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Cortical bone elasticity measured by resonant ultrasound spectroscopy is not altered by defatting and synchrotron X-ray imaging

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ABSTRACT

In the study of mechanical properties of human bone, specimens may be defatted before experiments to prevent contamination and the risk of infections. High energy synchrotron radiation micro-computed tomography (SR- μ CT) is a popular technique to study bone microstructure. However, little is known about the effects of defatting or irradiation during SR- μ CT imaging on different elastic coefficients including shear and longitudinal moduli in different anatomical directions. In this work, these effects are evaluated on a set of 24 samples using resonant ultrasound spectroscopy (RUS), which allows one to accurately measure the complete set of elastic coefficients of cortical bone non destructively. The results show that defatting with diethylether and methanol and irradiation up to 2.5 kGy has no detectable effect on any of the elastic coefficients of human cortical bone.

1. Introduction

Structure-function relationships of bone have been intensely studied in the past decades, particularly with the availability of X-ray-based micro-computed tomography (μ CT) techniques allowing a detailed quantification of bone microstructure. Among these, μ CT imaging techniques using X-ray synchrotron sources (SR) can deliver high resolution images of bone with increased signal-to-noise ratio and less beam hardening effects and less beam artifacts compared to laboratory-based μ CT imaging devices (Peter and Peyrin, 2011). In that vein, coupling mechanical tests with SR- μ CT is a powerful method to investigate the relationships between microstructure and mechanical properties in bone (Akhtar et al., 2008; Granke et al., 2011). Common limitations of such studies are the artifacts potentially introduced by the experimental protocol used, including sample preparation steps and measurements. These procedures may alter to a greater or lesser extent the tissue structure and, therefore, have impact on the final outcome of the studies.

To experimentally measure mechanical properties of human bone, specimens must be preserved and may be defatted to prevent contamination and the risk of infections. Generally, defatting is done by chemical fixation, using different products such as ethanol and formalin (Wieding et al., 2015; Stefan et al., 2010). Many studies have investigated the influence of these preservation methods on some

particular bone mechanical properties such as the Young's modulus (Haimi et al., 2008; Linde and Sørensen, 1993) and fracture toughness (Smith et al., 2011). Formalin affects post-yield behaviour, increases hardness and reduces toughness, while controversial effects on elasticity have been reported when ethanol was used (Sedin, 1965; Linde and Sørensen, 1993; Stefan et al., 2010). Nevertheless, in all these studies, different chemical composition was used and the properties were measured by destructive mechanical tests (Smith et al., 2011; Stefan et al., 2010).

The effect of irradiation is also a concern. Previous studies have reported that gamma irradiation doses of 25 kGy, i.e., the standard dose for sterilization, significantly reduce the high-cycle fatigue life of allograft bone tissues (Islam et al., 2016) and the strength of human cortical bone, while Young's modulus measured in three-point bending does not seem to be significantly affected (Currey et al., 1997; Nguyen et al., 2007). Similar results have also been reported using X-ray irradiation (Barth et al., 2011), being used in bone imaging techniques.

Most of the studies are limited to the investigation of the effect of irradiation or specimen preparation to the Young's modulus in the longitudinal direction (Stefan et al., 2010; Kaminski et al., 2012; Haimi et al., 2008; Currey et al., 1997; Barth et al., 2011). However, since cortical bone is a typical anisotropic material, quantifying a single modulus is not sufficient to fully characterize the elastic behavior. This is the aim of the present study to quantify the possible effects of typical

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defatting and SR- μ CT imaging conditions on human cortical bone elasticity. To this goal, resonant ultrasound spectroscopy (RUS) is used. RUS is a non destructive technique that allows one to characterize the same samples in native state (before any chemical or irradiation alteration), defatted state and after irradiation. Furthermore, RUS provides the elastic tensor of cortical bone (Bernard et al., 2013), that is, the five elastic coefficients of the transversely isotropic material, and not only Young's modulus as in previous studies.

