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High-resolution NMR-based metabolic detection of microgram biopsies using a 1 mm HRμMAS probe†

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A prototype 1 mm High-Resolution micro-Magic Angle Spinning (HRμMAS) probe is described. High quality ^{1}H NMR spectra were obtained from 490 μg of heterogeneous biospecimens, offering a rich-metabolite profiling. The results demonstrate the potential of HRμMAS as a new NMR analytical tool in metabolomics.

Today ^{1}H HRMAS (High-Resolution Magic-Angle Spinning) NMR (Nuclear Magnetic Resonance) spectroscopy finds great success in laboratory studies of the metabolome in heterogeneous biospecimens such as human and animal biopsies,[1–3] intact cells[4] and whole organisms,[5,6] owing to the nearly non-destructive nature of the technique and the straightforward data acquisition.[7] However, since NMR spectroscopy is an inherently insensitive analytical technique, HRMAS relies on a large sample mass, typically 10–20 mg per NMR data. For this reason, ^{1}H HRMAS analysis can be a real challenge (or even impossible) when samples – such as cells, organisms and tissue biopsies – are scarce. Moreover, analysis of 10–20 mg of heterogeneous specimens may prevent the investigation of a specific cell. In contrast, the high degree of homogeneity inside a microscopic specimen can offer a more direct NMR spectral analysis and enable a straightforward metabolic evaluation. The immediate advantages of small sample mass (*i.e.* microgram) analyses are: (1) they simplify the sample preparations such as cultivation and extraction; and (2) offer precise specimen-specific analyses for exploring the invisible phenotypes.

The most cost-effective approach to microscopic NMR detection is the use of a miniature detection coil (μcoil). With this technique, the coil is in close proximity to the microscopic sample optimizing the filling-factor (the ratio of the sample volume to the coil detection volume).[8] Fabricating a μcoil for HRMAS analyses is no easy task, especially without sacrificing detection sensitivity and spectral resolution.[9] The commercial μMAS systems currently available (*i.e.* 0.7 mm Bruker MAS and 0.75 mm JEOL MAS) are designed for solid materials but do not offer adequate spectral resolution (0.002 ppm) for metabolic investigations.

Today, the only approach to μMAS for metabolome analyses is the use of an inductively coupled High-Resolution Magic-Angle Coil Spinning (HRMACS) μcoil.[10] The HRMACS technique uses a secondary tuned circuit (*i.e.* μcoil-resonator), designed to fit inside a standard 4 mm MAS rotor, to convert the standard large volume MAS system into a high-resolution capable μMAS probe.[11,12] The use of HRMACS has shown some success in intact cells[13] and whole small organisms[14] for the metabolic profiling and differentiation of microgram biospecimens. However, great efforts are required to make and operate the HRMACS μcoil. For example, manually winding the μcoil is a strenuous task that requires good micro-engineering skills and patience; the μcoil is fragile and needs to be handled with great caution and care. In addition, the sample spinning frequency of the HRMACS μcoil is limited to 500 Hz in order to minimize the sample heating,[10] which originates from the eddy current.[15] As a result, the isotropic signal is dissipated into the dense spinning-sidebands diminishing the overall sensitivity. For this reason, special pulse-experiments (such as PASS) must be applied to acquire sideband-free isotropic spectra.

In this communication we present the first 1 mm high-resolution μMAS (denoted HRμMAS) NMR probe specially designed for the analyses of microscopic biospecimens. The prototype probe is modified from a solid-state μMAS probe. It features a stationary 10-turn μcoil solenoid 1 mm in diameter and 1.9 mm in length. The unloaded coil quality factor at 600 MHz is about 150. This value is considerably higher than

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†Electronic supplementary information (ESI) available: Sample preparation; NMR experimental details; photo illustration of a Kel-F MAS rotor; 2D TOCSY and COSY spectra of the brain extract. See DOI: 10.1039/c5an01810b

the quality factor of the manually made HRMACS μcoil, *i.e.* 30–50 at 500 MHz. Samples were packed in Kel-F rotors with 1.0/0.5 mm outer/inner diameters and 6.55 mm length. To ensure that the sample is inside the detection region, a Kel-F cap (each about 1.5 mm long) is inserted at each end of the rotor. The total sample detection volume is about 490 nl (see Fig. S1 in the ESI†). Using Kel-F rotors, instead of standard ZrO rotors, eliminates the anisotropic magnetic susceptibility broadening[16] – which cannot be completely averaged under MAS – caused by ZrO. Another advantage of Kel-F rotors is that they can be used as disposable rotors because the cost is substantially lower than ZrO (200 € *versus* 2000 €). The HRμMAS probe produces a very good B_1 homogeneity over the sample volume with an intensity ratio $I_{450°}/I_{90°}$ of about 95%; whereas <80% is found for HRMACS. A comparison of the probe properties and performances of the HRμMAS prototype and the existing HRMACS can be found in the ESI.†

