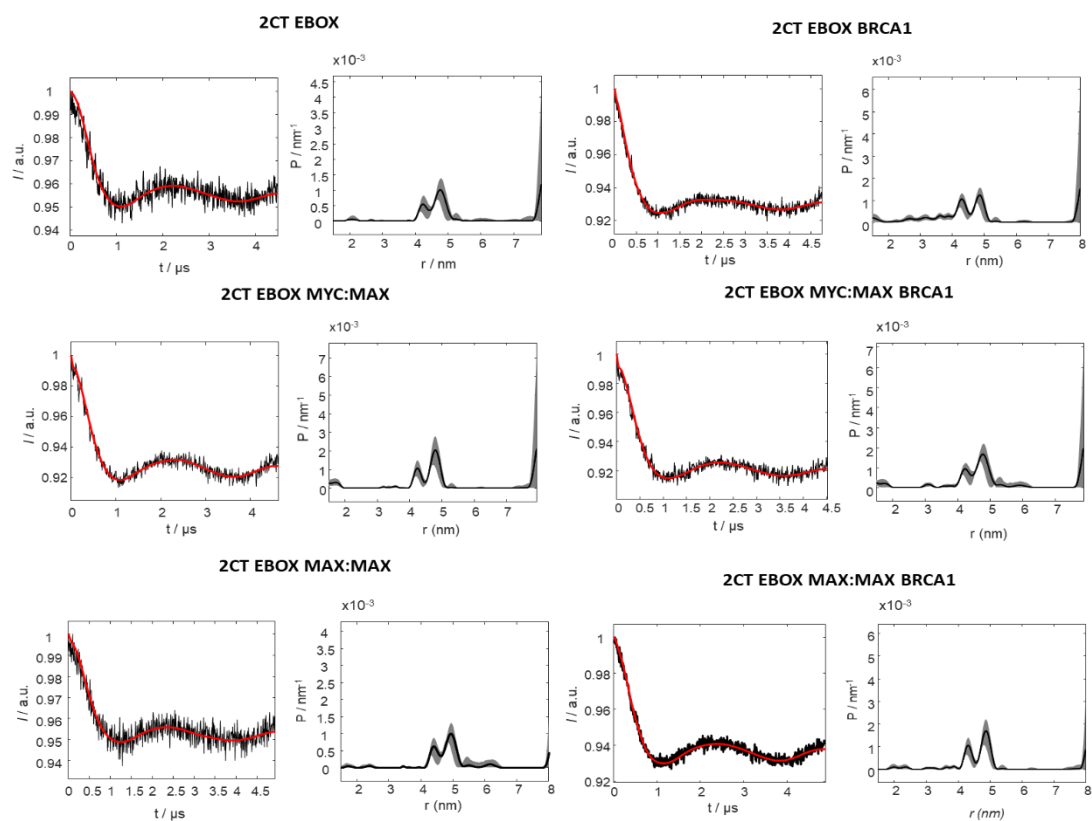


Figure SI 23. DEER form factors (black), fits (red) obtained by Thikonov-type regularization, and the corresponding distance distribution for the EBOX DNA labeled at the terminal cytosines.

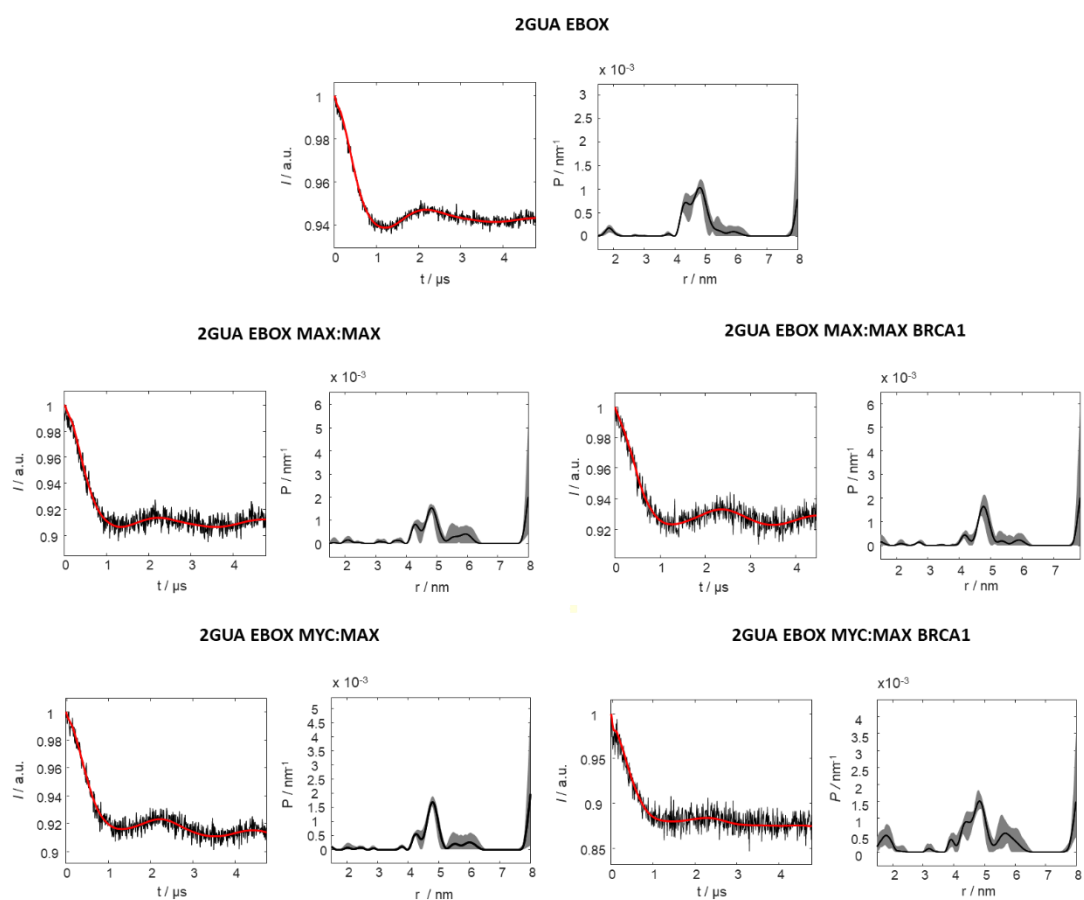


Figure SI 24. DEER form factors (black), fits (red) obtained by Thikonov-type regularization, and the corresponding distance distribution for the EBOX DNA labeled at the terminal guanosines. The results conform the distances found in Fig. SI 23.

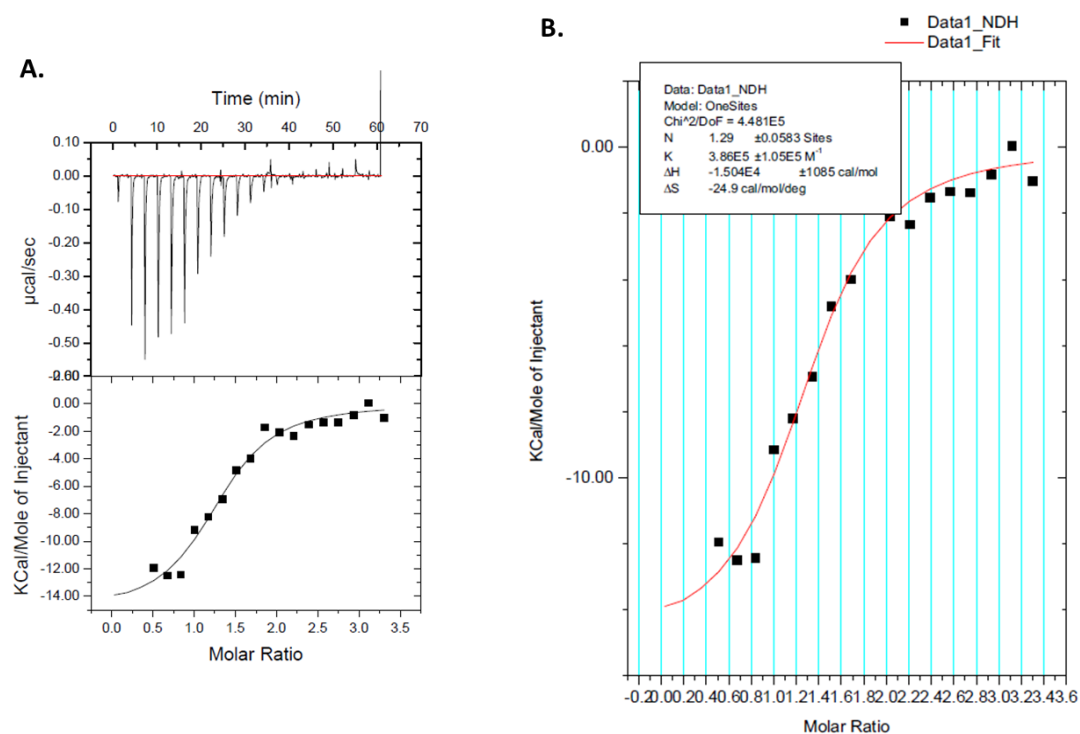


Figure SI 25. A.) ITC injection profiles and calculated heat of injectant (same as in the main text). B.) One-site model fit to the ITC titration profile. Enthalpy and entropy changes, the stoichiometry and dissociation constants are indicated in the legend.

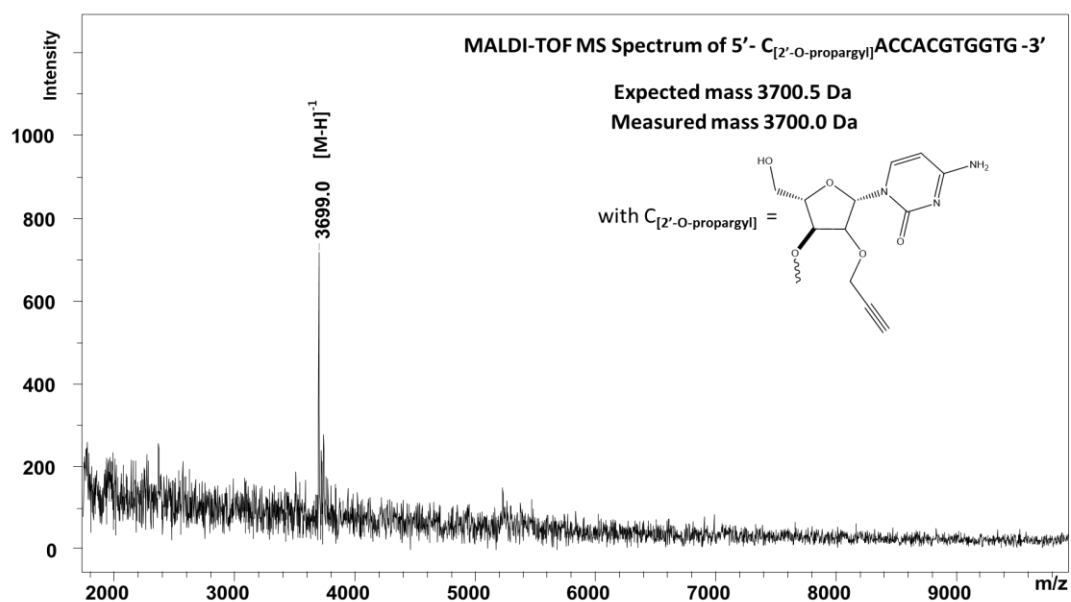


Figure SI 26. MALDI-TOF analysis: control experiment for oligonucleotide 5'-(C_[2'-O-Propargyl]ACCACGTGGTG)-3'.