2. Materials and methods

2.1. Specimens

Bone specimens were harvested from the left femur of 12 human cadavers. The femurs were provided by the Département Universitaire d'Anatomie Rockefeller (Lyon, France) through the French program on voluntary corpse donation to science. The tissue donors or their legal guardians provided informed written consent to give their tissue for investigations, in accord with legal clauses stated in the French Code of Public Health. Among the 12 donors, 8 were females and 4 were males (50 – 91 years old, 72.6 ± 13.0 , mean \pm SD). The fresh material was frozen and stored at -20 °C.

The samples were slowly thawed and then, for each femur, approximately a 10 mm thick cross section was cut perpendicular to the bone axis from the mid-diaphysis. Then, using a water-cooled low-speed diamond wire saw (Model 3241, Well, Lyon, France), two rectangular parallelepiped shaped specimens were prepared in the lateral and medial anatomical quadrants of each cross section, which led to a set of 24 specimens. The nominal specimen size was $3 \times 4 \times 5$ mm³ in radial (axis 1), circumferential (axis 2) and axial direction (axis 3), respectively, defined by the anatomic shape of the femoral diaphysis. All specimens were kept hydrated during sample preparation.

2.2. Elasticity measurements by resonant ultrasound spectroscopy

The elastic tensor, assuming a transversely isotropic symmetry was assessed by RUS. Here, the indices of the elastic tensor are written with the Voigt notation,

$$C_{ij} = \begin{pmatrix} C_{11} & C_{12} & C_{13} & 0 & 0 & 0 \\ C_{12} & C_{11} & C_{13} & 0 & 0 & 0 \\ C_{13} & C_{13} & C_{33} & 0 & 0 & 0 \\ 0 & 0 & 0 & C_{44} & 0 & 0 \\ 0 & 0 & 0 & 0 & C_{44} & 0 \\ 0 & 0 & 0 & 0 & 0 & C_{66} \end{pmatrix}, \quad (1)$$

where $C_{12} = C_{11} - 2C_{66}$ and (1 – 2) is the isotropy plane; C_{11} and C_{33} are longitudinal elastic coefficients and C_{44} and C_{66} represent shear elastic coefficients.

RUS was conducted following (Bernard et al., 2014, 2015). Briefly, the elasticity measurements by RUS are described as follows. A bone specimen was held by two opposite corners between two ultrasonic transducers (V154RM, Panametrics, Waltham, MA), one for emission and one for reception, to achieve a free boundary condition for vibration. The frequency response of the vibration in the frequency range 100–500 kHz, tuned so as to measure the 30-40 first resonant frequencies, was recorded by a vector network analyzer (Bode 100,

Omicron Electronics GmbH, Klaus, Austria) and a broadband charge amplifier (HQA-15M-10T, Femto Messtechnik GmbH, Berlin, Germany). Six consecutive measurements were performed on each specimen. Between each measurement, the specimen was turned with a slightly different orientation in order to maximize the number of detectable resonant frequencies. Then, the resonant frequencies of the specimen were extracted from the six measured responses using the method dedicated to high damping material (Lebedev, 2002). Finally, knowing the apparent mass and dimensions of each specimen, the elastic coefficients were automatically calculated by solving the inverse problem formulated in a Bayesian framework (Bernard et al., 2015). This RUS protocol usually yields a precision of 0.4% for C_{44} and C_{66} , 3% for C_{11} and C_{33} , 5% for C_{13} (Bernard et al., 2013).

2.3. Bone defatting

Bone specimens were defatted following a protocol that prevents the risk of infections and allows the sample conservation at room temperature. Briefly, the procedure consists of the following steps: (1) rinsing in saline at ambient temperature; (2) defatting for 18 h using a chemical bath of diethylether and methanol (1:1) at room temperature; (3) draining off the excess of chemical fluids on absorbent paper; and (4) rinsing by sonicating with distilled water in a temperature-controlled ultrasonic bath. After this procedure, defatted specimens were kept separately in a tube filled with saline and preserved in a fridge at 4 °C. This procedure was used previously in ex-vivo studies such as (Tang et al., 1996; Granke et al., 2011). In our experience, samples defatted using the above procedure can be measured in synchrotron facilities as they comply with safety regulations.

