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State-of-the-art accounts of hyperpolarized ¹⁵N-labeled molecular imaging probes for magnetic resonance spectroscopy and imaging

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Hyperpolarized isotope-labeled agents have significantly advanced nuclear magnetic resonance spectroscopy and imaging (MRS/MRI) of physicochemical activities at molecular levels. An emerging advance in this area is exciting developments of ¹⁵N-labeled hyperpolarized MR agents to enable acquisition of highly valuable information that was previously inaccessible and expand the applications of MRS/MRI beyond commonly studied ¹³C nuclei. This review will present recent developments of these hyperpolarized ¹⁵N-labeled molecular imaging probes, ranging from endogenous and drug molecules, and chemical sensors, to various ¹⁵N-tagged biomolecules. Through these examples, this review will provide insights into the target selection and probe design rationale and inherent challenges of HP imaging in hopes of facilitating future developments of ¹⁵N-based biomedical imaging agents and their applications.

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Introduction

1.1. Magnetic resonance spectroscopy and imaging – general information and limitations

Magnetic resonance spectroscopy and imaging (MRS/MRI) are powerful non-invasive molecular imaging modalities that provide biochemical and anatomical information about the human body. MR imaging mainly concerns the generation of anatomical images translated into spatial maps to distinguish healthy tissues from diseased areas.1 MR spectroscopy, performed along with MRI, analyzes chemical processes and metabolic contents of the scanned tissue. MRS offers qualitative and quantitative assessments of various MR-active nuclei (i.e., ¹H, ¹³C, ¹⁵N, and ³¹P) in metabolites using chemical shift assignments in the NMR spectra.^{2,3} Therefore, MRI and MRS have been routinely used in research and clinical practices as imperative diagnostic techniques that offer valuable biochemical and anatomical information. Despite these advancements, magnetic resonance spectroscopic technologies suffer from low sensitivity and clinical MRS/MRI are restricted to the most abundant proton (¹H) resonances as the signal source.

All MR scans are evolved from nuclear magnetic resonance (NMR) and the imaging sensitivity mainly relies on the abundance and polarization levels of nuclear spins. As thermal polarization levels of nuclear spins are small, traditional ¹H-MRI detects highly abundant ¹H signals in the form of water and fat to provide sufficient sensitivity. Yet scanning other MR-active nuclei found in biomolecules is challenging due to their

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low natural abundances. For example, carbon and nitrogen are among the most common elements found in the structures of biomolecules. The natural abundance for 13 C and 15 N is only 1.1% and 0.37%, respectively, in comparison to 99.99% for 1 H (Table 1). Other factors related to the MR signal intensity are the gyromagnetic ratio (γ) and concentration of the nuclei of interest. The γ value directly correlates with the NMR signal sensitivity; for instance, 13 C has a low gyromagnetic ratio, which is less than 1/4 of γ (1 H), and therefore has a lower relative sensitivity to 1 H. Furthermore, the ultra-low γ of 15 N (1/10 of γ (1 H)) translates into a significant decrease in sensitivity.

In addition to the low γ values, the sensitivity of isotopeenriched metabolites may suffer from the low concentration (sub-millimolar) of the interrogated metabolic species *in vivo*.⁵ Accordingly, it is challenging to observe these isotope signals, especially those of ¹⁵N nuclei, from biomolecules *in vivo* with the conventional MRS/MRI. Yet, several hyperpolarization techniques have emerged to tackle the challenge of MR sensitivity.

1.2. Hyperpolarization technique and current methods

The sensitivity of MR correlates with nuclear-spin polarization. The NMR signal intensity is governed by the population difference between two nuclear spin states, also referred to as the polarization level. The polarization is affected by the gyromagnetic ratio (γ) and the magnetic field strength (B_0). At thermal equilibrium, polarization levels of MR-active nuclei are low (only 10^{-6} to 10^{-4}), which is the reason for the low sensitivity of MRI/MRS.⁶

The hyperpolarization (HP) technique addresses the sensitivity problem and has revolutionized the field of MR

Table 1 Nuclear magnetic properties of ¹H. ¹³C and ¹⁵N nuclei⁴

Nucleus	Natural abundance (%)	$\gamma (10^7 \text{ rad } \text{T}^{-1} \text{ s}^{-1})$	Relative sensitivity ^a	Relative receptivity ^b
¹H	99.99	26.75	1.000	1.00
¹³ C	1.11	6.73	0.016	1.70×10^{-4}
¹⁵ N	0.37	-2.71	0.001	3.84×10^{-6}

^a At a constant magnetic field and equal number of nuclei. ^b The receptivity reflects the overall ease of acquiring an NMR signal relative to ¹H at the same magnetic field.

spectroscopy and imaging. Hyperpolarization artificially induces a nonequilibrium polarization of nuclear spins for a period of time (Fig. 1). The HP technique can enhance signal sensitivity by several orders of magnitude by increasing the spin state population difference. The dramatic signal enhancements allow real-time detection of both introduced hyperpolarized imaging agents and their metabolic products. Thus, HP-MR scans of isotope-labeled probes provide unparalleled ability to monitor complex biological processes through advantageous features of the NMR spectroscopy combined with its high structural specificity, non-invasiveness, and quantitative analysis.

Among several available hyperpolarization methods, two techniques have been used mainly for polarizing non-gaseous isotopes. The first technique is dynamic nuclear polarization (DNP), which is currently the most clinically advanced method that has been used for *in vivo* hyperpolarization studies.^{5,7} DNP relies on polarization transfer from electrons to the nuclei of interest dissolved in glass-forming solvents *via* microwave irradiation at low temperatures (1–2 K) for approximately 1–3 hours.⁸ After polarization build-up, the frozen pellet containing a hyperpolarized imaging agent is quickly dissolved with hot water (hence dissolution-DNP, d-DNP), generating a hyperpolarized solution ready for *in vivo* imaging. d-DNP is the most established polarization method used for preclinical and clinical imaging, as according to its principle, any molecule of interest can be hyperpolarized in water.

The second hyperpolarization method uses *para*-hydrogen as the polarization transfer source. ^{9,10} For example, *para*-hydrogen-induced polarization (PHIP) can be achieved by catalytic

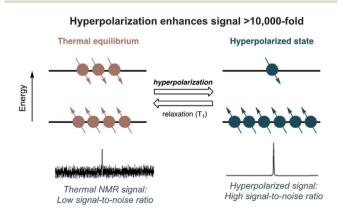


Fig. 1 Hyperpolarization of the nuclear-spin population to enhance NMR signals.

hydrogenation of para-H₂ across unsaturated bonds (e.g., alkene or alkyne) located near the MR-active isotope. Thus, the reduction of the unsaturated bond with a concomitant break of para-H₂ symmetry enables polarization transfer from ¹H to nearby ¹³C or ¹⁵N nuclei via networks of J-coupling. ¹¹ The PHIP method is not generally applicable as d-DNP as the substrate needs to have an unsaturated bond. Alternatively, para-H2 can be used to deliver polarization transfer by signal amplification by reversible exchange (SABRE) through reversible binding to a metal catalyst from both para-H₂ and the substrate. 12-14 Thus, SABRE can hyperpolarize a broader scope of substrates than the traditional PHIP method that relies on the irreversible hydrogenation reaction. The detailed mechanisms of these hyperpolarization techniques have been described in several review papers. 15-17 Overall, these developments in polarization techniques have significantly advanced simple proof-of-concept ideas of hyperpolarized MRS/MRI into clinical applications.