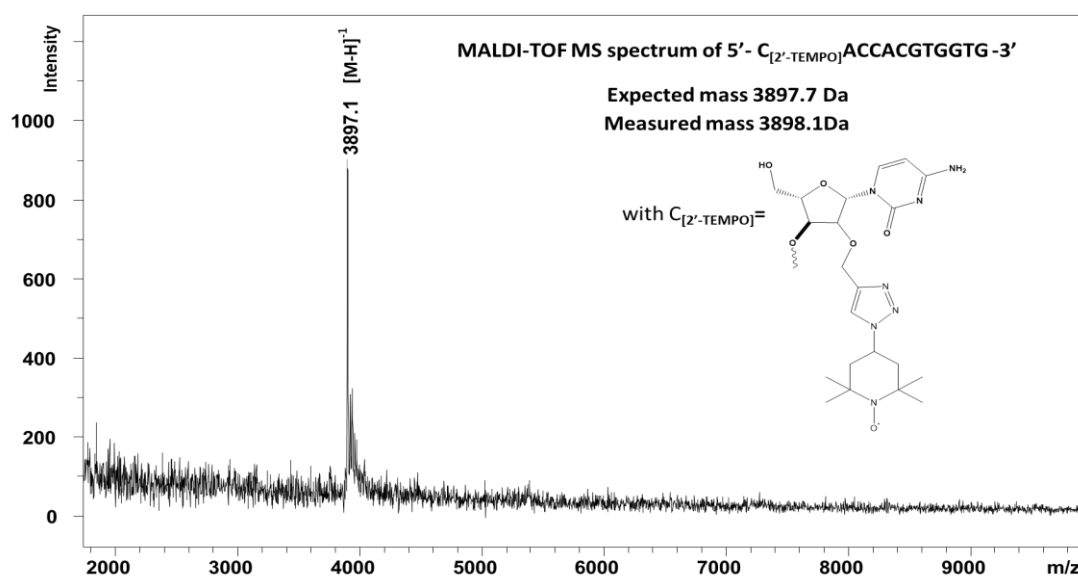


Figure SI 27. MALDI-TOF analysis: control experiment for oligonucleotide 5'-(C_[2'-O-Propargyl]ACCACGTGGTG)-3' labeled with azido TEMPO through a “click chemistry approach”.

LC-ESI MS analysis of 5'-(C_[2'-TEMPO])₂ACCACGTGGTG-3'

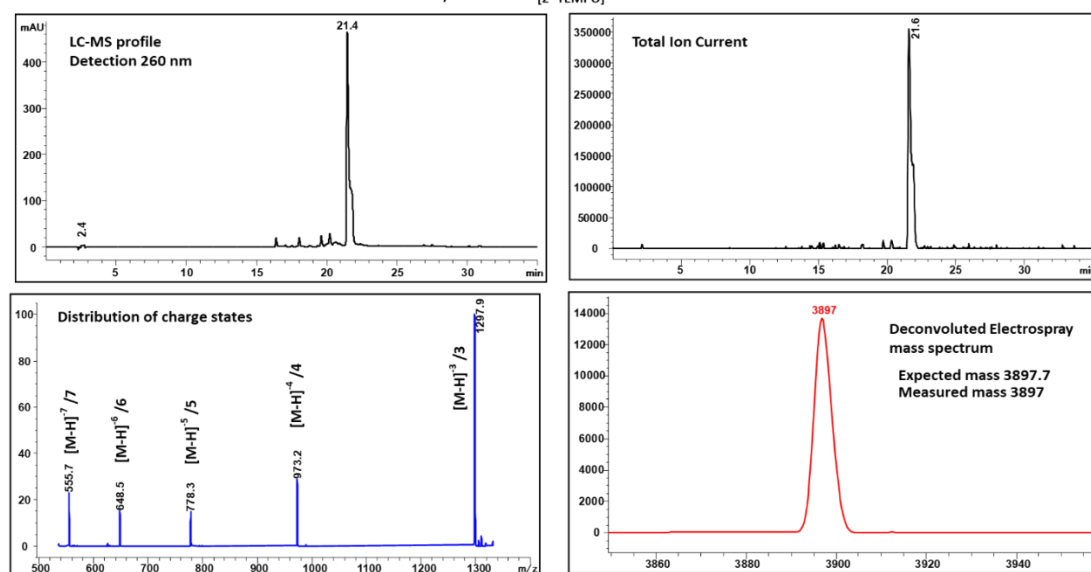


Figure SI 28. LC-ESI MS analysis: control experiment for oligonucleotide 5'-(C_[2'-TEMPO])₂ACCACGTGGTG-3'.

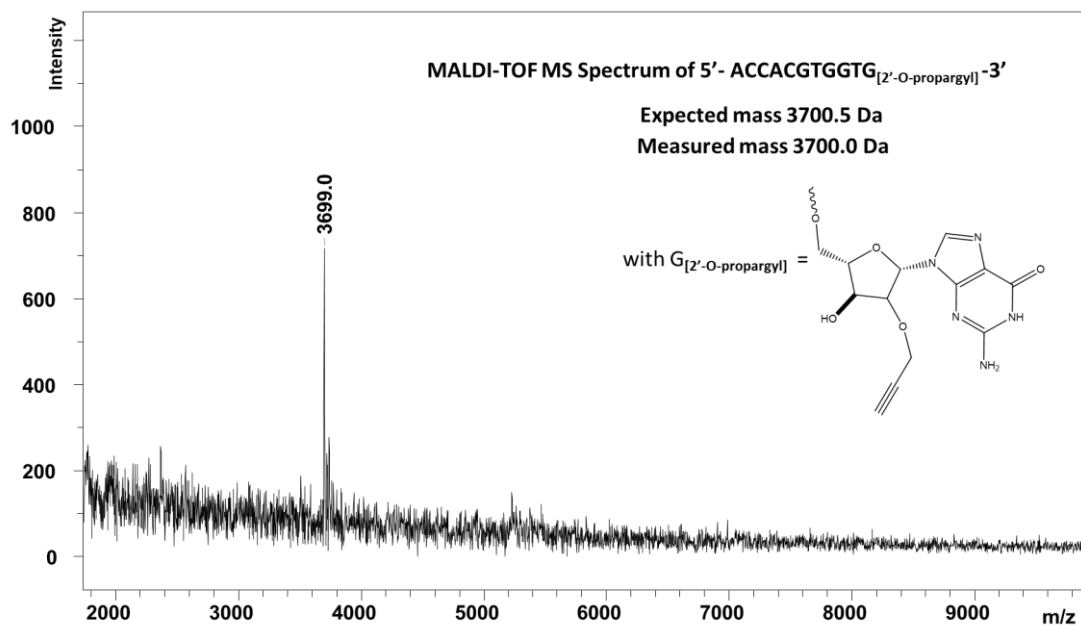


Figure SI 29. MALDI TOF analysis: control experiment for oligonucleotide 5'-(ACCACGTGGTG_[2'-O-Propargyl])-3'.

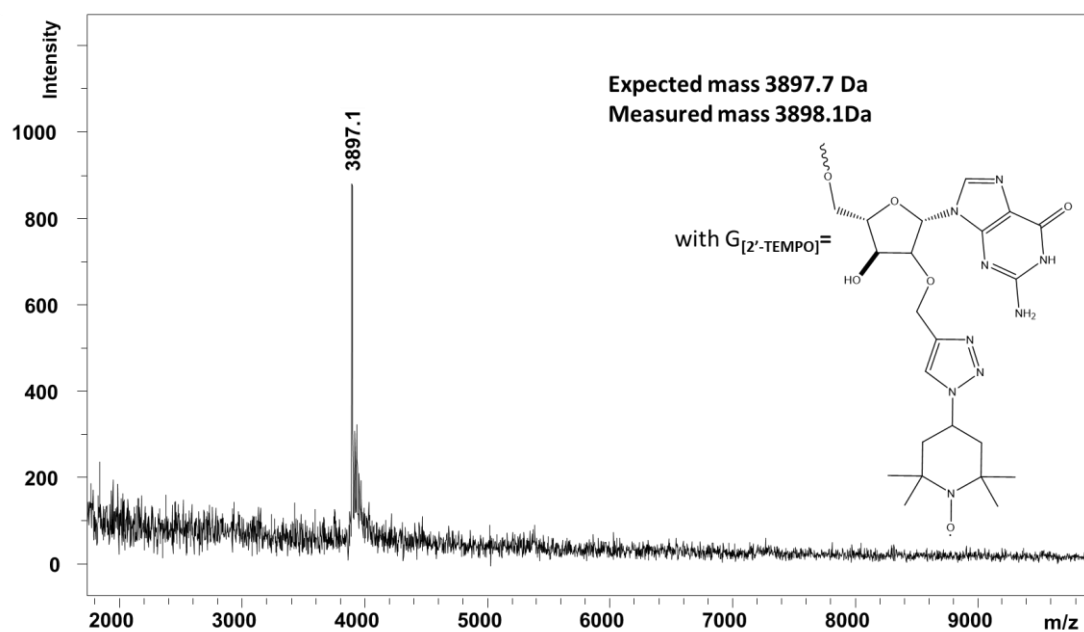


Figure SI 30. MALDI TOF analysis: control experiment for oligonucleotide 5'-(ACCACGTGGTG_[2'-O-Propargyl])-3' labeled with azido TEMPO through a "click chemistry approach".

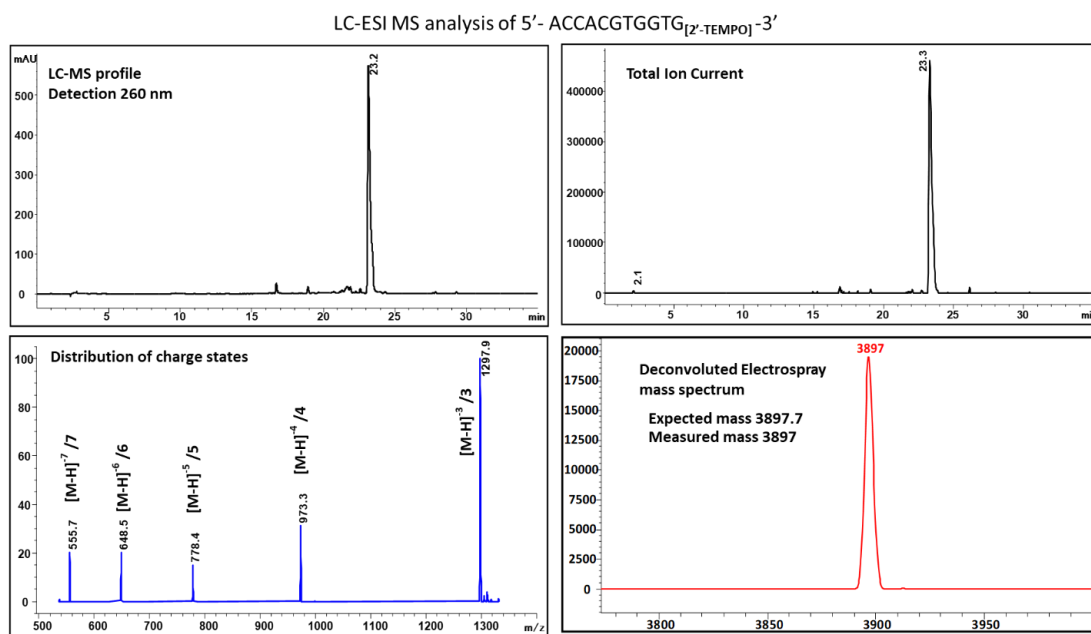


Figure SI 31. LC-ESI MS analysis: control experiment for oligonucleotide 5'-(ACCACGTGGTG_[2'-TEMPO])-3'.

