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COMMUNICATION

Preparation of highly and generally enriched mammalian tissues for solid state NMR

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Abstract An appreciable level of isotope labelling is essential for future NMR structure elucidation of mammalian biomaterials, which are either poorly expressed, or unexpressable, using micro-organisms. We present a detailed protocol for high level ^{13}C enrichment even in slow turnover murine biomaterials (fur keratin), using a customized diet supplemented with commercial labelled algal hydrolysate and formulated as a gel to minimize wastage, which female mice consumed during pregnancy and lactation. This procedure produced approximately eightfold higher fur keratin labelling in pups, exposed in utero and throughout life to label, than in adults exposed for the same period, showing both the effectiveness, and necessity, of this approach.

Keywords Carbon-13 · In vivo · Fur keratin · Bone collagen · Labelling · Mouse

Biomacromolecular structure determination by NMR has been revolutionized by the availability of target molecules, proteins and nucleic acids, enriched in NMR active (^{13}C, ^{15}N) or effectively "invisible" (^{2}H) isotopes (Saxena et al. 2012; Verardi et al. 2012; Wagner 2010). These are usually, and increasingly routinely, prepared by producing the requisite quantities of the molecular targets in a micro-organism expression system, genetically engineered as necessary, with subsequent isolation and purification. Hitherto the overwhelming focus has been on the production of pure biomolecules for structure elucidation and intermolecular interaction studies, and in the case of solid-state NMR, ordered ensembles of small and fairly pure biomolecules. There is an ongoing effort to increase the molecular weight limits of structures elucidated by solid-state NMR, either of proteins associated with lipid membranes, or fibrils and supramolecular structures formed from soluble subunits. There is increasing realization that, important and enlightening as the resultant information is, this approach cannot be directly applied to a huge body of biomaterials, such as the large (over 300 amino acids per chain) fibrous proteins, most importantly the collagens and keratins, comprising the extracellular matrices (ECM) of connective and structural tissues such as bone, cartilage, skin, and hair. These materials present formidable challenges for atomic level structure determination for other reasons besides their sheer size. Repetitive sequences and structural motifs exacerbate already serious issues of spectral overlap, and the commonly used micro-organism expression systems cannot reproduce the complex and specific post-translational modifications necessary for faithful assembly into native structures and cellular function, or the folding and chaperoning machinery essential for ultimate conformational integrity. To extend the power of

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NMR structure determination of such insoluble systems, high levels of isotope enrichment are absolutely necessary.

In principle, the challenge of preparing isotope enriched mammalian tissues and biomaterials is easily met: feed a suitable animal enough label for long enough, then harvest the metabolically labelled tissue. In practice, there are numerous obstacles; cost and availability of sufficient labelled diet in a palatable and ethically acceptable form, the not-entirely predictable effects of endogenous metabolic processes on the ultimate fate and destination of label (Berthold et al. 1991), and the feeding time necessary to label slow turnover tissues.

In this communication we describe a method for extensive enrichment of proteins in even slow turnover tissues in reasonable time and at acceptable expense. We use labelled animal feed formulated for easy administration with minimal wastage and in-cage spoilage. The success of our methods, especially with respect to slow turnover tissues, depends on timed pregnancy coinciding with the introduction of labelled diet; tissues harvested from pups at weaning thus will have been exposed to labelled metabolic precursors throughout gestation and free life.

