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Cellular Applications of DNP Solid-State NMR – State of the Art and a Look to the Future

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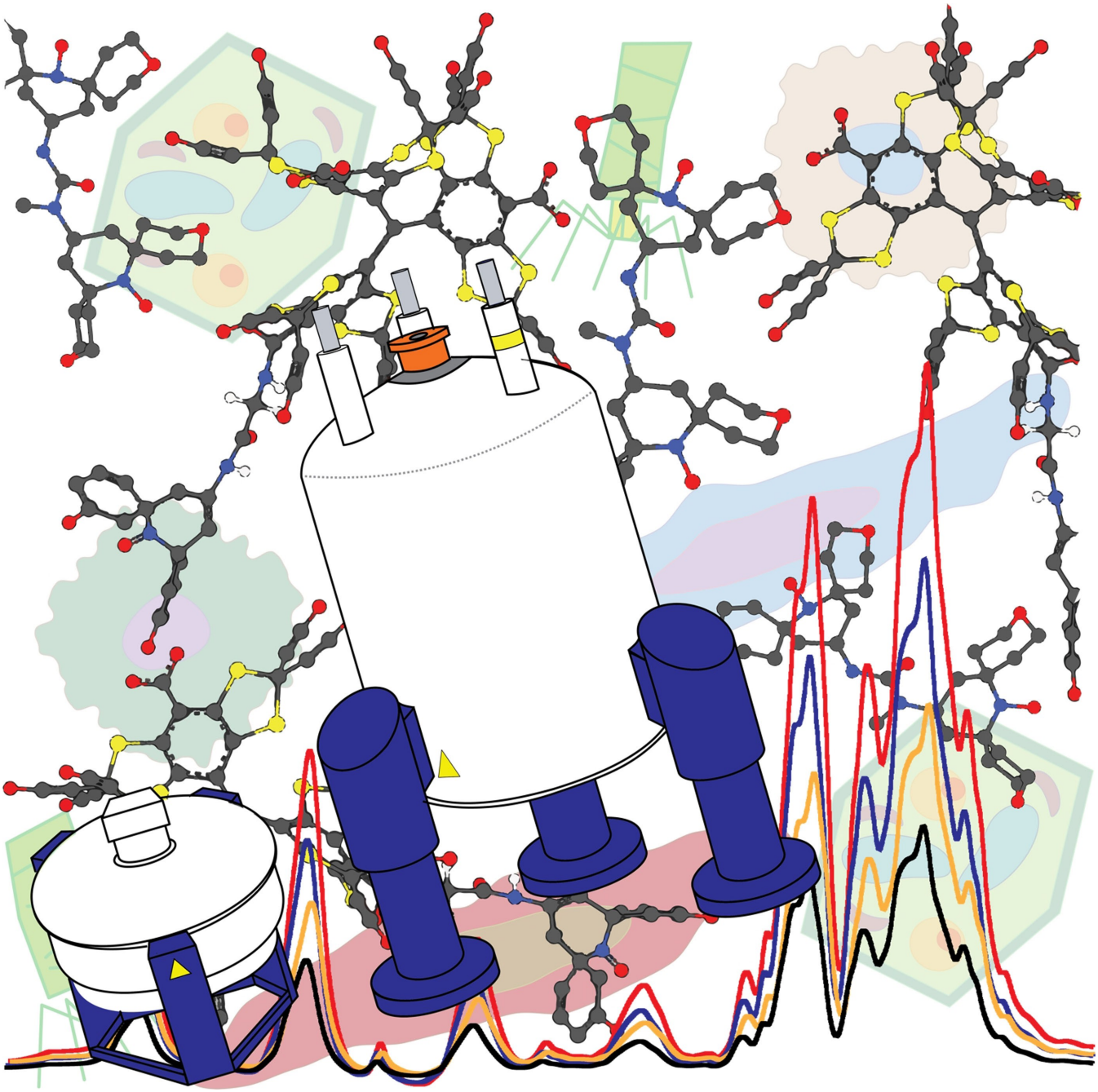

Sensitivity enhanced dynamic nuclear polarization solid-state NMR is emerging as a powerful technique for probing the structural properties of conformationally homogenous and heterogenous biomolecular species irrespective of size at atomic resolution within their native environments. Herein we detail advancements that have made acquiring such data, specifically within the confines of intact bacterial and eukaryotic cell a reality and further discuss the type of structural information that can presently be garnered by the technique's exploitation. Subsequently, we discuss bottlenecks that have thus far curbed cellular DNP-ssNMR's broader adoption namely due a lack of sensitivity and spectral resolution. We also explore possible solutions ranging from utilization of new pulse sequences, design of better performing polarizing agents, and application of additional biochemical/ cell biological methodologies.

1. Introduction

It is increasingly becoming evident that a biomolecule's structure and by extension function are influenced by its cellular surroundings.[1–4] Correspondingly, the last 20 years have seen a concerted effort to both develop new and adapt existing structure elucidation techniques for the express purpose of acquiring structural data *in vivo*, at all levels of resolution - from Å to μm.[5–18] As a result of these efforts, cryogenic electron microscopy (cryo-EM) and nuclear magnetic resonance (NMR) are increasingly being used to probe the structural properties of proteins, lipids, and nucleic acids at or near atomic resolution *in vivo*.[16,19–22] While cryo-EM based approaches, both *in vitro* and *in vivo*, excel at visualizing conformationally homogenous assemblies, detection of small ($<$100 kDA) and/or conformationally heterogenous species lies at the cusp or beyond the technique's current capabilities.[23,24] NMR on the other hand which has a rich history of visualizing conformationally homogenous or heterogenous species regardless of size *in vitro*[25–32] is increasingly proving capable of garnering similar data from within the cellular lumen. For example, in-cell NMR has been used to visualize α-synuclein's interactome,[15,16] discern a megadalton membrane-associated bacterial complex's flexible segments whilst embedded in native membrane extracts,[33] and further monitor ligand-riboswitch interactions.[34]

Attempts to utilize solution-state NMR for *in vivo* structural characterization began in the early-2000's,[6,7,35] being largely driven by the advent of high field spectrometers ($>$700 MHz) which boosted sensitivity to a level sufficient to visualize highly abundant and rapidly tumbling protein assemblies directly inside cells.[6,7] Now nearly 25 years on, progress is being made to further enhance *in vivo* solution-state sensitivity by exploring the use of ^{19}F NMR[36,37] and further overcoming intrinsic size limitations by exploiting cross-relaxation[38] and/or transverse relaxation-optimized pulse sequences[39] within cellular contexts.[40] Nevertheless, further efforts are required to obtain fundamental solutions addressing these limitations.

