MULTISCALE CHARACTERIZATION OF BONE (CARACTÉRISATIONS MULTIÉ-CHELLES DES TISSUS OSSEUX, DE L'IN VITRO À L'IN VIVO)

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1. INTRODUCTION

Bone is a porous mineralized tissue with a hierarchical organization. Bone mechanical properties are primarily investigated for a medical purpose. Also, from a material science point of view, bone is an interesting material because it is an optimal assembly of mineral and proteins able to bear heavy loads and resist moderate impacts (Fratzl and Weinkamer, 2007).

A healthy skeleton is the condition for normal locomotion and for the accomplishment of the motor tasks of daily living. Skeleton may be impaired as a consequence of trauma (fall, impact) or diseases such as osteoporosis which affect the mechanical competence of bone. Osteoporosis is a systemic disease characterized by a compromised bone strength and an increased risk of fracture. The social and economical burden of osteoporotic fractures is important. In 2010, in the 5 largest European countries, it was estimated that 21% of the women aged between 50 and 84 years old were osteoporotic, with an associated cost (fracture management and consequences, treatments) of about 30 billions of Euros. Furthermore, with the aging of the population, the number of osteoporotic cases is expected to increase in the next decades. Preventing the occurrence of fracture is thus an important medical and scientific challenge. Some medical treatments are available to slow down or stop bone mechanical alteration. However, diagnosing the subject who could best benefit of treatment is an issue. Also, new drug treatments are continuously developed to address the various forms of osteoporosis and the diversity of patients' responses to treatments. Also, millions of orthopedic surgeries are conducted each year worldwide, including more than a million of total hip replacement. The purpose of orthopedic surgery is to restore the function of an impaired bone, often by inserting a prosthesis or metallic pieces for internal fracture fixation. Bone research contributes to the continuous improvement of surgical procedures.

Both for the management of bone diseases and orthopedic research, it is necessary to characterize the mechanical behavior of bone at different scales. Indeed, a fracture of the proximal extremity of the femur (so-called « hip fracture ») is manifest at the scale of the organ but involves many hierarchical level : the microscopic damage, e.g. delamination of collagen-mineral arrays, develops into a crack propagating along a complex path influenced by the microstructure to finally form a macroscopic crack. The insertion of a prosthesis, for instance an uncemented hip implant anchored by press-fitting, modifies the stress-strain distribution in the bone, leading to remodeling of the bone surfaces which process involves several scales of the bone material.

Because bone mineral density alone (which can be assessed in vivo with X-rays) cannot accurately predict fracture risk, nor explain the therapeutic benefits of anti-resorptive agents in treating osteoporosis, there is a necessity of understanding how factors other than bone mineral density control fracture. Hence the large body of research aiming at elucidating those mechanical properties that might affect fracture, like elastic modulus, strength, and toughness. It is only recently that these properties have begun to be addressed quantitatively with regard to the microstructure of the tissue.

This paper is a short review of the main in vitro and in vivo techniques used for the mechanical characterization of bone. Characterization techniques are essentially used in one of the two following perspectives; (1) the identification of material behavior laws which could typically be used in predictive finite element models in the context of the prediction of the strength of a bone

(e.g., diagnosis of osteoporosis) or in the context of the prediction of the stresses and remodeling activity around an implant (orthopedic application) ; (2) the measurement of so-called *biomarkers* for diagnosis of bone health, i.e., the measurement of some physical quantities which are related to bone mechanical competence and to the status of a bone pathology. Because bone, as we shall see, is an organized material at several scales, characterization techniques have been developed for scales from a few hundreds of nanometers (nm) to the centimeter (cm) scale.

The paper is organized as follows : Section 2 is devoted to a description of bone anatomy at the different scales. Sections 3 to 6 describe the techniques for the in vitro mechanical characterization of bone at the so-called ultrastructural level (< 10 μm), microstructural level (~100 μm), mesocale (~ 1 mm), and macroscale (whole bone). Section 7 is devoted to the imaging of the porosities of bone which are found at different hierarchical levels. Section 8 presents the main measurement techniques available to assess in vivo the mechanical competence of bones.