Appendix B

Toward protein NMR at physiological concentrations by hyperpolarized water—Finding and mapping uncharted conformational spaces

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Supporting Information

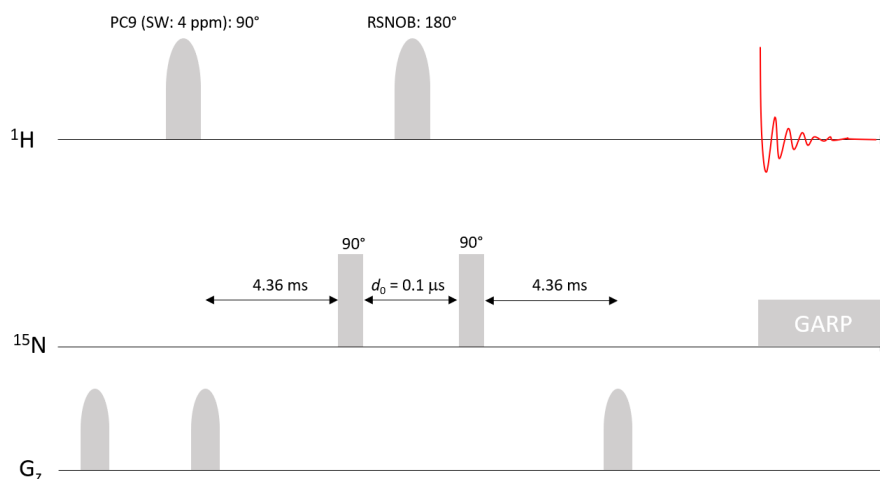


Figure S1. Pulse Sequence for DDNP Detection. Pulse sequence used for detection in the DDNP experiments. We used PC9 and RSNOB41 selective 90° and 180° pulses covering a bandwidth of 4 ppm centered around a carrier frequency of 9.5 ppm to excite and invert the protons. The pulse lengths were 3495 and 1165 μs, respectively. The 90° pulse for the ¹⁵N channel was 15 μs long. Heteronuclear decoupling was achieved using the GARP42 scheme as preinstalled in Bruker TOPSPIN 4.

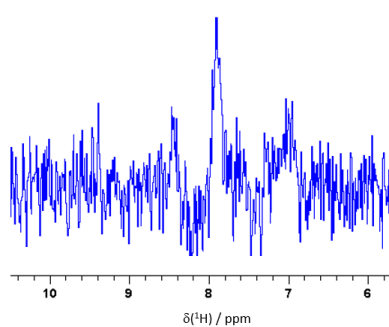


Figure S2. Thermal equilibrium spectrum. The thermal equilibrium spectrum recorded directly after the DDNP experiments in 98% deuterated buffer. This spectrum was used to calculate the signal enhancement.

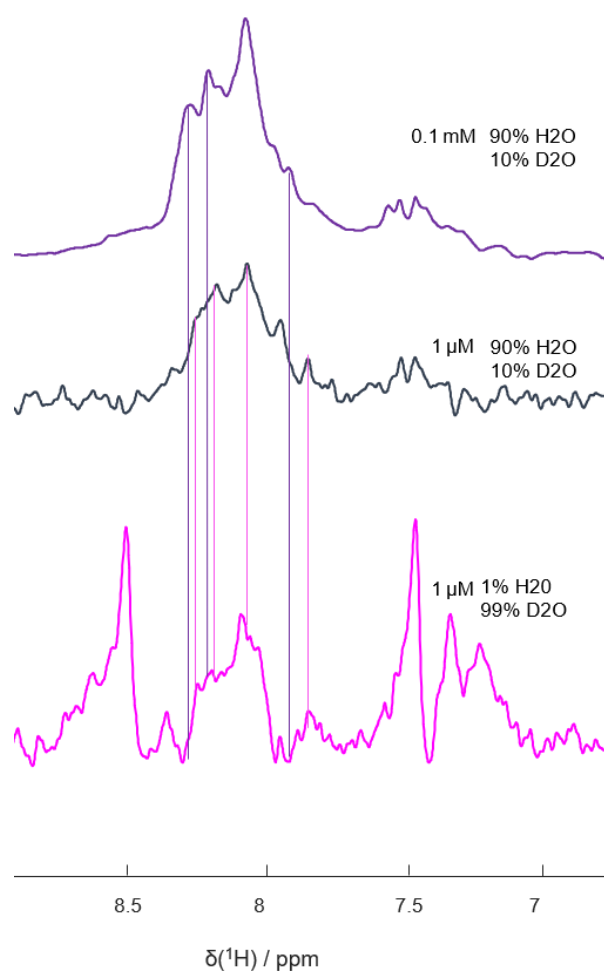


Figure S3. Enlarged spectra. Zoom onto the spectra of Fig. 1d. The magenta lines show similarities between the low concentration spectra, while the purple lines show differences between the high and low concentration spectra.

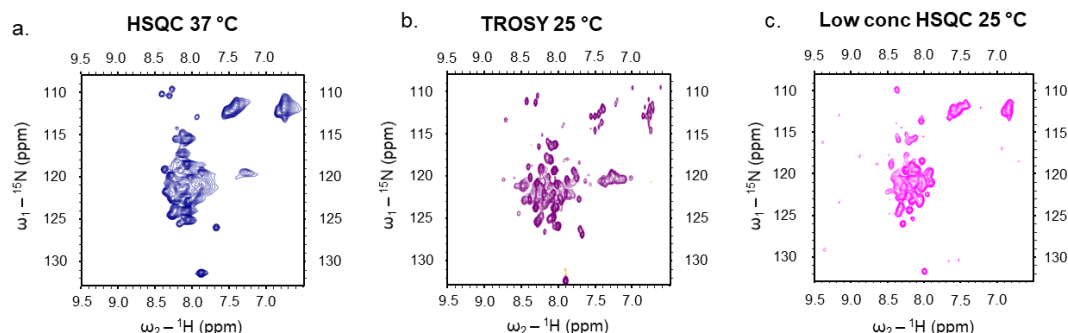


Figure S4. Thermal Equilibrium 2D NMR. MAX ^{15}N spectra at high concentration (0.3 mM) and low concentration (1 μM). a) HSQC experiment of 0.3 mM MAX ^{15}N collected at 37 $^{\circ}\text{C}$ on a 700 MHz Bruker NEO spectrometer. Most signals overlap, indicating that MAX mainly populates its unfolded state in these conditions. b) 0.3 mM MAX ^{15}N TROSY experiment at 25 $^{\circ}\text{C}$. on a 700 MHz Bruker NEO spectrometer At this temperature, the folded conformation of MAX dominates the spectrum. c) HSQC spectrum of MAX ^{15}N at 1 μM recorded at 25 $^{\circ}\text{C}$. The spectrum indicates a conformation that is different from both the spectra collected at high concentrations. Note that this spectrum was recorded on a high-field spectrometer (600 MHz) equipped with a IHe-cooled TXI cryogenically cooled probe (compared to a 500 MHz spectrometer equipped with a BBFO prodigy probe used for the DDNP experiments) to obtain a sufficiently high signal-to-noise ratio. In addition, we did not use the DNP-buffer (98% D_2O and contaminated with TEMPOL) but switched to a radical-free MES buffer in 90% H_2O /10% D_2O at pH 5.5. These changes significantly boosted the signal amplitudes in thermal equilibrium and enabled recording the spectrum. (Note that the samples for the thermal equilibrium spectra were prepared conventionally, such that pressure or temperature shocks can be excluded.)

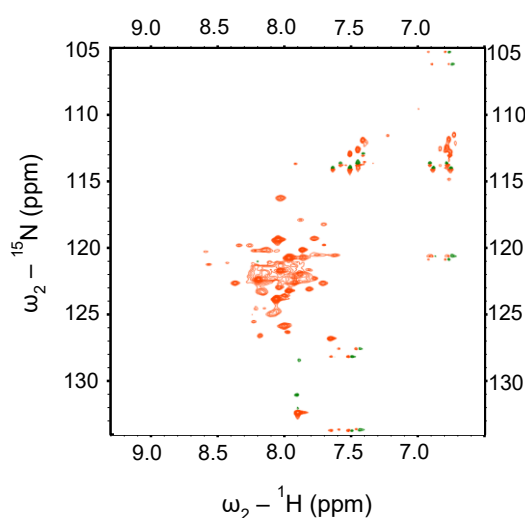


Figure S5. Influence of Buffer Strength. TROSY experiment on a 700 MHz Bruker NEO spectrometer of MAX ^{15}N at high concentration (0.3 mM) in 30-fold diluted MES buffer (0.83 mM).

mM NaCl, 0.83 mM MES, 3.33 mM ArgHCl in ddH₂O) performed on a 700 MHz Bruker spectrometer at 25 °C. Buffer effects can be excluded.

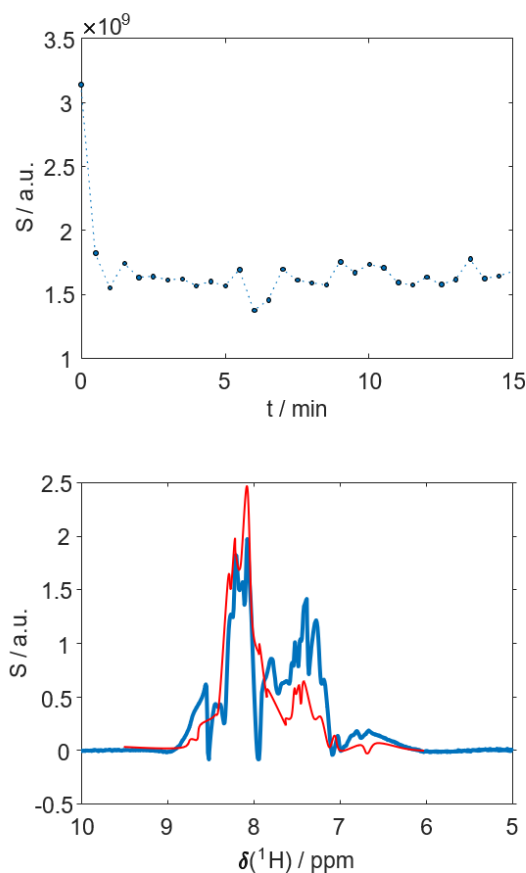


Figure S6. Reference DDNP Experiment at High Concentrations. Signal decay (top) and line shape (bottom) of MAX in hyperpolarized water at a final concentration of 0.1 mM. The elongated coiled-coil folded dimer does not receive as high signal boosts from the solvent as the monomer likely due to its short relaxation times. The spectrum (blue line, bottom) resembles that of the MAX dimer (red line) showing that injection of hyperpolarized water does not perturb the protein structure although the signal enhancement is not homogeneous across the spectrum (as expected when dissolving a protein in hyperpolarized water).