In the late 2000's, early 2010's, the potential of magic angle spinning (MAS) supported-solid state NMR (ssNMR) to study largely immobilized yet abundant proteins *ex vivo*, *in situ*, and *in vivo* was demonstrated.[8,41,42] Given that ssNMR suffers from the same spectral insensitivity, attention subsequently turned to utilizing hyperpolarization in conjunction with ssNMR for studying biomolecules regardless of their cellular abundance, with no molecular size limitations *in vivo*.

Dynamic Nuclear Polarization (DNP) is one such hyperpolarization technique that . It can increase NMR sensitivity by several orders of magnitude. In the context of ssNMR, DNP proceeds through transfer of radical electron spin polarization to NMR active nuclei with radical electrons being doped into samples in the form of carefully engineered polarizing agents (PAs).[43–53] The combination of DNP and ssNMR is increasingly proving powerful and has been used to study the structural properties of anti-microbial peptides, globular proteins, membrane associated proteins within bacterial, eukaryotic membrane vesicles (see Refs. [9,10,33,54,55–59]), and nucleic acids directly within intact/lysed bacterial/eukaryotic cells.[11,12,58,60,61] Additionally, the methodology has demonstrated usefulness for pharmaceutical applications.[62]

Despite these advancements, the full potential of DNP-ssNMR could so far not be realized due to limited spectral resolution, especially when acquiring data at low/medium magnetic fields, where homogenous line broadening effects can give rise to excessively broadened correlations,[63,64] is forced due to a lack of PAs with sufficient performance at high magnetic fields.[50,65] Given the great potential of cellular DNP-ssNMR to probe biological systems at atomic resolution against a native background, we herein detail research efforts aimed at improving spectral sensitivity and resolution predominately in cellular contexts. The work discussed includes novel PA design/ delivery,[48–51,66] optimized sample preparation (e.g., use of selective isotope labelling[67] & cellular fractionation[52]), application of novel pulse sequences including ^{1}H-detection,[68] chirped-DNP.[69] Lastly, we discuss the types of biological questions that can be answered by cellular DNP-ssNMR today and possibly in the future.

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2. Optimizing DNP-ssNMR Specific Aspects of Sample Preparation

Sample preparation is a critical aspect of cellular-based DNP-ssNMR, one that involves fulfilling both sample and DNP-ssNMR specific requirements. From the viewpoint of DNP-ssNMR achieving superior spectral sensitivity as well as resolution requires ensuring sample stability at cryogenic temperatures (typically around 100 K) and efficient PA delivery especially for whole cell applications.

The need to ensure sample stability at cryogenic temperatures arises from pioneering work performed by the Griffin group,[70] who observed that suspension of T4 lysozyme in a 6:4 v/v mixture of glycerol and water supplemented with 40 mM of the PA TEMPO, yielded a 100-fold increase in carbon correlation sensitivity at 211 MHz/140 GHz. An important aspect of this work was that it demonstrated that the glycerol/water mixture acted as both a cryoprotectant, preventing ice crystal induced protein damage, and readily formed a homogenous glassy matrix upon flash cooling.[70,71] The mixture's formation of a homogenous matrix was of particular importance given that ice has up to 15 known polymorphic states[72] with concomitant formation of even several polymorphs likely resulting in interfacial PA partitioning, thereby diminished PA performance.[73] As such, the mixture of 6:4 v/v glycerol/water, now colloquially known as "DNP juice", is a mainstay in biological DNP-ssNMR studies. However, recent reports implicate high glycerol concentrations in perturbation of protein hydration and structure.[74] Consequently, the suitability of alternative cryo-protectants (dimethyl sulfoxide – DMSO or trehalose[58,75,76] – Figure 1a) and a variation of "DNP juice" recipe in terms of proportions is currently being investigated, particularly in the context of cellular applications.[61,76]

Of the alternative cryo-protectants, DMSO has thus far received the most attention from the DNP-ssNMR community,[58,61] possibly due to its routine use in the long-term storage of immortalized cell lines. However, increasing reports suggest that cells preserved in DMSO can suffer from epigenetic changes[77] and/or other biological alterations that are seemingly more significant than when using glycerol.[78] Given that trehalose is safe for human consumption[79] efforts to investigate its suitability for cellular DNP-ssNMR studies may be more worthwhile.[75] However, it is important to note that cryo-protectants give rise to strong correlations in DNP-ssNMR spectra thus complicating spectral analysis. Luckily, glycerol correlations can effectively be suppressed by using ^{13}C, ^{1}H depleted glycerol.[11] Additionally, the use of double quantum filtering (see Ref. [11,50,52]), short cross polarization contact times,[49] and ligating PAs directly to biomolecules of interest[60] are also all viable approaches to suppressing glycerol correlations. For trehalose, production of such an NMR-active isotope

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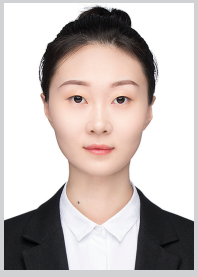

Jiaxin Zhou obtained her bachelor's degree in Pharmacy in 2019 from Tianjin Medical University. She is pursuing her Ph.D. degree under the supervision of Prof. Yangping Liu in Tianjin Medical University. Her research interest is focused on the development of polarizing agents for DNP-ssNMR.

Yangping Liu obtained a bachelor diploma in chemistry in 2001 from Nanchang University, Nanchang, China and a Ph.D. in physical chemistry in Institute of Chemistry, Chinese Academy of Sciences, Beijing, China. After postdoctoral training at the Ohio State University, he moved back to China and joined Tianjin Medical University as a full professor in 2013. His research interest is mainly focused on development of stable (bi)radicals for electron paramagnetic resonance spectroscopy/imaging as well as dynamic nuclear polarization (DNP)-enhanced NMR spectroscopy.

Gert E. Folkers obtained his doctoral degree from the Hubrecht Institute in 1998, working on retinoic acid receptors. Following this, he worked as a postdoc at the NMR spectroscopy research group in Utrecht University. During this time, he worked on biochemical characterization and structure determination of several proteins. In 2006, he was appointed as an associate professor in the life science faculty at Utrecht University. Since then, he has worked on diverse areas of research including studying DNA-damage repair mechanisms and developing novel expression methods for in situ NMR.

Marc Baldus studied Physics at the TU Darmstadt and at the University of Florida. He completed his PhD at ETH Zurich in 1996. After postdoctoral research at MIT and a lecturer position at Leiden University, he worked as tenured group leader at the MPI for Biophysical Chemistry. In 2008, he joined Utrecht University as full professor. His current research focuses on establishing structure–function relationships in complex biomolecular systems using NMR in combination with other spectroscopic, biophysical and cell biology methods.

