Magnetic Resonance Cryoporometry Analysis of Red Deer (Cervus elaphus) Antler Bone

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Abstract
Deer antler is a unique example of mammalian long bone because it regenerates annually with an extremely rapid rate of growth. This study describes a low-field NMR technique combined with cryoporimetric calibration for the estimation of pore size distribution in antler bone from spin-spin ($T_2$) relaxation data. Pore sizes determined by the NMR method have been compared with the more traditional methods of gas adsorption and mercury intrusion experiments. The NMR method has the advantage of being rapid, non-destructive and non-invasive. It is superior to image analysis because the whole sample is used rather than a cross-section. This technique is particularly useful for the determination of fine pore structure including pore dimensions at the nanometre level and may be applied to other bioceramic materials where pore size and interconnectivity of the pores is important.

Key words: antler bone, NMR spectroscopy, pore size distribution, hydroxyapatite.

INTRODUCTION
Deer are even-toed ungulates (hoofed animals). With the exception of the reindeer and the caribou, antlers are grown exclusively by the male of the species. Antlers are dimorphic appendages serving as weapons and display organs in the social order of the deer [1]. The branches on antlers are called tynes, while the trifurcate (three-forked) branching at the end of the antler constitutes the crown. The antler tynes contain hyperplastic tips responsible for the rapid growth of the structure – up to two centimetres per day including nerve cells and vascular channels [2]. Massive ossification occurs prior to full antler maturity in preparation for the rutting season which occurs in March/ April in the southern hemisphere. Like all bone samples, the primary inorganic component of mature deer antlers is a carbonated apatite interwoven with a collagenous organic matrix [2, 3]. Deer antler must be extremely tough; before casting, fully calcified antler can withstand applied stresses of over 300 MPa, at least 100 MPa higher than that of mammalian femur [4]. The complex array of cavities within mineralized structures is thus usually oriented to optimize fracture mechanics of the material.

Up to seven hierarchical levels of structure have been identified in bone [5] with typical dimensions ranging in scale from 1.5 nanometres for the diameter of the triple helical tropocollagen filament through to millimetre or larger dimensions for the bone macrostructure [4]. Within these tissues, pore size and interconnectivity of the pores are vitally important since they affect the strength and other mechanical properties of the pores in addition to allowing nutrient diffusion and cell attachment during growth and in natural repair processes.

In this study, nuclear magnetic resonance spin-spin relaxation times ($T_2$'s) have been measured on the antler bone from a mature male of the species Cervus elaphus. The distribution of $T_2$’s has been interpreted in the first instance as pore size distributions. The effects of removing both lipid and proteinaceous material on pore size distributions have also been studied.

MATERIALS AND METHODS
Sample collection
Fully hardened antlers were removed from one mature red stag as part of the animal husbandry programme at Western Sydney University, Hawkesbury Campus. In line with RSPCA requirements, the antlers were removed after sedation with xylazine (0.8 mg/kg body weight). Samples were immediately wrapped in food grade plastic and stored at 0 - 1°C. For this study, the antler crown and tynes were subsequently removed.
using a diamond saw and only main beam tissue was used for analysis.

**Light microscopy**
To elucidate the gross anatomy of antler bone, transverse sections approximately 4 mm thick were embedded in epoxy resin, polished and examined under a Leica EZ4D light microscope.

**Sample treatment**
Samples of the main beam approximately 8 cm long were allowed to fully dry in a vacuum desiccator. Once the dry mass of tissue had been accurately determined, the sample was analysed using solid state NMR to measure the residual water retained in the sample after the drying procedure. The samples were then saturated with deionised water by immersing the sample in approximately 500 mL of water, applying a vacuum (rotary pump) for 20 minutes and then purging with nitrogen for a further 20 minutes. This process was repeated several times. The sample was then removed quickly, the external surfaces dried and the sample weighed. The entire procedure was repeated until the mass of the water in the antler was constant. The saturated sample was then wrapped in teflon tape to prevent water loss during the measurements, and immediately placed in the NMR chamber for analysis.