1.3. Development of hyperpolarized MRI/MRS agents: considering factors and current progress

Hyperpolarized imaging studies rely on molecular probes, which are isotope-enriched chemical agents used to visualize, characterize, and quantify biological processes. 18,19 These probes can be fine-tuned to characterize a specific molecular or cellular process of interest for diagnostic or therapeutic applications. Developing an effective hyperpolarized molecular imaging probe is challenging, particularly in addressing several important considerations that are specific to hyperpolarized imaging. First, the labeled nuclei should have a long longitudinal relaxation time, denoted as T_1 . The MR signal detection window strictly depends on the T_1 value, which represents approximately 1/3 of polarization decay back to the thermal equilibrium of the spin population. Therefore, great efforts have been devoted to extending the polarized state in the hyperpolarization process and identifying isotope-labeled functional groups and centers with long T_1 lifetimes. For example, 13 C centers without directly attached protons, such as 13C centers in carbonyl groups, benefit from the decreased dipolar relaxation and commonly have longer T_1 values.²⁰

In addition to the dipolar contribution, the magnetic field strength also affects the T_1 value. The magnetic field strength, commonly measured in tesla (T), correlates with the signal-to-noise ratio – a stronger magnetic field yields stronger signals over background noise and consequently, provides a better image. Routinely used clinical MRI scanners have field strengths of 1.5 and 3.0 T, while research MRI and laboratory

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NMR spectrometers commonly have field strengths of 7.0, 11.7, and 14.1 T. Generally, the T_1 has an inverse correlation with the magnetic field, so the higher fields result in shorter T_1 values.

Second, the design of HP-MR probes should consider the difference in the chemical shift between the probe and its reaction product (i.e., metabolite). A larger chemical shift difference in the NMR spectra will provide more distinguishable peak identification and quantification, especially in lower magnetic fields (for example, typically 5-40 ppm for ¹³C).²¹

In the current field of HP imaging, ¹³C tracers are the most explored for studying metabolic processes, largely because carbon serves as a backbone for nearly all organic biomolecules. Several comprehensive review papers delineate hyperpolarized ¹³C probes exploited for preclinical and clinical research, ^{20–28} which is beyond the scope of this review paper. The success in hyperpolarized ¹³C imaging has validated the applicability of HP MRI/MRS technology in clinical settings for monitoring disease progression and therapy response. At the same time, ¹³C-labeled agents often manifest short polarization lifetimes, with T_1 values of only tens of seconds, presenting a limitation for imaging slower biological processes beyond rapid metabolic

Compared to ¹³C-based probes, hyperpolarized ¹⁵N agents have proved to offer much longer T_1 lifetimes and are well suited for sensor designs.29 This review will present current accounts of hyperpolarized 15N-labeled biomolecular probes studied in the literature, the advantages and challenges associated with ¹⁵N-probes, and how ¹⁵N-agents can provide unique directions in the field of hyperpolarized imaging.

Hyperpolarized ¹⁵N probes 2.

2.1. Introduction on ¹⁵N-labeled probes: unique properties and potential in molecular imaging

Nitrogen atoms are present ubiquitously in bioorganic molecules, and in principle, any nitrogen center can be isotopeenriched with 15N nuclei.30 As 15N has a gyromagnetic ratio lower than those of ¹H and ¹³C, the ¹⁵N-NMR signal suffers from poorer sensitivity. However, the reduced interaction of ¹⁵N with an external magnetic field allows longer polarization lifetimes of 15N centers in the order of several minutes. Such long polarization lifetimes expand the imaging window of the hyperpolarized species and dramatically broaden the potential applications in biomedical imaging beyond rapid metabolism tracing restricted by the shorter T_1 lifetime of 13 C metabolites.

Besides potentially long hyperpolarized lifetimes of the ¹⁵N nucleus, ¹⁵N-NMR has a wider range of chemical shifts. This warrants a greater sensitivity of 15N chemical shift to its environment. The development of non-1H-based MRI and MRS agents has been partially motivated by the difficulty in deconvoluting many metabolite resonances in the narrow chemical shift range of ~10 ppm of the ¹H spectrum. In comparison, peaks corresponding to 13C metabolites of interest occur over a much wider range of approximately 200 ppm. A wider range of chemical shifts provides hyperpolarized ¹³C-based probes with greater qualitative analysis capability to trace complex biochemical processes. In this respect, the 15N spectrum

provides an even more comprehensive range up to 900 ppm,²⁹ thus providing hyperpolarized ¹⁵N probes with an even higher detection accuracy and an extended scope of chemical complexity. These favorable features of hyperpolarized 15Nprobes offer promising biomedical and clinical imaging applications.

This review presents the up-to-date progress in the development of various ¹⁵N agents for hyperpolarized bioimaging. The HP ¹⁵N agents reported so far are classified into three main categories in this review: (1) 15N-enriched endogenous molecules and drugs, (2) 15N sensors designed for specific physiological parameters, and (3) biomolecules labeled with 15N molecular tags. The discussion on these probes generally covers the design principles, considerations, and imaging performances in each molecular probe category.

¹⁵N-Enriched endogenous molecules and drugs

Isotope enrichment is the most straightforward approach in designing HP agents, including 15N-labeled endogenous metabolites and drug molecules. Ideally, HP agents should have low toxicity and high cellular uptake for in vivo imaging. Considering that the imaging agents are typically hyperpolarized ex vivo and injected intravenously into animals, high concentrations of HP agents (generally 10-100 mM) are often needed, taking into account the dilution in the blood, to produce sufficiently detectable NMR signals in vivo. The cytotoxicity profiles of endogenous metabolites and drug molecules are readily available, which expedited the in vivo applications of ¹⁵N-enriched hyperpolarized probes. So far, several successful probes in this regard have been reported, including ¹⁵N-choline, ¹⁵N-permethylated amino acids, ¹⁵N-carnitine, azidothymidine (AZT), and 15N-heterocycle-based drugs.

2.2.1. ¹⁵N-Choline. Choline (Cho) is an endogenous molecule involved in phospholipid metabolism. Elevated metabolism of choline to phosphocholine (PCho) catalyzed by choline kinases is a known characteristic of cancer, making choline an ideal biomarker for tumor imaging.31,32 None of the carbon centers in the natural choline molecule (CH₃)₃N⁺CH₂CH₂OH would retain a long T_1 lifetime if 13 C-enriched. However, the quaternary amine is suitable for 15N-enrichment to achieve long-lasting polarization, benefiting from the absence of proton-based dipole relaxation.