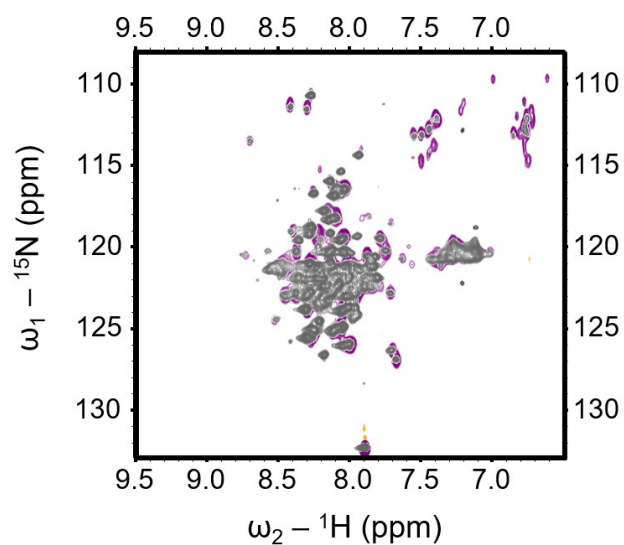


Figure S7. Protein Stability Check. Overlay of a ^1H - ^{15}N TROSY of MAX at 0.1 mM in MES buffer at 25°C before (purple) and after re-concentration in TEMPOL-free MES after the dissolution experiment (grey). The spectra are identical showing that the protein wasn't damaged during the DDNP experiment.

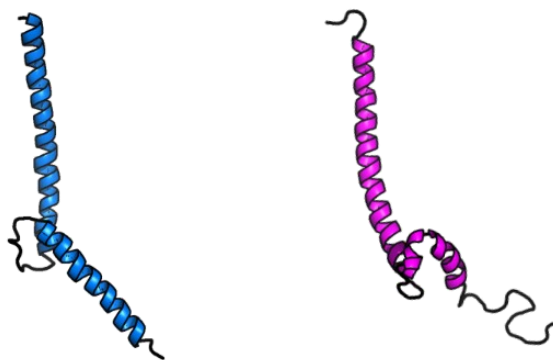


Figure S8. MD Starting Structure Validation. Comparison between the monomeric MAX structure predicted by Phyre2 (in blue) and the monomer extrapolated from the known structure of MAX dimer (in magenta). Despite slight differences at the N-terminus, both structures show the documented basic-helix-loop-helix-ZIP conformation.

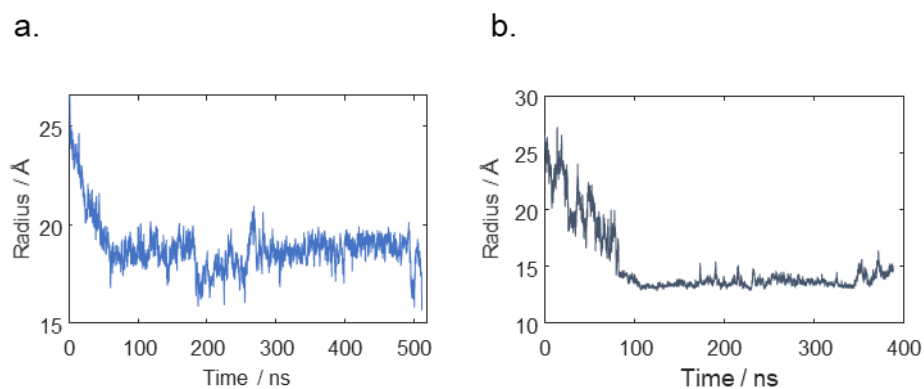


Figure S9. R_h Trajectories. Radius of hydration over time for the two MD replica (next to the data in Fig 2 of the main text).

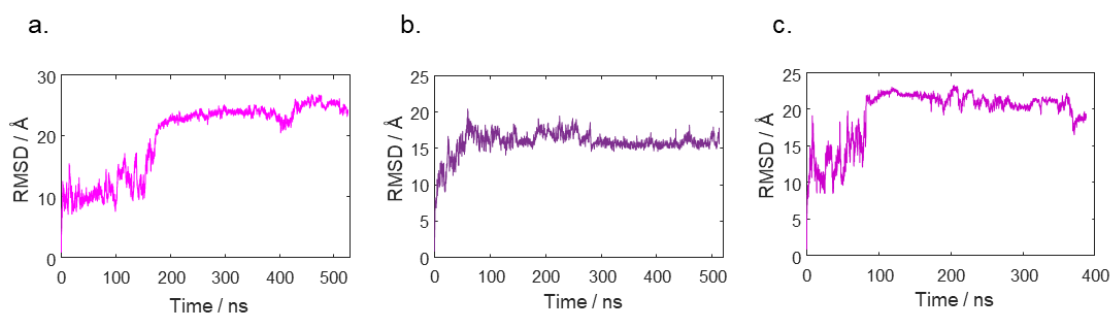


Figure S10. RMSD Trajectories. Average root-mean-square deviation from the starting structure coordinates of the first (a.), second (b.) and third (c.) MD replica.

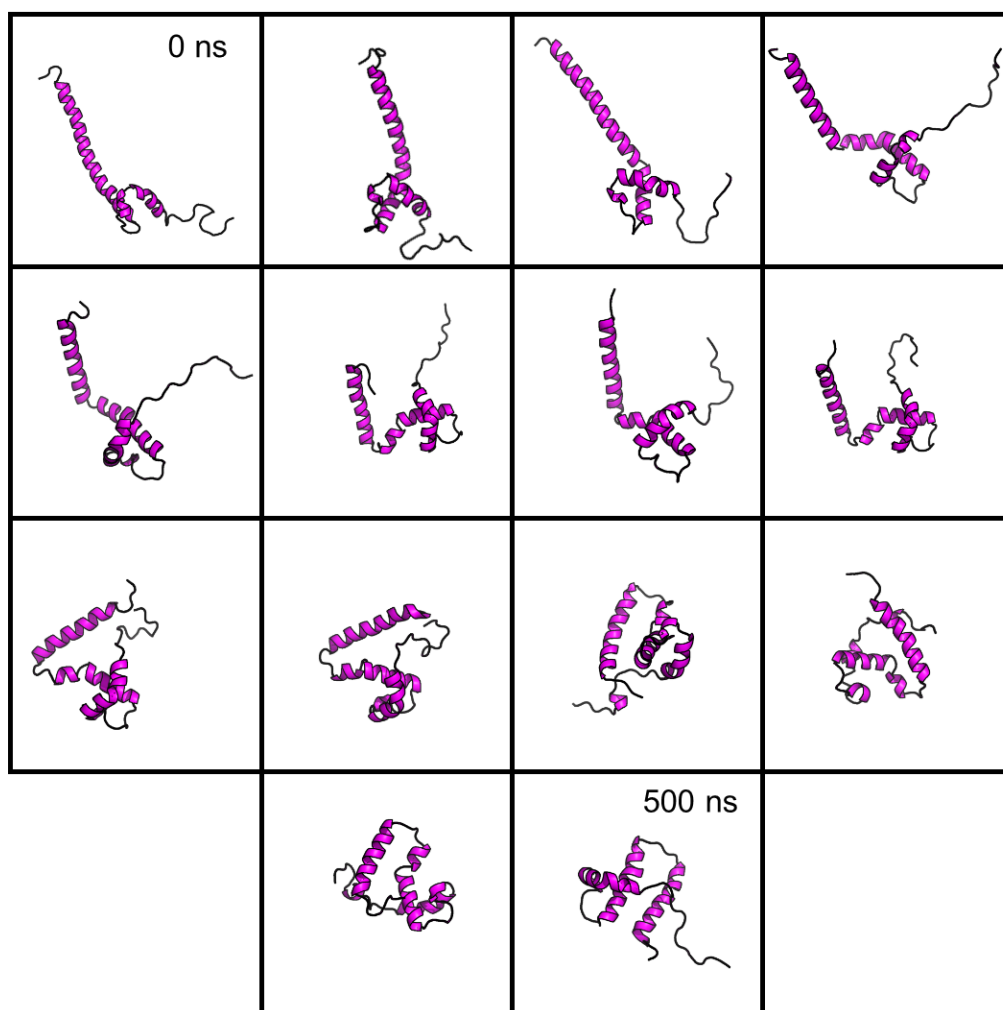


Figure S11. Conformations Sampled in MD run 1. Snapshots of the first MD simulation of MAX.

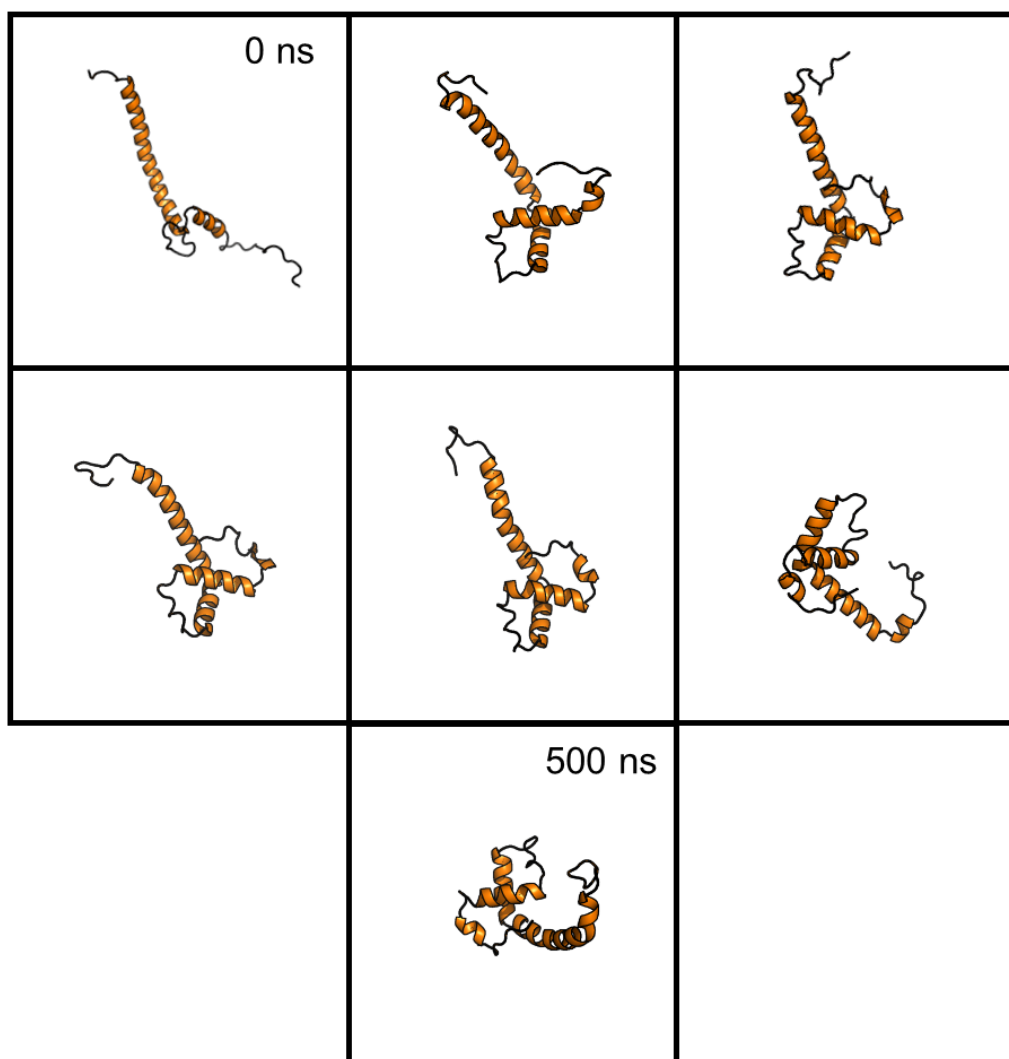


Figure S12. Conformations Sampled in MD run 2. Snapshots of the second MD simulation of MAX.

