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Targeted DNP for biomolecular solid-state NMR

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High-field dynamic nuclear polarization is revolutionizing the scope of solid-state NMR with new applications in surface chemistry, materials science and structural biology. In this perspective article, we focus on a specific DNP approach, called *targeted* DNP, in which the paramagnets introduced to polarize are not uniformly distributed in the sample but site-specifically located on the biomolecular system. After reviewing the various targeting strategies reported to date, including a bio-orthogonal chemistry-based approach, we discuss the potential of *targeted* DNP to improve the overall NMR sensitivity while avoiding the use of glass-forming DNP matrix. This is especially relevant to the study of diluted biomolecular systems such as, for instance, membrane proteins within their lipidic environment. We also discuss routes towards extracting structural information from paramagnetic relaxation enhancement (PRE) induced by *targeted* DNP at cryogenic temperature, and the possibility to recover site-specific information in the vicinity of the paramagnetic moieties using high-resolution *selective* DNP spectra. Finally, we review the potential of *targeted* DNP for in-cell NMR studies and how it can be used to extract a given protein NMR signal from a complex cellular background.

Introduction

The function of biological systems, *e.g.* proteins, nucleic acids, lipid membranes and carbohydrates is rooted in the details of their dynamical structures. Thus, structural insights in terms of stability, conformational dynamics and intra-/inter-molecular interactions can not only provide answers regarding why and how challenging biological systems perform their function, but

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Ons Dakhlaoui received her Master in Integrated Structural Biology in 2019 from the University Grenoble Alpes, France. She performed her master internship in biomolecular liquid-state NMR at IRIG-IBS under the supervision of Drs. Beate Bersch and Paul Schanda. She is currently a PhD student at IRIG-MEM and CERMAV under the supervision of Drs. Sabine Hediger, Gaël De Paëpe and Anne Imberty, working on improving the SelDNP methodology for the study of protein binding sites.

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also reveal the reasons for a loss of functionality (inhibitor binding, aggregation, *etc.*).

For this reason, different methods were developed to elucidate the structure of biomolecules, including X-ray crystallography, cryo-electron microscopy (Cryo-EM), neutron diffraction, small-angle X-ray scattering (SAXS), and nuclear magnetic resonance (NMR). While all these methods complement each other, the specific strength of NMR is its capability to provide site-specific structural details, to reveal molecular interactions, and also to give insights into conformational dynamics, particularly relevant for the study of low-populated excited states.[1–7]

Despite its uniqueness, specificity and versatility, the Achilles' heel of NMR lies in its inherent low sensitivity that comes from the low interaction energies involved and the resulting small nuclear spin population differences, which are directly proportional to the NMR signal. In addition, most of the NMR active isotopes, except for ^{1}H and ^{31}P, are only present at very low natural abundance (*e.g.* 1.108% for ^{13}C, 0.365% for ^{15}N). As a result, advances in molecular biology to produce isotopically labelled systems have led to a major leap in the capability of NMR to provide structural insights on biomolecules. This triggered the development of innovative multidimensional NMR methods making use of the multiple spin interactions present in isotopically labeled systems. Over the years, improving sensitivity and resolution has been a constant motivation for biomolecular NMR, necessary for the structural study of ever larger and more complex systems.[8]

In this battle for more sensitivity, we have witnessed, for instance, the successful development of intense magnetic fields, cryogenic solution-state probes and ultra-fast magic angle sample-spinning (MAS) solid-state probes, as well as advanced pulse sequence engineering and optimized sample preparation. Finally, the successful development of a hyperpolarization approach called high field Dynamic Nuclear Polarization (DNP), pioneered by the Griffin group, is currently revolutionizing MAS solid-state NMR (ssNMR) enabling several orders of magnitude of sensitivity gains.[9–11] Such unprecedented improvement in sensitivity enabled data to be recorded using experiments that were previously deemed infeasible. In addition, MAS-DNP can be applied to a broad range of systems with emerging new applications in surface chemistry, materials science and structural biology.[11–21]

In this perspective article, we will discuss a specific DNP approach, called *targeted* DNP (*t*DNP), in the context of biomolecular applications. We will first explain why it differs from conventional DNP experiments. We will then review various strategies to implement *t*DNP experiments and discuss foreseen benefits in terms of sensitivity and selectivity improvement. Next, we will report on the feasibility of obtaining structural information through paramagnetic relaxation enhancement (PRE) induced by *t*DNP at cryogenic temperature, and the possibility to recover high-resolution information using the recently introduced *selective* DNP (*Sel*DNP) methodology. Finally, the development of *t*DNP in the context of in-cell DNP-enhanced NMR will be discussed.

Ildefonso Marín-Montesinos is researcher at CICECO-Universidade de Aveiro, Portugal. He received his M.Sc. from Universidad de Sevilla, Spain, and his Ph.D. from University of Southampton, UK. He then moved to University of Birmingham for a postdoctoral position. In 2010, he received the "Juan de la Cierva" grant from the Science Ministry of Spain to work with Prof. M. Pons at Universitat de Barcelona. He then moved to IRIG at CEA Grenoble to work with Drs. Sabine Hediger and G. De Paëpe, before settling in Aveiro in 2019. His research focus on application of solid-state NMR and MAS-DNP to characterize biomolecular systems and porous materials for CO_2 adsorption.

Sabine Hediger received her M.Sc. and Ph.D. from the Swiss Federal Institute of Technology in Zurich (ETHZ), under the supervision of Profs. R. R. Ernst and B. H. Meier. She was then hired as a research scientist in charge of solid-state NMR at the Nestlé Research Centre in Lausanne, Switzerland. In 1999, she obtained a research position at the National Center for Scientific Research (CNRS) in France, first at the Ecole Normale Supérieure de Lyon and then at CEA Grenoble, where she develops her research interests in solid-state NMR and DNP methodology, specifically towards biomolecular applications.

Gaël De Paëpe is currently leading the DNP-NMR group at IRIG (MEM, CEA/Univ. Grenoble Alpes), France. He received his M.Sc. and Ph.D from the ENS Lyon, France, under the supervision of Prof. L. Emsley. In 2004, he moved to MIT for a postdoctoral work with Prof. R. G. Griffin. He returned to France in 2008 with an ANR "Chaire d'Excellence" to set up high-field MAS-DNP at CEA. His group focuses on MAS-DNP method and technology development for applications in materials science, chemistry, and biology. He received the Vold Memorial Prize (2013) and a consolidator grant funded by the European Research Council (2015).