To address the question 'is it possible to construct a μMAS probe with high spectral resolution and high detection sensitivity that is suitable for ^{1}H NMR-based metabolomics studies?' we have modified a JEOL 1 mm solid-state MAS probe with ^{1}H frequency at 600 MHz. This probe was originally designed for the ultra-fast sample spinning (up to 80 kHz) of rigid-solids to achieve a high spectral resolution for solid materials.[17] The optimal spectral resolution is however not adequate for ^{1}H NMR metabolomics studies. As shown in Fig. 1a, the ^{1}H NMR spectrum of a 20 mM sucrose solution displays broad lines in all resonances (FWHM of about 0.01 ppm) that prevent any detailed and precise analyses of the metabolome in specimens. The observed line-broadenings are mainly attributed to the large magnetic susceptibility gradients between the sample and the nearby stationary (non-spinning) components inside the probe, such as the MAS stator and the copper-wire. To minimize these gradients, we have changed the air-bearing inside the MAS stator from Zirconia to Vespel® and the copper wire μcoil to a susceptibility-matched wire (copper/aluminum) which has susceptibility similar to air. These changes result in a drastic improvement of the resolution that passes from 0.01 ppm (FWHM) to about 0.002 ppm (Fig. 1a). The small residual broadening in the HRμMAS spectrum is attributed to the susceptibility gradient originating from the leads of the μcoil, which are made of copper wire. We note that these can be replaced with a copper/aluminum wire to eliminate the residual broadening. Nonetheless, the spectral resolution obtained with the HRμMAS probe offers nearly ideal spectral conditions (superior to the solid-state μMAS probe) for high-precision metabolic analyses.

One of the reasons why ^{1}H NMR spectroscopy, including ^{1}H HRMAS, has found great success in metabolomics is because it provides a stable and repeatable analytical platform. To evaluate the spectral data repeatability with HRμMAS, 24 consecutive spectra of the same 20 mM sucrose solution were acquired with the following experimental procedures: (1) manually inserting the Kel-F rotor containing the sample into the probe head, (2) spinning the sample at 2255 ± 5 Hz, (3) applying the ^{2}H lock (using the X-channel), (4) acquiring the ^{1}H spectrum, (5) stopping the sample spinning, (6) ejecting the sample and repeating the above procedures for 24 acquisitions. Fig. 1b shows the obtained 24 spectra and clearly illustrates the good stability and data repeatability of the HRμMAS probe.

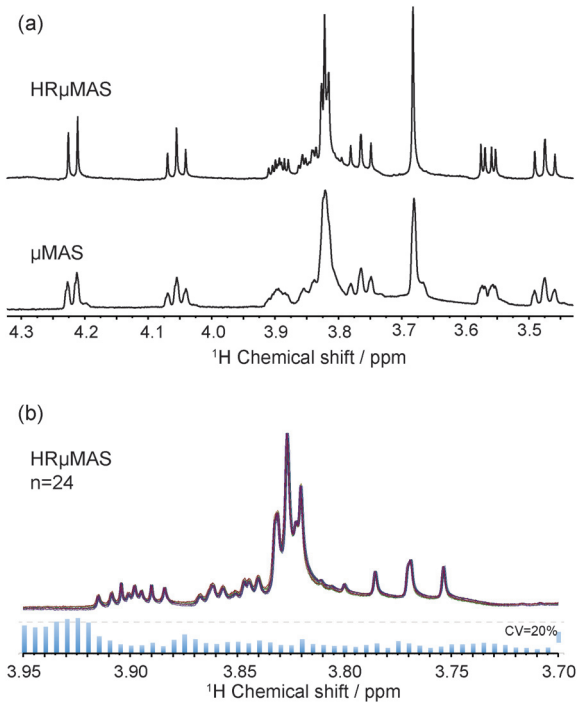

Fig. 1 (a) ^{1}H MAS NMR spectral comparison of 490 nl 20 mM sucrose solution using an unmodified solid-state 1 mm μMAS probe [bottom] and the 1 mm HRμMAS probe [top]. (b) Overlay of 24 spectra of sucrose solution acquired with the HRμMAS. The blue columns represent the Coefficient of Variant (CV) of each of the chemical shift bucket (0.005 ppm) intensities (average sum). The CV values can be found in the ESI.† All spectra are processed without apodization.