In order to determine the effects of lipid and proteinaceous material on the pore size and interconnectivity, these components were sequentially removed by chemical extraction and the water saturation and NMR analysis repeated after each extraction process. For lipid removal, the sample was treated with diethyl ether in a soxhlet extractor for approximately 12 hours. Protein was removed according to the method of Frayssinet et al. which uses hydrogen peroxide extraction followed by treatment with 1M NaOH solution [6]. The efficacy of these treatments on fallen deer antler have been reported previously using infrared spectroscopy [3].

**Nuclear magnetic resonance spectroscopy**
Proton relaxation processes were recorded on a Resonance Maran 2 broadline NMR spectrometer. This instrument utilizes a large permanent magnet producing a low intensity field with a Larmor frequency of 2.1 MHz. Relaxation measurements were recorded using the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence [7, 8] with a series of pulses separated by a delay time, \( \tau \), of 150 \( \mu \)s. The number of scans collected was 1024. After an initial \( \pi/2 \) pulse, a series of \( \pi \) pulses at \( \tau \), 3\( \tau \), 5\( \tau \), 7\( \tau \) \( \mu \)s etc. were applied to produce a series of echoes at 2\( \tau \), 4\( \tau \) and 6\( \tau \) etc. which decrease exponentially with time constant, \( T_2 \).

**Pore size calibration by melting point depression**
Magnetic resonance cryoporometry can be used to determine the pore size distribution in the mesopore range since this method relies on the fact that a pure material in a confined space will have a melting point that is depressed from the bulk melting point by a temperature difference that is inversely proportional to the pore diameter [9].

To calculate the pore size from a knowledge of the melting point depression of water, a modified Kelvin equation (also known as the Gibbs-Thompson equation) was used [10, 11]:

\[
\Delta T = T_m - T_m^x = \frac{4\sigma l_m^3}{\Delta H_f \rho_s x}
\]

Where \( T_m \) is the melting point of bulk water (273.15 K), \( T_m^x \) is the melting point of water in the pores of diameter, \( x \), \( \sigma \) is the surface tension of water (75.64 mN m\(^{-1}\) = 7.564 \times 10\(^{-2}\) J m\(^{-2}\)), \( \Delta H_f \) is the bulk enthalpy of fusion of water (333.6 J g\(^{-1}\)), \( \rho_s \) is the density of ice (at the temperature measured) and \( x \) is the pore diameter. If \( \rho_s \) has units of g cm\(^{-3}\), then a value of \( x \) will be obtained with units of \( \mu \)m. It may be seen from the above equation that as long as the temperature is held constant below normal (bulk) melting point, then \( \Delta T \) will be inversely proportional to pore diameter. A proportionality constant relating the relaxation time, \( T_2 \), to pore diameter can then be determined from the NMR spectrum obtained at the depressed temperature by noting the \( T_2 \) value separating frozen from nonfrozen water. This method has been used previously to calibrate pore sizes in biogenic (fallen antler [3]), nonbiogenic (sandstone [12]) and bio ceramic (hydroxyapatite [13]) samples.

In the current study, calibration was performed using delipidated/deproteinated samples. These samples were saturated with water and then frozen at a number of known temperatures. During the NMR measurements, the temperature of the sample was monitored with a platinum resistance thermometer to ensure that constant temperature was being maintained. After recording the NMR spectra, the \( T_2 \) value which separates the frozen and liquid water was noted for calibration purposes. Errors associated with this methodology have been discussed elsewhere [12].

To follow water loss from the antler by evaporative processes, NMR analyses of the untreated sample were repeated as a function of time over a 22 hour period.
Gas adsorption and mercury porosimetry
Gas adsorption and desorption isotherms using nitrogen (73.35 K) and a Micrometrics TriStar 3000 instrument were conducted by Particle & Surface Sciences Pty. Ltd on the untreated cortical bone of red deer antler. The desorption data was analysed using the Barrett, Joyner and Halenda (BJH) method [14] to obtain the pore size distributions. To gain a second estimate of pore size distribution, mercury intrusion experiments were also performed by Particle & Surface Sciences Pty, Ltd. using a Micrometrics AutoPore IV 9500 instrument.