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Targeted DNP *versus* conventional DNP

In MAS-DNP experiments, the NMR sensitivity enhancement is achieved by transferring the polarization from unpaired electrons, contained in dopant molecules called Polarizing Agents (PAs), to the surrounding nuclear spins of the system of interest. PAs are typically composed of stable organic mono- or biradicals, although DNP with metals has also been demonstrated.[22–37] The transfer of polarization is driven by high-power high-frequency microwave (μw) irradiation while spinning the sample at the magic angle at cryogenic temperature, usually around 100 K using cold nitrogen gas[38] or at lower temperature using closed-loop cryogenic helium spinning systems.[39–41]

In conventional biomolecular MAS-DNP experiments, PAs are typically dissolved in a glass-forming matrix (usually a mixture of glycerol or DMSO, D_2O and H_2O) and randomly distributed around the sample of interest. The polarization is transferred from the electron spin system to surrounding nuclei (typically ^1H, ^{13}C, ^{15}N in the case of biomolecules), then to more distant nuclei, either directly through-space using hyperfine couplings to the electrons, and/or relying on spin-diffusion mechanism among nuclear spins.

In *t*DNP approaches, on the contrary, the PAs are not randomly distributed, but located or anchored at specific molecular sites. It is important to note that the use of localized paramagnetic species is not new to the field of biomolecular magnetic resonance. A large part of proteins naturally bears paramagnetic centers in the form of metal ion binding sites or naturally occurring radical moieties. These endogenous paramagnetic centers can directly be observed by electron paramagnetic resonance (EPR),[42–44] whereas NMR can be used to indirectly detect the influence of the paramagnets through resonance broadening and shifting.[45–47] Extension of this methodology to diamagnetic systems has led to the development of various spin-labeling strategies,[48–50] enabling the use of EPR[51,52] and paramagnetic NMR[53–56] to study structure and dynamics of biomolecules.

Making use of spin labels in the context of MAS-DNP was thus a natural next step in the development of the technique, and several groups in the last years have investigated the benefits of tagging their biomolecular system with the PA required for hyperpolarization.[57,58] The motivation and objectives of the different approaches were diverse, going from optimization of the sample preparation for best sensitivity[59,60] and biocompatibility,[61–63] investigation of the bleaching/quenching effect of the paramagnetic tag[64–66] under DNP conditions for its potential use as a structural probe,[67–69] selection of a particular protein in a complex environment,[62,63,70–75] to the detection of protein–protein interactions,[76] and the development of high-resolution localized techniques through *selective* DNP.[77]

Targeting strategies

Covalent binding and site-directed spin labeling of PAs

Considering that the replacement of monomeric TEMPO by the bis-nitroxide biradical TOTAPOL has been one of the major milestones in the development of high-field MAS-DNP,[23] it is not surprising that the first attempt towards *t*DNP in 2011 has been conducted using a TOTAPOL moiety covalently linked to the system of interest, a decapeptide in this case (see Fig. 1a).[59] More recently, in the context of antimicrobial peptides, Sani *et al.* introduced by solid-phase synthesis one or two spin label amino acid TOAC (2,2,6,6-tetramethyl-*N*-oxyl-4-amino-4-carboxylic acid, see Fig. 1b) at the N-terminus of a maculatin mutant in order to investigate with MAS-DNP the interaction of this antimicrobial peptide with the bacterial cell membrane of *E. coli*.[74] Although neither the monoradical TOAC nor the biradical TOAC–TOAC are optimized PAs for MAS-DNP, DNP enhancement factors of 10 and 13, respectively, were obtained on cell samples.

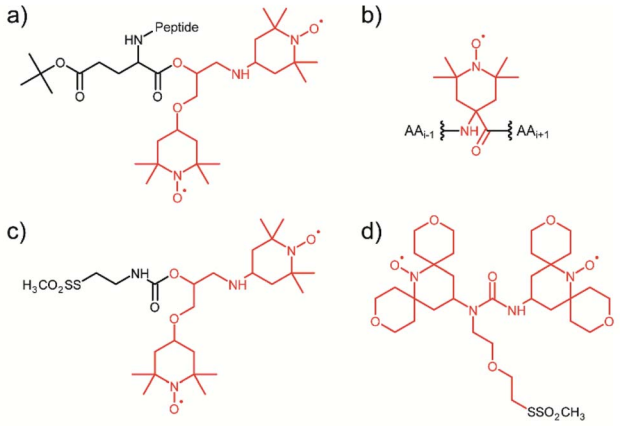

Fig. 1 Illustration of the several *t*DNP spin labeling approaches based on the covalent binding of the PA onto the targeted system: (a) TOTAPOL is covalently attached to the terminal CO of a decapeptide.[59] (b) Spin-label amino acid TOAC compatible with solid-phase peptide synthesis.[74] (c) and (d) MTS derivatives of TOTAPOL and AMUPol, respectively, which reacts with accessible cysteine thiol groups of proteins.[64,67] The original radical or biradical moiety is highlighted in red throughout the figure.

Targeted DNP was also implemented on proteins using the site-directed spin-labeling (SDSL) strategy, combining site-directed mutagenesis and sulfhydryl chemistry. For this purpose, Voinov *et al.*[64] and van der Cruijsen *et al.*[67] synthesized methanethiosulfonate (MTS) derivatives of the DNP biradicals TOTAPOL[23] and AMUPol[31] (Fig. 1c and d), respectively, which can react selectively with thiol groups of surface exposed cysteines through the formation of a covalent disulfide bond. This approach is rather robust, with efficient coupling efficiencies, and also versatile, since the position of the tagged PA can be chosen using cysteine mutagenesis. In addition to MTS, protein thiol groups are known to react specifically as well with the maleimide and iodoacetamide functional groups, which can broaden the chemical possibilities for targeting PAs in the future. When used in the context of large protein complexes and membrane proteins in their lipidic environment, this strategy definitively leads to a non-uniform dispersion of PA, ensuring proximity between the diluted biomolecule of interest and the hyperpolarizing agent.