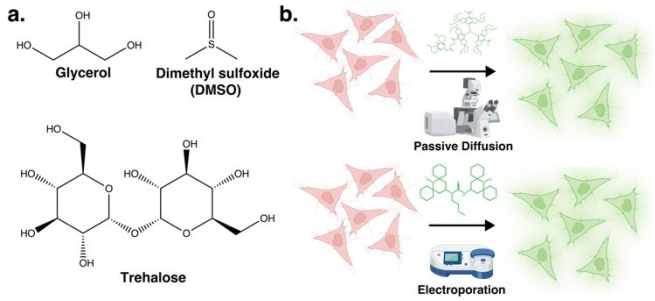

Figure 1. DNP-ssNMR cryo-protectants and polarizing agent delivery methods. **Panel a**. Structures of currently used (glycerol, DMSO) and future cryoprotectants (trehalose). **Panel b**. Polarizing agents delivered into eukaryotic cells (HEK 293T, HeLa, etc) by passive diffusion (top) and electroporation (bottom).

depleted version should also be possible but may come with an increased cost.

Efficient PA delivery and the discernment of PA distribution is a significant consideration for cellular based DNP-ssNMR studies. Both the Barnes[61] and Baldus groups[11,52] have independently demonstrated that PAs of various molecular weights (AMUPOL[80] and SNAPol-1[50]) can be delivered to cells via passive diffusion (Figure 1b). The latter process maybe in part facilitated by glycerol induced membrane alterations.[81] Recently, Ghosh *et al.*, showed that electroporation (Figure 1b) is also a viable delivery mechanism that may yield higher enhancements with significantly lower starting PA concentrations.[58] In addition to electroporation, other deliver methods such as a liposomal[82] and cell penetrating peptides[7] could also be exploited for intercellular PA delivery.

Further, given that PA enhancement is distance dependent[47] it is paramount to determine the PA distribution post-delivery so as to gauge enhancement homogeneity. PA spatial distribution has been successfully assayed by fluorescently tagging PAs[52,61,83] and/or comparing experimental polarization build-up times.[51,52,58] While all delivery methods used thus far seem to distribute PAs homogenously throughout cells, recent reports suggest that the method of PA delivery can influence intracellular PA distribution.[58,83]

3. Designing Polarizing Agents for High-Field Cellular DNP-ssNMR Application

While optimizing sample preparation is important, the development of PAs yielding larger enhancements at high magnetic fields, where homogenous line broadening is significantly diminished, is currently a major focus in the drive to improve both sensitivity and resolution. NMR signal enhancement factors obtained thus far by DNP-ssNMR at high magnetic fields are still far from the theoretical value and strongly dependent on the polarizing agents (PAs) used. The rational design of PAs is inseparable from the understanding of the polarization transfer mechanisms (i.e., DNP mechanisms). DNP mechanisms suitable for the continuous microwave irradiation include the Overhauser effect (OE),[84] the solid effect (SE),[85] the thermal mixing (TM) and the cross-effect (CE),[86] and the last has been proven to be the most effective at high magnetic fields. Different from the two-spin process involving a coupled electron and nucleus in OE and SE, CE relies on a three-spin system consisting of two electron and one nuclear spins.[87] In general, the CE-DNP condition is satisfied when the difference between the Larmor frequencies of the two coupled electron spins matches that of the target nucleus. According to this feature, biradicals have become the most potential PAs for high-field DNP-ssNMR.

Nitroxide biradicals (Figure 2a) are the first class of PAs[88] which have opened a new chapter in high-field DNP. A variety of homogenous dinitroxide-based PAs have been synthesized through the optimization of electron relaxation times, the relative orientation of the g-tensors of the two electron spins, electron-electron coupling interactions, and water solubility.[46] The representative water-soluble PAs such as TOTAPol,[89] AMUPol[80] and bcTol[90] exhibit excellent DNP performances with DNP enhancement factors ($\varepsilon_{on/off}$) of up to ~300 at intermediate magnetic fields (at 9.4 T and ~100 K). Regrettably, the DNP efficiency of these dinitroxide-based PAs suffers from the unfavorable field and magic angle spinning (MAS) rate dependencies. For instance, the $\varepsilon_{on/off}$ values of AMUPol decreased from 235 at 9.4 T to 30 at 18.8 T[45,65] and the overall sensitivity gains were further reduced by a factor two from 5 to 40 kHz MAS due to the depolarization effect.[66] On the other hand, the ε values for complex biomolecules such as membrane proteins, fibrils, and macromolecular aggregates were even in the single digits, much lower than the corresponding values for small molecules. Recently, the heterogenous dinitroxide-based PAs (e.g., AsymPolPOK[66] and M-TinyPol[48]) with enhanced electron-electron interactions have been shown to be more promising than AMUPol for high-field DNP applications, but their application potentials have to be tested for complex biomolecules such as proteins. Taken together, the cellular applications of the dinitroxide-based PAs are mostly confronted with three challenges: (i) the unfavorable magnetic field dependence which greatly reduces the DNP enhancements at high magnetic fields; (ii) significantly lower DNP enhancements for biological macromolecules as compared to those for small molecules; (iii) fast bioreduction of the currently available PAs in cellular environments.

In 2015,[91] some authors in this review collaborated with the Griffin group to introduce a new class of heterogenous trityl-nitroxide (TN) biradical-based PAs (Figure 2b), TEMTriPols, which represents a significant step forward to address the issues from the field and MAS rate limitations. TEMTriPols in which a trityl radical was tethered with a nitroxide via different spacers showed several advantages over the dinitroxide-based biradicals including (i) the ideal EPR frequency separation between the nitroxide g_{yy} component and the almost isotropic g value of trityl radical;[92] (ii) their favorable relaxation times, which allow simultaneous microwave saturation and polarization turnover; and (iii) moderate electron – electron exchange interactions (*J*) which are beneficial for their DNP performance at high magnetic fields.[93] As such, the DNP

