Acta Biomaterialia xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Multimodal correlative investigation of the interplaying micro-architecture, chemical composition and mechanical properties of human cortical bone tissue reveals predominant role of fibrillar organization in determining microelastic tissue properties

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ARTICLE INFO

Article history:
Received 16 May 2016
Received in revised form 3 July 2016
Accepted 2 August 2016
Available online xxxx

Keywords:
Lamellar bone
Collagen fibril orientation
Chemical composition
Mass density
Elastic stiffness

ABSTRACT

The mechanical competence of bone is crucially determined by its material composition and structural design. To investigate the interaction of the complex hierarchical architecture, the chemical composition and the resulting elastic properties of healthy femoral bone at the level of single bone lamellae and entire structural units, we combined polarized Raman spectroscopy (PRS), scanning acoustic microscopy (SAM) and synchrotron X-ray phase contrast nano tomography (SR-nanoCT). In line with earlier studies, mutual correlation analysis strongly suggested that the characteristic elastic modulations of bone lamellae within single units are the result of the twisting fibrillar orientation, rather than compositional variations, modulations of the mineral particle maturity, or mass density deviations. Furthermore, we show that predominant fibril orientations in entire tissue units can be rapidly assessed from Raman parameter maps. Coexisting twisted and oscillating fibril patterns were observed in all investigated tissue domains. Ultimately, our findings demonstrate in particular the potential of combined PRS and SAM measurements in providing multi-scalar analysis of correlated fundamental tissue properties. In future studies, the presented approach can be applied for non-destructive investigation of small pathologic samples from bone biopsies and a broad range of biological materials and tissues.

Statement of Significance

Bone is a complex structured composite material consisting of collagen fibrils and mineral particles. Various studies have shown that not only composition, maturation, and packing of its components, but also their structural arrangement determine the mechanical performance of the tissue. However, prominent methodologies are usually not able to concurrently describe these factors on the micron scale and complementary tissue characterization remains challenging. In this study we combine X-ray nanoCT, polarized Raman imaging and scanning acoustic microscopy and propose a protocol for fast and easy assessment of predominant fibril orientations in bone. Based on our site-matched analysis of cortical bone, we conclude that the elastic modulations of bone lamellae are mainly determined by the fibril arrangement.

1. Introduction

Bone is a hierarchically organized composite material with remarkable mechanical properties – it combines high stiffness and strength with toughness, while maintaining light weight. The mechanical properties of bone are attributed to a multitude of

Abbreviations: CTPR, carbonate-to-phosphate-ratio; FWHM, full width at half maximum; IT, interstitial tissue; MTMR, mineral-to-matrix-ratio; OT, osteonal tissue; PRS, polarized Raman spectroscopy; qBEI, quantitative backscattered electron imaging; SAM, scanning acoustic microscopy; SR-μCT, synchrotron micro computed tomography; SR-nanoCT, synchrotron nano computed tomography.

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http://dx.doi.org/10.1016/j.actbio.2016.08.001
1742-7061/

material and structural properties. Key factors determining the mechanical competence at the micron scale are the complex three-dimensional (3D) architecture, the chemical composition, the tissue mineralization and mass density, and the size and shape of the mineral particles [1–4]. However, current insight into the complex interplay between material composition, structure and resulting mechanical properties at the micrometer and sub-micrometer level is still limited.

Bone consists of an interwoven framework of collagen fibrils reinforced by mineral particles and embedded in an extrafibrillar mineralized matrix [5,6]. As a result of the continuous remodeling process, human cortical bone is organized in structural units of different maturity levels, young osteons and older interstitial tissue domains. Osteons are cylindrical shaped units, built of periodic lamellae which are concentrically arranged around a central Haversian canal [1]. They are embedded in interstitial tissue domains, remnants of the initially built general lamellae or older secondary osteons that have been partially replaced during the remodeling process. Similar to osteons, interstitial domains are structures of quasi-parallel layered lamellae. However, due to their increased tissue age, interstitial domains exhibit on average a higher degree of mineralization and an increased level of mineral maturity than directly adjacent osteons [6–8].

The lamellar appearance of cortical bone is attributed to the complex multilayer architecture of mineralized collagen fibrils. The majority of previous studies investigating the lamellar structure describe the lamella as a staggered array of sublayers, each consisting of unidirectional aligned fibrils. The fibrils of each sublayer have a distinct angle of twist φ in the lamellar plane and with respect to the osteonal axis x_3 (Fig. 1) [9–11]. Many recent studies confirmed the existence of the twisted plywood pattern, first described by Giraud-Guille [9,12], as the predominant organizational motif, which is characterized by a smooth, continuous, and full fibril rotation in the lamellar plane. The concept was further developed by (i) the proposition of an asymmetrical rotated plywood structure with specific angles of precession between lamellar sublayers and individual sublayer thickness values [10,13], and (ii) by the suggestion of a helicoidal plywood arrangement with spiraling fibril orientation [11]. Recent findings described (iii) oscillating fibril patterns with twist angles φ smoothly oscillating around the osteon axis x_3 with a distinct maximum angular deflection [14]. Furthermore, the coexistence of oscillating, asymmetrical oscillating and twisted plywood patterns within the same osteon was demonstrated [14,15]. In addition, (iv) the existence of disordered sublayers with partial randomly oriented fibrils and decreased packing density was observed [14–16].