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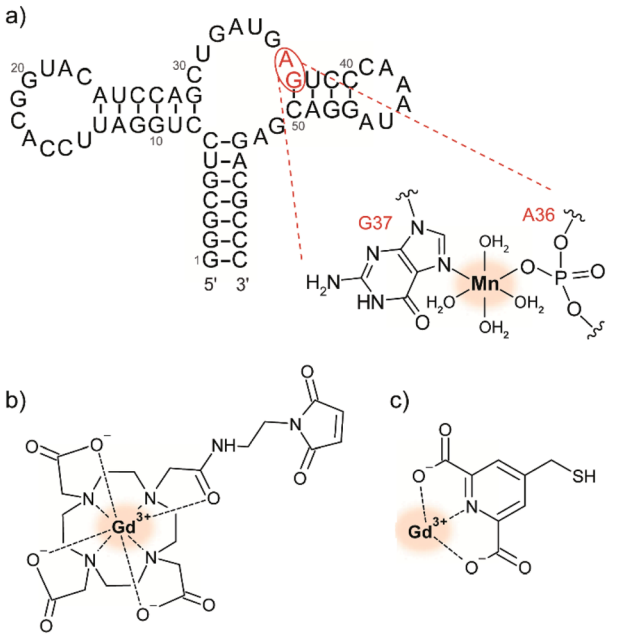

Fig. 2 (a) Predicted secondary structure of the full-length hammerhead ribozyme with the proposed selective binding site for divalent ions. Adapted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, *Journal of Biomolecular NMR*, dynamic nuclear polarization of nucleic acid with endogenously bound manganese, P. Wenk *et al.*, Copyright Springer Science + Business Media Dordrecht (2015).[65] Chemical structure of (b) maleimide derivatized Gd-DOTA and (c) Gd-4MMDPA spin labels, which can react with the thiol group of exposed cysteines, forming either a carbon–sulfur or a disulfide bond, respectively.[68] The metal ion used as PA is highlighted with a red shadowed circle.

Endogenous metal DNP and site-directed spin labeling of metal PAs

Paramagnetic metal ions are ubiquitous in biology and can thus be leveraged for *t*DNP experiments. In particular, Mn^{2+} and Gd^{3+} form high-spin complexes ($S = 5/2$ for Mn^{2+} and $S = 7/2$ for Gd^{3+}) whose magnetic properties are compatible with DNP at high magnetic fields.[29] While Mn^{2+} has biological roles enabled by its redox reactivity, Gd^{3+} is interesting due to its high redox stability which makes it a favorable candidate for in-cell DNP. The first demonstration of their use as PA has been published in 2011.[28] In contrast to bis-nitroxide radicals, MAS-DNP with paramagnetic metal ions rely on the solid-effect (SE) mechanism. Irradiation on the narrow central EPR transition of the half-integer electronic spin of the metal ion leads to polarization transfer to the hyperfine coupled nuclear spins.[29]

The use of paramagnetic metal ions for *t*DNP has been developed so far by the Corzilius group. A first study investigated the use of endogenous Mn^{2+}, whose presence is essential in hammerhead ribozymes for their self-cleavage function (see Fig. 2a).[65,69] Extension of this approach for diamagnetic biomolecules was obtained *via* chelator ligands compatible with site-directed spin-labeling of cysteines, like DOTA-M or 4MMDPA (see Fig. 2b and c).[68]

Ligand-binding PAs

Most of the *t*DNP spin-labeling approaches presented so far are based on a covalent bond attaching the PA to the protein of interest, with the exact position of the spin label being chemically engineered. Alternatives have been proposed where the location of the spin label is determined through protein–ligand interactions, with the PA being covalently attached to the ligand. In that case, the position of the PA is determined by the ligand-binding site. In addition, some selectivity is introduced through the specificity of the interaction, which might allow the targeting of a specific protein in the presence of other biomolecules.

A first example in that direction was proposed by the Etzkorn group in 2016. With the aim to selectively enhance the 20 kDa Bcl-x_L protein in cell lysates, they covalently attached

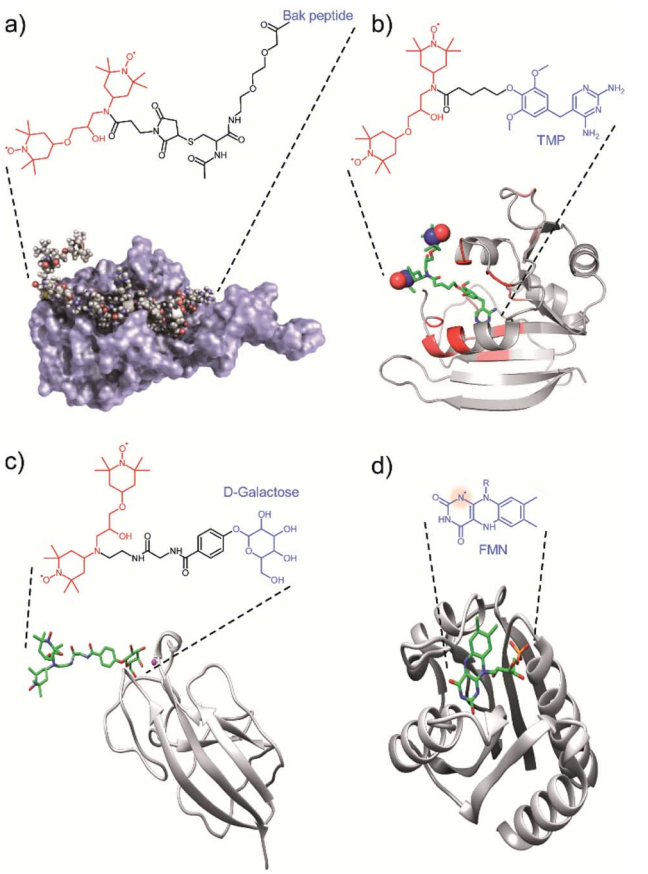

Fig. 3 Examples of *t*DNP approaches that uses (a–c) the specific affinity of a ligand-derivatized PA with the protein of interest, and (d) an endogenous paramagnetic cofactor. (a) Protein Bcl-x_L interacting with the Bak peptide tethered to TOTAPOL. Adapted with permission from ref. 71. Copyright 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (b) DHFR protein interacting with TMP tethered to TOTAPOL. Adapted with permission from ref. 66. Copyright (2017) American Chemical Society. (c) LecA interacting with galactose tethered to TOTAPOL.[77] (d) Protein flavodoxin with its endogenous stable semiquinone radical (flavin mononucleotide).[61] The biradical and the ligand moiety are highlighted respectively in red and blue throughout the figure. The nitrogen radical in (d) is highlighted with a red shadowed circle.

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a TOTAPOL moiety to the Bak peptide, the binding partner of Bcl-x_L (see Fig. 3a).[71] A similar approach was used by the group of McDermott who tagged a TOTAPOL moiety (or a derivative) to trimethoprim (TMP), which has a high affinity to *E. coli* dihydrofolate reductase (DHFR) (Fig. 3b), in order to investigate the signal attenuation induced by the presence of the paramagnetic spin-label[66] and the possibility to maintain effective DNP efficiencies in the case of dilute samples.[60] Finally, our group also reported the use of a galactose ligand tethered to a TOTAPOL moiety with the goal to selectively probe the galactose binding site of the lectin LecA with MAS-DNP (Fig. 3c).[77]

Whereas all these examples made use of exogenous PA tethered to protein ligands, some biomolecular systems naturally possess endogenous transient or stable organic radical like flavins or quinones, which can be used for *t*DNP. Such an example has been shown by Maly *et al.*[61] who used the semiquinone (SQ) form of the flavin cofactor in the protein flavodoxin to obtain DNP sensitivity enhancement of proton spins by MAS-DNP (Fig. 3d). The appeal of this approach is that it allows the study of an intact biomolecular system. However, it is also limited since monomeric flavodoxins are far from being competitive in terms of DNP efficiency compared to optimized PAs, especially at high magnetic fields and fast sample spinning.