2. BONE ANATOMY

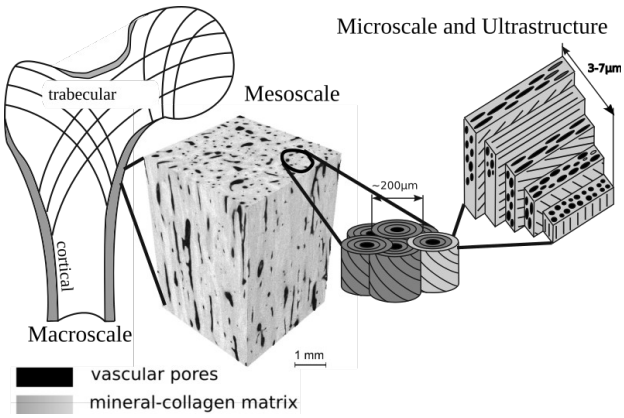

Illustration 1: Hierarchical cortical bone structure. After Rho et al, 1998 Med Eng. Phys 20:92 and Reisinger et al 2010 Biomech Model Mechanobiol 9:499.

This section gives a brief overview of bone anatomy. Details may be found in the books of Cowin (2001), Currey (2002), and Martin et al (1998). Bone is essentially a biphasic medium, that is, a solid phase (mineralized tissue) and pores. However, the solid phase has a complex hierarchical microstructure that can be considered at several dimensional scales (Rho et al 1998) and there are several levels of porosities. Here, bone tissue is described resorting to four scales (ultrastructure, microstructure, mesoscale and macroscale). However other authors may choose a different partitioning.

Ultrastructural level. The basic building bloc is a compound of type-I mineralized collagen fibers (up to 15 nm in length, 50–70 nm in diameter) bound and impregnated with mineral (apatite nanocrystals) measuring tens of nm in length and width, and 2–3 nm in thickness. These mineralized fibers are organized at a micrometer length-scale into a lamellar structure with various orientations in adjacent lamellas (3–7 micrometer thick) (Giraud-Guille 1988, Weiner et al 1999, Reznikov et al 2014).

Microstructural level. The lamellar structure is the basic material of cortical bone (dense tissue, 80 % of skeleton mass) and trabecular bone (spongious tissue). Cortical bone forms the envelope (cortex) of bones and trabecular is mostly a filling material. The primary focus of this papers is cortical bone; nevertheless, both the cortical and trabecular bone have a significant contribution the mechanical function of bone. The lamellar structure is arranged in cylindrical or hemi-cylindrical structures called osteons or Haversian systems (200–300 μm diameter) oriented roughly along the long axis of the bone shaft in cortical bone. Osteons are surrounded by a thin “cement line” from the surrounding tissue. One osteon is the product of a remodeling cycle, hence mature bone tissue is a compound of entire osteons of different ages and so-called “interstitial tissue” (old partial osteons) filling the space between entire osteons. Importantly, the degree of mineralization increases with tissue age, causing a certain level of heterogeneity of material properties at the microstructural level. A network of pores permeates the organized lamellar tissue. The vascular pores are found in the osteons (Haversian channels on the osteon axis, diameter ~50 μm) and perpendicular to them

(Volkman channels). Smaller pores are lacunae which contain the body of osteocytes (bone cell buried in the tissue, ellipsoidal cavities ~10 μm) and canaliculi which contain the cell processes. Lafage-Proust et al (2015) have recently reviewed the role of vascularization in bone and its relationship to porosities.

Mesoscale level. This is the scale of a representative volume element (RVE) of bone material, at the scale just below the organ scale. In trabecular bone, a mesoscale RVE contains a sufficient number of trabeculae (Pahr and Zysset, 2008), and in cortical bone, a sufficient number of osteons (Grimal et al 2011). For cortical bone, the mesoscale characteristic size is about 1 mm and mesoscale properties vary accross the organ due varying levels of mineralization and, to a larger extent, variations of porosity (Rohrbach et al 2015).

Macroscale. This is the scale of the whole bone.

3. SOME CHALLENGES FOR BONE MECHANICAL CHARACTERIZATION

At all scales, bone is an anisotropic and viscoelastic material. Although the full anisotropic tensor of bone has never been assessed at any scale, it seems that, at most scales, the assumption of an orthotropic material is reasonable. Due to viscoelasticity (Garner et al 2000, Lakes 2001), the mechanical properties are a priori rate-dependent at all scales. The viscoelastic properties are also anisotropic (Iyo et al 2004 ; Bernard et al 2015). Because bone contains defects such as pores and cracks at several scales, the elastic properties assessed for a given scale may depend on the sample size (Choi et al 1990).