Due to their transverse isotropic mechanical properties, the orientation of the collagen fibrils is an important factor regarding the micro-mechanical properties of bone tissue. So far, the micro-mechanical elastic properties of lamellar bone have been investigated by means of nanoindentation experiments [17–19] and scanning acoustic microscopy (SAM) [20,4,3]. Both, nanoindentation and SAM measurements revealed periodically oscillating modulations of the measured elastic properties (i.e. indentation modulus and acoustic impedance for nanoindentation and SAM, respectively) along the radial direction of the osteon.

In order to explain the observed micro-mechanical undulations, several approaches combined compositional and mechanical analysis methods and attributed the undulating lamellar elastic properties (i) to variations of the tissue composition and/or (ii) to changes of the fibrillar orientation. Experiments using quantitative backscattered electron imaging (qBEI) and nanoindentation [19] suggested locally varying mineral contents in osteonal lamellae and concluded that compositional variations may contribute to the oscillating lamellar mechanical properties, as well. Similarly, intralamellar variations of the mineralization were observed in a study based on backscattered electron microscopy [21]. Contrary to these propositions, experimental evidence on the homogeneity of the lamellar composition was presented by means of polarized Raman spectroscopy (PRS) [4,22,23] and synchrotron X-ray phase nano tomography (SR-nanoCT) [14,24]. Recently, a combined approach using SAM, small angle X-ray scattering and synchrotron X-ray micro tomography [3] concluded a predominant determination of the modulated apparent elastic properties by variations of the fiber orientation in single osteons and to a lesser extent by variations of the mineral particle size and density. However, these findings were in contrast to the results of another study based on combined qBEI, nanoindentation and quantitative polarized light microscopy measurements [25]. Interestingly, in their approach, no substantial correlation of indentation moduli with mineralization and fiber orientation was found.

The variety of different proposals on the link between micro-mechanical properties, structural fiber arrangement and chemical composition underlines the difficulty to analyze the structure-function relationship of lamellar bone tissue at the micron scale. To shed light on this intricate relationship, we propose a multimodal approach, combining for the first time PRS, SAM and synchrotron X-ray phase contrast micro tomography (SR-μCT) and SR-nanoCT in serial measurements. As a result of our site-matched analysis with these high-resolution and non-destructive techniques we directly correlate organizational, compositional and mechanical properties in regions of different tissue age and at two hierarchical levels of bone structure. Furthermore, we present a novel method using a histogram related kernel density estimation to analyze predominant fibril orientations and corresponding value distributions of elastic properties of entire tissue domains. Finally, a validation of the fibril orientation information assessed by means of SR-nanoCT and PRS is presented.

2. Materials and methods

2.1. Sample preparation and measuring protocol

Four human femoral cortical bone samples from four donors (Table 1) were obtained from the Centre of Anatomy and Cell Biology, Medical University Vienna. Ethical approval for the collection of the samples was granted by the Human Ethics Committee of the Medical University of Vienna. The tissue donors or their legal guardians provided informed written consent to donate their tissue for research purposes. In a previous study, areal bone mineral density (aBMD) was measured by means of Dual-energy X-ray Absorptiometry (DXA) on the entire proximal femur [26]. A 10 mm thick cross section of each femur shaft was cut perpendicular to the bone long axis at approximately 1/3 of the total bone length (Fig. 1a) using a diamond-coated band saw (Exakt 300, EXAKT Advanced technologies GmbH, Norderstedt, Germany). After dissection, the bone specimens were kept frozen at −20 °C until further processing. Cylindrical samples with a diameter of approximately 500 μm and a length of 5 mm (Fig. 1b) were fabricated from the anterior-medial location of the cross section by means of a high-precision lathe and stored in 70% ethanol.

The serial measuring protocol is schematically illustrated in Fig. 1c. First, low dose SR-μCT images were acquired to provide a 3D overview of the entire sample volume. The overview scans were used to identify volumes of interest which were then scanned at nanometer resolution by means of SR-nanoCT. These SR-CT images refer to the same set of measurements presented in the study by Varga et al. [27]. Subsequently, samples were fixed and dehydrated in a graded series of alcohol (70%, 96% and 100% ethanol, immersion for 24 h in each solution) and embedded in polymethylmethacrylate (PMMA). Next, the samples were carefully ground

Please cite this article in press as: S. Schrof et al., Multimodal correlative investigation of the interplaying micro-architecture, chemical composition and mechanical properties of human cortical bone tissue reveals predominant role of fibrillar organization in determining microelastic tissue properties, Acta Biomater. (2016), http://dx.doi.org/10.1016/j.actbio.2016.08.001