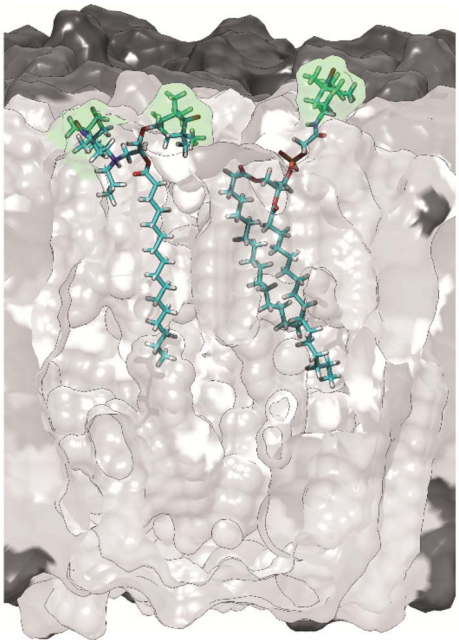

Fig. 4 Molecular model illustrating the embedding of the biradical *N*-propyl-PALMIPOL[62] (on the left) and the TEMPO-functionalized phosphatidylcholine TEMPO-PC[70] (on the right) in the phospholipid bilayer. The green shadow highlights the location of the radical moieties.

PAs with unspecific affinity: the case of lipid-anchored PAs

Unspecific affinity of the PAs with the sample of interest have been reported in several studies.[78–82] In the context of matrix-free (MF) sample preparation, which circumvents the use of a glass-forming matrix, we showed that unspecific affinity of the PA for the system of interest can be used to preserve a uniform distribution of PA molecules.[78,83]

In the context of *t*DNP, this MF approach is particularly interesting in the case of heterogeneous biomolecular samples with domains of different polarities like liposomes or cells. For instance, the use of DMSO or glycerol in higher concentrations, usually required for DNP samples, is known to perturb the lipid bilayer structure. In addition, it is not straightforward to predict the location of water-soluble PAs such as TOTAPOL,[23] AMUPol[31] or AsymPolPOK[25] in such multi-component biomolecular systems. This was recently illustrated in the work by the Hong group that uses paramagnetic relaxation enhancement of lipid signals to investigate the partitioning of AMUPol and TOTAPOL in the lipid bilayer.[84]

To avoid perturbation of such complex and fragile biomolecular systems, and to control the location of the PA, it was relevant to investigate alternative protocols and to design new specific PAs. Thus, a lipid-anchored biradical, *N*-propyl-PALMIPOL, was developed in our group (see Fig. 4).[62] It consists of a TOTAPOL moiety functionalized with a palmitate chain, which is able to localize to the lipidic membrane, keeping the biradical function at the surface of the liposomes (see Fig. 4). This leads to appreciable DNP enhancement despite the absence of excess cryoprotectant (DMSO/glycerol) as usually introduced in DNP samples. A similar idea was also proposed by the Long group who compared different commercial spin-labeled phospholipids, with the TEMPO moiety located either on the phosphate head group (see Fig. 4), in the middle or at the end of the lipid choline chain.[63,70] Subsequently, other groups have further explored this direction and reported improved matrix-free DNP efficiency using other lipid-anchored biradicals.[72,73]

Bio-orthogonal chemistry for in-cell *t*DNP applications

The extension of the *t*DNP approach to in-cell NMR applications is highly desirable since the main limitation of such studies lies in the difficulty of detecting an "endogenous" amount of protein within a large cellular background. *t*DNP could certainly be useful to preferentially enhance the protein of interest over the cellular background. However, it is not straightforward to specifically target a given protein inside the cellular environment. Approaches based on cysteine chemistry are limited because of their lack of selectivity, and methods relying on protein–ligand interactions are only possible for systems with known and very specific ligands.

Alternative strategies require the incorporation of an unnatural amino acid (UAA)[85] and the development of bio-orthogonal chemistry.[86] This path has proven very successful for the efficient and specific incorporation of optical probes into proteins of the cellular milieu.[87,88] In the context of DNP experiments, Debelouchina and coworkers have reported the first successful example of targetable tetrazine-based PAs for biological systems.[75] In their work, the unnatural amino acid norbornene-lysine is introduced in the protein sequence through amber suppression.[85] DNP spin labeling is then

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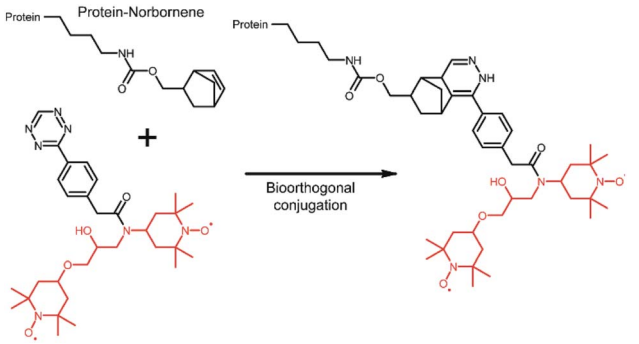

Fig. 5 Tetrazine-derivatized TOTAPOL designed to perform a bioorthogonal conjugation with the unnatural amino acid norbornene-lysine genetically introduced in the mutant protein.[75] The TOTAPOL moiety is highlighted in red.

obtained through biorthogonal conjugation of a TOTAPOL-tetrazine radical (TTz) as illustrated in Fig. 5. The approach has been demonstrated on several proteins and in cell lysates.[75]