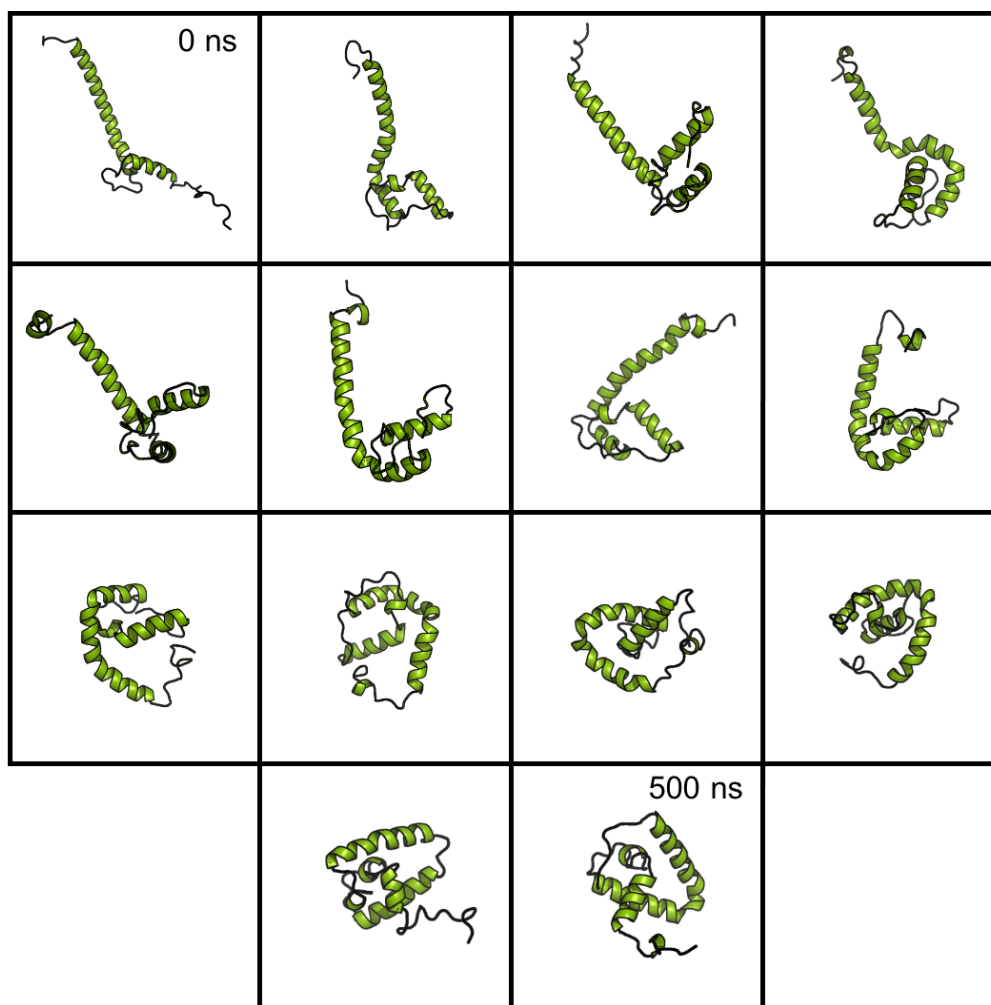


Figure S13. Conformations Sampled in MD run 3. Snapshots of the third MD simulation of MAX.

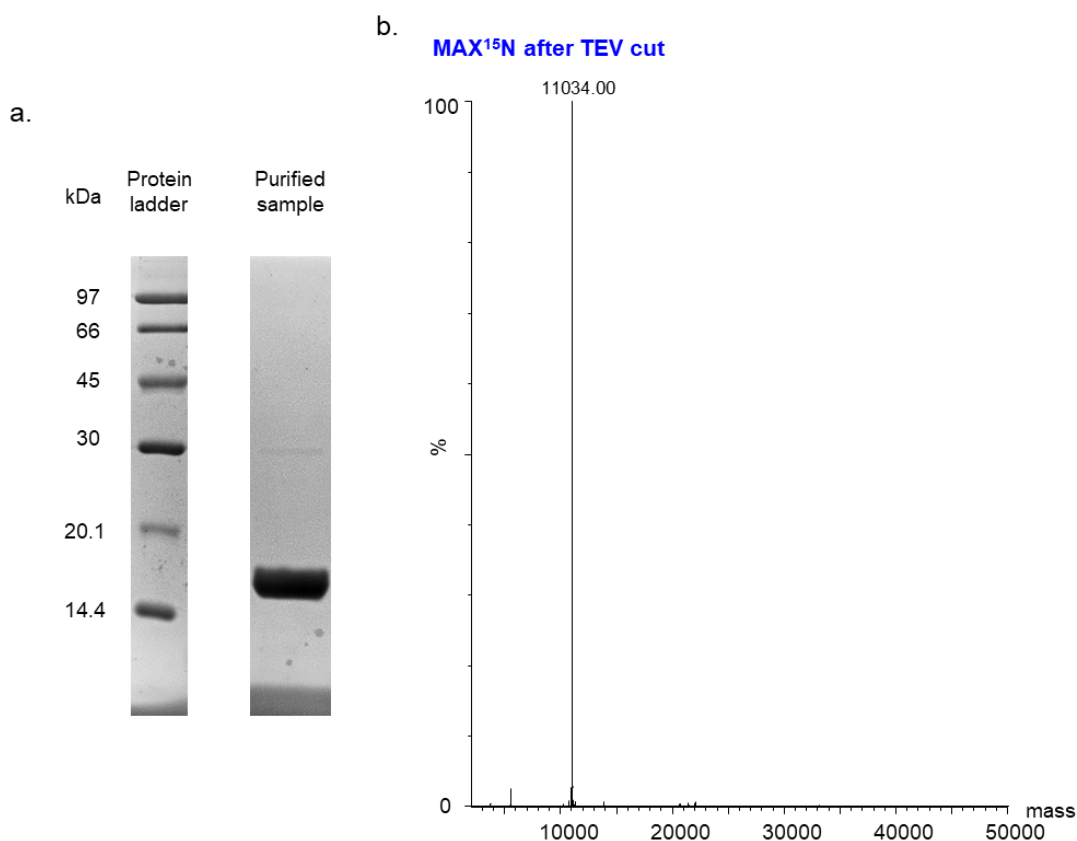


Figure S14. Protein Purification. Elution profile of MAX ¹⁵N after affinity chromatography purification. a) SDS-PAGE of purified His-tagged MAX (¹⁵N labeled). The protein mass should be approximately 15 kDa, while the expected uncut protein has a MW of ~13.99 kDa. The 1 kDa discrepancy is due to the high net charge of MAX even in the presence of SDS. b) Deconvolute mass spectrum of a purified MAX ¹⁵N sample. The mainly detected mass (11.034 ± 0.05 kDa) is consistent with the expected mass of a homogeneously labeled MAX ¹⁵N (11.085 kDa).

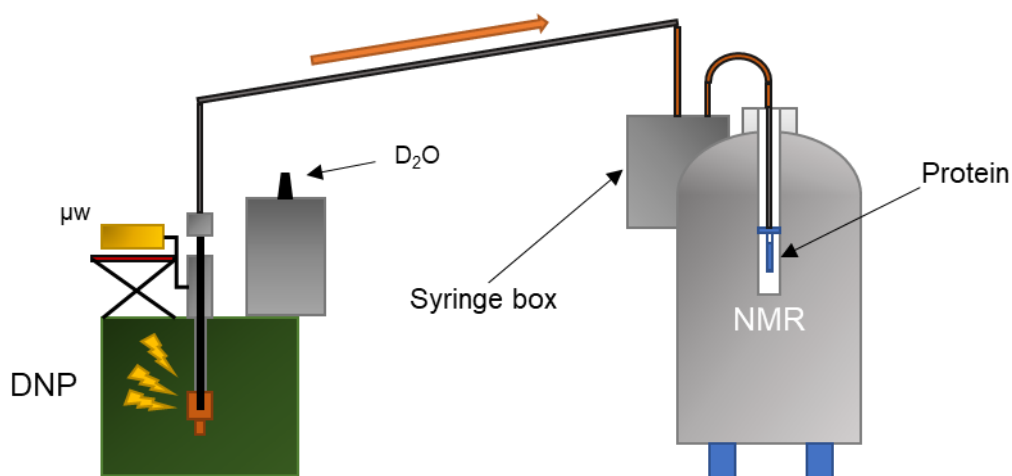


Figure S15. DDNP Setup. Schematic representation of the DDNP set up at the University of Vienna. The DNP sample containing TEMPOL is hyperpolarized in a cryostat by microwave irradiation. After polarization, hot D_2O at high pressure is injected into the polarizer, dissolving the sample that is consequently transferred through a magnetic tunnel into a “syringe box” injection system that controls the injected volume.

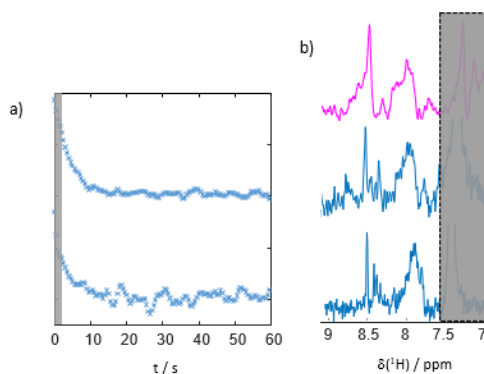


Figure S16. Reproducibility checks. Reproductions of the DDNP experiments. a) Signal intensity decay for two repetitions. b) Spectra obtained from the repetition experiments (blue) compared to the one in the main text (magenta). The grey boxes indicate regions excluded from the analysis as in the main text.

Table S1. Sparta+ results. Averaged signal intensity in dependence of the chemical shift from the SPARTA+ predictions for the first, second and third MD simulation run.

first simulation			second simulation			third simulation	
CS	S / a.u.		CS	S / a.u.		CS	S / a.u.
6.800	0.000		6.740	0.000		6.740	0.042
6.828	0.000		6.769	0.000		6.769	0.000
6.857	0.120		6.798	0.000		6.798	0.000
6.885	0.000		6.827	0.000		6.827	0.000
6.913	0.000		6.856	0.000		6.856	0.000
6.941	0.000		6.884	0.000		6.884	0.083
6.970	0.000		6.913	0.000		6.913	0.000
6.998	0.000		6.942	0.000		6.942	0.000
7.026	0.000		6.971	0.000		6.971	0.000
7.055	0.000		7.000	0.000		7.000	0.000
7.083	0.000		7.029	0.000		7.029	0.083
7.111	0.000		7.058	0.000		7.058	0.000
7.139	0.000		7.087	0.000		7.087	0.000
7.168	0.000		7.116	0.000		7.116	0.000
7.196	0.000		7.144	0.000		7.144	0.167
7.224	0.000		7.173	0.000		7.173	0.000
7.253	0.080		7.202	0.000		7.202	0.292
7.281	0.000		7.231	0.000		7.231	0.083
7.309	0.000		7.260	0.043		7.260	0.083
7.337	0.040		7.289	0.000		7.289	0.042
7.366	0.000		7.318	0.000		7.318	0.167
7.394	0.040		7.347	0.000		7.347	0.083
7.422	0.120		7.376	0.000		7.376	0.208
7.451	0.040		7.404	0.000		7.404	0.125
7.479	0.240		7.433	0.130		7.433	0.417
7.507	0.080		7.462	0.000		7.462	0.375
7.535	0.120		7.491	0.000		7.491	0.500
7.564	0.160		7.520	0.000		7.520	0.333
7.592	0.000		7.549	0.304		7.549	0.208
7.620	0.240		7.578	0.174		7.578	0.458
7.648	0.480		7.607	0.174		7.607	0.333
7.677	0.240		7.636	0.391		7.636	0.625
7.705	0.440		7.664	0.261		7.664	0.333
7.733	0.200		7.693	0.565		7.693	0.333
7.762	0.320		7.722	0.261		7.722	0.292
7.790	0.400		7.751	0.870		7.751	0.708
7.818	0.320		7.780	0.478		7.780	0.625