2.4. Synchrotron radiation microtomography

SR- μ CT imaging was performed on the beamline ID19 at the European Synchrotron Radiation Facility (ESRF, Grenoble, France). This SR- μ CT setup is based on a 3D parallel beam geometry acquisition (Salomé et al., 1999; Weitkamp et al., 2010).

A full set of 2D radiographic images were recorded using a CDD detector (Gadox scintillator, optic lenses, 2048 \times 2048 Frelon Camera) by rotating the sample in 1999 steps within a 360° range of rotation. The detector system was fixed to get a pixel size of 6.5 μ m in the recorded images in which a region of interest of 1400 \times 940 was selected to fit the sample. The aim was to assess bone mineral density and the vascular network of the microarchitecture of cortical bone. Then, the bone images were binarized by selecting a single threshold to assess bone porosity.

The beam energy was tuned to 26 keV. Due to the limited beamline time at the ESRF facilities, half of the specimens were scanned in monochromatic configuration by using a (Si111) double crystal monochromator (total scan time \sim 55 min/specimen) and the rest were scanned in pink beam configuration (total scan time \sim 15 min/specimen). The total dose received by the specimen was estimated to be 0.5 kGy for monochromatic scanning and 2.5 kGy for pink scanning.

2.5. Experimental protocol

The experimental protocol is depicted in Fig. 1 and includes: (1) RUS measurements of the elastic coefficients (C_{ij}^N) on the 24 native specimens right after sample preparation; (2) the same set of specimens

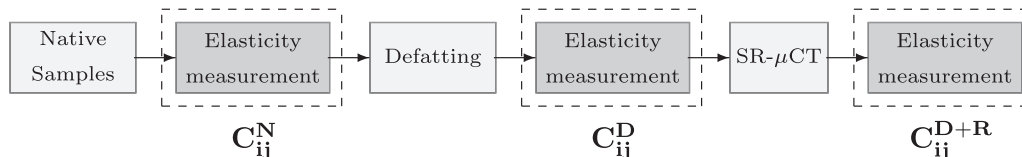


Fig. 1. Experimental protocol.

were defatted as detailed in Section 2.3; (3) RUS measurements of elastic coefficients on the defatted specimens (C_{ij}^D); (4) SR- μ CT imaging on the defatted specimens as described in Section 2.4; and (5) RUS measurements of the elastic coefficients on the defatted and irradiated specimens (C_{ij}^{D+R}).

RUS measurements were conducted on fully hydrated bone specimens at room temperature (monitored between 20 and 24 °C). In the range of the monitored temperature, the variations of the elastic coefficients measured by RUS are expected to be small. The mass density ρ (mg/mm³) was deduced from the measured mass (precision \pm 0.1 mg) and dimensions (precision \pm 0.01 mm).

2.6. Statistics

Data was analyzed to reveal possible differences of elastic coefficients in the three measurement states: native (N), defatted (D), and after defatting and SR- μ CT imaging (D+R). Elastic coefficients were analyzed using a mixed linear model with state (N, D, D+R) as the fixed effect and specimen as the random effect. Homoscedascity was checked and variance components were extracted to evaluate inter-specimen and inter-state variability as well as possible interactions. At last Bland-Altman plots were used to report any possible bias (Bland and Altman, 1986).

Statistical analysis were carried out using Matlab Statistics Toolbox Release 2014a (The MathWorks Inc., Natick, Massachusetts, United States) and JMP[®] Pro, Version 12 (SAS Institute Inc., Cary, NC, 1989-2007). All results are expressed as mean \pm standard deviation. Data were considered statistically significant for $p < 0.05$.

3. Results

Mass density of the specimens ranged from 1.75 mg/mm³ to 1.98 mg/mm³. Elasticity was successfully assessed by RUS in all groups. An average of 23 resonant frequencies were obtained from the measured spectra of each specimen. The mean error between predicted and measured frequencies was 0.5%.

Mean and standard deviation of the measured elastic coefficients are given in Table 1. Multiple comparison of means did not show significant difference between the different states either for any of the elastic coefficients. We may observe that variance in elastic coefficients are mostly explained by the inter-specimen variability (Table 2). In addition, the Young's modulus and Poisson ratios of the different states also show no significant difference.