Targeted DNP for improved sensitivity

As stated before, a conventional MAS-DNP experiment requires the PA to be distributed as homogeneously as possible inside the sample. This has been conventionally obtained by co-dissolving the PA and the system of interest in a glass-forming solvent mixture commonly dubbed DNP juice (for biomolecules, often a glycerol– or DMSO–water mixture).[18,89] The analyte is then studied under DNP conditions in the form of a frozen solution. The major drawback of this glassy matrix approach lies in the fact that the solvent occupies a non-negligible part of the fixed and limited volume of the ssNMR sample rotor. This impacts significantly the amount of analyte inside the rotor, and consequently the sensitivity of its signal. In most cases, simply reducing the amount of solvent lead to PA aggregation and its phase separation from the system of interest with detrimental effects on the DNP efficiency. For organic and biomolecules, this phase separation can be avoided when some affinity of the PA for the analyte is present, allowing thus the preparation of matrix-free samples.[78] This kind of non-specific affinity with commonly-used PAs has been described for several biomaterials and biomolecules like cellulose,[78] proteins,[79,80] fibrils,[81] and bacterial cell wall polymers.[82] In the absence of such an affinity, Takahashi *et al.* has shown that an intermediate molecule like trehalose can be used to bridge the affinity between the PA and the investigated system (see Fig. 6).[83]

As the PA is always in close proximity to the system of interest in MF samples and in *t*DNP applications, its concentration should be reoptimized, rather than relying on the same concentration as commonly used for frozen solution preparations. For instance, the supernatant solution of only 1 mM in TOTAPOL was found to be optimum for the study of extracted bacterial cell wall polymers due to the affinity of the PA for the peptidoglycan,[82] whereas standard concentrations of about

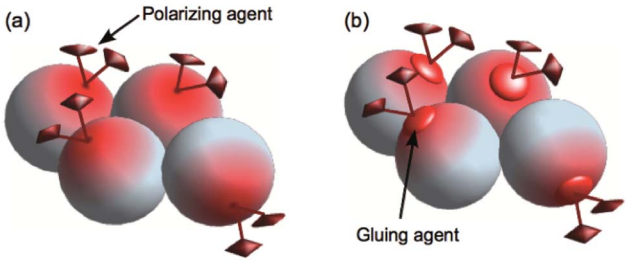

Fig. 6 Schematic illustration of the matrix-free approach developed by Takahashi *et al.*,[83] using either direct (a) or indirect (b) affinity of the PA with the system of interest. Reproduced from ref. 83 with permission from The Royal Society of Chemistry, copyright 2013.

10 mM are usually required in samples prepared with the conventional frozen solution method.

Similar observations were reported in the work from Voinov *et al.*,[64] where the biradical TOTAPOL is directly attached to a solvent exposed cysteine of the heptahelical transmembrane protein, *Anabaena* sensory rhodopsin (ASR) embedded in a multilamellar liposome mixture (DMPC : DMPA 9 : 1). For a given mass of protein sample, *t*DNP with a matrix-free deuterated buffer provided similar DNP sensitivity as the conventional frozen solution DNP buffer with TOTAPOL dissolved into a glycerol-d_8/D_2O/H_2O matrix. Interestingly, removing the glassy matrix *via* the *t*DNP approach enabled more sample to be packed inside the rotor, which translated into a 4-fold sensitivity increase.

Consistent observations were recently made in the work from Good *et al.* aiming at optimizing matrix-free sample preparation for membrane proteins with a biradical-tagged phospholipid as PA.[73] The ability to maximize the NMR sensitivity of membrane proteins reconstituted in lipids by *t*DNP is an important direction since the very small amount of protein present in lipid sample (to mimic relevant physiological condition) severely limits sensitivity for conventional NMR approaches. Nevertheless, the loss of NMR resolution induced by sample freezing typically requires multiple isotopic labeling schemes to be used for obtaining intermolecular information.

Another advantage of the *t*DNP approach, recently highlighted by Rogawski *et al.*, lies in the fact that sub-millimolar concentration of bound PA can be used. For instance, the authors use a biradical-derivatized trimethoprim ligand (TMP-T) of dihydrofolate reductase (DHFR) to report 2D correlation experiments of 1 : 1 DHFR : TMP-T complexes at 0.5 mM, allowing characterization of DHFR with only ∼15 nmol of proteins.[60]

Targeted DNP and paramagnetic enhanced relaxation

Introduction of paramagnetic tags to the protein of interest, covalently bonded or not, induces paramagnetic effects that can translate into contact shifts, pseudo-contact shifts and paramagnetic relaxation enhancement (PRE) effects.[47,53] These effects are well-known in solution-state NMR[46,90,91] and have

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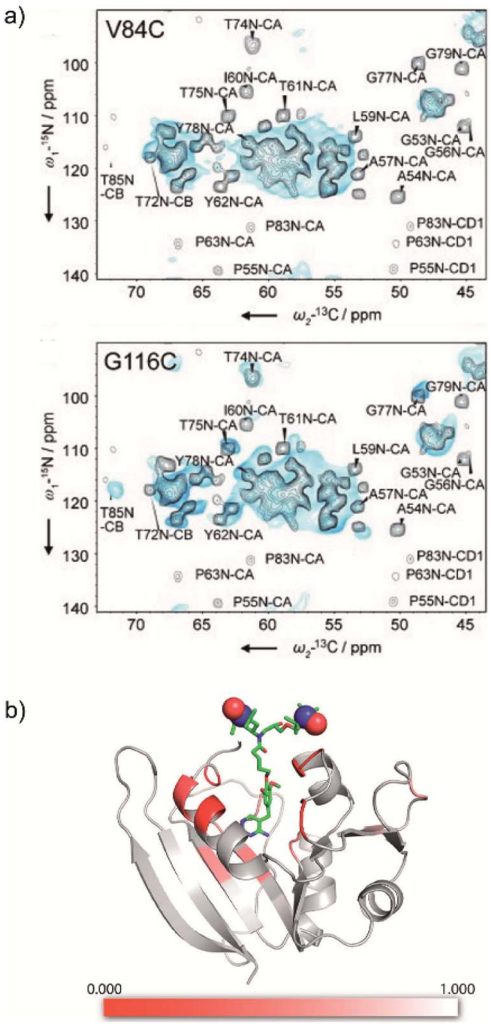

Fig. 7 (a) 800 MHz low-temperature DNP 2D NCA of U-[^{13}C, ^{15}N] spectra (blue) of KcsA mutants V84C (top) and G116C (bottom), spin labeled with AMUPol-MTSSL (see Fig. 1d), compared to room temperature 700 MHz solid-state NMR spectra (black) of wild-type KcsA (black). The presence of the spin label in the mutants leads to paramagnetic bleaching at cryogenic temperature. Reproduced with permission from ref. 67. Copyright 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (b) Model of the protein DHFR complexed with the high affinity ligand TMP derivatized with TOTAPOL (see as well Fig. 3b), with heat map showing the bleaching information obtained from low temperature DNP spectra. The strongest reduction in signal intensity (in red) is observed for residues proximal to the TMP-radical binding pocket. Reproduced with permission from ref. 66. Copyright (2017) American Chemical Society.