¹⁵N-Enriched choline has been hyperpolarized and studied to monitor in vitro choline metabolism to ¹⁵N-PCho for the first time by Gabellieri et al. (Fig. 2A).33 The non-basic and symmetrical environment of the quaternary 15N center in choline led to exceptionally long T_1 values of 285 \pm 12 s in water and 120 \pm 10 s in blood at 37 $^{\circ}\text{C}$ in a magnetic field of 11.7 T. A reduction of T_1 in the blood is a documented phenomenon and can result from the increased relaxation caused by the viscosity of blood, presence of red blood cells, and hydrogen-bonding with biomolecules. This study monitored the in vitro enzymatic conversion of hyperpolarized ¹⁵N-Cho to ¹⁵N-PCho, with a maximum buildup of ¹⁵N-PCho observed at 114 s. The initial rate of ¹⁵N-PCho buildup was estimated to be 1.45 mM min⁻¹ using choline kinase (2 µM). Such kinetics information

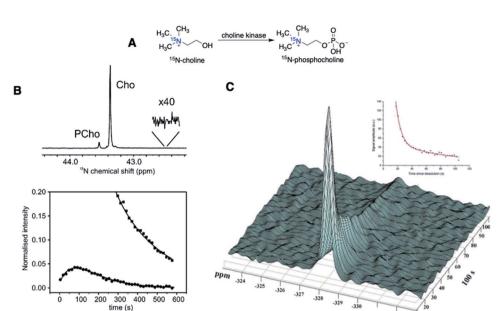


Fig. 2 First hyperpolarization experiments of 15 N-choline. (A) Schematic conversion of 15 N-choline to 15 N-phosphocholine. (B) (Top): Enzymatic conversion of hyperpolarized 15 N-Cho to 15 N-PCho, scanned at the maximum buildup PCho (t=114 s, Δ^{15} N = \sim 0.2 ppm), and (bottom): peak integral plotted against imaging time in seconds, with squares = 15 N-Cho and circles = 15 N-PCho. (C) First *in vivo* polarization decay graph of 15 N-Cho spectra, with the 15 N peak referenced to nitromethane. (B) Adapted with permission from ref. 33. Copyright 2008, American Chemical Society. (C) Adapted with permission from ref. 35. Copyright 2010, The Royal Society of Chemistry.

obtained using the plotted hyperpolarization signal over time is vital for estimating substrate buildup rates and enzyme activity. Most encouragingly, the $^{15}{\rm N}$ signal remained after 10 min, substantially exceeding the longevity compared to deuterated $^{13}{\rm C}$ -choline with a T_1 of $\sim\!30$ s (11.8 T). 34 On the other hand, the $^{15}{\rm N}$ spectra of $^{15}{\rm N}$ -Cho and $^{15}{\rm N}$ -PCho showed a chemical shift difference of only $\sim\!0.2$ ppm. Such a small difference presents a challenge for practical *in vivo* imaging of choline kinase activity, especially with low sensitivity of the $^{15}{\rm N}$ nucleus at clinically relevant MRI (3 T) (Fig. 2B). $^{33}{\rm N}$ Nonetheless, the exceptionally long relaxation time of $^{15}{\rm N}$ -choline in these earlier studies showed great promise in hyperpolarized $^{15}{\rm N}$ imaging and has drawn scientific attention to exploring a new range of biological applications.

In 2012, Cudalbu *et al.* performed MRS of HP 15 N-Cho to monitor 15 N-Cho build-up in a rat brain. This *in vivo* study has established the feasibility of detecting hyperpolarized 15 N signals in the animal model for the first time. Injection of 15 N-choline infusate at \sim 90 mM was tolerated without severe toxicity, although previous work has reported that MRI of choline was problematic due to its toxicity at high doses. As shown in Fig. 2C, hyperpolarized 15 N-Cho provided a T_1 of 126 ± 15 s *in vivo* (9.4 T) and the 15 N-Cho signals were detectable well over 100 s, possibly over 300 s based on the T_1 value. However, the choline kinase activity was not observed in the animal model, possibly owing to the decreased sensitivity of the 15 N signal *in vivo* and slow Cho uptake.

Promising potential of hyperpolarized choline in diverse applications has also attracted efforts to improve hyperpolarization efficiency and lifetimes of ¹⁵N-Cho, for example, by a deuteration strategy (Fig. 3). A study by Sarkar *et al.* showed

that naturally abundant choline-d₉ with deuterated methyl groups showed a $T_1(^{15}{\rm N})$ of 390 \pm 110 s, and $^{15}{\rm N}$ -Cho showed a T_1 of 189 \pm 2 s (both in D₂O at 7 T).³⁷ Note that compared to 285 \pm 12 s (11.7 T) in Gabellieri *et al.*, ³³ the shorter relaxation of $^{15}{\rm N}$ -Cho shown in Sarkar's study was due to the addition of free radicals used for d-DNP hyperpolarization. In another study by Kumagai *et al.*, the fully deuterated $^{15}{\rm N}$ -choline-d₁₃ showed a T_1 of 580 \pm 10 s (9.4 T).³⁸

Similarly, a ¹⁵N-choline analog has been hyperpolarized using PHIP in aqueous media, achieving a T_1 of 348 \pm 10 s and 494 \pm 13 s for protonated and deuterated substrates, respectively (9.4 T).³⁹ Longer relaxation times of hyperpolarized ¹⁵N signals in these deuterated choline analogs were rationalized by the reduced dipolar relaxation pathway with every neighboring proton (spin = $\frac{1}{2}$) replaced with deuterium (spin = 1). These studies also demonstrate that the deuteration of nearby protons can increase the polarization lifetime of ¹⁵N ammonium centers up to 3-fold.

2.2.2. Permethylated, perdeuterated ¹⁵N-amino acids. ¹⁵N-Enriched derivatives of amino acids, another type of endogenous molecules, have been studied extensively for long-lasting hyperpolarized perfusion imaging. Specifically, the ¹⁵N-

Fig. 3 Structures of 15 N-cholines with various degrees of deuteration, showing deuteration of the methyl and methylene groups of choline elongates the T_1 lifetime.

enriched amino acids can perform as tracers to study renal functions, such as filtration rates and tubular properties that are vital for diagnosing metabolic disorders.

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Chiavazza *et al.* have first prepared permethylated, perdeuterated derivatives of glutamine, glutamate, and lysine.⁴⁰ The design of perdeuterated glutamine compounds was based on the long relaxation time previously observed for deuterated ammonium centers that benefit from the reduced dipolar interaction with neighboring protons. The α -glutamine-¹⁵N, prepared by ¹⁵N-enrichment of α -amine (¹⁵NH₂) in naturally occurring amino acids, had a T_1 value of merely 8 s (14.1 T). In comparison, perdeuteromethylation of α -¹⁵N-amine in amino acids as a strategic approach dramatically increased the T_1 values up to 220–250 s (Fig. 4A).