Fig. 2 shows four 1D ^{1}H NMR spectra of different 490 μg biospecimens (refer to the ESI† for the sample preparation) obtained with the HRμMAS probe at 600 MHz and with stable sample spinning (±5 Hz) between 2000 and 2500 Hz. The spectra were recorded with a standard t_2-edited CMPG pulse-experiment to suppress signals from the large biomolecules (*i.e.* proteins and lipids). The gain in sensitivity from the μcoil permits short acquisitions. In just 10–30 minutes, the NMR spectra display a good signal-to-noise ratio (SNR) over the metabolite-rich ^{1}H chemical shift region (3–4 ppm): 82 for chicken liver, 38 for pig liver, 32 for brain biopsy and 20 for brain extract. We note that the aforementioned residual susceptibility broadening (top spectrum in Fig. 1a) is negligible in Fig. 2. The combination of good SNR and resolution offers precise and detailed metabolic profiling. About 25 to 30 metabolites are identified from the spectra, including the low signal intensities of aromatic metabolites (6–9 ppm). The spectral profile (*i.e.* metabolic profile) for each specimen is clearly different from one another. For example, the lipids (1.28 ppm)

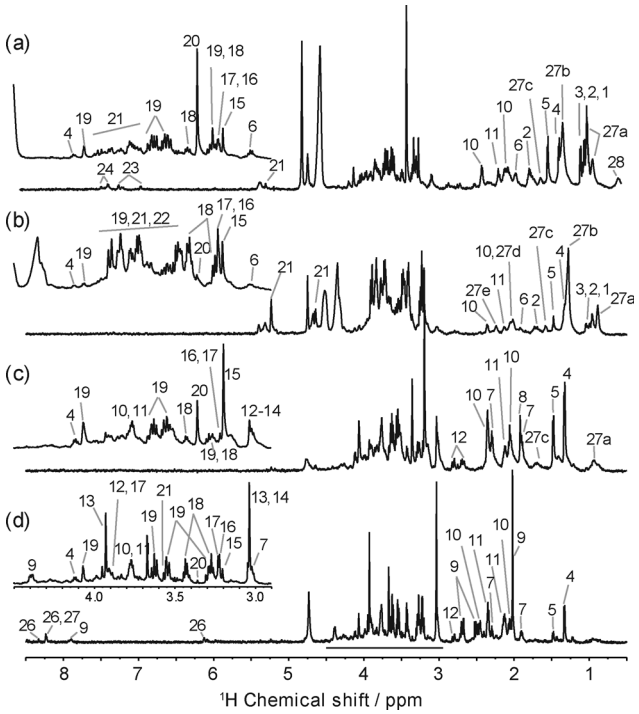

Fig. 2 ^{1}H HRμMAS NMR metabolic profiling of four 490 μg biospecimens (a) chicken liver, (b) pig liver, (c) rat brain biopsy and (d) rat brain extract. The inserted spectra are the chemical shift expansions, 2.9–4.5 ppm, which are indicated by a horizontal bar in (d). The spectra were acquired with a t_2-edited CPMG pulse experiment at 600 MHz and with sample spinning between 2000 and 2500 Hz using a Kel-F rotor. See the ESI† for more NMR experimental details. The metabolite assignments are as follows: 1 isoleucine; 2 leucine; 3 valine; 4 lactate; 5 alanine; 6 lysine; 7 γ-aminobutyric acid; 8 acetone; 9 *N*-acetyl aspartate; 10 glutamate; 11 glutamine; 12 asparagine; 13 creatine; 14 phosphocreatine; 15 choline; 16 phosphocholine; 17 glycerophosphocholine; 18 taurine; 19 *myo*-inositol; 20 *scyllo*-inositol; 21 glucose; 22 glycerol; 23 tyrosine; 24 phenylalanine; 25 adenosine; 26 adenosine triphosphate; 27 lipids: (a) CH_3, (b) $-(CH_2)_n-$, (c) $-(CH_2)_nCO$, (d) $CH_2C{=}C$, (e) CH_2CO, (f) $C{=}CCH_2C{=}C$.