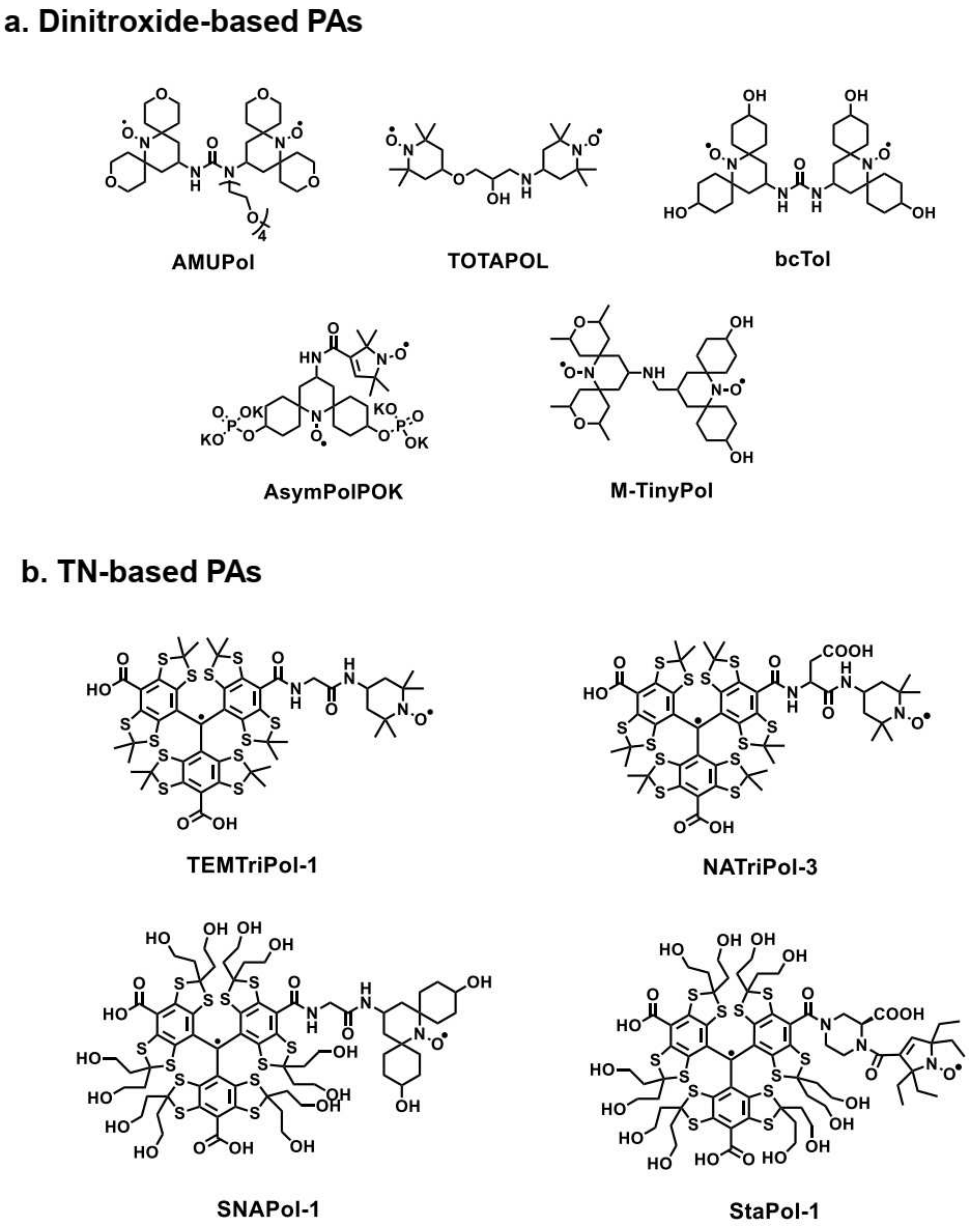

Figure 2. Molecular structure of various polarizing agents. **Panel a**. Dinitroxide-based PAs. **Panel b**. Trityl-nitroxide (TN) biradical-based PAs.

enhancements of TEMTriPols exhibit favorable magnetic-field dependence and the optimal value is displaced toward higher magnetic fields. For example, TEMTriPol-1, the best in the series, shows DNP enhancement factors for ^{13}C-labeled urea of 50, 87 and 65 at 5.0, 14.1 and 18.8 T, respectively, thus yielding a record ^{1}H NMR signal (^{13}C-urea) enhancement at 18.8 T at that time. This work demonstrates that TEMTriPols are very promising to overcome the unfavorable field dependency of the DNP enhancements of the biradical-based PAs. Importantly, as shown by De Paepe *et al.*,[93] in contrast to dinitroxides, TEMTriPol-1 did not induce significant depolarization effects at the chosen MAS settings due to the moderate electron-electron exchange interaction. The crucial importance of the magnitude of the exchange interaction in the DNP process was further confirmed by a subsequent work using the chiral TN biradicals in which the two radical parts were tethered by a proline linker.[94] Since TEMTriPol-1 has good DNP performance for ^{13}C-labeled urea at high magnetic fields, it is very desirable to test its performance for biological macromolecules. Unexpectedly, only a 3-fold signal enhancement for ^{13}C,^{15}N-ubiquitin was observed for TEMTriPol-1 due to self-aggregation under the DNP conditions and hydrophobic interactions with biomolecules. Thus, with the purpose of addressing the second challenge as mentioned above, Zhai *et al.*[95] reported the analogues of TEMTriPol-1, dubbed NATriPols, by structural modification at the α position of the amino acid linker. NATriPols have almost identical dipolar and exchange interactions but distinct hydrophilicity. Owing to their relatively high hydrophilicity, NATriPol-5 ($\varepsilon_{on/off} = 15$), NATriPol-3 ($\varepsilon_{on/off} = 30$) afford 5-, 10-fold DNP improvements at 18.8 T, respectively,

compared to TEMTriPol-1 ($\varepsilon_{on/off} = 3$) when applied to the globular protein ubiquitin. The improved DNP performance of NATriPol-3 has been further confirmed by its application to a membrane peptide Nisin. Therefore, these findings suggest a direct relationship between hydrophilicity and DNP enhancement when using NATriPols for biological applications and that TN biradicals are one of the most promising candidates of PAs for high-field DNP applications.