Durst *et al.* applied the amino acid derivative (CD₃)₃¹⁵N⁺Gln to compare the hyperpolarized imaging performances of ¹⁵N-probes and ¹³C-urea in HP-MRI perfusion studies (Fig. 4B and C).⁴¹ The signal from the ¹⁵N-glutamine analog was localized to the kidney area and detectable for more than 5 minutes. In contrast, the ¹³C signal from [¹³C, ¹⁵N₂]urea was delocalized around the tissue and disappeared within 90 s (Fig. 4B). In practice, the hyperpolarized signal of ¹⁵N had a lower SNR than that of ¹³C, as the SNR correlates with the gyromagnetic ratio (Fig. 4C). However, this was offset by the slow signal decay of the ¹⁵N-glutamine analog in the order of several minutes.

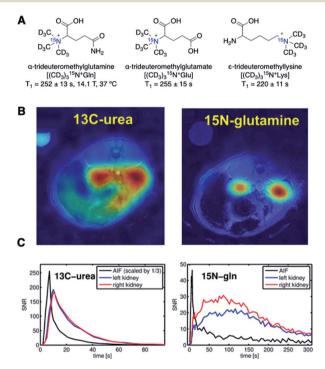


Fig. 4 (A) Structures of perdeuteromethylated 15 N glutamine, glutamate, and lysine analogs. T_1 values of all three analogs were measured at 14.1 T and 37 °C. (B) HP-MRI of $[^{13}$ C, 15 N $_2]$ urea (left) and (CD $_3$) $_3$ 15-N+GIn (right) at the peak of signal accumulation. Image laid over 1 H MRI, demonstrating the localized 15 N-glutamine signal in the kidneys. (C) Plot of the signal-to-noise ratio (SNR) of 13 C-urea and 15 N-glutamine signals over a time course from kidney and blood vessel regions. (B and C) Adapted with permission from ref. 41. Copyright 2016, John Wiley and Sons.

These permethylated, perdeuterated amino acid analogs had long signal retention and showed minimal toxicity in an animal model, meeting the requirements for *in vivo* imaging applications of hyperpolarized probes. The high T_1 values and strong localization properties make perdeuteromethylated ¹⁵N probes promising candidates for perfusion imaging. These examples also reinforce design principles to increase $T_1(^{15}N)$ by reducing the dipolar interaction with neighboring protons and installing a symmetrical environment of the ¹⁵N nucleus.

2.2.3. ¹⁵N-Carnitine. The ideal HP properties of quaternary ¹⁵N centers are further illustrated by ¹⁵N-labeled L-carnitine, ⁴² an endogenous metabolite involved in acetyl-coenzyme A and fatty acid metabolism (Fig. 5A). The T_1 times of L-¹⁵N-carnitine-d₉ were determined to be 210 s in water and 160 s *in vivo* (4.7 T) (Fig. 5B). Furthermore, the MR spectroscopic imaging of HP ¹⁵N-carnitine in the rat abdomen three minutes after injection showed ¹⁵N signals localized in the liver and kidney area, proving the feasibility of imaging the biodistribution of an ¹⁵N-agent for an extended period (Fig. 5C–E). However, no downstream ¹⁵N-acetyl-carnitine metabolites were detected in this study due to magnetic isolation of the ¹⁵N-quaternary atom.

The ¹⁵N-labeled choline, amino acids, and carnitine studies show several benefits of simple isotope-enrichment of endogenous molecules, such as ease of synthesis, high aqueous solubility, and low cytotoxicity. Nonetheless, ¹⁵N-labeled endogenous molecules do not present detectable chemical reactions and thus cannot capture real-time physicochemical activities. Discovery of imaging agents that undergo an enzymatic or chemical reaction with significant ¹⁵N chemical shift differences will provide even greater analytical appeal in terms of structure determination and quantification.

2.2.4. ¹⁵N-Azidothymidine (AZT). ¹⁵N-Enrichment of nitrogen-containing drug molecules may offer the capability of monitoring the drug's location and metabolism by HP imaging. A good example of this category is azidothymidine (AZT), an azide-containing antiviral drug that prevents reverse transcriptase from forming viral DNA.43 Shchepin et al. have reported the synthesis of AZT using sodium-15N14N2 azide to yield singly labeled 15N14N2-AZT as a mixture of 1-15N and 3-15N isotopomers. This mixture of 1-15N and 3-15N labeled AZT provided two distinct hyperpolarized ¹⁵N NMR peaks. ⁴⁴ SABRE hyperpolarization provided T_1 values of 1-15N and 3-15N azides as 45 \pm 1 and 37 \pm 2 s, respectively (9.4 T) (Fig. 6A). In another study by Bae et al., triply labeled ¹⁵N₃-AZT and singly labeled ¹⁵N¹⁴N₂-AZT hyperpolarized by d-DNP showed T_1 values of 2.5-5.3 min (1 T).45 In this study, the singly labeled 1-15N center was affected by the scalar relaxation with neighboring ^{14}N (I=1), leading to unmeasurable T_1 at 1 T. These studies exemplify the synthesis of 15N-labeled drug molecules with the potential to monitor drug activities.

2.2.5. ¹⁵N-Nicotinamide and ¹⁵N-dalfampridine. Hyperpolarized ¹⁵N-heterocycles have been explored as potential drug contrast agents. Nicotinamide, also known as vitamin B3 amide, is a drug that is used for the treatment of *M. tuberculosis*, HIV and cancer. ^{46,47} Shchepin *et al.* have demonstrated an efficient synthesis of ¹⁵N-enriched nicotinamide with high isotopic purity. ⁴⁸ SABRE-SHEATH (SHield Enables Alignment Transfer to

Fig. 5 (A) Structures of endogenous L-carnitine and its acetylated product. (B) T_1 lifetimes of L- 15 N-carnitine- d_9 in water and *in vivo*. (C) Spectral grid used for MR imaging overlaid on the 1 H anatomic image. (D) 15 N spectra of each spectral grid (E) hyperpolarized 15 N-carnitine signals in color overlaid on the anatomic image, illustrating the biodistribution of 15 N-carnitine in the liver and kidney. (C-E) Adapted with permission from ref. 42. Copyright 2020, John Wiley and Sons.

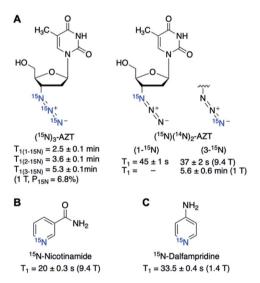


Fig. 6 Structures and hyperpolarized lifetimes of (A) singly and triply labeled 15 N-AZT, (B) 15 N-nicotinamide, and (C) 15 N-dalfampridine.

Heteronuclei) hyperpolarization of 15 N-nicotinamide provided a T_1 of 20.2 \pm 0.3 s (9.4 T), presenting the possibilities of synthesized 15 N-heterocycles as hyperpolarized drug contrast agents. Similarly, dalfampridine (4-aminopyridine) is another pyridine-based drug used to treat the symptoms of multiple sclerosis. 49 In a study by Chukanov et~al., 15 N-enriched dalfampridine has been synthesized and hyperpolarized with SABRE-SHEATH to afford a T_1 of 33.5 \pm 0.4 s (1.4 T). 50 These studies illustrate the significance of 15 N-enrichment methodology development for biomedical applications.