The degree of agreement between native and defatted specimens and between native and irradiated specimens is also described by the Bland-Altman plots. Fig. 2 shows the corresponding Bland-Altman plots for the coefficient C_{33} . There was no bias and the mean and standard deviation of differences appeared to be constant throughout the range of elasticity values. Similar results were obtained for the rest of the elastic coefficients (results not shown).

Table 1

Elastic coefficients (in GPa) and mass density (in mg/mm³) measured on native, defatted and irradiated specimens and their respective analysis.

	N Mean \pm SD	D Mean \pm SD	D+R Mean \pm SD
C_{11}	19.88 \pm 1.94	19.77 \pm 1.79	19.81 \pm 1.90
C_{33}	28.89 \pm 1.85	28.92 \pm 2.16	28.70 \pm 2.08
C_{13}	11.96 \pm 0.96	12.04 \pm 0.87	11.96 \pm 0.95
C_{44}	5.83 \pm 0.57	5.84 \pm 0.61	5.81 \pm 0.60
C_{66}	4.41 \pm 0.63	4.40 \pm 0.62	4.42 \pm 0.64
ρ	1.885 \pm 0.090	1.893 \pm 0.064	1.895 \pm 0.069

Table 2

Proportion of variance of the elastic coefficients explained by each component (state, specimen and interaction). For all the elastic coefficients, the mixed linear model shows a significant influence of specimen ($^{**}p < 10^{-4}$), and no significant effect of state and interaction.

	Variance components		
	State	Specimen	Interaction
C_{11}	0.06%	98.77%**	1.17%
C_{33}	0.24%	97.15%**	2.61%
C_{13}	0.17%	94.51%**	5.32%
C_{44}	0.05%	99.35%**	0.60%
C_{66}	0.01%	99.40%**	0.59%

4. Discussion

Linking the bone mineral density and micro-architecture assessed by SR- μ CT with bone mechanical properties is a necessary step to understand structure-function relationships in bone. When the mechanical test is destructive, imaging is preferentially conducted first. Comparing the quantitative results of different studies using different conservation (sterilization) and irradiating imaging protocols necessitates a documentation of the possible effects of the steps of the protocol on mechanical properties.

In this study the entire set of elastic coefficients of 24 cortical bone samples were measured by RUS at the three different states: native, defatted and irradiated. Our results show that the used defatting protocol and irradiation dose have no detectable effect on any of the elastic coefficients of cortical bone measured by RUS.

Finding a general answer to the influence of the chemical defatting methods on bone elastic properties is difficult because different chemical products and bones have been used by different authors. The most used chemical products in bone fixation include formalin and alcohol (ethanol or methanol). There is a lack of consensus about the effects on the mechanical properties (Cowin et al., 2001). Fixative solutions like formalin was reported to make bone tissue stiffer (Burkhart et al., 2010), or less stiff (Öhman, 2008), or to have no effect on elastic properties (van Haaren et al., 2008; Wieding et al., 2015). Fixation using ethanol (Linde and Sørensen, 1993) or alcohol-glycerine (Stefan et al., 2010) was found to have no significant effect on trabecular and cortical bone elastic properties. It has also been noted that elephant dentin samples fixed with methanol, compared to ethanol, have a closer mechanical behavior to the unfixed samples and as revealed by ultraviolet Raman spectroscopy, the chemical compositions spectra of the methanol-fixed ones are closer to the unfixed ones (Nalla et al., 2005).

In this study, we investigated the influence of a simple defatting procedure using diethylether and methanol, that has been used for several years in our laboratory. In our experience, the samples processed following this protocol could be stored at room temperature for a long time without damage. While we did not check the fat content after defatting, we believe the procedure is sufficient to remove the lipid from the samples. Our results show that our defatting protocol does not alter anisotropic elasticity of cortical bone measured in a hydrated state.