7.846	0.200		7.809	0.783		7.809	0.792
7.875	0.600		7.838	0.565		7.838	0.500
7.903	0.280		7.867	0.609		7.867	0.458
7.931	0.240		7.896	0.391		7.896	0.583
7.960	0.840		7.924	0.391		7.924	0.458
7.988	0.360		7.953	1.000		7.953	0.458
8.016	0.560		7.982	0.652		7.982	0.292
8.044	0.560		8.011	0.870		8.011	0.583
8.073	0.640		8.040	0.522		8.040	0.458
8.101	0.680		8.069	0.739		8.069	0.125
8.129	0.680		8.098	0.652		8.098	0.542
8.158	0.760		8.127	0.870		8.127	0.417
8.186	0.360		8.156	0.652		8.156	0.417
8.214	0.520		8.184	0.522		8.184	0.583
8.242	0.560		8.213	0.652		8.213	0.458
8.271	0.480		8.242	0.174		8.242	0.500
8.299	0.720		8.271	0.783		8.271	0.500
8.327	0.400		8.300	0.391		8.300	0.375
8.356	0.520		8.329	0.652		8.329	1.000
8.384	0.320		8.358	0.565		8.358	0.958
8.412	0.160		8.387	0.435		8.387	0.625
8.440	0.400		8.416	0.130		8.416	0.292
8.469	1.000		8.444	0.522		8.444	0.583
8.497	0.600		8.473	0.304		8.473	0.375
8.525	0.720		8.502	0.957		8.502	0.458
8.554	0.520		8.531	0.478		8.531	0.458
8.582	0.320		8.560	0.522		8.560	0.292
8.610	0.600		8.589	0.174		8.589	0.083
8.638	0.440		8.618	0.478		8.618	0.292
8.667	0.480		8.647	0.609		8.647	0.375
8.695	0.360		8.676	0.391		8.676	0.333
8.723	0.440		8.704	0.696		8.704	0.250
8.752	0.160		8.733	0.304		8.733	0.125
8.780	0.160		8.762	0.217		8.762	0.333
8.808	0.320		8.791	0.000		8.791	0.292
8.836	0.200		8.820	0.087		8.820	0.125
8.865	0.360		8.849	0.261		8.849	0.167
8.893	0.000		8.878	0.174		8.878	0.125
8.921	0.160		8.907	0.304		8.907	0.000
8.949	0.080		8.936	0.174		8.936	0.000
8.978	0.200		8.964	0.000		8.964	0.042
9.006	0.000		8.993	0.043		8.993	0.167
9.034	0.120		9.022	0.130		9.022	0.125

9.063	0.120		9.051	0.261		9.051	0.000
9.091	0.240		9.080	0.174		9.080	0.000
9.119	0.280		9.109	0.261		9.109	0.000
9.147	0.080		9.138	0.000		9.138	0.000
9.176	0.000		9.167	0.087		9.167	0.000
9.204	0.000		9.196	0.000		9.196	0.000
9.232	0.040		9.224	0.087		9.224	0.000
9.261	0.000		9.253	0.043		9.253	0.000
9.289	0.000		9.282	0.000		9.282	0.000
9.317	0.000		9.311	0.087		9.311	0.000
9.345	0.000		9.340	0.000		9.340	0.000
9.374	0.000		9.369	0.000		9.369	0.000
9.402	0.000		9.398	0.000		9.398	0.083
9.430	0.000		9.427	0.000		9.427	0.000
9.459	0.000		9.456	0.000		9.456	0.000
9.487	0.000		9.484	0.000		9.484	0.000
9.515	0.000		9.513	0.000		9.513	0.000
9.543	0.000		9.542	0.000		9.542	0.000
9.572	0.000		9.571	0.000		9.571	0.000
9.600	0.000		9.600	0.000		9.600	0.000

Appendix C

Nuclear Overhauser spectroscopy in hyperpolarized water - chemical vs. magnetic exchange†

Supplementary Information

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Jörg,^{b,c} Christian Schröder^c and Dennis Kurzbach^{a,*}

1. Experimental materials and methods

1.1 DNP

Spin hyperpolarization of 150 μL of a flash-frozen (vitrified) 15 mM 4-Hydroxy-2,2,6,6-tetramethylpiperidinyloxy (TEMPOL) solution containing 10% H_2O , 40% D_2O , and 50% glycerol- d_8 was performed at 1.4 K and a magnetic field of 6.7 T in a cryostat-magnet apparatus, purchased from Cryogenic Ltd. The sample was irradiated with microwaves at a frequency of 188.08 GHz using a Virginia Diodes Inc. (VDI) microwave source and frequency amplifier. The hyperpolarization build-up was monitored through a 400 MHz Bruker Avance III spectrometer modified to operate at 285.3 MHz. A homemade tune and matching system was combined with the 400 MHz console. 1° detection pulses every 5 s were applied to monitor the proton polarization build-up. For DNP and sample dissolution, the system described in Ref¹ was used.

After polarizing for 3 h, 5 mL of preheated D_2O were injected at a pressure of 1.5 MPa onto the sample for dissolution. The solvent was pressurized in a homemade pressure heater combined with an Arduino system, controlled by a home-written MATLAB code. The hyperpolarized solution was then transferred with pressurized helium gas at 0.6 MPa to an NMR tube placed into a 500 MHz Bruker NEO spectrometer, equipped with a Prodigy BBFO cryo-probe. In the NMR tube, the DNP sample mixed rapidly with 200 μL of 2-(N-morpholino)ethanesulfonic acid (MES) buffer (25 mM NaCl, 25 mM MES, 100 mM arginine hydro chloride (ArgHCl), pH 5.5), reconstituted in D_2O waiting inside the NMR spectrometer. Therefore, the MES buffer was diluted up to a volume of 5 mL, with a final ArgHCl concentration of 4 mM. The detection was carried out employing using 1° hard pulses every

second using the 'zg2d' pulse sequence implemented in Bruker TopSpin 4. The dDNP data were baseline corrected and fitted to a Lorentzian function using the MATLAB R2019a software to extrapolate signal intensities.

1.2 NMR

The series of conventional ^1H - ^1H nuclear Overhauser enhanced spectroscopy (NOESY) spectra was acquired on a 600 MHz Bruker NEO spectrometer equipped with a cryogenically cooled TCI probe. The "noesyfpgpphrs190" pulse sequence was used as implemented in the Bruker TopSpin 4 pulse program library. Twelve NOESY experiments with different mixing times (10, 20, 30, 40, 50, 70, 100, 150, 200, 300, 500, and 1000 ms) were collected on a 100 mM arginine solution in 600 μL MES buffer (pH 5.5.) containing 10% D_2O as field lock solvent. Quadrature detection was achieved using the States-Time Proportional Phase Incrementation (TPPI) method.

All NOESY spectra were measured with spectral widths of 9615.3 Hz in the F2 dimension and 7202.1 Hz in the F1 dimension. We recorded 32 scans. The processing and analysis of the collected data were carried out on Topspin 4.0.7, SPARKY, and MATLAB R2019a. The NOESY spectra were zero-filled and Fourier transformed prior to peak assignment. The arginine peaks were assigned according to SDBS entry 1143.

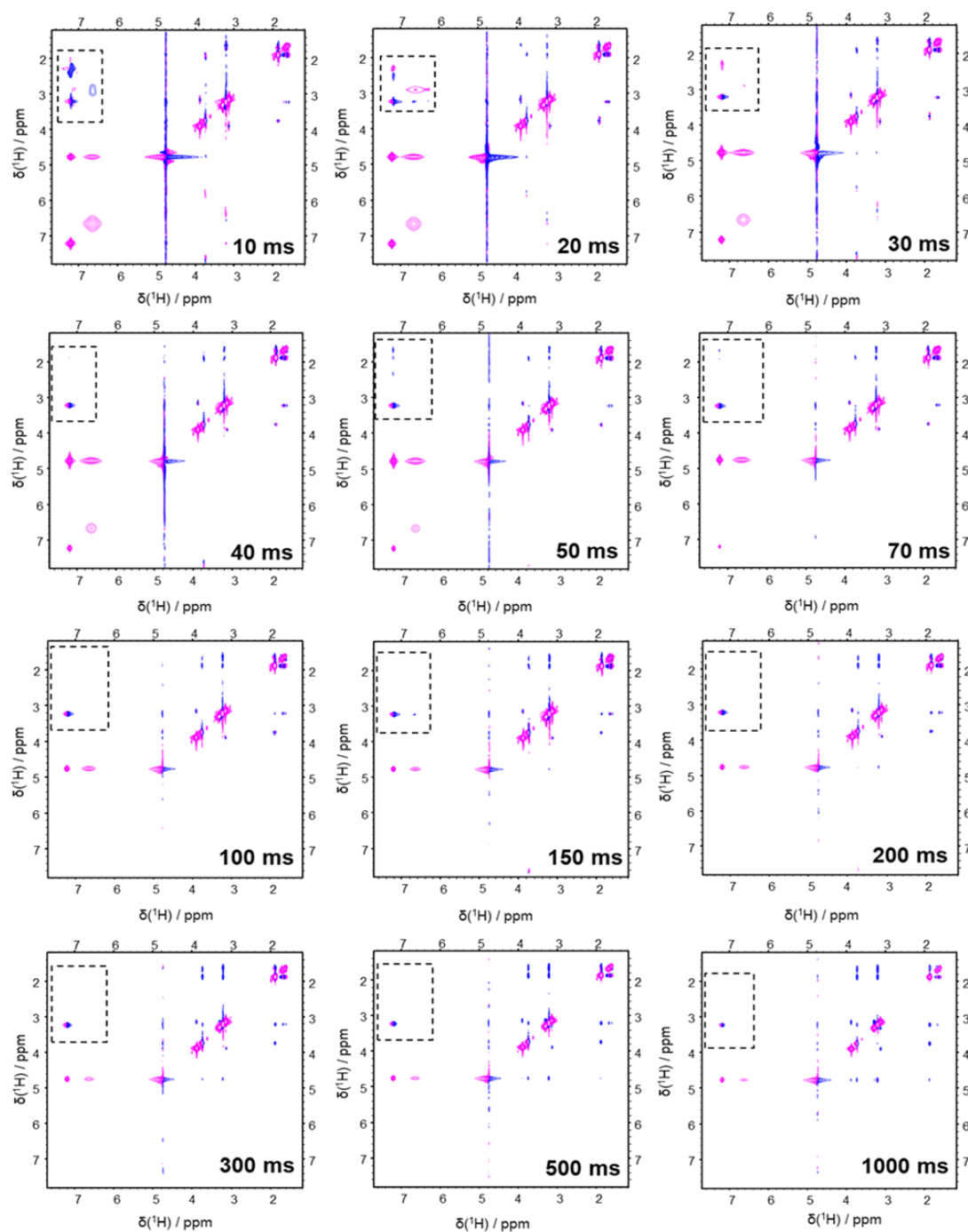


Figure S1: Thermal equilibrium NOESY experiment on a 100 mM ArgHCl solution with different mixing times (indicated on the bottom right of each spectrum).