The feasibility of hyperpolarized ¹⁵N-drug imaging is yet to be confirmed with *in vivo* studies. In addition to hyperpolarization efficiency, factors such as drug metabolism rate, cellular

uptake, and cytotoxicity of the probe of interest need to be scrutinized to meet the criteria for preclinical applications.

2.2.6. ¹⁵N-Nitrate. Hyperpolarized ¹⁵N-nitrates (¹⁵NO₃⁻), bioactive ions that mediate physiological processes, have been explored as contrast agents for HP-MRI. D-DNP hyperpolarization of ¹⁵N-nitrate in D₂O, H₂O and saline provided T_1 values of ~100 s for each solvent at a temperature range of 34–44 °C, which was reduced to a T_1 of 29 \pm 1 s in blood samples. The metabolic conversion from ¹⁵N-nitrate to ¹⁵N-nitrite was undetectable in blood and saliva, making this molecular probe suitable as an MR tracer for perfusion or tissue retention imaging.⁵¹

15 N-Labeled molecular sensors for detecting the biological environment

Most nitrogen centers in biomolecules are proton-bound amines or amides, in which hyperpolarized 15 N signals would suffer shortened lifetimes, owing to the dipole relaxation pathway. This challenge associated with short T_1 has limited the range of HP 15 N-labeled endogenous molecules to quaternary permethylated 15 N-centers, such as the 15 N-choline and amino acid derivatives. However, $de \ novo \ ^{15}$ N molecular probes not restricted to endogenous biomolecules present great promise as chemical sensors. Several examples of 15 N-labeled chemical sensors have been reported so far for the detection of intracellular pH, signaling molecules, and enzymatic activity as potential disease biomarkers.

2.3.1. ¹⁵N-Heteroatom bases as pH sensors. Imbalanced intracellular pH is closely related to pathological processes and is a hallmark for diseases such as cancer. ⁵² Developing pH sensors for effective cancer diagnosis has attracted continuous interest, including isotope-labeled hyperpolarized pH sensors. Several ¹³C-pH sensors have been developed, such as [1-¹³C]-bicarbonate ^{53,54} and ¹³C₂-zymonic acid, ⁸ allowing for pH detection *via* the proton exchange of ¹³C-carboxylic acids.

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Alternatively, ¹⁵N-based pH sensors have been developed for direct ¹⁵N-protonation-based chemical shift imaging because sp²-hydridized, aromatic nitrogen centers can be protonated near physiological pH and cause significant electronic changes in the ¹⁵N atom.

Jiang *et al.* first illustrated hyperpolarized ¹⁵N-pyridine and pyridine derivatives as potential pH sensors (Fig. 7A and B). ⁵⁵ ¹⁵N-Pyridine demonstrated pH-sensitive chemical shift changes up to 90 ppm at a pH range of 2.1–8.5. Sharper chemical shift changes were observed in pH near a ¹⁵N-pyridine p K_a of 5.17, and the pH sensitivity was further altered by adding substituents to the pyridine derivatives. Yet the ¹⁵N-pyridines suffered from a short hyperpolarization lifetime, with a T_1 value of 41 s for non-protonated ¹⁵N-pyridine (pH 8.4) that decreased to 11 s in plasma (9.4 T). The reduced T_1 is due to an added relaxation pathway from proton exchange between the ¹⁵N atom (H–¹⁵N⁺) and water.

A study by Shchepin *et al.* examined $^{15}\mathrm{N}_2$ -imidazole as a pH sensor by SABRE-SHEATH hyperpolarization (Fig. 7C and D). 56 $^{15}\mathrm{N}_2$ -Imidazole, with a p K_a of \sim 7.0, showed higher sensitivity near physiological pH than that of $^{15}\mathrm{N}$ -pyridine, with a chemical shift change of \sim 15 ppm within the range of 6.5–7.5 (1.5 ppm/0.1 pH unit). $^{15}\mathrm{N}_2$ -Imidazole demonstrated a T_1 value of only 24 s in 1 : 1 MeOH : H₂O (9.4 T). Although the T_1 measurements at the physiological pH were not disclosed, $^{15}\mathrm{N}_2$ -imidazole is expected to have faster signal decay upon protonation, based on the results from $^{15}\mathrm{N}$ -pyridine. Similarly, a simultaneous hyperpolarization of cleavable $^{15}\mathrm{N}_2$ -imidazole and $^{13}\mathrm{C}$ -acetate has been reported, exemplifying the possibility of dual $^{15}\mathrm{N}/^{13}\mathrm{C}$ -labeled HP agents for metabolic and pH sensing. 57

These studies use isotope-enriched substrates because of the low natural abundance of 15 N (0.37%). Notably, high levels of 15 N polarization of naturally abundant substrates (*i.e.*, pyridine, metronidazole and acetonitrile) up to $P_{15N} = 51\%$ have been achieved using SABRE hyperpolarization in the presence of amines as coligands of the SABRE catalyst. Such a study will allow simple and efficient hyperpolarization of nitrogencontaining pH sensors and relevant biomolecules for 15 N-MRI. 58

2.3.2. ¹⁵N-TMPA for detection of ROS and enzyme activity. Unlike the above-mentioned ¹⁵N-based pH sensors designed with an all-in-one ¹⁵N-sensing and signaling unit, the probes can be designed with a separate sensing unit and a signal unit. In this alternative design, the sensing unit surveys a biological system of interest while a remote ¹⁵N signaling unit provides chemical shift changes as a readout.

Nonaka et al. exemplified this design strategy in [15N]trimethylphenylammonium (15N-TMPA) as a versatile platform for developing 15N-based sensors that can potentially adapt any sensing of interest.⁵⁹ At the same time, ¹⁵N-TMPA can provide a long polarization lifetime of the quaternary permethylated ¹⁵N center, with minimal influence on T_1 from the environment. In this study, the 15N-TMPA imaging platform was examined for a reaction-based detection of H2O2 and carboxyl esterase, the representative reactive oxygen species and enzyme commonly elevated in diseases (Fig. 8A and C). Both probes showed H₂O₂ concentration or enzyme-activity-dependent ¹⁵N-chemical shift changes. Deuterated [15 N, d₉]-TMPA offered a T_1 of over \sim 7 min (9.4 T). Such a long polarization lifetime allowed for an extended ¹⁵N signal detection of up to 40 min, considering that the T_1 value is approximately 37% of the total hyperpolarization decay. However, both H2O2 oxidation or carboxyl esterase

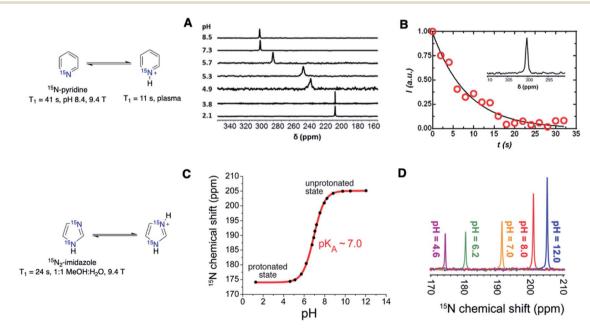


Fig. 7 (A) pH-dependent 15 N chemical shifts of free-base and protonated 15 N-pyridine. (B) Hyperpolarization signal decay of 15 N-pyridine in rat plasma with a T_1 value of \sim 11 s. (C) Determination of 15 N₂-imidazole p K_a using 15 N chemical shifts. (D) Chemical shifts of thermally polarized 15 N₂-imidazole in water at various pH values. (A and B) Adapted with permission from ref. 55. Copyright 2015, Springer Nature. (C and D) Adapted with permission from ref. 56.

