COMMUNICATION

Hyperpolarized NMR of plant and cancer cell extracts at natural abundance†

Jean-Nicolas Dumez,[a] Jonas Milani,[b] Basile Vuichoud,[b] Aurélien Bornet,[b] Julie Lalande-Martin,[c] Illa Tea,[c,d] Maxime Yon,[c,e] Mickaël Maucourt,[f,g] Catherine Deborde,[f,h] Annick Moing,[f,h] Lucio Frydman,[e] Geoffrey Bodenhausen,[b,i,j,k] Sami Jannin*[b] and Patrick Giraudeau*[c,l]

Natural abundance ^{13}C NMR spectra of biological extracts are recorded in a single scan provided that the samples are hyperpolarized by dissolution dynamic nuclear polarization combined with cross polarization. Heteronuclear 2D correlation spectra of hyperpolarized breast cancer cell extracts can also be obtained in a single scan. Hyperpolarized NMR of extracts opens many perspectives for metabolomics.

Lying at the interface of chemistry and biology, metabolomics is one of the youngest sprouts of the sprawling tree of "omic" sciences. The last decade has witnessed a growing interest in mapping metabolic pathways, in identifying new biomarkers, and in modeling metabolic fluxes.[1,2] Nuclear Magnetic Resonance (NMR) spectroscopy is a major analytical technique in this field, offering unambiguous information about the identity of metabolites and their time-dependent concentrations in complex samples such as biofluids, extracts and tissues. However, proton NMR spectra have a limited range of chemical shifts and often suffer from overlapping signals. Carbon-13, which has a larger chemical shift dispersion, can benefit from sensitive detectors[3] or from isotopic enrichment,[4] albeit at the cost of an enhanced complexity of the spectra due to [13]C–[13]C couplings. Heteronuclear ([1]H→[13]C) correlation spectroscopy can in principle offer a satisfactory dispersion.[5,6] So far, ^{13}C NMR is not routinely used in metabolomic studies, but hyperpolarization by dissolution dynamic nuclear polarization (D-DNP) combined with cross polarization (CP) could boost its popularity in this field.

Hyperpolarization techniques are capable of generating spin polarization levels that are about 50 000 times above Boltzmann equilibrium at room temperature.[7–9] Once prepared and transferred to a solution-state NMR spectrometer, the resulting magnetization can be exploited within a limited time-span that depends on the longitudinal relaxation time T_1 of the hyperpolarized nuclei. Among various methods for hyperpolarization, dynamic nuclear polarization (DNP) is least affected by the chemical substrate and sample preparation.[10] For solution-state NMR, particularly promising results can be obtained by dissolution DNP, where the sample to be analyzed is mixed with free radicals in a glass-forming solution, frozen to liquid helium temperatures, and hyperpolarized by irradiating in the vicinity of the electron spin resonance (ESR) of the unpaired electrons of the radicals.[11] Spins of ^{13}C nuclei may be polarized either directly or *via* cross-polarization from protons to [13]C.[12] Rapid melting and shuttling of the sample from the polarizer to a routine NMR spectrometer enables conventional solution-state NMR spectroscopy with a sensitivity that can be boosted by a factor of about 50 000. Most applications of D-DNP have been restricted to the injection of pure hyperpolarized ^{13}C-labeled molecules such as pyruvate into living organisms.[13] D-DNP has hardly been applied to complex mixtures of metabolites,[14] but only to a few well-chosen small molecules.[15–18] The detection of 2D spectra after D-DNP has been reported on such mixtures of small mole-