Despite the improved DNP performance of the hydrophilic NATriPol-3, its enhancement factor for ubiquitin is only approximately one twentieth of the theoretical value (~658). Thus, there is still much room to improve the performance of TN biradicals, especially for cellular applications. This stimulated the development of highly efficient SNAPols[50] by greatly enhancing hydrophilicity and extending electron relaxation times. To do so, the highly hydrophilic trityl radical OX063 was tethered with the spirocyclohexanoyl nitroxide via the optimal glycine linker. The resulting SNAPol-1 exhibits extremely high hydrohilicity due to the presence of the 14 hydroxy groups (12 from trityl and 2 from nitroxide), and two negatively charged carboxylates. Meanwhile, the relatively large size of SNAPol-1 together with replacement of the methyl groups in the trityl and nitroxide parts (in the case of TEMTriPol-1) by the CH_2CH_2OH and spirocyclohexanoyl groups, respectively, effectively lengthen both its longitudinal and transverse relaxation times. Gratifyingly, SNAPol-1 exhibits outstanding DNP performance with the $\varepsilon_{on/off}$ value of 133 for $^{13}C,^{15}N$-proline at 18.8 T, 8 kHz MAS frequency and 103 K. Moreover, this PA provides a new record DNP enhancement of 110 for the globular soluble protein $^{13}C,^{15}N$-ubiquitin at ultra-high magnetic fields, which is 3.7-fold higher than that of AMUPol. More importantly, using SNAPol-1, the enhancement factors of 45–80 were obtained for membrane proteins and cellular samples at 18.8 T, comparable to those obtained by using other PAs at 9.4 T. Our recent work has also shown that SNAPol-1 enables high field DNP-ssNMR for cellular applications with superior sensitivity and spectral resolution when utilized inside Hela cells, and on isolated cell nuclei electroporated with $^{13}C,^{15}N$- labeled ubiquitin.[52] In addition, SNAPol-1 also outperformed M-TinyPol and NATriPol-3 at 18.8 T, thus enabled revealing hidden side chains in amyloid fibrils by MAS-DNP ssNMR at 18.8 T.[96] These results consistently demonstrate that SNAPol-1 is an outstanding PA for various biological samples at ultra-high magnetic fields.

As mentioned above, SNAPol-1 exhibits outstanding DNP performance for different biological samples at ultra-high magnetic fields, but its maximal cellular DNP applications may be limited due to the potential bioreduction in cellular reducing environments. While the trityl part in SNAPol-1 is relatively inert to biological redox reactions, the nitroxide part is highly sensitive to bioreduction.[97] As such, these TN biradicals were initially used for measurement of redox status in biological systems.[58,98] The bioreduction of the dinitroxide-based PAs such as AMUPol[58] and TOTAPol[99] has been a critical issue confronted with their cellular applications. To address this issue and further enhance the potential of TN biradical-based PAs in cellular DNP applications, Yao *et al.*[51] reported a highly bioresistant and hydrophilic TN biradical-based PA (StaPol-1) comprising the trityl radical OX063 ligated to the highly bioresistant *gem*-diethyl pyrroline nitroxide via a rigid piperazine linker. EPR experiments demonstrate the high reduction resistance of StaPol-1 to ascorbate and in HeLa cell lysates. Importantly, the use of a relatively high concentration of StaPol-1 (e.g., 30 mM) owing to its high hydrophilicity achieved a uniform DNP enhancement of 183 at 18.8 T for $^{13}C,^{15}N$-ubiquitin in HeLa cell lysates even after incubation at room temperature for 1.5 h. Thus, StaPol-1 represents the first PA which integrates outstanding DNP performance for complex biomolecules at high/ultra-high magnetic fields with long-term stability in cellular environments.

4. Selective Stable Isotope Labelling, Intercellular Biomolecule Delivery, and Fractionation Approaches to Boost Sensitivity and Resolution

Stable isotope labelling has been extensively used in conventional NMR as a means of increasing sensitivity and reducing spectral crowding.[100–104] It has also been employed in cellular-based DNP NMR studies albeit with several additional considerations arising from the need to maintain cellular homeostasis.

Considering that established stable isotope labelling strategies were designed with the intention of downstream target purification, a chief contention for cellular NMR studies becomes distinguishing between overexpressed and background signals. In 2001, the Dötsch group[35] demonstrated that correlations originating from a protein overexpressed in *E.coli* cells, grown on ^{15}N-enirched media, could by and large be distinguished from background correlations as overexpression significantly slowed down the host's metabolic and catabolic processes. Nevertheless, background ^{15}N correlations were still evident appearing in spectrally crowded regions. To combat this, the Dötsch group removed background correlations by performing a spectral subtraction which required acquisition of an equivalent "blank" spectrum i.e., on non-induced *E. coli* grown on ^{15}N-labelled media.

While the procedural ease of indiscriminate labelling methods and difference spectroscopy is undoubtedly a major contributor to their popularity,[38,102,103] the joint methodology imposes significant limitations; firstly, spectral sensitivity becomes dependent on a respective protein's expression level and secondly it is difficult to achieve overexpression at a biologically relevant concentration. To overcome these issues the Baldus group has sought to introduce minor procedural modifications that would largely retain procedural ease but significantly increase both labelling selectivity and sensitivity. Firstly, they demonstrated that background labelling could be suppressed near entirely in bacteria if the overexpression vector made use of a non-bacterial polymerase (e.g., the T7 system) by treating cells with the bacterial RNA polymerase inhibitor rifampicin (Figure 3a).[53,105,106] An even more selective spectrum could be achieved for membrane-embedded proteins by employing a protein sequence optimized labelling scheme with