A B + H₂O₂ 6.18 mM

2.50 mM

1.25 mM

0.25 mM

0.49 mm

1.25 mM

0.49 mm

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Fig. 8 (A) Scheme of H_2O_2 detection probe reaction. (B) Scans of the hyperpolarized H_2O_2 detection probe in the presence of various concentrations of H_2O_2 (in PBS, 50 s after mixing). (C) Scheme of carboxyl esterase detection probe reaction. (D) Scans of the hyperpolarized carboxyl esterase detection probe in the presence of esterase (125 units mL^{-1} in PBS). (B and D) Adapted with permission from ref. 59. Copyright 2013, Springer Nature.

 $\delta/p.p.m$

reaction of $[^{15}N, d_9]$ -TMPA resulted in a ^{15}N shift difference of merely \sim 1.5 ppm (Fig. 8B and D). Such a small chemical shift difference corresponds to a ^{15}N frequency of only 60 Hz at 9.4 T and even smaller at clinically relevant magnetic fields, with 19 Hz at 3 T and 9.7 Hz at 1.5 T, which would be insufficient for signal distinction. These results suggest that a ^{15}N chemical shift change of larger than 1.5 ppm is needed to distinguish the peaks for accurate analysis of the signals.

The excitingly long T_1 values in these ¹⁵N-based probes significantly broaden the HP imaging possibilities for *in vivo* characterization of slower biochemical reactions, such as enzymatic reactions, redox activities, and cellular signaling pathways, which would be otherwise challenging with a short signal lifetime of HP ¹³C-probes.

2.3.3. ¹⁵N-Metronidazole and ¹⁵N-nimorazole as hypoxia sensors. ¹⁵N-Labeled probes for hypoxia sensing have been developed as an imaging model of the tumor microenvironment. Hypoxia, a condition with inadequate oxygen supply in tissues, is a common feature in solid tumors and a diagnostic marker for therapy-resistant tumors.⁶⁰ Thus, non-invasive and reliable hyperpolarized hypoxia sensors offer valuable tools for cancer diagnosis and predicting therapy efficacy.

Nitroimidazoles have been widely used as hypoxia markers through immunohistochemistry and PET imaging. Under hypoxic conditions, the nitro group of these nitroimidazole compounds is expected to undergo sequential bioreduction to form nitroso, hydroxylamine, and amine derivatives (Fig. 9A). These hypoxia-based reactions can potentially provide significant ¹⁵N chemical shift changes and make ¹⁵N-nitroimidazoles suitable candidates for MRS/MRI probes. So far, two types of

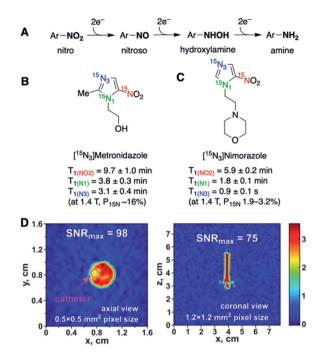


Fig. 9 (A) Schematic illustration of sequential nitro reduction under hypoxic conditions. (B) T_1 lifetimes of the three 15 N centers in 15 N-labeled metronidazole. (C) 15 N-Labeled nimorazole as a hyperpolarized imaging agent of hypoxia. (D) 2D sub-second 15 N MRI visualization of HP [15 N $_3$]nimorazole in a 5 mm NMR tube (9.4 T). Axial (left) and coronal (right) projections of the first scan of 15 N MRI. (D) Adapted with permission from ref. 63. Copyright 2020, John Wiley and Sons.

¹⁵N-labeled nitroimidazoles have been investigated as hypoxia sensors.

Metronidazole is an FDA-approved nitroimidazole-type antibiotic drug. It can be administered safely at high doses, which well suits the use of hyperpolarized solution at high concentrations for HP-MR studies. Efficient hyperpolarization of naturally abundant metronidazole61 as well as 15N-enriched [15N3]-metronidazole62 has been demonstrated using SABRE--SHEATH. In the work by Shchepin et al., all three 15N sites had high polarizations of \sim 16% and long polarization lifetimes (Fig. 9B).62 Among the three 15N centers, 15NO2 had an extraordinarily long T_1 value of 9.7 min (1.4 T), and the two aromatic 15 N-1 and 15 N-3 centers in the imidazole ring had T_1 values of 3.1 and 3.8 min, respectively. Nimorazole is another imidazole-based radiosensitizer drug for head and neck cancer. [15N₃]-Nimorazole has also been studied as a potential HP sensor for tumor hypoxia. Salnikov et al. reported hyperpolarized [15N3]-nimorazole as a potential theranostic agent for dual therapy and imaging of tumor hypoxia (Fig. 9C).63 Hyperpolarization of [15N3]-nimorazole using SABRE-SHEATH provided long T_1 lifetimes, especially for $^{15}NO_2$ (5.9 min, 1.4 T). Such remarkably long-lasting polarizations open opportunities for hyperpolarized hypoxia MR imaging for over tens of minutes.

Although neither of these two studies have reported metabolic imaging of ¹⁵N-nitroimidazoles, the *ab initio* calculations Chemical Science Review

revealed that the sequential hypoxic reduction processes shown in Fig. 9A were expected to provide significant $^{15}\mathrm{N}$ chemical shift differences, with nearly 800 ppm difference for the $^{15}\mathrm{N}$ -nitro center. 63 Such a dynamic chemical shift range of the $^{15}\mathrm{N}$ -sites bodes well for future *in vivo* imaging of nitroimidazole metabolism. One challenge is that the $^{15}\mathrm{NH}_2$ metabolite from hypoxic reduction would deliver a short T_1 because of the proton-coupled relaxation pathway. A possible alternative readout to monitor hypoxia is the other two $\mathrm{sp^2}_-^{15}\mathrm{N}$ atoms that may also lead to chemical shift changes upon $^{15}\mathrm{NO}_2$ reduction.

While *in vivo* imaging has not been demonstrated in these studies, the 2D 15 N MRI visualization of $[^{15}N_3]$ -metronidazole⁶⁴ and $[^{15}N_3]$ -nimorazole⁶³ displayed high spatial and temporal resolution (Fig. 9D), highlighting the prospects of high-resolution 15 N-imaging.