recently been extended to biomolecular solid samples at room temperature.[92,93] In the case of nitroxide radicals, the main contribution is PRE. It translates into a variation of the longitudinal and transverse coherence nuclear decay times that can be modelled to extract distance information. Even though the usual solution state PRE theory has not yet been adapted and validated for MAS NMR at cryogenic temperature, it is directly linked to the bleaching phenomenon observed in DNP, where signal from spins close to the PA are strongly attenuated and/or broadened beyond detection.[78,94,95]

Recently the Baldus and McDermott groups reported evidence of site-specific signal bleaching for residues close to the tagged PA (Fig. 7).[66,67] In the latter, the PRE induced signal attenuation of a PA-functionalized ligand was investigated with solution-state NMR and compared to 100 K DNP-enhanced data.[66] Using specifically-labeled proteins, the authors measured the bleaching of the NMR signal intensity for numerous sites in the protein and showed a correlation with the distance to the tagged PA. They discuss three cases: (i) residues at 10 Å or closer to the unpaired electrons are fully bleached; (ii) residues which are 10 to 25 Å in distance are strongly affected by the presence of the paramagnets; (iii) above 30 Å, much less bleaching effect is observed.[66] A more precise quantification of the distance-dependency of the bleaching effect is not straightforward, mostly because of the heterogeneous broadening at 100 K, which limits site-specific information, and the conformational disorder of the paramagnetic tag.

It is also worth pointing out that Corzilius and co-workers have also investigated similar questions in the context of paramagnetic metal centers. They notably reported signal attenuation of the full-length hammerhead ribozyme (HHRz) complexed with the paramagnetic Mn^{2+} divalent ion, when compared to the diamagnetic reference with Mg^{2+}.[65] Nevertheless, access to site-specific information was limited due to significant spectral overlap of RNA resonances. An approach to recover resolution in the proximity of the paramagnetic tag while indirectly detecting the PRE effect is described in the following section.

Targeted DNP with *Sel*DNP: improving sensitivity and resolution

In 2019, our group reported a novel approach that combines the use of localized PRE and difference spectroscopy in order to recover high-resolution DNP spectra from uniformly labeled biomolecules by means of spectroscopic selection.[77] This approach, called *Sel*DNP, requires the introduction of a Paramagnetic Tag (PT) on the protein of interest and was first demonstrated to reveal the carbohydrate-binding site of the protein LecA (see Fig. 3c). It enabled recovery of site-specific information from the interaction site thanks to high-quality multidimensional data. It nicely complements other NMR methodologies that can be used to locate binding sites and solve their structures.[96–98] Very importantly, *Sel*DNP does not require the use of advanced non-uniform isotopic labeling strategies to produce resolved data from residues located within a 30 Å sphere from the PT. In addition, the method has no inherent limitations with respect to the size of the biomolecular system.

High-resolution DNP-enhanced *Sel*DNP spectra of protein binding sites

*Sel*DNP requires the acquisition of the same NMR experiment on two samples. The first sample is used to produce the reference dataset, for which it is assumed that all resonances are uniformly polarized by DNP. The sample can be prepared using standard

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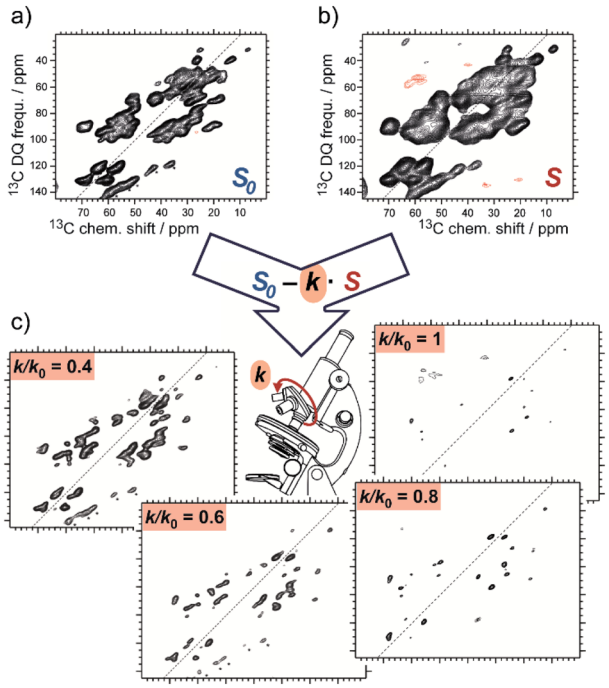

Fig. 8 Schematic principle of the *Sel*DNP approach. (a) Reference dataset S_0 recorded on the sample prepared with the protein and its native ligand together with a non-specific PA. (b) *t*DNP dataset S recorded on the sample prepared with the protein and the ligand functionalized with the PA (acting as PT). The difference of the two datasets using a variable relative scaling factor k (selectivity factor) produces a set of *Sel*DNP spectra given in (c) for $k = 1$, 0.8, 0.6, and 0.4, with increasing number of peaks for lower values of k. These peaks correspond to residues at increasing distances from the PT position. Spectra (a) and (b) are 2D ^{13}C–^{13}C double-quantum single-quantum correlation spectra. They were acquired on the protein LecA as described in ref. 77.

DNP sample preparation protocols where the PA is randomly distributed around the protein molecules. The second sample is prepared following a *t*DNP approach. For instance, Marin-Montesinos *et al.* have used a functionalized ligand tethered to a PA moiety as PT in order to selectively introduce PRE effects around the binding site and to polarize the rest of the protein. *Sel*DNP spectra are obtained by subtracting the two datasets. Resonances from residues which are close to the PT are more bleached in the *t*DNP spectrum while they are of similar intensity to the rest of the residues in the reference spectrum; those resonances are therefore revealed by taking the difference of the two spectra. In contrast, resonances from residues that are far enough to be unaffected by the presence of the PT will appear similarly in both datasets and will thus be minimized or even suppressed by the difference spectroscopy. *Sel*DNP results in high-resolution spectra with a reduced number of peaks, all originating from sites close to the PT, and thus to the binding site. Examples of *Sel*DNP spectra can be found in Fig. 8. For each sample, a ^{13}C–^{13}C double-quantum (DQ) dipolar correlation spectrum was acquired with a mixing time set to produce one-bond correlations. Note that only positive intensities are retained in the 2D *Sel*DNP spectra, and those positive peaks originate from the protein with its native ligand.