RESULTS AND DISCUSSION

Light microscopy
The anatomy of antler bone at the gross level shows regions which are analogous to cortical (compact) and trabecular (spongy) bone found in other mammalian bone structures (Fig. 1). Trabecular bone has a very open structure with some voids ranging from 200 to 400 µm. This is outside the range of the NMR technique presented here since water in such large pores behaves in the same way as bulk water. Thus the NMR results reported here reflect smaller pores found in the cortical region of antler bone (< 100 µm).

Fig. 1: Transverse section of main beam red deer antler bone (magnification = 8X).

Fig. 2: CPMG spin relaxation decay signal from main beam of red deer antler bone (bottom (blue) trace = untreated sample, middle (pink) trace = delipidated sample, top (green) trace = delipidated and deproteinated sample).
NMR relaxation studies

Fig. 2 shows an example of a NMR Carr-Purcell-Meiboom-Gill (CPMG) relaxation decay signal for the main beam sample of red deer antler. The decay data demonstrates exponential relaxation behaviour. The highest signal (green trace) corresponds to the deproteinated and delipidated sample while the lowest signal (blue trace) is for the untreated material. The pink trace corresponds to the delipidated sample only. The relaxation differences in intensity are proportional to the volume of mobile $^1$H protons present within the bone sample.

Pore size calibration

Fig. 3 shows a sample $T_2$ distribution obtained for the delipidated and deproteinated sample of the red deer antler which had been saturated with water then equilibrated at 251.81 K overnight. The highest $T_2$ value of 3477.7 µs corresponds to liquid water which had not been frozen in the pores of the antler. Water in pores larger than this would have been frozen at this temperature. The modified Kelvin equation was used to calculate the pore diameter (4.24 nm), at which water would freeze at this temperature. This was used to convert the $T_2$ distribution to a pore size distribution. The average proportionality constant using this method was found to be $249 \pm 26 \, \mu s \, nm^{-1}$.

Pore size distribution

NMR spectroscopy of the untreated antler, analysed with the natural fluids present in the antler, showed a distribution of five peaks for the red deer antler. The $T_2$ value of 594 µs was the largest peak for the sample, due to water trapped in very small pores (about 2 nm). After vacuum desiccation, no NMR signal was recorded, confirming that this peak was due to trapped water and not from organic material. Other studies have found signals of 50 µs may be attributed to bone collagen [15].

Fig. 4 indicates the quasi-continuous $T_2$ relaxation distributions for main beam antler cortical bone from red deer. This relaxation data gives information on the geometry and connectivity of the pore microenvironment. These $T_2$ relaxation times are distributed across a wide time range, from sub-millisecond to hundreds of milliseconds, corresponding to a wide pore size distribution as shown in fig. 5.

Fig. 3: Modal distribution peak for nonfrozen water in the pores of the delipidated and deproteinated sample at 251.81 K.
Fig. 4: NMR signal intensity vs. relaxation time ($T_2$) for red deer antler (blue trace = untreated sample, pink trace = delipidated sample, green trace = delipidated and deproteinated sample).

Fig. 5: NMR signal intensity vs. pore size for red deer antler bone (blue trace = untreated sample, pink trace = delipidated sample, green trace = delipidated and deproteinated sample.)
Following delipidation of the sample, notable differences in the $T_2$ distribution were observed throughout the intermediate range of 10 to 100 ms. These peaks underwent a shift to longer times and increased in intensity, presumably as some pores increase in diameter following the treatment and new pores are exposed by removal of lipids (fig. 4). These occurred at $T_2$ values of 21.8, 52.06 and 458.6 ms corresponding to diameters of 0.08, 0.21 and 1.84 $\mu$m respectively as shown in fig. 5.