2. Computational analysis of the NOE

We used the CHARMM36 (Chemistry at Harvard Macromolecular Mechanics 36) force field,²⁻⁵ as implemented in CHARMM-GUI⁶ for MD simulations of ten arginine and ten chloride counter ions were surrounded by 6788 water molecules (TIP3P model⁷⁻⁸). The initial

intermolecular geometries were optimized by energy minimization, before equilibration for 5 ns as isothermal-isobaric ensembles (NpT ; $T = 300$ K, $p = 1$ atm) until the simulation box lengths converged at $a = 60$ Å. The trajectories were produced as isothermal-isochoric ensembles (NVT , $T = 300$ K) using a Nosé-Hoover thermostat⁹⁻¹⁰ with periodic boundary conditions in OpenMM.¹¹ High-frequency vibrations of covalent bonds involving hydrogen atoms were constrained using the Shake algorithm.¹² Non-bonded interactions were cut off at 12 Å, and electrostatic interactions were treated using the Ewald method. The simulation trajectories were read into a Python3-based analysis program using the MD-Analysis module.¹³⁻¹⁴ Computational bottlenecks were accelerated using the high-performance hybrid language Cython.

The NOE is a magnetization transfer phenomenon mediated by the nuclear dipoles and is a function of the distance and not the chemical connectivity of the interacting nuclei I and S . Using Redfield theory¹⁵ and Solomon's differential equations¹⁶

$$\frac{d\langle\Delta I_z\rangle}{dt} = \rho_{II}\langle\Delta I_z\rangle + \sigma_{SI}\langle\Delta I_z\rangle \quad (1)$$

$$\frac{d\langle\Delta S_z\rangle}{dt} = \sigma_{IS}\langle\Delta S_z\rangle + \rho_{SS}\langle\Delta S_z\rangle \quad (2)$$

the kinetics of the two spins I and S can be related to the self- (ρ) and cross- (σ) relaxation rates. Following Redfield theory, these rates can be obtained from the spectral density function $J(\nu)$:

$$\rho_{II} = 0.6J(\nu_I + \nu_S) + 0.3J(\nu_S) + 0.1J(\nu_I - \nu_S) \quad (3)$$

$$\sigma_{IS}^{NOE} = 0.6J(\nu_I + \nu_S) - 0.1J(\nu_I - \nu_S) \quad (4)$$

$$\sigma_{IS}^{ROE} = 0.3J(\nu_S) + 0.2J(\nu_I - \nu_S) \quad (5)$$

with $\rho_{II} = \sigma_{IS}^{NOE} + \sigma_{IS}^{ROE}$. As shown in ¹⁷, the ratio $\sigma_{IS}^{NOE}/\sigma_{IS}^{ROE}$ is not plagued by long-range contributions whereas the individual σ_{IS}^{NOE} and σ_{IS}^{ROE} are affected.

A similar effect is also visible in the water residence times. In Table S1, the residence times of water molecules within four Å and six Å of the respective arginine hydrogen are displayed.

Table S1: Experimental initial signal enhancements $1/\epsilon_i$ and computational values for the arginine hydrogens. For the coordination number and the residence time τ only water molecules with hydrogens closer than four/six Å to the corresponding arginine hydrogen were taken into account in the simulation.

	[a. u.]	[a. u.]		# H ₂ O	τ [ps]	# H ₂ O	τ [ps]
H ^{α}	—	4.98	0.986	4.6	4.0	24	8.3
H ^{β}	2.30	4.95	0.984	3.7	1.8	24	7.6
H ^{γ}	2.22	4.70	0.981	3.9	1.9	23	7.2
H ^{δ}	1.24	4.55	0.981	4.2	2.9	23	6.5

Using the larger distance criterion $r < 6$ Å, the cross-relaxation rate $\sigma^{NOE} = \sigma_L$ shows the same trend as the residence times: The longer σ_L , the slower τ . Increasing the distance criterion results in a larger overlap of the water neighborhood of the arginine hydrogens and consequently blurs the trend.

Interestingly, the ranking of the residence times is reversed for the shorter distance criterion of four Å (see Table S1.). Here, only more or less direct water neighbors of the arginine hydrogens are taken into account. This situation resembles the short-range effect of the ratio $\sigma_L^{NOESY}/\sigma_R^{ROESY}$ which has been used for protein surface hydration mapping.¹⁸ However, since arginine is a small molecule and all the water residence times are all similarly short, the ratio $\sigma_L^{NOESY}/\sigma_R^{ROESY}$ becomes almost uniform as has been previously found for disaccharide hydration.¹⁹

It should be noted that the detected samples contained 1.4% glycerol-d8, which was used as (necessary) glassing agent in the dDNP experiments. It is well-documented that glycerol can influence the hydration shell of biomolecules. However, concentrations of >10% v/v are typically required to observe such effects.²⁰ Hence, in our MD simulations, the presence of glycerol was neglected. The correspondence between the computed and experimentally observed relaxation rates justifies this simplification. Nevertheless, this potential bias of possible changes in hydration shells should be considered when precise quantifying computed NOE rates is required. Likewise, the presence of D₂O in the hyperpolarized samples was not considered during the MD simulations. However, deuteron exchange and dilution of water by D₂O can indeed impact NOE rates as well as proton exchange efficiencies. This potential bias could experimentally yet not be confirmed as only hyperpolarized protons

were detected. However, again, it should be kept in mind that the degree of deuteration could potentially bias the MD simulations when aiming to extract precise NOE rates.

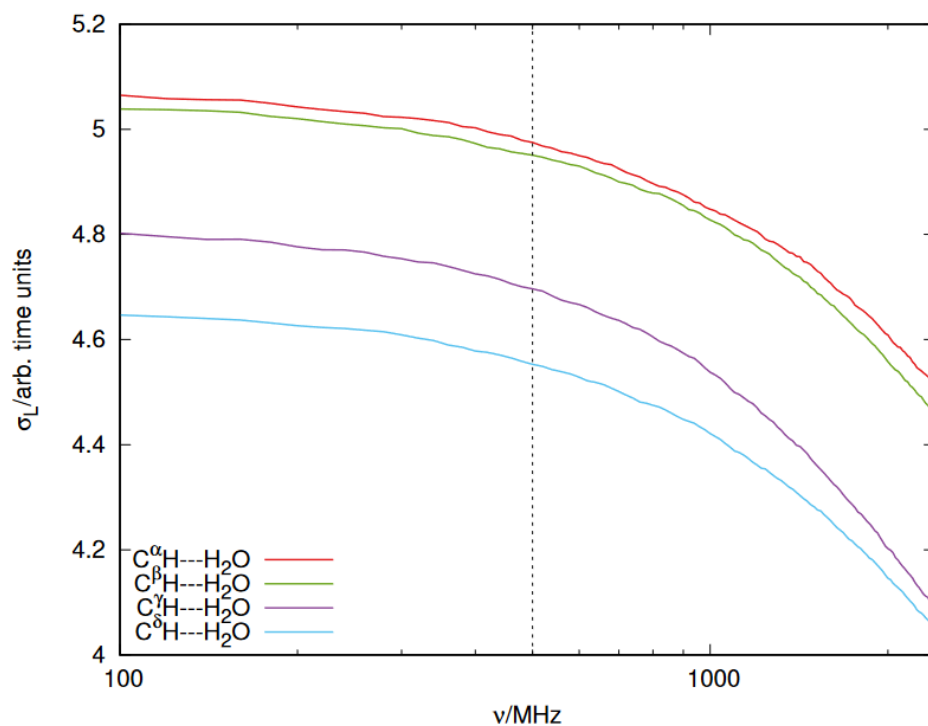


Figure S2: Predicted solvent NOE rates (colored solid lines) at different resonance frequencies for each non-exchangeable arginine proton. The black dashed line indicates the experimental Larmor frequency.

3. Additional experimental data

To showcase the application of our method to other biological systems and verify that the approach is suitable for different amino acids, too, we performed similar dDNP experiments to study the transfer of polarization from water to polyaspartate (pAsp) at a concentration of 1 mg / mL. We followed the same protocol outlined above. We observed that, while the signal of the H^β protons is readily identified at 2.6 ppm, the signal of the H^α proton was again covered by the broad water peak. The enhanced signals of the amide protons were detected at 8.2 ppm. The hyperpolarized proton signals decay within approximately 20 seconds. As for the arginine dDNP experiments, $t = 0$ corresponds to the start of acquisition. The amide proton signal decays monotonic, as expected since the enhancement is based primarily on

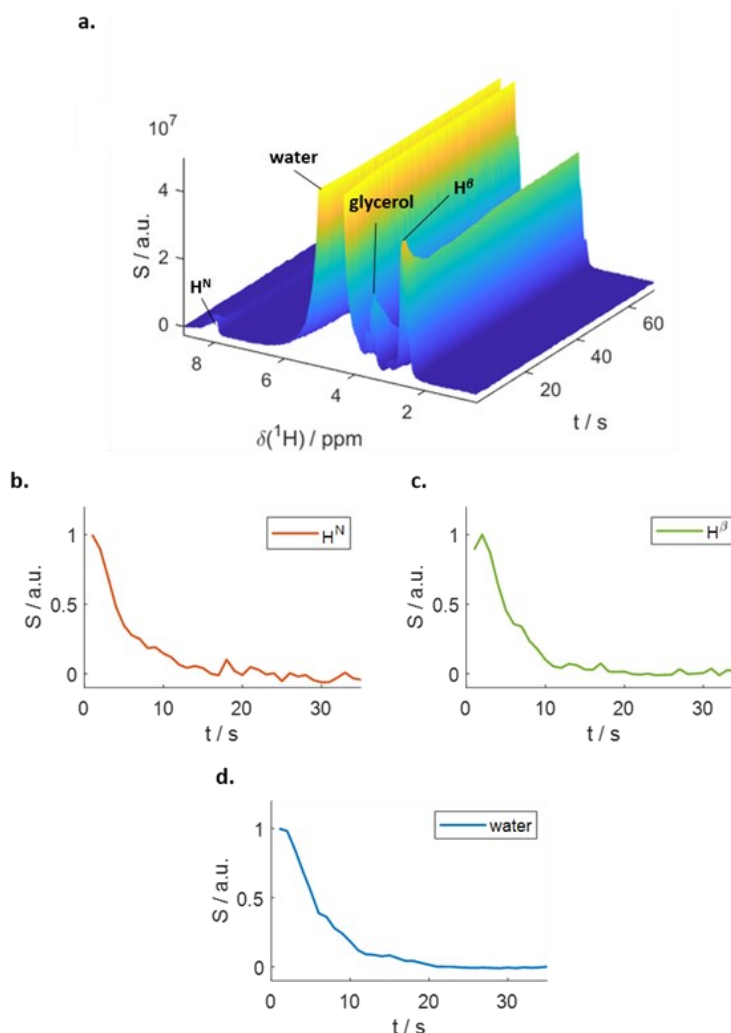


Figure S3: Hyperpolarized water experiments with polyaspartate. a) Time series of proton NMR spectra of pAsp in hyperpolarized water. At $t=0$, signals of the amide protons, the water, the glycerol, and the aliphatic protons are discernible. b) Intensities of the pAsp amide protons. The signal decay is strictly monoexponential. c) Intensities of the H^β signal. The maximum intensity of the signal is reached two seconds after start of the detection, indicating the contribution of NOE to the pAsp system. d) Intensity of the water signal.

chemical proton exchange. Contrarily, the H^{δ} signal reaches its maximum intensity two seconds after the beginning of the detection period as a result of the slower exchange-relayed NOE rate. Importantly, the observed signal build-ups and decays differ from those observed for arginine. As the hydration shells of a single amino acid and a biomacromolecule necessarily differ, such an observation can be expected. Therefore, the possibility arises to determine intramolecular interactions or conformational changes from different solvent interaction.