[a]Institut de Chimie des Substances Naturelles, CNRS UPR 2301, F-91190 Gif-sur-Yvette, France
[b]Institut des Sciences et Ingénierie Chimiques, Ecole Polytechnique Fédérale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland. E-mail: sami.jannin@epfl.ch
[c]Université de Nantes, CNRS, CEISAM UMR 6230, F-44322 Nantes Cedex 03, France
[d]Cancer Metabolism and Genetics Group, The John Curtin School of Medical Research, The Australian National University, Canberra, ACT2601, Australia
[e]Department of Chemical Physics, Weizmann Institute of Science, IL-76100 Rehovot, Israel
[f]Plateforme Métabolome Bordeaux – MetaboHUB, Centre de Génomique Fonctionnelle Bordeaux, IBVM, Centre INRA Bordeaux, F-33140 Villenave d'Ornon, France
[g]Université de Bordeaux, UMR 1332 Biologie du Fruit et Pathologie, Centre INRA Bordeaux, F-33140 Villenave d'Ornon, France
[h]INRA, UMR 1332 Biologie du Fruit et Pathologie, Centre INRA Bordeaux, F-33140 Villenave d'Ornon, France
[i]Département de Chimie, Ecole Normale Supérieure (ENS)-Paris Sciences Lettres (PSL) Research University, F-75005 Paris, France
[j]Laboratoire de Biomolécules (LBM), Université Pierre et Marie Curie (UPMC) – Paris 06, Sorbonne Universités, F-75005 Paris, France
[k]Laboratoire de Biomolécules (LBM), Unité Mixte de Recherche (UMR) 7203 Centre National de la Recherche Scientifique (CNRS), F-75005 Paris, France
[l]Institut Universitaire de France, F-75005 Paris, France.
*E-mail: patrick.giraudeau@univ-nantes.fr*
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cules, either relying on small-angle 2D pulse sequences[15,19] or on single-scan approaches.[16,17] Recently, the ^{13}C detection of a hyperpolarized mixture of up to 10 metabolites at natural abundance was reported,[18] however such experiments remain to be applied to complex samples of genuine interest for metabolomics.

In this communication, we show that by using a recently developed methodology combining D-DNP with ^{1}H→^{13}C CP, one can rapidly achieve a high hyperpolarization of ^{13}C spins at natural abundance even in complex samples. The great advantage of CP assisted D-DNP is that its performance is not sample specific and simply relies on transferring the polarization of the protons in a glass-forming DNP solvent (here water/glycerol), to all dissolved metabolites by multiple ^{1}H→^{13}C CP transfers. As reported here, the acquisition of 1D and single-scan HMBC-type heteronuclear (^{1}H→^{13}C) 2D correlation NMR spectra of biological extracts with natural ^{13}C abundance is possible, including measurements of metabolite signals that could not be detected without DNP even after hours of signal averaging. The potential of this approach is discussed, as well as perspectives for metabolomics.

In our initial attempts to obtain ^{13}C NMR spectra boosted by D-DNP, we investigated extracts of human breast cancer cell lines either in natural abundance or with partial enrichment, using protocols that are typical for other metabolomic studies (see ESI†), and using a commercially available D-DNP Hypersense® machine that was not equipped for ^{1}H→^{13}C transfer by cross-polarization. The experimental conditions were similar to those described for model mixtures of metabolites.[18] However, no ^{13}C signals could be detected using direct polarization of ^{13}C nuclei with a trityl radical. This was attributed to the inefficiency of ^{13}C–^{13}C spin diffusion. One option would be to optimize spin diffusion in the polarization matrix using abundant ^{13}C-labeled additives as suggested by Ludwig et al.[15] We decided not to adopt this strategy so as not to contaminate the samples nor to unnecessarily crowd the ^{13}C spectra with intense background signals. Another issue of using abundant ^{13}C-labeled additives is the uncontrolled interfering effect on the solubility of the metabolites. Here, we propose to overcome this problem by simply using cross-polarization from ^{1}H to ^{13}C nuclei.[12] The metabolite DNP samples are based on water–glycerol mixtures doped with nitroxides with a high ^{1}H concentration (typically 20% H_2O) so as to enable fast ^{1}H–^{1}H spin-diffusion and high ^{1}H polarization, regardless of the concentration of dissolved metabolites. The ^{1}H polarization is then transferred to ^{13}C of the metabolites of interest by CP, leading to a high and uniform ^{13}C polarization of all metabolites in about 30 minutes. The large advantage of ^{1}H→^{13}C CP *versus* ^{13}C direct polarization is clearly illustrated in the ESI Fig. S1† for tomato and cancer cell extracts.