In vivo labelling was maximized by feeding labelled diet to female mice during the entire periods of pregnancy and lactation. This was achieved with 2000 g of a gel diet (modified Classic A03 Geldiet, SAFE, Augy, France), to minimize in-cage spillage. This comprised 50 g ^{13}C,^{15}N-labelled Celtone powder (Cambridge Isotope Laboratories, Andover, MA, USA), 67 g standard animal feed fish hydrolysate, 410 g protein free diet (mainly corn starch), 72.9 % water, and 2.1 % preservatives and texture additives. The diet was packaged in 100 g packs for convenience and irradiation sterilized at 10 kGy. The manufacturer's certificate of analysis showed that the Celtone consisted of 70 % peptides or amino acids. Another batch of gel diet identical in all respects, except that it contained unlabelled and not labelled Celtone, was similarly prepared, to familiarize the animals with the diet (which they consumed avidly), and for feeding during the initial phase of the study before conception. Two young adult female C57Bl/6 mice were housed together with a stud male and fed unlabelled gel diet until mating was confirmed by vaginal plugging. The male was removed and both females were fed ad libitum on labelled diet during pregnancy (20 days) and until weaning of the first litter of pups (after 19 days from birth), at which point all mice (both adult females and the four, and two, pups surviving from each litter) were humanely euthanized using a Schedule 1 method and tissues harvested and stored at −80° C until acquisition of NMR data. Comparison of spectra from tissue from the two mothers, and spectra from the six pups, showed no differences in spectral profile or relative intensity among individual samples within either group. The in vivo phase of the project complied with the institutional Ethical Review Process, and the UK Animals (Scientific Procedures) Act 1986.

Tissue labelling was confirmed qualitatively using 1D ^{13}C-^{13}C DQ filtered NMR, which selectively detects only ^{13}C atoms which are strongly dipole–dipole coupled, in practice directly bonded, to neighbouring ^{13}C atoms. DQ filtering was achieved using CP preparation with the addition of a subsequent POST-C7 (Permutationally Offset Stabilized C7) sequence filter (Hohwy et al. 1998); results are summarized in Fig. 1.

The inherent efficiency of the DQ editing relative to standard CP, which detects all carbons irrespective of neighbours, is difficult to quantify in a rational manner. The DQ editing efficiency is sample dependent, and depends on the chemical (e.g. methyl, methylene, methine, quaternary) and probably dynamical (e.g. backbone vs. sidechain) properties of specific carbons. Accordingly we derived a quantitative measure of the degree of labelling in bone collagen and fur keratin by comparing the intensities of CP spectra of the same mass of unlabelled tissue, labelled maternal tissue, and labelled pup tissue, using otherwise identical acquisition conditions (Fig. 2). Enhancements in the various labelled tissues relative to enhancements in the unlabelled tissues are summarized in Table 1.

In previously published work (Chow et al. 2014) we achieved levels of ^{13}C labelling in some tissues, sufficient for high quality 2D NMR, by simply feeding labelled diet to an adult mouse for about three weeks. Using this protocol, however, the level of labelling varied widely between different tissue types, being among the highest in bone collagen, and barely detectable in slower turnover biomaterials such as fur keratin, among others, precluding any structurally informative 2D experiments in the latter. The strategy described here maximizes labelling of even slow turnover tissues, with the costs of labelling the diet contained within reasonable limits. We chose a gel rather than pellet formulation to minimize wastage due to the unavoidable pelleting machine "dead" volume of commercial pelleting machines, and in-cage spillage and soiling of pelleted diet. Two adult females were used to avoid single animals being alone in a cage, thus keeping the experiment as an unregulated procedure under the UK legislation. By feeding the pregnant animals labelled diet from conception, through parturition and lactation, and only at weaning culling both mother and pups, we ensured that the latter were exposed to a high proportion of labelled amino acids for their entire in utero and free living existence. This resulted in efficient labelling in the pups of even slow turnover tissue materials, such as the fur keratin described here.