2.3.4. Coordination-based detection of Ca²⁺ and Zn²⁺ metal ions. MR probes have also been designed for sensing biologically important metal ions. Free metal ions, such as calcium and zinc, participate in essential cellular ionic signaling cascades and oxidative balance. The importance of metal ion homeostasis suggests the promise of *in vivo* metal ion concentrations as diagnostic markers for analyzing diseases associated with metal ion imbalance. So far, hyperpolarized sensors for metal ions have been developed by designing chelators that can coordinate to metal ions to induce electron localization and chemical shift changes.

[15 N, d₉]-TMPA has been studied as a potential sensor for calcium ions (Ca^{2+}). Calcium ions are ubiquitous signaling molecules that control various cellular functions, and abnormal Ca^{2+} concentrations are responsible for several pathological

processes. The design of [15 N, d₉]-TMPA used (CD₃) $_3^{15}$ N⁺ as the signaling unit and triacetic acid branches as the Ca²⁺ chelator. Unfortunately, small 15 N chemical shift changes up to 1.5 ppm were inadequate for unambiguous Ca²⁺ detection (Fig. 10A). To address this limitation, another Ca²⁺ sensor 15 N-o-aminophenol-N, N, O-triacetic acid (15 N-APTRA) was designed with the 15 N center positioned close to the Ca²⁺ coordination site. Encouragingly, 15 N-APTRA provided chemical shift changes up to 5.2 ppm with the addition of 2 equivalence of Ca²⁺. On the downside, 15 N-APTRA showed a T_1 of only 37 s (pH 7.4, 9.4 T), a 3.5-fold decrease from T_1 = 130 s of [15 N, d₉]-TMPA, presumably from protonation of 15 N-aniline (Fig. 10B).

¹⁵N-Labeled sensors for Zn²⁺ metals have also been reported. Elevated cellular Zn2+ levels are highly toxic and linked to cancer and neurodegenerative disorders. Imaging labile zinc ions as biomarkers presents a promising approach for diagnosing these diseases. 67,68 15 N-labeled tris(2-pyridylmethyl)amine (TPA) was developed by Suh et al. using chemical shift changes resulting from pyridine-Zn²⁺ coordination for the detection and quantification of free Zn²⁺ metal (Fig. 10C).⁶⁹ ¹⁵N-TPA showed several promising spectral features, including a favorable 15N signal linewidth, a large chemical shift of 20 ppm, and a linear relationship of peak area to zinc concentration. T_1 values for $[^{15}N]TPA-d_6$ and $Zn^{2+}-[^{15}N]TPA-d_6$ were 71 s and 57 s, respectively (9.4 T). Excitingly, the hyperpolarized [15N]TPA-d₆ probe was able to measure physiological levels of Zn²⁺ (0-200 μM) in human prostate tissue homogenate and intact human prostate epithelial cells (Fig. 10D).

These studies show versatile ¹⁵N design principles through metal-ligand coordination-based chemical shift changes for

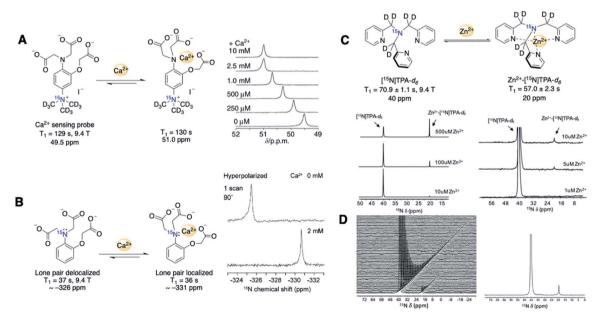


Fig. 10 (A) 15 N-TMPA based Ca²⁺ detection probe and Ca²⁺ level-dependent 15 N chemical shifts (measured in HEPES buffer, 40 s after mixing). (B) 15 N-APTRA based Ca²⁺ detection probe and 15 N NMR spectra with and without Ca²⁺. (C) [15 N]TPA-d₆ based Zn²⁺ detection probe and 15 N NMR spectra of hyperpolarized [15 N]TPA-d₆ (1.2 mM) with various concentrations of Zn²⁺ (1–500 μ M). (D) Time-dependent 15 N spectra collected using intact PNT1A cells after addition of 2.8 mM of HP-[15 N]TPA-d₆ (left) and its first 15 N spectrum showing the detection of *in vitro* Zn²⁺ (right) (pH 7.4, 9.4 T). (A) Adapted with permission from ref. 59. Copyright 2013, Springer Nature. (B) Adapted with permission from ref. 66. Copyright 2015, The Royal Society of Chemistry. (C–E) Adapted with permission from ref. 69. Copyright 2020, Springer Nature.

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Fig. 11 Selected examples of 15 N₂-diazirine-tagged endogenous and drug molecules. Hyperpolarized with d-DNP and all T_1 lifetimes were measured at 1 T.

hyperpolarized imaging of labile metal ions. Although a limited number of 15 N probes have been developed so far, these studies of exogenous 15 N sensors provide valuable lessons, including the effects of 15 N center placement on chemical shifts and T_1 values. These principles will expedite the design of more effective 15 N-molecular imaging probes in future studies.

2.4. 15N-Molecular tags and biomolecules

An alternative to isotope enrichment, an attractive new strategy in designing HP ¹⁵N-labeled agents, is to install biocompatible and long-lasting polarized ¹⁵N-molecular tags onto biologically relevant molecules. Such a molecular tagging strategy can potentially introduce a ¹⁵N-signaling moiety into any target of interest. In the probes mentioned above, long-lived ¹⁵N signals rely on permethylated ¹⁵N-ammonium or ¹⁵N-heterocycles as the common ¹⁵N-centers. In comparison, the ¹⁵N-molecular tags can constitute various nitrogen-containing functional groups that are non-proton bound and symmetrical, selected for optimal polarization efficiency and long-lived polarization states.

2.4.1. ¹⁵N₂-Diazirine tags. ¹⁵N₂-Diazirines are one of the first ¹⁵N-based molecular tags explored for HP MRS/MRI. ⁷⁰ Structurally, ¹⁵N₂-diazirines are three-membered rings containing a nitrogen–nitrogen double bond (Fig. 11). Diazirines have desirable physicochemical properties for a molecular tag, including small size, biocompatibility and stability under physiological conditions, and minimal effects on the physicochemical properties of biomolecules. ^{71,72} Particularly attractive for HP-MR detection, ¹⁵N₂-diazirines have a unique symmetrical molecular structure that stores polarization for an extended period through a singlet state. The singlet state ($T_{\rm s}$) has a zero magnetic moment, so the symmetry has to be broken to be NMR-detectable. This also means that the singlet spin order is

immune to many relaxation mechanisms and polarization is long-lived. In particular, SABRE-SHEATH hyperpolarization of $^{15}\rm{N}_2$ -diazirine-labeled compounds had a long singlet relaxation of $T_{\rm s}=23$ min. 70 Furthermore, several $^{15}\rm{N}_2$ -diazirine-labeled biomolecules have been hyperpolarized by SABRE-SHEATH 73 and d-DNP 74 methods. Examples include the $^{15}\rm{N}_2$ -diazirine tagged analogs of amino acids, glucose, and drug molecules. Hyperpolarization by d-DNP showed that all provided T_1 values in the 3–4 min range (1 T) (Fig. 11). The study showed the considerable influence of the solubility of the $^{15}\rm{N}$ -tagged molecules on their hyperpolarization efficiencies. High solubility of the hyperpolarized probes in the aqueous glassing solvent (at least 100 mM) is crucial for effective hyperpolarization of non-polar endogenous or drug molecules for practical applications.