The first results obtained with this approach for biological extracts are shown in Fig. 1, which compares spectra recorded with two identical extracts of tomato fruit pericarp. The thermal spectrum (Fig. 1a) was acquired using conventional sample preparation, while the D-DNP spectrum was obtained by polarizing the extract in a partially deuterated water–
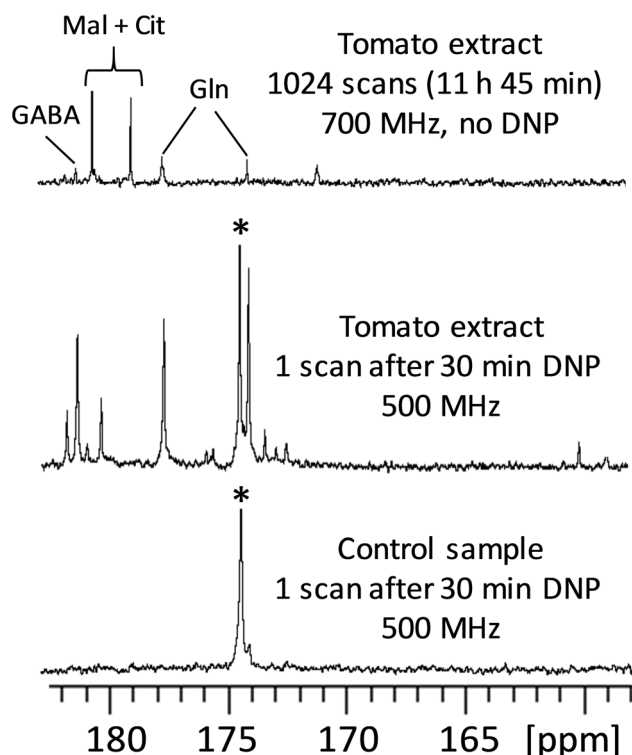
Fig. 1 ^{13}C NMR spectra (quaternary region) of mature green tomato fruit pericarp extracts. (a) Conventional (non-hyperpolarised) spectrum of 20 mg extract (prepared from 20 mg lyophilized grounded tissue) dissolved in 700 µL D_2O, recorded with 1024 scans (11 h 45 min) at 700 MHz with a cryogenic probe. (b) Spectrum of an identical extract recorded with D-DNP combined with CP. The extract was first dissolved in a 200 µL mixture of H_2O/D_2O/glycerol-d_8 (2 : 3 : 5) doped with 25 mM TEMPOL, then polarized for 30 min at 1.2 K and 6.7 T, and finally dissolved with 5 mL of hot D_2O and transferred in about 10 seconds to a 500 MHz spectrometer equipped with a cryogenic probe. (c) Same as (b), but the tomato extract was replaced by a control sample prepared under strictly identical conditions without any biological material. The hyperpolarized spectra result from the sum of six consecutive acquisitions using 30° pulses spaced by 7.7 s. *Indicates the signal of a ^{13}C-labeled pyruvate impurity from the D-DNP setting. Cit: citrate; GABA: γ-aminobutyrate; Gln: glutamine; Mal: malate. Experimental details are given in the ESI.†

glycerol mixture with nitroxide radicals. The sample was polarized for 30 min at 1.2 K and 6.7 T with microwave frequency modulation,[20] with 1 ms ^{1}H→^{13}C CP applied every 5 min, and followed by dissolution with hot D_2O and transfer to a 500 MHz NMR spectrometer (11.7 T) for detection[12] (see ESI† for further details). The spectra in Fig. 1 focus on the quaternary ^{13}C region, where the sensitivity gain is the most remarkable and where the polarization is best preserved because of favourably long T_1's. The signals of protonated ^{13}C nuclei were below the noise level despite hyperpolarization, because their short T_1's led to excessive losses during transfer and injection that required about 10 s.