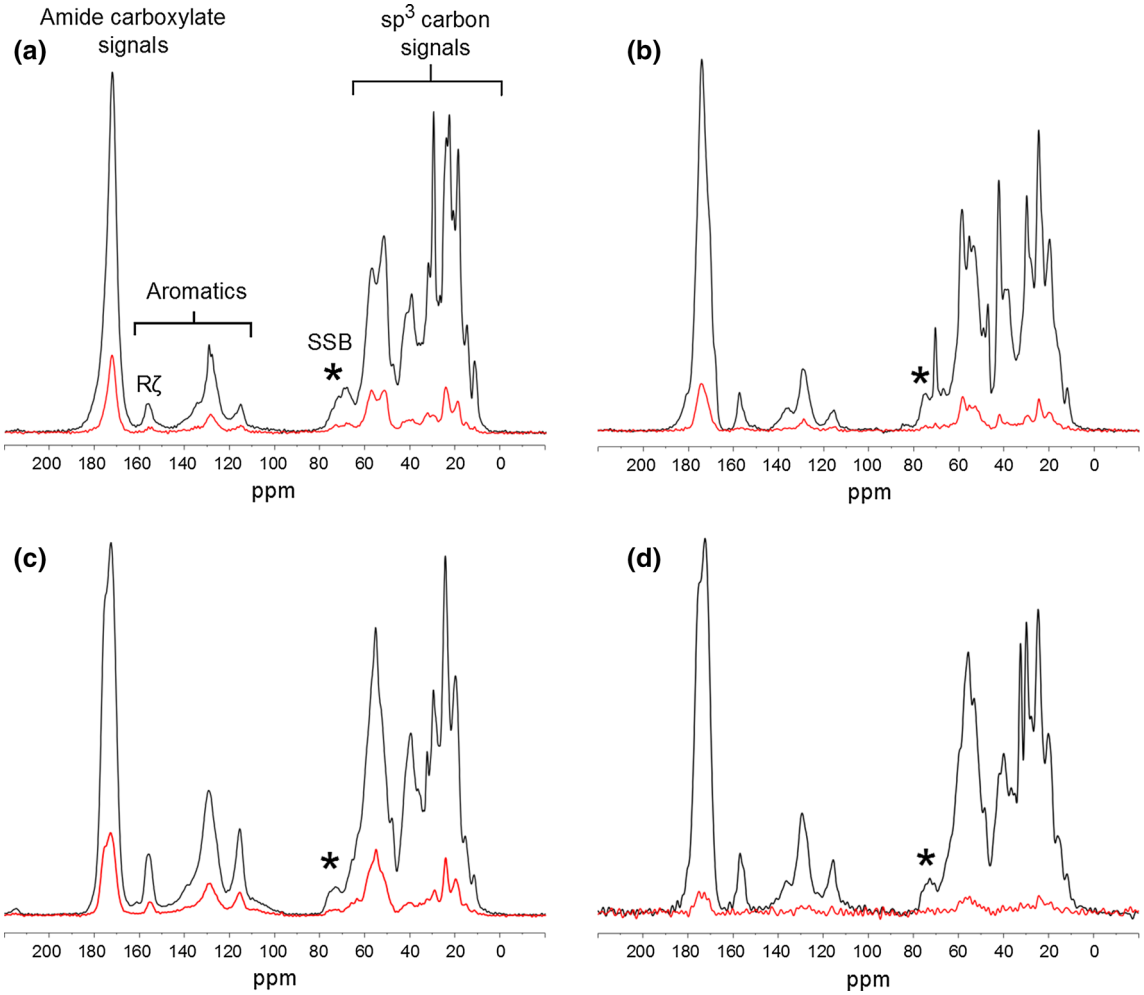

Fig. 1 Standard ^{13}C CP (*black*) and 1D post-C7 DQ filtered (*red*) spectra, acquired with identical scan numbers, of **a** labelled limb bone collagen from a mouse pup and **b** from its mother (*right hand panel*), and **c**, **d** of fur keratin from the same respective animals. *Asterisks* denote spinning side bands. Pertinent experimental parameters were: NMR—Bruker Avance I, 9.4T, 400 MHz ^{1}H, 100 MHz ^{13}C, MAS 10 kHz, ^{1}H $\pi/2$ pulse 2.5 μs, ramped CP 2.5 ms at 70 kHz matching RF fields, DQ filtering (when applied) with 70 kHz POST-C7 pulse sequence (Hohwy et al. 1998) on ^{13}C to excite DQ coherence in 0.4 ms, returned to zero quantum by another 0.4 ms POST-C7, 100 kHz Lee-Goldberg ^{1}H decoupling during DQ evolution, 100 kHz ^{1}H spinal64 decoupling during acquisition, repetition time 2 s, chemical shifts relative to external α-glycine methylene signal at 43.1 ppm, sample temperature 18 C. Cleaned limb bones were broken into small chips and packed into kevlar inserts fitted into standard zirconia 4 mm rotors; shaved fur was tightly packed directly into 4 mm rotors. Spectra showed insignificant interindividual and inter-litter variation. All samples were run at native hydration levels; neither linewidth nor intensity changed noticeably over time so we assume insignificant dehydration during the course of all NMR experiments. Comparison of the intensity of the DQ filtered with the CP spectra gives a rough measure of labelling efficiency of the different feeding regimes (in-life, adult vs. in utero followed by in-life, pup), and in the different tissues