Following deproteination of the sample, the $T_2$ distribution of the system underwent a significant change to a trimodal quasi-continuous distribution. A shift in the $T_2$ time occurred for water trapped in the smallest pores from a time of 0.59 ms (pore size of 2 nm) to $T_2$ value of 1.38 ms (pore size of 6 nm). It can be concluded that removal of the proteins and lipids has increased the diameter of the pores. It is interesting to note that pores with a $T_2$ value of 458 ms showed little change after the extraction procedures. This time corresponds to a pore diameter of 1.84 $\mu$m which may possibly be associated with osteocyte lacunae since these structures have diameters in the 4 – 5 $\mu$m range [16].

Pore size distributions of the samples after each extraction step are given in table 1. The results indicate that most pores are associated with nanometre dimensions which are probably associated with porosity arising from spaces around the organic matrix fibres. Much larger holes within the bone microstructure are lacunae and canaliculae that contain bone cells (osteocytes) and their protrusions. While average diameters of osteocyte lacunae are 4 – 5 $\mu$m, the interconnecting canaliculae tunnels are an order of magnitude smaller. Thus lacuno-canalicular dimensions are well within the range discernable using the current methodology.

The $T_2$ distribution curves for the untreated sample at various time intervals are given in fig. 6. The results show that water loss commences from the largest pores.

Table 1: List of $T_2$ distribution times and corresponding pore size for each sample and extraction step.

<table>
<thead>
<tr>
<th>sample</th>
<th>$T_2$</th>
<th>pore size ($\mu$m)</th>
<th>% distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated</td>
<td>594.0 $\mu$s</td>
<td>0.002</td>
<td>88.0</td>
</tr>
<tr>
<td></td>
<td>4.2 ms</td>
<td>0.017</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>20.1 ms</td>
<td>0.081</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>71.8 ms</td>
<td>0.289</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>198.8 ms</td>
<td>0.799</td>
<td>2.0</td>
</tr>
<tr>
<td>delipidated</td>
<td>0.580 $\mu$s</td>
<td>0.002</td>
<td>57.4</td>
</tr>
<tr>
<td></td>
<td>4.4 ms</td>
<td>0.018</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>21.8 ms</td>
<td>0.088</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>52.1 ms</td>
<td>0.209</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>458.6 ms</td>
<td>1.842</td>
<td>22.7</td>
</tr>
<tr>
<td>delipidated &amp; deproteinated</td>
<td>1.4 ms</td>
<td>0.006</td>
<td>71.9</td>
</tr>
<tr>
<td>deproteinated</td>
<td>38.9 ms</td>
<td>0.156</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>458.6 ms</td>
<td>1.842</td>
<td>18.5</td>
</tr>
</tbody>
</table>
Fig. 6: $T_2$ distribution curve for the progressive drying of the untreated sample. The blue trace corresponds to the fully saturated sample with progressive drying shown by the black (8 hours) and then green (22 hours).

Fig. 7: Pore size distribution from nitrogen adsorption/desorption (BJH) and mercury intrusion (Hg) experiments.
Gas adsorption and mercury porosimetry
Results of the gas desorption and mercury intrusion experiments are shown in fig. 7. Both gas desorption and mercury intrusion experiments indicated that nanosized pores are present in the samples of the cortical bone of the antler sample. The BJH desorption analysis gave a maximum percentage distribution for pore diameter of 3 nm, while mercury intrusion gave a maximum percentage distribution for diameter of 4 nm. These results correlate well with the pore sizes found in the NMR experiments in which the smallest pore size was 2 nm.

CONCLUSIONS
This study has described a low-field NMR technique combined with cryoporimetric calibration for the estimation of pore size distribution in bone from spin-spin relaxation data. Pore sizes determined by the NMR approach correlate well with the more traditional methods of gas adsorption and mercury intrusion. The NMR method has the advantage of being rapid, non-destructive and non-invasive. It is superior to image analysis because the whole sample is used rather than a cross-section. Mercury porosimetry is destructive since the pores are destroyed upon mercury intrusion, and only connected pores are measured. NMR analysis is independent of this limitation and shows the results of all fluids within the sample. This technique may be applied to other bioceramic materials where pore size distributions and interconnectivity of the pores is important.

These experiments have also given useful insights into the distribution of pores in freshly cut antler bone and the influence of organic material on pore dimensions, particularly at the level of nanometre dimensions.

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REFERENCES