In X-ray synchrotron tomography experiments on bone, the radiation doses can vary a lot depending on the synchrotron source and the experimental imaging conditions selected on the beamline (insertion device, energy bandwidth, exposure time, spatial resolution and scintillator...). The effect that X-ray synchrotron-source irradiation has on the structure and mechanical properties of human cortical bone has been previously studied at a wide range of irradiation doses, from 0.05 to 630 kGy (Barth et al., 2010, 2011). It was found irradiation doses higher than the called safe threshold dosage for bone allografts (~25–35 kGy), can have a deleterious impact on the post-yield

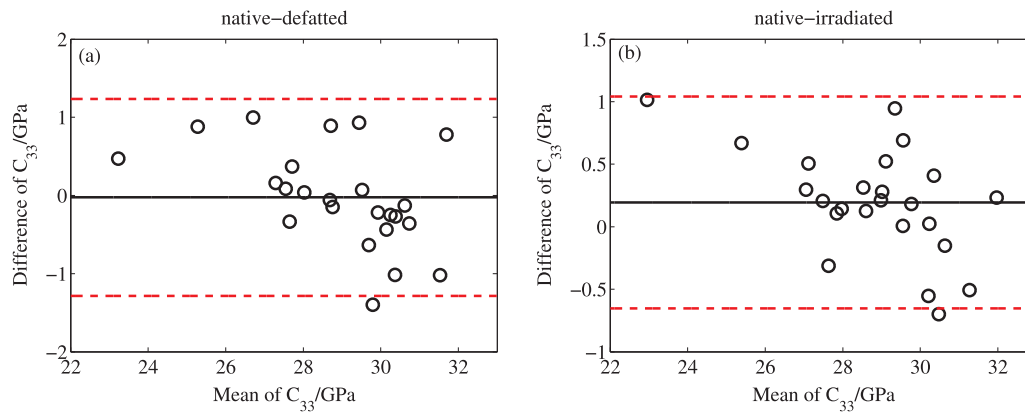


Fig. 2. Bland-Altman of C_{33} measured on (a) native and defatted specimens, (b) native and irradiated specimens.

behavior, such as bending strength and toughness and after exposure to an irradiation dose of 210 kGy the fracture toughness can be reduced by five times (Barth et al., 2011). However, the Young's modulus measured in compression/tension or three-point bending, does not seem to be significantly affected. In our SR- μ CT experiments, the irradiation dose received for imaging bone porous network was estimated to be 0.5 kGy for monochromatic scanning and 2.5 kGy for pink scanning, which is in the lower range of the doses investigated by Barth et al. (2010, 2011). Consistent with their results, we found no significant effect of the irradiation on the Young's modulus, nor on the other elastic constants.

The main constituents of bone material, i.e., water, organics (mainly collagen) and mineral, determine the mechanical properties of bone. Any physical and/or chemical damage to these constituents are likely to be reflected by the alteration of the mechanical properties. It was reported that damaged collagen is associated to a degradation of toughness and strength (Currey, 2003). Also, the high-cycle fatigue life of human bone was found to decrease by more than 15-fold after Gamma radiation sterilization (dosage between 25 and 27 kGy) which was attributed to the damage to the collagen matrix (Islam et al., 2016). Indeed, Barth et al. (2011) found that, after X-ray irradiation, the collagen environment (Amide I-III) is significantly modified after enduring 70 kGy of radiation which was associated with an increased incidence of specific cross-linking caused by the loss of bounded water molecules. However, the irradiation dose smaller than 70 kGy is unlikely to have much effect on mineral (Currey, 2003). As is evident from the elastic moduli of collagen (~ 1.5 GPa) and hydroxyapatite (~ 114 GPa) (Ziopoulos et al., 1999), some have suggested that collagen may not be a critical contributor to bone stiffness (Burr, 2002). In the present study, the mineral content of the samples are unlikely changed during SR- μ CT scanning or defatting which may explain why bone elasticity measured by RUS has not been modified.

5. Conclusion

We have shown that cortical bone elastic properties are not significantly affected by the defatting protocol and a X-ray synchrotron radiation dose of less than 2.5 kGy used for imaging bone vascular pores and quantifying mineral content at $6.5 \mu\text{m}$ voxel size. These findings complete previous results limited to the effects on elasticity in one direction, i.e. one Young's modulus, and also confirm that the defatting protocol and SR- μ CT imaging can be performed before measuring bone elastic properties, if necessary.

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