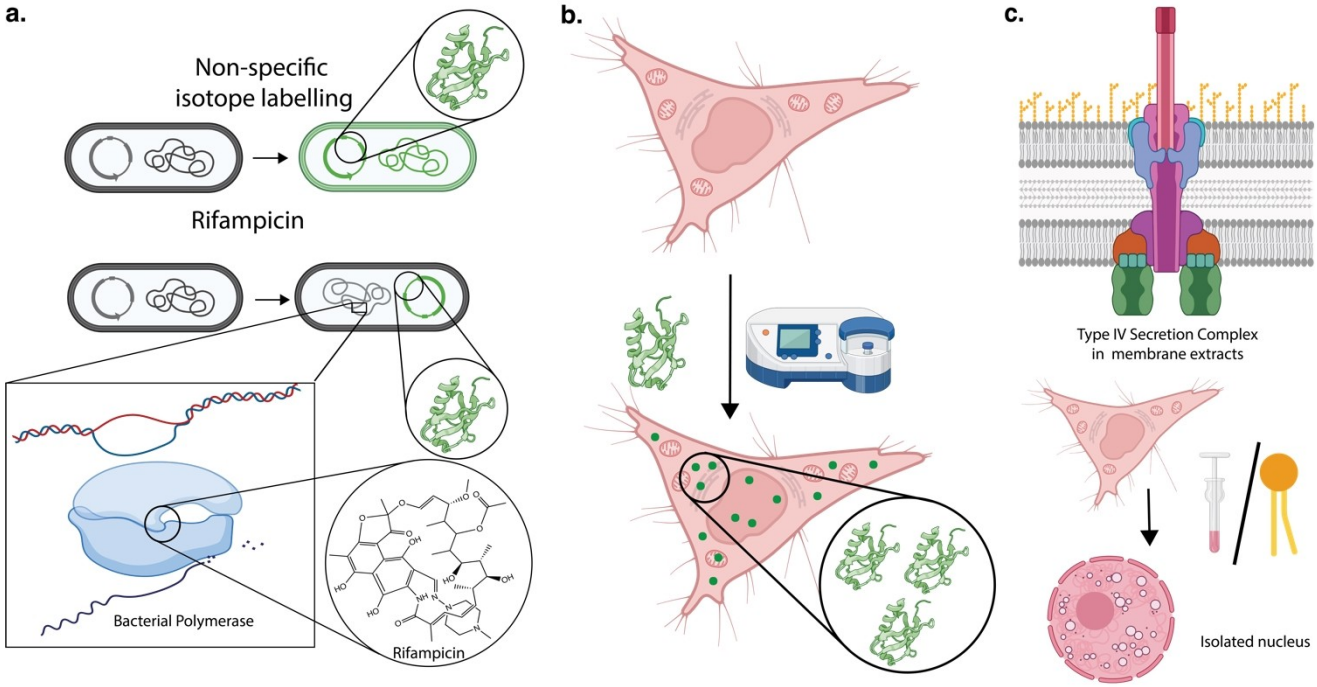

Figure 3. Strategies to improve sensitivity and resolution by optimizing sample preparation. **Panel A**. Depiction of the rifampicin specific stable isotope labelling strategy. In the case where no rifampicin (top) is used all biomolecules are labelled (coloured a green). In the case where rifampicin is used, only the protein encoded for by the non-bacterial polymerase promoter plasmid (such a T7 RNA polymerase) is stable isotope labelled. The bottom panel depicts the inhibition of bacterial polymerases by rifampicin. **Panel B**. Depiction of protein delivery by electroporation. After electroporation, the protein is treated endogenously being proportioned amongst the various cellular compartments as is customary. **Panel C**. Cellular fractionation allows to both decrease spectral crowding by removing signals originating from peripheral biomolecules and increases sensitivity by allowing for increased sample packing. The top image depicts the Type IV secretion complex in cell envelope extracts and the bottom image depicts isolation of cellular nuclei that can be achieved via mechanical (Dounce) and/or detergent based isolation.

pairs of adjacent amino acids, unique to the protein of interest, being labelled in a reverse manner and subsequently detected via a pulse sequence selective for correlations arising from adjacent residues (*i*, *i* + *1*).[10,33] Recently, all these strategies have been used in tandem to facilitate membrane-protein structure determination within native membrane extracts.[107]

Given the difficulty of recombinantly producing biomolecules in eukaryotic cells without background labelling,[108] in-cell NMR studies attempting to investigate the structural aspects of molecules in such systems have sought to employ delivery systems. The idea of delivering exogenous molecules to cells is highly attractive as it permits to simultaneously ensure selective stable isotope labelling and exert control over the intercellular molecule concentration. The first forays into delivery were made by the Shirakawa group who showed that the labelled globular protein ubiquitin could be delivered into *Xenopus laevis* oocytes[6] via injection and into somatic eukaryotic cells (HeLa cells)[7] by ligation to cell penetrating peptides (CPPs).

Considering that injection is not viable at scale and that significant efforts are required to produce CPP conjugated biomolecules that retain biological activity, the Selenko group pioneered the delivery of proteins by electroporation (Figure 3b) a method extensively used for delivery of nuclei acids.[15,19,34,109] The Selenko, Shektman,[40] Baldus groups,[11,52] and others[110,111] have all subsequently demonstrated that a variety of proteins (both intrinsically disordered, globular) can be delivered via electroporation at near endogenous concentrations with a relatively homogenous distribution amongst somatic host cells. Most importantly the delivered proteins are folded and remain functional even being treated as their endogenous counterparts. As such, electroporation presents as a versatile and easy to use method for homogenous intercellular delivery of stable isotope labelled nucleic acids and proteins that standout against the unlabelled background of host cells. However, a caveat with electroporation is that the technique induces cellular stress, reduces cellular viability (up to 30%) with the former being overcome by long (>3 hrs) recovery times.[112] As a result less damaging delivery approaches, like those based on microfluidics, are currently being investigated.[112]

In addition to selective labelling and exogenous delivery, sample enrichment is also emerging as a viable means of boosting sensitivity whilst reducing spectral crowding. For example, membrane extracts (Figure 3c) have been utilized instead of intact cells to study membrane proteins as a means of significantly enhancing rotor packing.[10,33] Further, cellular fractionation has been used to acquire spectra of proteins with sub-cellular specificity.[52,113] Recently our groups showed that isolation of cellular nuclei (Figure 3c) facilitated a 4-fold increase in spectral sensitivity[52] and, from a DNP standpoint, membrane removal facilitated increasing PA entry yielding a further 2-fold increase in sensitivity.

5. Solid-State NMR Methods

Like in conventional NMR, sensitivity and spectral resolution are also influenced by the pulse sequence and detected nuclei. Thus far, cellular DNP-ssNMR data have predominately been acquired with 3.2 mm low-temperature MAS (LT-MAS) probes with maximal MAS rates of 12–14 kHz. As such, $^{13}C/^{15}N$-detected multidimensional cross polarization (CP[114]) based pulse sequences including proton driven spin diffusion,[115] frequency switched Lee-Goldburg heteronuclear correlation spectroscopy (FSLG-HETCOR),[116,117] SPECIFIC-CP,[118] 2D/3D ^{13}C double quantum filtered experiments (DQSQ)[119,120] and transferred echo double resonance (TEDOR)[121] have seen extensive use. Additionally, 2D/3D pulse sequences facilitating inter-residue assignment have also been performed with carbon detection.[33,105]

While these experiments have provided valuable insights for both carbon and nitrogen spins, DNP-ssNMR spectra can suffer from poor resolution because of cryogenic temperature induced freezing out of conformational dynamics.[52,63,64,122,123] This is especially an issue for nitrogen detected experiments and is routinely overcome by using ^{15}N filtered pulse sequences.[33,105,124]

To further increase spectral sensitivity and resolution the DNP-ssNMR community has begun to explore the potential of ^{19}F NMR[125] and ^{1}H-detection especially in recent years with the advent of commercial faster-spinning (low temperature) LT-MAS probes.[68,126] While the utility of ^{1}H-detection under DNP conditions has presently only been tested on small molecules, early *in vitro* results seem very promising[68,126] particularly because fast-MAS not only unlocks the benefits traditionally associated with ^{1}H-detection, but also permits the use of smaller diameter rotors which allow for vastly improved microwave distribution[127] facilitating a significant boost in PA enhancement.[48,49] However, it should be noted that application of ^{1}H-detection for studying intact cellular systems will require significant care to ensure cellular integrity at the associated higher g-forces.[128]

As discussed earlier the most effective PA agents exploit the cross effect which requires continuous microwave (CW) irradiation which necessitates that experiments are performed at cryogenic temperatures where spectral resolution is influenced by both homogeneous, inhomogeneous broadening. Efforts are now underway to apply microwaves selectively, possibly leading to DNP performed at ambient temperatures. Thus far the application of chirped DNP to whole Jurkat cells boosted PA enhancement by over 20%.[69]

6. Applications of DNP-ssNMR to Study Complex Biological Systems

While methodological developments are vital, application of these innovations to obtain new biological insights is also crucial especially in demonstrating the technique's utility to the broader scientific community. To this end, the following sections provide a detailed discussion of insights thus far garnered by applying DNP-ssNMR in the study complex bacterial, fungal, plant, and eukaryotic systems.