The comparison of Fig. 1a and b clearly shows the sensitivity gain. The single-scan ^{13}C D-DNP spectrum (where the most intense peak has a SNR of 65) shows many more peaks
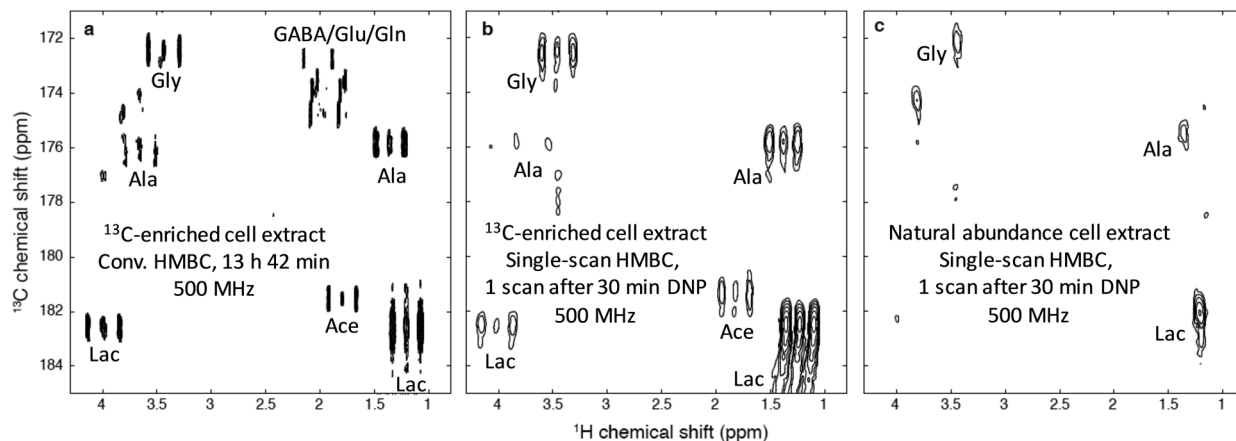
Fig. 2 ^{1}H$\rightarrow$^{13}C HMBC-type spectra of extracts of SKBR3 human breast cancer cell lines. (a) Conventional HMBC spectrum, recorded in 13 h 42 min at 500 MHz with a cryogenic probe, on a partially enriched extract (*ca.* 57 million extracted cells) dissolved in 700 μL D$_2$O. (b) Hyperpolarized single-scan spectrum. The cell extract was dissolved in 200 μL of a mixture of H$_2$O/D$_2$O/glycerol-d_8 (2 : 3 : 5) with 25 mM TEMPOL and polarized for 30 min at 1.2 K and 6.7 T, and finally dissolved with 5 mL D$_2$O. A fraction of 700 μL of the hyperpolarized sample was injected in a 500 MHz spectrometer equipped with a cryogenic probe where the spectrum was recorded in a single scan. (c) Same as (b), but with a natural abundance extract (*ca.* 113 million cells) obtained from the same SKBR3 cell line. Ace: acetate; Ala: alanine; GABA: γ-aminobutyrate; Gln: glutamine; Glu: glutamate; Gly: glycine; Lac: lactate. Experimental details are given in the ESI.†

than the thermal spectrum (where the most intense peak has a SNR of 30), although the latter was recorded with 1024 scans at 41 s intervals in *ca.* 12 h and at a higher magnetic field (16.4 T). A confirmation that the additional peaks provide a spectral signature of the plant extract comes from Fig. 1c, recorded under the same conditions as Fig. 1b but on a control sample which underwent the same extraction and preparation steps as the plant extract.