4. References

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Appendix D

SUPPORTING INFORMATION

Formation and evolution of nanoscale calcium phosphate precursors under biomimetic conditions

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Figure S1

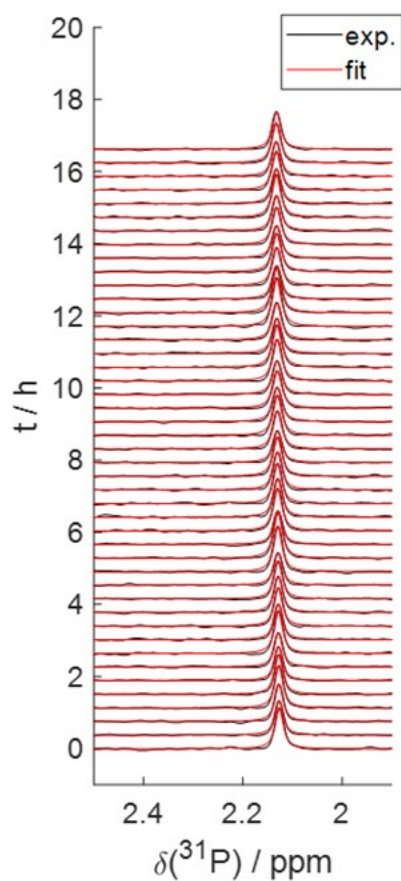


Figure S1. Negative control experiment of 1 mM Pi solution in which all salts (except Pi) from mSBF have been replaced by NaCl at equivalent concentrations to keep ionic strength constant (in HEPES buffer at pH 7.4). The ^{31}P NMR time traces are constant over time and exhibit one single resonance of similar position and line width to mSBF at $t = 0$. This control allows us to assign unambiguously the 2.18 ppm ^{31}P resonance of mSBF to free Pi.

Please note that mSBF solution without Pi cannot be used as a control because of calcite precipitation in these conditions (see Oyane *et al. J. Biomed. Res. A* **2003**, 64A, 339-348).

Figure S2

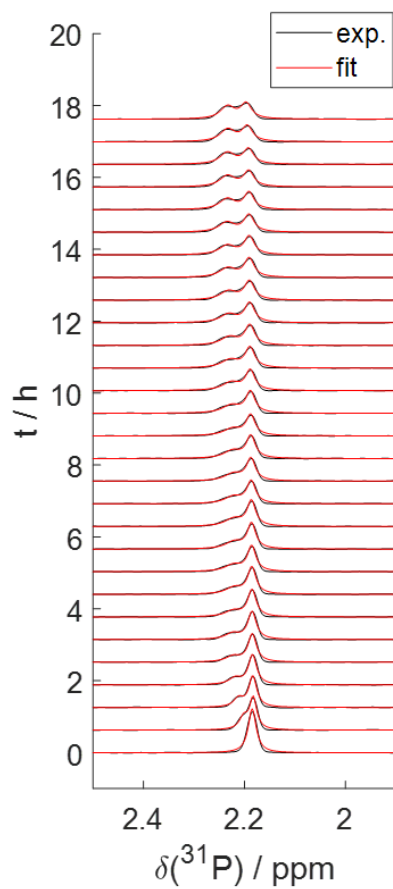


Figure S2. ^{31}P NMR time traces of 5 mM P_i in mSBF as used in the DOSY experiments. The evolution is qualitatively the same as in the case of neat mSBF. However, the evolution is slowed down due to the excess in phosphate, which enables the detection of longer DOSY experiments in the slow exchange regime.

Figure S3

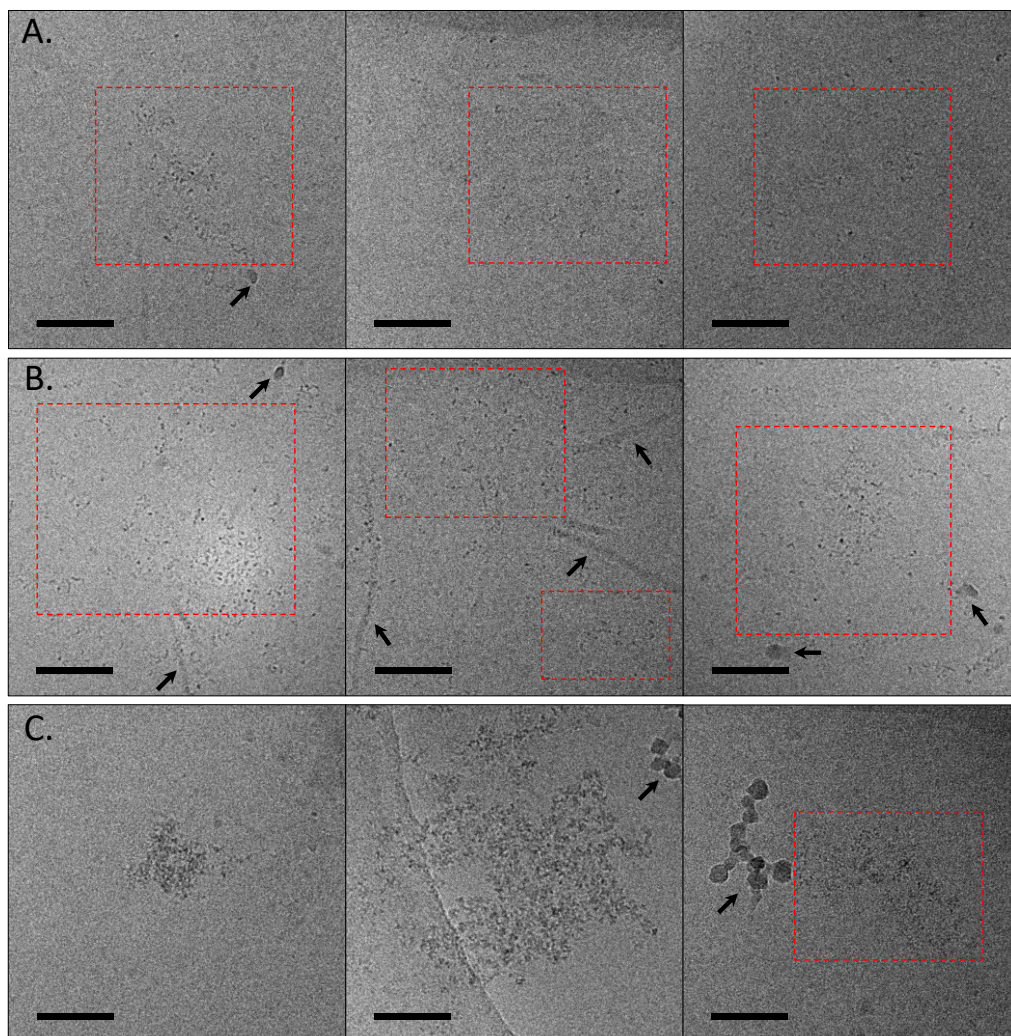


Figure S3. Evolution of PNS formation in mSBF over time revealed through cryo-TEM. (a) 1h, (b) 5.5h and (c) 23h after preparation. Scale bars = 50 nm. Red rectangles surround PNS units, black arrows point pollutions due to the freezing process, the plasma treatment of the grid or the solution container

Figure S4

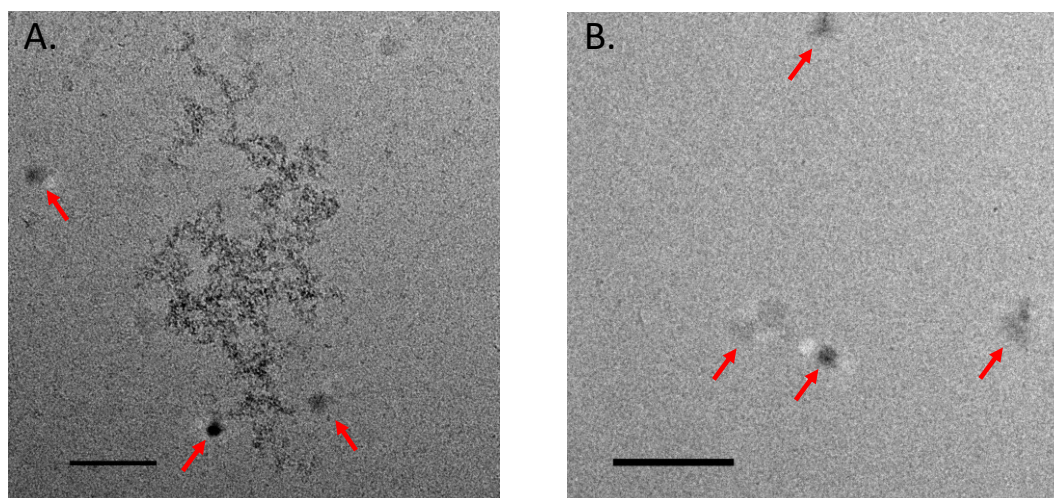


Figure S4. Cryo-TEM micrographs corresponding to the zones analyzed through EDX. (A) zone containing PNS. (B) zone empty of PNS (B.). Scalebar = 100 nm. Red arrows exhibit pollutions due to the freezing process and the plasma treatment of the grid.

Figure S5

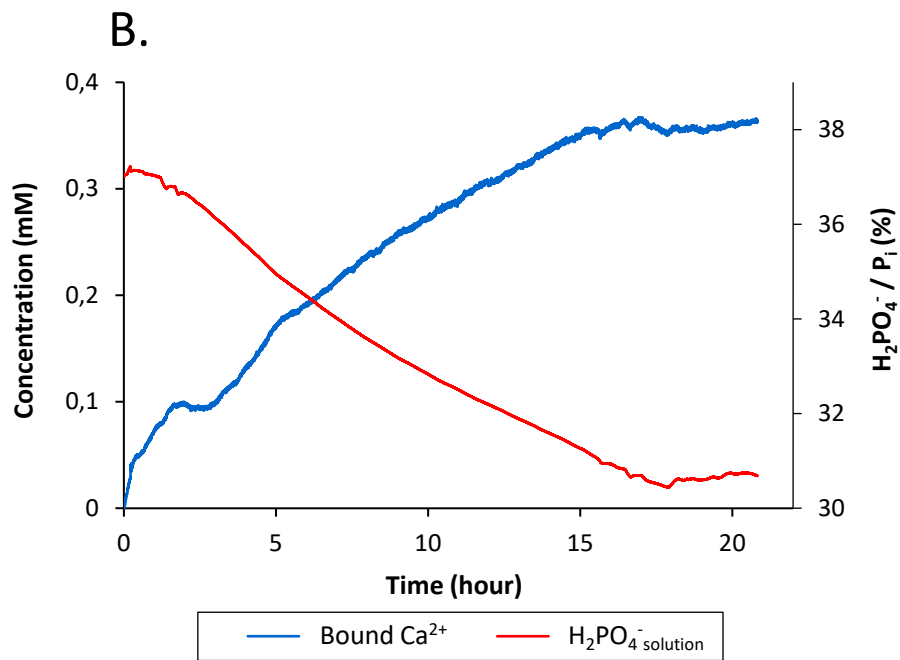


Figure S5. Calculation of bound- Ca^{2+} involved in PNS (blue curve) and H_2PO_4^- concentration (red curve) in mSBF over time.