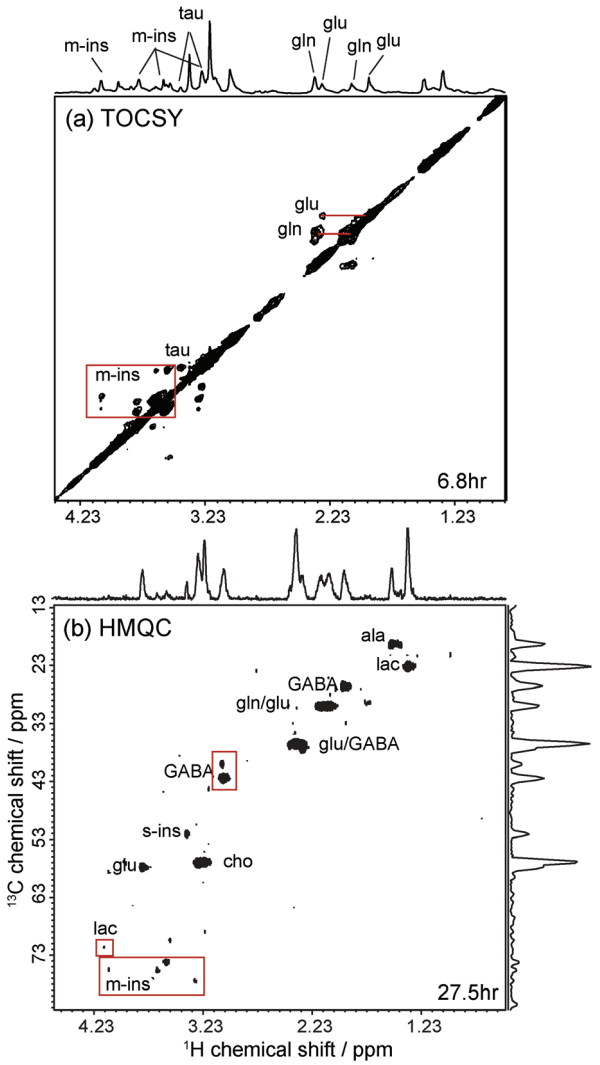

Fig. 3 (a) ^{1}H–^{1}H TOCSY and (b) ^{1}H–^{13}C HMQC HRμMAS spectrum of a [3-^{13}C]lactate infused brain biopsy, demonstrating the feasibility of performing homonuclear and heteronuclear 2D experiments with HRμMAS. See the ESI† for NMR experimental details.

are absent in the brain hydrophilic extracts, whereas a high content of lipids is found in the pig liver biopsy. A much higher content of *scyllo*-inositol (3.35 ppm) is found in the chicken liver as compared to the pig liver. On the other hand, a greater content of glucose (5.23 ppm) is found in the pig liver.

Similar to the standard large volume 4 mm HRMAS probe, multi-dimensional experiments can be readily performed with HRμMAS, enhancing the ability of metabolic identification and annotation. Fig. 3a displays a 2D ^{1}H–^{1}H TOCSY HRμMAS spectrum of a brain biopsy. The cross-signals in the 2D spectrum indicate the presence of glutamate, glutamine, taurine and *myo*-inositol. It is noteworthy that 2D TOCSY and COSY experiments with excellent spectral quality have also been acquired for the brain extract. These 2D spectra reveal numerous metabolites (see Fig. 2S in the ESI†).

The brain biopsy in Fig. 3a was previously infused with [3-^{13}C]lactate during the brain simulation by a continuous whisker movement (1 h).[18] Spectral identification of the [3-^{13}C] lactate and its relevant metabolites using ^{1}H–^{13}C NMR spectroscopy, permits the investigation of the lactate metabolisms in astrocytes in the nervous system. The 2D ^{1}H–^{13}C HMQC spectrum in Fig. 3b identifies a few metabolites – alanine, lactate, glutamate, glutamine, γ-aminobutyric acid – associated with [3-^{13}C]lactate in the lactate metabolisms. These results are in agreement with the previous HRMAS study[18] obtained using 20–30 mg biopsy for one NMR data. The observed broad ^{1}H signal (~2 Hz) in HMQC is attributed to the small B_0 drift over the 27 hours of experimental time, since the HMQC spectrum was recorded without the ^{2}H-lock. This is because the X-channel was used for the ^{13}C resonance. Nonetheless, the results demonstrate that performing double resonance NMR experiments with the HRμMAS probe is straightforward. Conversely, the HRMACS μcoil would need a more elaborate experi-

mental setup.[19] Moreover, the fast sample spinning applied with the HRμMAS probe (2000–2500 Hz) eliminates the sideband manifold 2D spectra offering cleaner spectral data than that with the HRMACS.

Conclusions

The 1 mm HRμMAS probe described here is a prototype. It was modified from a solid-state 1 mm μMAS probe to demonstrate the possibility of constructing a high resolution and high sensitivity μMAS probe for microgram biospecimen applications in a convenient, reliable and repeatable manner. The high quality spectra reported here illustrate the potential of investigating small quantity biopsies or other biospecimens. Also, new studies and experiments with the HRμMAS probe can be explored. For example, HRμMAS could be coupled with microfluidic-based cell (or small organism) sorting and manipulating techniques for a potent micro-scale NMR screening pipeline;[20,21] it could also be used for investigating the metabolic profiles of scarce specimens (*i.e.* neurons cells and small diseases tissues), or exploring the *invisible phenotypes* of specimens that cannot be studied with standard large volume HRMAS NMR spectroscopy.

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