Early tissue isotope in vivo labelling successes were achieved by injecting (Sarkar et al. 1983, 1985) or feeding (Torchia et al. 1985) rodents with enriched single amino acids; the latter study anticipates our approach by feeding to a lactating mother and harvesting enriched tissue from pups. The experimental enrichment paradigm, whereby developing organisms are exposed to labelled feedstock from as early a phase of life as possible, has been used to label the connective tissue from molluscs (blue mussels) (Arnold et al. 2013) for solid state 2D NMR. Similar methods to ours have also been used to effect high levels of ^{15}N labelling of various soft tissues in rats (McClatchy et al. 2007a; Wu et al. 2004) and chicks (Berthold et al. 1991) for mass spectrometric proteomic quantification (McClatchy et al. 2007b). ^{19}F labels can also be introduced into mammalian tissue by feeding mice with specifically fluorinated amino acids (Sarkar et al. 1987). A study of $^{13}C_6$ lysine enriched mouse bone has been reported although it was not stated how labelling was achieved (Nikel et al. 2013). In the proteomics studies the protein component of the diet was effectively totally enriched only with ^{15}N; we supplemented the more expensive ^{13}C, ^{15}N

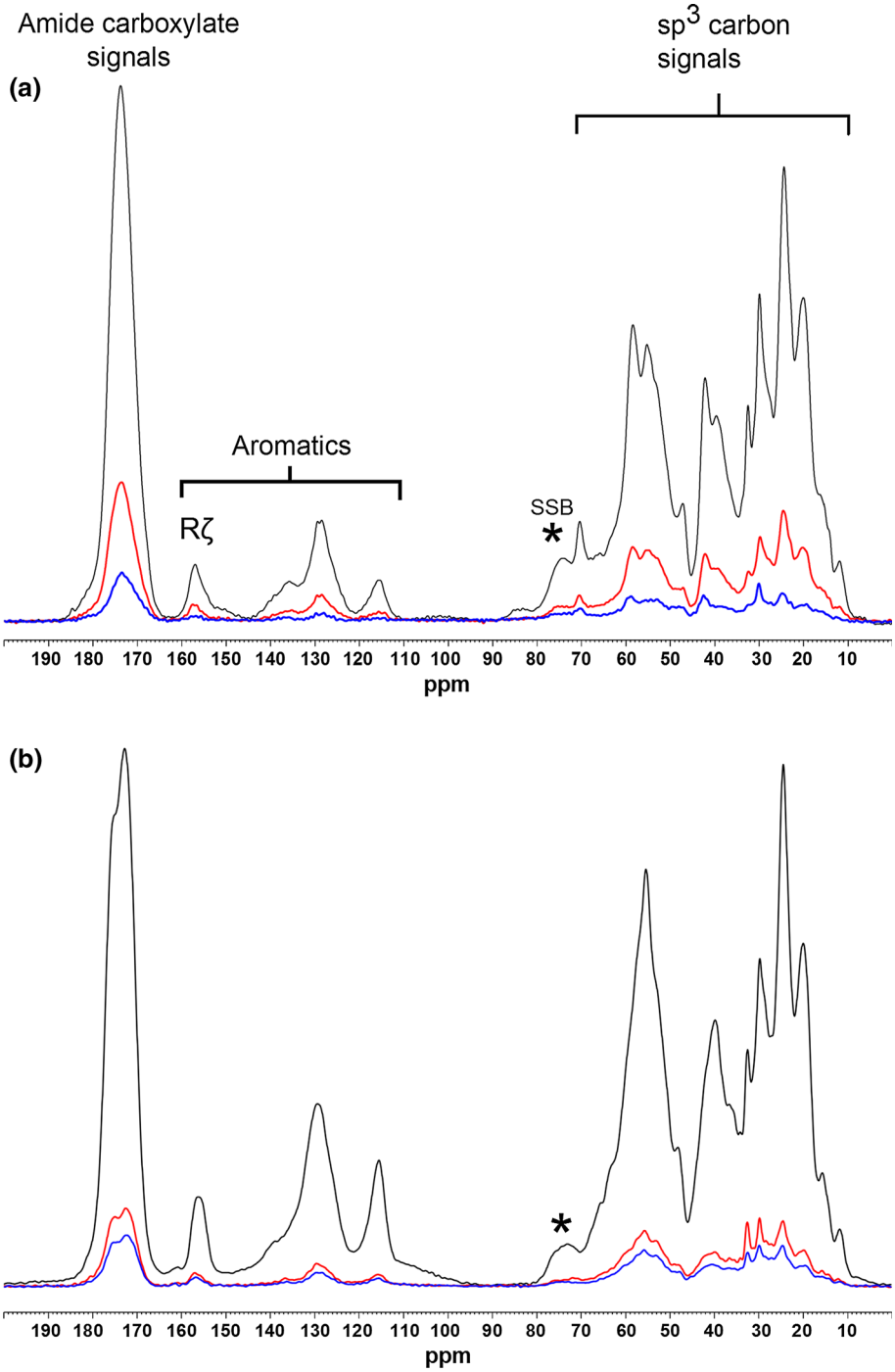

Fig. 2 Overlays of standard ^{13}C spectra (same parameters as described in legend to Fig. 1) of **a** bone (ca. 22 mg in all cases) from: labelled pup (*black*), labelled mother (*red*), and unlabelled adult (*blue*), and **b** fur keratin (ca. 9 mg in all cases) from the same respective animals. Relative intensities were estimated by integrating over the entire spectral region (including the high frequency spinning side bands which are not shown). The extent of spectral enhancement in labelled tissue was estimated relative to the overall intensity of the unlabelled tissue as shown in Table 1