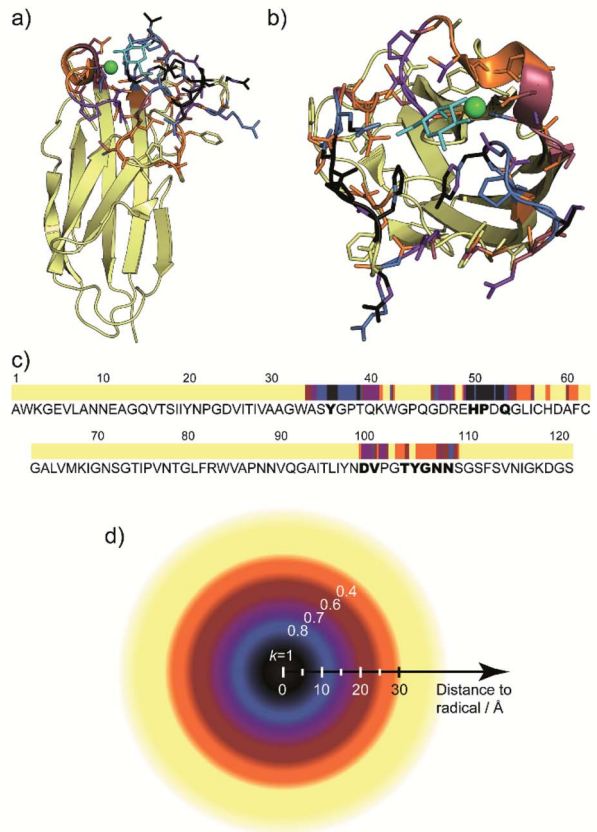

Fig. 9 (a) and (b) Lateral and top views of the protein LecA with residues present in the *Sel*DNP spectra of Fig. 8c) highlighted in colour, depending in which spectrum they first appear: black for $k = 1$, blue for $k = 0.8$, purple for $k = 0.7$, burgundy for $k = 0.6$, orange for $k = 0.4$. The galactose ligand is in cyan and the calcium ion in green. (c) LecA protein sequence with the residues assigned from *Sel*DNP spectra highlighted using the same colour code as in (a). Residues identified in the crystal structure to be in interaction with the ligand are identified in bold. (d) Illustration of the cut off distance to PT of residues appearing in the *Sel*DNP spectrum for a given selectivity factor k. Adapted from ref. 77 with permission from the Royal Society of Chemistry, copyright 2019.

Tuning the spectral selectivity and its relationship to the distance to the paramagnetic tag

By varying the scaling or weight factor k between the two spectra, one can tune the number of positive cross-peaks displayed in the *Sel*DNP spectra. This corresponds to adjusting the focus around the PT by selecting resonances as a function of the PRE magnitude, which is related to the distance between the corresponding nuclei and the electron spins of the PT. As discussed in the work from Rogawski *et al.*,[66] the direct analysis of this relationship requires the use of selectively labeled proteins. In the work from Marin-Montesinos *et al.*,[77] a solution to this problem is proposed. Not only can one recover spectral resolution by selecting residues close to the PT, but it is also possible to relate the selectivity factor k to an approximate distance cut-off from the PT (Fig. 9d), and thus to introduce long-range constraints with respect to the PT.

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Resonance assignment of *Sel*DNP spectra: locating the binding site

The one-bond 2D ^{13}C–^{13}C *Sel*DNP correlation spectra reported in Fig. 8c are ^{13}C NMR fingerprints of the residues around the binding site. A residue-type assignment can be obtained by comparing the resonances to tabulated values for the different amino acids in proteins. Using minimal prior information known about the protein, *i.e.*, that the detected residues should be grouped in only a few stretches of the protein sequence and that ^{51}Pro and ^{50}His are involved in the galactose binding of LecA, Marin-Montesinos *et al.* were able to propose a sequence assignment of the residues contained in the set of *Sel*DNP spectra from LecA, in agreement with location of the binding site as determined in the crystal structure of the protein (Fig. 9).[77] *Sel*DNP can, in principle, also be applied to ^{15}N–^{13}C correlation experiments in order to extract sequential information. Nevertheless, the poor resolution in the ^{15}N dimension might make it challenging. Therefore, it is a good strategy to favor multidimensional experiments that provide side-chain ^{13}C correlations, even for sequential assignment, due to the wider inherent spectral distribution of such correlations.[99] We are currently developing computational algorithms for guided resonance assignment to extend *Sel*DNP to more complex biomolecular assemblies.

Targeted DNP for in-cell NMR: improving selectivity in complex environments

NMR applications related to structural biology have mostly been performed so far under *in vitro* conditions. Although there are growing evidences that atomic-scale studies should also be performed under conditions closer to the cellular environment,[100–102] it has proven very challenging. Examples of NMR studies on proteins embedded in their natural membrane or surrounded by their natural cellular environment have clearly highlighted many challenges ahead, from the production of isotopically labeled samples to the limited sensitivity resulting from the diluted conditions. As a consequence, background signal rapidly becomes a major obstacle to extract site-specific information from the biomolecular system of interest.[71]

The field of in-cell NMR, pioneered in the liquid-state by Selenko and coworkers,[100] is now expanding to solid-state NMR[103,104] with the main argument being that large biomolecular assemblies become impractical for solution-state NMR due to resonance broadening. In addition, studying proteins at their physiological concentration strongly limits the amount of active material. For instance, in a recent work, Narasimhan and Scherpe *et al.* estimated that only 10–30 μg of labeled ubiquitin is contained in a 3.2 mm rotor filled with cells, when the protein is present at physiological concentrations.[105] The resonances of such diluted amounts of protein, even when labeled, face significant overlapping and crowding from signals contributed by the cellular background, which constitutes a tremendous limitation. Therefore, a hyperpolarization approach such as DNP is necessary and potentially more suitable for these complex biological environments, especially if combined with innovative on-going methodological developments.[101,102,105–107]

Introducing selectivity using *t*DNP for in-cell NMR studies

The first attempt towards in-cell DNP uses methodology that was developed in standard in-cell solution and solid-state NMR. The ^{13}C, ^{15}N isotopically labeled protein is introduced into unlabeled or even isotopically depleted cells (*i.e.* ^{2}H, ^{12}C labeled) using for example electroporation or pore-forming peptides. The DNP PA is then added to the cells in order to record DNP-enhanced NMR data. More details can be found in the recent review from the Frederick group.[108] DNP experiments need to be performed relatively rapidly after addition of the PA in order to limit radical reduction.[109] In this approach, the challenge is to detect the labeled protein at μM concentration, which can be overcome using multiple-quantum filtered pulse sequences that only select NMR-active nuclei that are close in space. This works well for the study of soluble proteins (*e.g.* ubiquitin, alpha-synuclein) inside cells, to track *e.g.* structural changes or complex formation.