The results of Fig. 1 also highlight two limitations of the current experiments. First, as mentioned above, the dissolution DNP NMR experiment yields most of its gain for quaternary carbons. Protonated ^{13}C nuclei having short relaxation times (typically T_1's of few seconds) lose most of their hyperpolarization during the transfer from the polarizer to the NMR spectrometer. We shall attempt to increase the speed of the transfer to overcome this issue.[21] Still, the fact that only quaternary ^{13}C signals can be exploited is not necessarily problematic, as most metabolites contain at least one quaternary ^{13}C. The second drawback comes from a lack of a-priori knowledge of the metabolites present in the extracts, and hence the difficulty of assigning the quaternary ^{13}C peaks. The few peaks observed in the thermal spectrum could be assigned based on conventional HMBC experiments (data not shown) and based on previous experience with similar samples. However, an unambiguous peak assignment for the resonances in Fig. 1b was not straightforward because (i) the chemical shifts were different from those of the thermal spectrum due to different sample preparation conditions and (ii) the fast decay of the ^{13}C hyperpolarization is not compatible with the recording of a conventional HMBC spectrum after dissolution. Yet even without assignments, 1D DNP experiments could still be of interest for metabolomics, since one can systematically compare spectra of samples of different origins

recorded under identical conditions. In the latter case, a normalization of the peak intensities would be necessary to account for variations in polarization and losses during dissolution.

Single-scan 2D (also known as ultrafast) NMR offers a powerful solution to this problem. Single-scan 2D NMR spectra can be recorded by transferring the polarization of hyperpolarized ^{13}C nuclei to scalar-coupled ^{1}H after spatial encoding, and followed by an echo-planar detection scheme.[22] In particular, single-scan HMBC-type experiments allow one to assign quaternary carbons.[16,23] Here, this strategy was applied to extracts from SKBR3 human breast cancer cell lines, both at natural abundance and with partial isotopic enrichment (Fig. 2).

Fig. 2a and b show a conventional ^{1}H$\rightarrow$^{13}C HMBC 2D NMR spectrum measured at thermal equilibrium (*ca.* 14 h) with the single-scan HMBC recorded after D-DNP (30 min for DNP and 100 ms for acquisition) on the same ^{13}C enriched extract partitioned into two identical aliquots. The first one was dissolved in 600 μL D$_2$O, and the second one was prepared for DNP as described for the plant extract (see ESI†). Both spectra show similar isotopic patterns, characteristic of ^{13}C-enriched samples. The GABA/Glu/Gln region has disappeared because of T_1 losses. However, all other peaks are present in the two spectra. This highlights the considerable time saving offered by DNP. More importantly, Fig. 1c shows that the same acquisition on a similar extract but at natural ^{13}C abundance is also feasible. At thermal equilibrium, such experiments would not have been possible within a reasonable experiment time. Although some of the peaks have disappeared because of T_1 relaxation during transfer, the missing peaks can in principle be recovered if the transfer were sufficiently fast (<2 s). We are currently upgrading our fluid transfer system.[21]

The results shown in Fig. 2 demonstrate that the assignment of quaternary carbon signals of biological extracts is now possible at natural abundance after building up DNP in about 30 minutes. Natural abundance spectra are simple since there are no multiplets due to homonuclear $J(^{13}C-^{13}C)$ couplings. On the other hand, enriched samples can in principle yield information on ratios of isotopomers in mixtures. This would be of interest for fluxomics, where heteronuclear 2D spectra are commonly used to quantify site-specific isotopomers.[24,25].

Conclusions

Our observations show that one can obtain information on the metabolites of biological samples by combining D-DNP with CP followed by detection of 1D or single-scan 2D spectra. The resulting approach is robust, general, and not sample specific thanks to the implementation of CP. The gain in time and sensitivity offers promising perspectives for metabolomics, where sensitivity is inevitably limited. Moreover, in enriched samples, single-scan heteronuclear 2D spectra provide interesting isotopic information for fluxomics. While the application to large-scale metabolomics studies will require to improve and validate the reproducibility of the method, the impact of hyperpolarization on analytical metabolomics appears promising.

Acknowledgements

Notes and references

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