enriched Celtone with standard animal feed fish protein (at an approximate ratio of 1:2 by protein weight respectively) both to keep the experiment affordable and to supply any possible dietary deficiencies resulting from low levels of essential amino acids e.g. tryptophan, in the algal hydrolysate (see Supplementary Table S1 for comparison of amino acid contents of the two feedstocks). In the rapid turnover bone collagen, feeding with labelled diet during adulthood only produces 2.9-fold spectral enhancement, which corresponds to sufficient labelling for structurally meaningful 2D spectroscopy (Chow et al. 2014). The meagre isotope enrichment of only 1.6-fold produced by adult feeding in fur keratin (typifying a slow turnover tissue) is quite insufficient for this purpose. However, the most meaningful and telling comparison between the two tissue types is the ratio of total spectral enhancement in pup tissue to that in maternal tissue. In the more rapid turnover bone tissue simply feeding to an adult produces enough

Table 1 CP spectral intensity ratios in maternal, and pup, bone collagen, and fur keratin

Tissue	Labelled:Native (CP)	Pup:Maternal (CP)
Bone		
Maternal	3:1	3.7:1
Pup	11:1	
Fur		
Maternal	1.6:1	7.3:1
Pup	12:1	

enrichment to make structure-dependent 2D experiments possible in bone collagen, but not in slower turnover materials such as fur keratin (and others). In the latter, our currently reported protocol produces a 7.3-fold increase in spectral signal (with corresponding increase in labelling) relative to adult feeding (compared to only 3.7-fold in bone), providing sufficient labelling and ^{13}C-^{13}C proximities to permit 2D ^{13}C-^{13}C correlation spectroscopy.

Achieving even higher enrichment levels is only constrained by the cost of labelled feedstock, and the practical necessity of supplementing protein with unlabelled starch which inevitably incorporates unlabelled non-essential amino acids resulting from de novo biosynthesis in vivo.

All raw experimental data relevant to this communication is available at https://www.repository.cam.ac.uk/handle/1810/249109.

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Compliance with ethical standards

Conflict of interest None of the authors have any conflicts of interest in regard to this work.

Human and animal rights statement No human subjects or tissues were used. All animal procedures complied with institutional ethical guidelines, and the UK Animals (Scientific Procedures) Act 1986.

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