A second approach requires producing the protein of interest directly inside the cells (*E. coli*, insect cells, and more recently mammalian cells) and is thus more suited for the study of non-soluble proteins such as membrane proteins, or proteins that require post translational modification in order to be functional (*e.g.* glycosylation or phosphorylation).[110] Even though the labeled cell medium is usually introduced just before triggering the protein overexpression, partial isotopic enrichment of the rest of the cell cannot be avoided. In this second approach, selecting the protein signal out of the partly isotopically labeled background can be problematic even when employing multiple-quantum filters.

For this reason, DNP methods that can selectively hyperpolarize low amounts of a target protein in a large cellular background were recently introduced. The first demonstration of such a background suppression approach was presented by Viennet *et al.*[71] It relies on a *t*DNP approach combined with deuteration of the DNP matrix. Specifically, the authors demonstrated first that 50 μM of a TOTAPOL-modified binding peptide was enough to selectively hyperpolarize about 50 μM of a 20 kDa protein (Bcl-x_L, see Fig. 3a) dissolved into a deuterated buffer, that was essential to prevent ^{1}H–^{1}H spin diffusion whereby hyperpolarization would be lost to the solvent. In a second step, they validated their approach on non-purified cell lysates, which were previously suspended in D_2O buffer and then concentrated. The end pellet was estimated to contain a total protein concentration of ~35 mg ml^{-1}, including about 10% of Bcl-x_L. *t*DNP experiments performed with the TOTAPOL-modified binding peptide revealed a non-uniform protein-specific DNP enhancement compared to conventional DNP experiment with a non-specific PA. Assuming that the binding peptide only interacts with Bcl-x_L, the cellular background contribution could be removed by subtracting a scaled uniformly polarized spectrum (see Fig. 10a).[71]

More recently, the Debelouchina group demonstrated the first use of bioorthogonal chemistry in the context of *t*DNP

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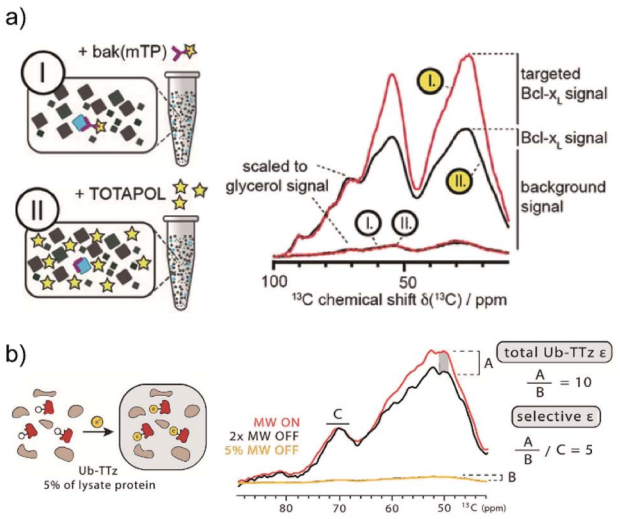

Fig. 10 Examples of *t*DNP applied to selectively enhance proteins at close to endogenous concentration in isotopically labeled bacterial lysates. (a) In the work from Viennet *et al.*, two samples are prepared (left panel) using two PAs: either Bak-mTP, a ligand functionalized with TOTAPOL (sample I), or "free" TOTAPOL (sample II). The corresponding ^{13}C CPMAS spectra (red and black lines, respectively) are shown on the right-hand side. Spectra with white and yellow labels correspond to data obtained without and with microwave irradiation, respectively. Normalizing the background signal from the two on-spectra using the glycerol resonance reveals a selective enhancement of the targeted protein when using the functionalized PA. The different signal contributions are indicated next to the scaled spectra. Adapted with permission from ref. 71. Copyright 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (b) In the work from Lim *et al.*, the tetrazine-functionalized PA reacts directly in the isotopically labeled bacterial lysate with the overexpressed ubiquitin mutant containing the UAA norbornene-lysine (left panel). Normalizing the spectra obtained with and without microwave irradiation according to the glycerol resonance (right panel) allows the estimation of left-over enhancement denoted as A. Considering that ubiquitin accounts for 5% of the protein signal (estimated using biochemical assays), the total DNP enhancement for ubiquitin is obtained by scaling A with 5% of the microwave off signal intensity (denoted B). The resulting DNP enhancement for the targeted ubiquitin is about 10, much larger than the background signal enhancement of ~2 (denoted C). Adapted with permission from ref. 75. Copyright 2019 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.

experiments as described above.[75] Their approach was first tested on three purified mutant proteins (ubiquitin, heterochromatin protein HP1α, and the structural maintenance of chromosomes protein SMC) at concentrations of only tens of μM. As a proof of concept, *t*DNP using the tetrazine-based PA was then applied to isotopically labeled bacterial lysates containing overexpressed ubiquitin mutants without purification. Similar to the work from Etzkorn and co-workers, the data show a clear selectivity towards the targeted protein (see Fig. 10b).[75] Both approaches show clear promises for future in-cell *t*DNP applications, if sufficient spectral resolution can be obtained.

Perspectives and conclusions

*t*DNP is a rapidly developing approach which show great promises for the study of biomolecules by DNP-enhanced NMR. As detailed in this review, reasons to further develop the *t*DNP methodology are multiple, both for *in vitro* and in-cell structural studies. The combination of hyperpolarization and PRE measurements enables residue-specific structural characterization of challenging biomolecular complexes. This is clearly an appealing approach to combat the limited sensitivity of NMR while accessing long range distance constraints (10 to 30 Å or more). Unfortunately, the low temperature required for DNP often induces significant spectral resolution degradation which is usually overcome with samples comprising of multiple isotopic labeling schemes. Nevertheless, as shown in this review, this resolution limitation can be mitigated by the *Sel*DNP approach. This promising method can deliver resolved site-specific information for resonance assignment and also can provide distance information (including PRE-type constraints). Finally, we envisage that the *t*DNP approach will be useful for the emerging field of in-cell solid-state NMR by selectively improving the sensitivity of the targeted protein over the large cellular background.

Of course, many new developments are still necessary to move from the proof-of-concept stage to a robust and widely applicable solution. This includes developing DNP at higher magnetic fields, combining DNP-NMR data with computational approaches,[111] but also accessing sustainable helium spinning at ultra-low temperature (<100 K), and developing chemically persistent radicals for in-cell *t*DNP applications.

Author contributions

Diego Gauto contributed to the outline and wrote the first draft of the manuscript. Ons Dakhlaoui and Ildefonso Marin-Montesinos participated in the writing of the following versions of the document. Diego Gauto and Ons Dakhlaoui designed and produced the figures. Sabine Hediger and Gaël De Paëpe designed the outline of the review and were involved in all stages of the writing process. All authors contributed to the editing of the paper.

Conflicts of interest

There are no conflicts to declare.

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