6.1. DNP-ssNMR to Study Macromolecular Assemblies in Bacterial Cells

As mentioned before, cellular DNP-ssNMR was first used for characterizing bacterial systems. In 2012, Renault *et al.*, demonstrated that both membrane proteins and nucleic acids could be visualized at atomic resolution directly within intact *E.coli* by DNP-ssNMR at 400 MHz/227 GHz.[42] In the former case, correlations unique to PagL, which was overexpressed on $^{13}C,^{15}N$-labelled media, could be discerned from general background signals; such unique signals were also apparent in isolated cellular envelope preparations. In the latter case, deoxyribose/ribose nitrogenous base connections and imino protons signals arising from base pairing interactions were evident for DNA and RNA (at endogenous concentrations). Subsequently, the Reif group used DNP-ssNMR (400 MHz/227 GHz), protein overexpression, amino acid selective-labelling, membrane extraction, and 3D carbon detected experiments to acquire inter-residue assignments (*i*, *i-1)* which revealed that the protein Mistic was membrane-embedded and structurally homogenous in *E.coli*.[54] The methods pioneered above were subsequently used to characterize a variety of bacterial membrane proteins,[107,129,130] with a shift to characterizing larger complexes like the pentameric 200 kDa β-barrel assembly machine (BAM),[131] the megadalton type IV secretion complex,[33] and more recently the 231 residue aquaporin 2.[107] In the case of the secretion complex, it was demonstrated that DNP-ssNMR was capable of detecting signals from highly dynamic segments that were previously poorly resolved by other structure elucidation techniques. Efforts have also been made to visualize globular bacterial proteins within both bacterial lysates and intact cells.[12,55,56,60,132] The Etzkorn group demonstrated that protein-peptide interactions could be discerned *ex vivo* with high sensitivity and at near endogenous concentrations by ligating PAs directly to the molecules of interest.[60] Our group recently provided the first *in vivo* atomic evidence of artificial metalloenzyme assembly within *E coli* cells.[12] Detailed workflows developed in our group for both membrane associated proteins and globular proteins alike have also been published.[53] Presently, bacterial-based DNP-ssNMR is increasingly being used as a means of validating antimicrobial peptide-lipid, peptide-peptide and protein-lipid structures determined *in vitro*, *ex vivo*.[55,56,132–134] A noteworthy aspect of this work has been made by the Weingarth group, who in conjunction with our group, have visualized peptide-lipid, peptide-peptide and peptide-water interfaces[135] by employing magnetization transfer between differentially (^{13}C, ^{15}N) labelling species.[136]

6.2. Applications of DNP-ssNMR to Fungal and Plant Cells

Another area where DNP extends the capabilities of solid-state NMR in studying complex biomolecules within a native setting

refers to studies of fungal and plant cells. While conventional ssNMR methods, in combination with isotope-labeling, offer insight to characterize such systems at chemical, structural, and dynamical detail[137,138] DNP studies have expanded the applications[139] by improving sensitivity in structural studies.[137] Complementary to the use of ^{1}H detection,[140,141] the use of DNP-based ssNMR experiments also enabled studies of unlabeled cell and cell materials, as well as establishing interfacial contacts.[137,142,143]

6.3. Applications to Eukaryotic Membrane Vesicles and Cellular Lysates

Like the in-cell solution state NMR community, the DNP-ssNMR studies have also progressed to studying eukaryotic systems. The first efforts in that direction were made in 2015 by the Griffin group, who via DNP-ssNMR (211, 700 MHz) observed that isotope labeled Sup35, an intrinsically disordered protein exogenously added to yeast cell lysates at endogenous concentrations underwent structural changes in regions previously revealed to be biologically important.[9] In 2016, our group used DNP-ssNMR to describe at an atomic-level of resolution the conformational dynamics of EGFR whilst embedded in native eukaryotic membrane vesicles.[10]

6.4. Application of DNP-ssNMR to Study Biomolecules Directly within Intact Eukaryotic Cells

Presently, a frontier of cellular DNP-ssNMR lies in the structural characterization of biomolecules directly within eukaryotic cells. In 2019, the Baldus group utilized electroporation to deliver recombinantly produced ^{13}C, ^{15}N-labelled ubiquitin (Ub) into the lumen of HeLa cells and visualize its correlations *in vivo* at 400 MHz. More recently, the Baldus group extended that approach to acquire Ub spectra at 800 MHz, within cellular nuclei isolated from whole cells electroporated with Ub in manner similar to what was previously done to study soluble mitochondrial proteins *in situ* by solution-state NMR.[113] This organelle-specific approach revealed significant spectral changes between Ub in nuclei and whole cells suggesting that the DNP-ssNMR spectra acquired at 800 MHz captured Ub's purported conformational plasticity.[144,145]

7. Summary and Outlook

The increasing need to characterize the functional and structural aspects of biomolecular systems in native surroundings has in a large part spurred on the development of biological DNP-ssNMR.[11,58,61] As discussed above, a critical aspect of those endeavours was to increase sensitivity and resolution through optimization of sample preparation, PA development (Figure 4), and strictly spectroscopic considerations.