2.4.2. ¹⁵N₃-Azide tags. Azides, unique linear species containing three nitrogen atoms, have been known as bioorthogonal reactive partners and possess desired features for a molecular tag.75,76 15N-Azides have been demonstrated as another class of 15N-molecular tags for hyperpolarized imaging by Bae et al. 45 Triply labeled 15N3-azides have been incorporated into choline, glucose, and tyrosine analogs for investigation. Hyperpolarization of all these ¹⁵N₃-tagged molecules by d-DNP demonstrated long lifetimes up to 9.8 min (1 T) (Fig. 12). The terminal nitrogen, 15Nγ, retained the longest HP signal, followed by $^{15}N\beta$ and $^{15}N\alpha$, in which the long T_1 corresponds to increased distance from the nearest protons. The 15N3-azide tag is especially interesting as three distinct ¹⁵N signals can be monitored simultaneously. Additionally, the extended imaging time window opens possibilities for ¹⁵N₃-azide bioconjugation reaction in vivo (i.e., azide-alkyne cycloaddition) for hyperpolarized secondary labelling.

The 15 N-tagging strategy demonstrated in 15 N-azide and 15 N-diazirine compounds will broaden the application of HP 15 N

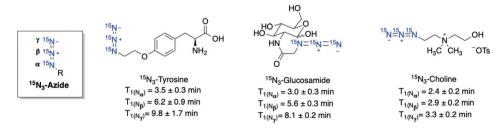


Fig. 12 Selected examples of $^{15}N_3$ -azide-tagged endogenous and drug molecules. Hyperpolarized with d-DNP and all T_1 lifetimes were measured at 1 T.

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imaging beyond nitrogen centers in heteroarenes and permethylated amines. Moreover, these ¹⁵N-tag motifs can be readily introduced into a broad range of biomolecules, allowing for preparing a variety of hyperpolarized imaging probes with a long polarization lifetime. Of note, in some examples where the ¹⁵N-tags are generally installed several bonds away from the metabolic sites, significant ¹⁵N chemical shift changes may not be observed upon metabolism. Nevertheless, the ¹⁵N-azide and ¹⁵N-diazirine-tagged molecules are of great interest for future studies on their applications in monitoring cellular uptake and accumulation.

3. Summary and outlook

This review provides the current state of development of HP 15 N-probes, including their hyperpolarization performances in relation to design principles. As an emerging molecular imaging technique, hyperpolarized 15 N MRS/MRI shows promising potential for biomedical applications. Several 15 N-labeled endogenous and *de novo* molecular probes delivered long hyperpolarization lifetimes in the order of several minutes. Such substantial hyperpolarization lifetimes allow an extended imaging period to capture slower biochemical reactions that are useful for disease diagnosis. At the same time, long T_1 lifetimes of HP 15 N agents can compensate for the low sensitivity issues, as shown in the MRS/MRI scans of 15 N-amino acids and 15 N-carnitine acquired over several minutes.

Despite recent progress and increased interest in ¹⁵N-based imaging in the past decade, hyperpolarized ¹⁵N MR has not gained widespread use to enter the preclinical stage. As reflected in the analysis of currently studied HP ¹⁵N-probes in this review, advancing ¹⁵N MRS/MRI into a practical imaging tool requires advancements in multiple aspects such as new probe design, extensive animal imaging studies, and improved MR technology.

Fundamental considerations for the design of novel ¹⁵Nprobes include the factors of T_1 lifetime, chemical shift differences, and toxicity. First, the discussion on the reported ¹⁵N probes in this review reveals that the ¹⁵N signal lifetime can be greatly extended by the probe design to reduce dipole-relaxation pathways (i.e., deuteration of neighboring protons). Compared to commonly observed ¹³C carbonyl centers, the ¹⁵N centers of the HP probes in the literature have greater structural diversity, such as quaternary amine, diazirine, and azides. All these 15N centers warrant a long T_1 lifetime. So far, most studies have presented polarization lifetimes at high B_0 (7-11.7 T). Future work on demonstrating T_1 in clinically relevant magnetic fields (1-3 T) will be important to accurately predict the performances of HP 15N probes in in vivo imaging. Second, accurate measurement of chemical reactions would require significant chemical shift differences. A serviceable chemical shift difference needed for HP imaging is affected by the magnetic field, polarization levels, and spectral resolution. Finally, the probe candidates must be biocompatible and non-toxic in living systems. The cytotoxicity profiling is critical for exogenous ¹⁵Nmolecular agents, especially at high concentrations (mM range). The current exogenous ¹⁵N-probes solely demonstrate

spectroscopic analysis, and only endogenous compounds (*i.e.*, ¹⁵N-choline) have advanced to *in vivo* MRI studies.

Extensive characterization of ¹⁵N-labeled agents must be performed to understand the potential use of hyperpolarized ¹⁵N imaging in clinical studies. Cellular experiments of ¹⁵N-labeled HP-NMR agents can provide information on the membrane permeability of probes and cellular reaction kinetics. Additionally, *in vivo* imaging should be conducted to validate the hyperpolarization measurements and sensitivity threshold of the ¹⁵N probes. So far, most studies have demonstrated MRS experiments. The conjunction of MRS with MRI in small animal model imaging is desirable, which will provide not only pharmacokinetic data to quantify the rate of substrate buildup and metabolite conversion but also anatomical distribution of ¹⁵N signals for accurate and quantitative analysis in preclinical studies.

Developing hyperpolarized ¹⁵N imaging for preclinical studies requires addressing several technical challenges of MR scanners' technical challenges. For instance, ¹⁵N imaging requires dedicated ¹⁵N radiofrequency coils, which are not widely available in conventional MR scanners.⁶⁹ Parallel efforts in improving pulse sequences and multichannel coils may be crucial. Advances in hyperpolarization techniques can increase polarization efficiency and address the sensitivity issues associated with ¹⁵N imaging.

Overall, the insights into the chemical and physical properties of ¹⁵N-molecular probes gained through the up-to-date examples will assist in more effective designs for future hyperpolarized ¹⁵N-based probes. Along with the advancement in MRI/MRS techniques, emerging next-generation probes are expected to foster hyperpolarized ¹⁵N-sensors as widespread molecular imaging technology in the future.

Author contributions

Both H. P. and Q. W. contribute to the writing of this manuscript.

Conflicts of interest

There are no conflicts to declare.

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