As it currently stands, signals originating from stable isotope labelled proteins delivered to cells via electroporation, yielding low μM (10–30) intracellular concentrations can now be routinely detected in by DNP-ssNMR. This level of sensitivity compounded with recent reports showing that electroporation[109] and other techniques[112] can be used to deliver proteins of larger sizes (upwards of 100 kDA) to cells opens up the gates to studying a large proportion (>70%) of the 40 most abundant proteins including chaperones, the 14-3-3 family and 26 S proteosome constituents within HeLa cells.[146] Probing the binding interactions of such proteins with greater

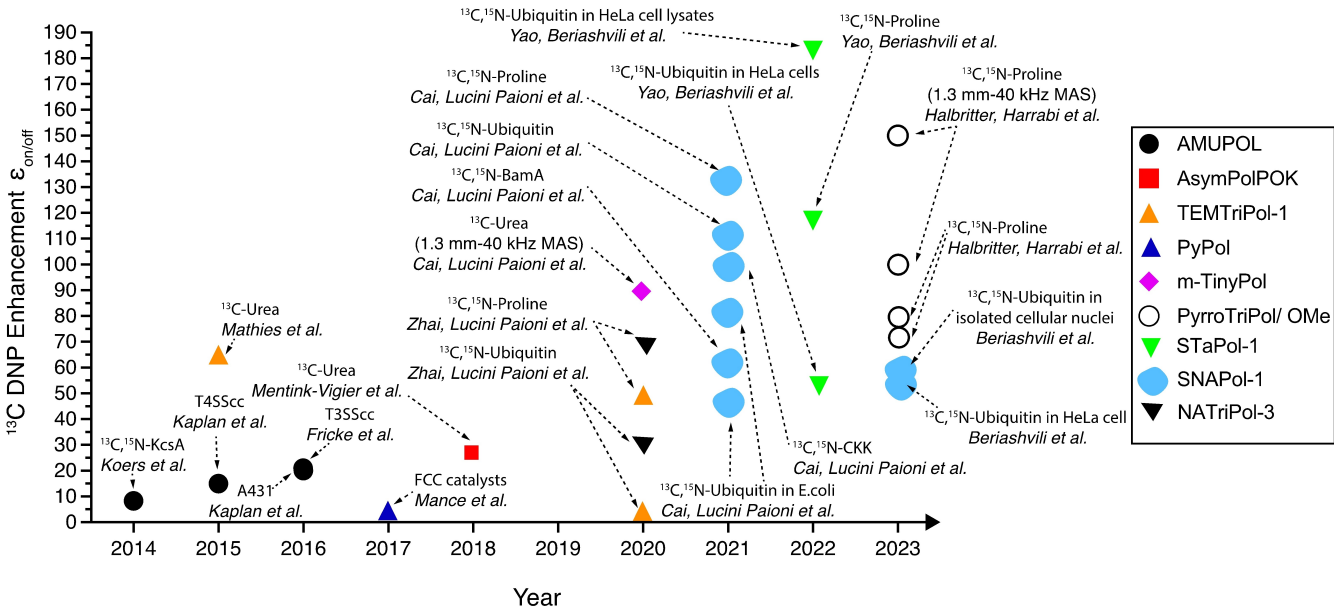

Figure 4. ^{13}C enhancements obtained thus far at 18.8T at a range of 8 to 11 kHz MAS and 100 K on predominately biological samples. An increasing ^{13}C enhancement is evident over the years.

specificity may also be achieved by tagging them with PAs, maybe directly within the cellular lumen by employing click chemistry[147] and codon expansion[148] in combination with selective isotope labelling.

However, although signals originating from proteins at low μM concentrations within cells are readily evident interpreting the resultant DNP-ssNMR spectra remain difficult due to the presence of broadened and thus overlapping correlations. The spectroscopic linewidth in ssNMR is influenced by homogenous and inhomogeneous line broadening effects, with homogeneous line broadening being caused by incomplete averaging of dipolar interactions under MAS and with inhomogeneous line broadening reflecting intrinsic structural disorder (barring technical issues of course for the latter).[63,64,122,149,150] Under DNP conditions structural disorder is substantially increased because the biomolecule's conformational states are frozen out by the cryogenic conditions necessary for data acquisition (Figure 5). While inhomogeneous line broadening is intrinsic, homogenous line broadening can be readily reduced by acquiring spectra at higher magnetic fields[63] and/or MAS rates[151].

This taken together with the desire to characterize less abundant species makes it evident that currently available levels of sensitivity and resolution benefit from further improvements. To overcome these issues, it is therefore desirable to acquire cellular DNP-ssNMR data at high magnetic fields, which is now increasingly becoming possible due to the advent of new PAs (see section 3) which provide significant DNP enhancements at high magnetic fields and increased spectral resolution. For example, the recently designed TN biradical-based PAs by our groups such as SNAPol-1 and StaPol-1 are highly attractive for a broad range of cellular DNP applications. We anticipate that the molecular design of these PAs will be instrumental for the further development of even more efficient high-field compatible dinitroxide-based PAs. Additionally, given emerging evidence that PAs can engage in endogenous biological processes it is also important that future generations of PAs are designed to be fully biological inert[152] and that additional efforts are devoted to further improve intercellular PA delivery whose intracellular concentrations remain undetermined.

Moreover, calculations suggest that existing TN-biradical scaffolds will also perform well at even higher magnetic fields including 1.0, 1.2 GHz for which DNP setups are currently being developed. Such setups are also expected to further improve the reach of cellular DNP in the future. In parallel, research is also being undertaken to conduct ssNMR in combination with DNP or other hyperpolarization methods at ambient temperatures which would further broaden the applicability of cellular ssNMR.

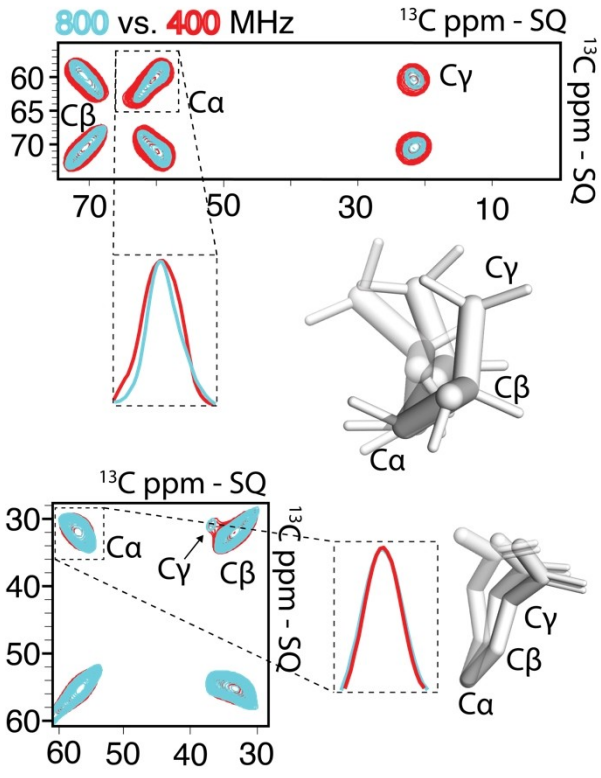

Figure 5. Cellular protein conformational dynamics detected by DNP-ssNMR experiments at low (400 MHz- cyan), high (800 MHz- red) magnetic fields. Detected are 2 residues one (top panel) that experiences a reduction in linewidth at 800 MHz, one (bottom panel) that experiences no change indicating that intrinsic structural disorder is sampled at both field strengths by the latter residue.

Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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