Bone is also an hydrated material, accordingly assessed mechanical properties are different in hydrated and dry measurement conditions. This is an issue for certain techniques, such as those requiring a scanning electron microscope necessitating vacuum.

Bone from animals is often considered as a surrogate of human bone. However, the bone microstructure differs between large and small mammals (mice, rats, rabbits). Also one characteristic of bone from human adults is that it is the result of a large number of remodeling cycles, which is not the case for most animals who are killed rather young (bovine and ovine bone). While pilot studies may be conducted on animal bone, it is important to document specifically human bone for clinical relevance.

Finally, for the assessment of mesoscale properties (the bulk homogenized material constituting the compartments of the whole bone), scientists must cope with specimens of relatively small size (a few millimeters for human bones). The problem of retrieving bone properties from three-point bending of whole small animal bones as recently been pointed out by Wallace et al (2014).

4. MECHANICAL CHARACTERIZATION OF THE ULTRASTRUCTURE AND THE MICROSTRUCTURE

Nanoindentation (NI) has been widely used to assess ultrastructural properties, that is, the properties of the arrays of collagen-mineral compound building the lamellae. Pioneering works are those of Rho et al (1997) and of the group of Ph. Zysset (Zysset 1999 , Hengsberger 2001). The problem of measuring an anisotropic material with NI has also been addressed (Swadener et al 2001 ; Franzoso and Zysset 2009) in order to estimate the stiffness tensor of ultrastructural bone. The depths of indentations in bones is typically 0.5-1 μm. The indentations are load-controlled with maximum loads of about 10 mN. The order of magnitude of the indentation elastic modulus is 20 GPa.

Scanning acoustic microscopy (SAM) has also been used with frequencies between 50 MHz and 1 GHz to measure stiffness. Pioneering works are by Katz and collaborators (Meunier et al 1988) and Turner and collaborators (Hasegawa et al 1995). Most recent contributions are by the group of Raum and the group of Laugier (Raum 2008 ; Granke 2013 ; Saied 2008).

Overall, NI and SAM studies indicate that the ultrastructure of the bone tissue differs substantially among lamellar types (different patterns of relative angles of adjacent lamellae in osteons), anatomical sites and individuals. NI and SAM reveal tissue heterogeneity that is of potential importance in bone fragility and remodeling.

Recently, a variant on nanoindentation, called micropillar compression, has been used to measure bone ultrastructural properties (Schwiedrzik et al 2014). The technique uses a nanoindenter with a flat punch to compress a small cylindrical volume (1 mm diameter by 2 mm length cylinders) to obtain an uniaxial stress-strain behavior. Micropillars are machined by millingusing focused ion beam (FIB). Compared with the micro-indentation test, the interpretation of micro-compression is more straightforward because the stress/strain field is relatively uniform in the micropillar. Micropillar tests seems to be a promising technique to identify ultrastructural post-yield properties. Schwiedrzik et al (2014) found that isolated lamellae exhibit a plastic behavior, with higher yield stress and ductility than at the mesoscale.

The behavior of isolated osteons has been investigated essentially in A Ascenzi's works starting in the 1960' (Ascenzi and Boucci 1964).

The microstructure and heterogeneity of the mineralized tissue in cortical bone implies a complex parttern of stress/strain distribution in the tissue. Strain field heterogeneity has been investigated by two-dimensional digital image correlation techniques (Nicolella 2001, 2006 ; Hoc et al 2006, Granke 2012).

5. MECHANICAL CHARACTERIZATION OF THE MESOSCALE

All standard mechanical testing methods have been used to measure mesoscale properties on specimens of characteristic dimensions of a few millimeters : torsion, bending, traction and compression (Cowin 2001).

As regards elastic properties, ultrasound have also been a method of choice to retrieve anisotropic properties since the work of Lang (1969). Typically, the time-of-flight of longitudinal or shear waves is measured in the different directions of a rectangular parallelepiped specimen, from which the coefficients of the (orthotropic) stiffness tensor are deduced.. A large amount of the data on bone elastic anisotropy was obtained with such methods (Yoon and Katz 1976, Ashman and Rho 1988, Pithioux et al 2002, Espinoza Orías 2009, Grimal et al 2009, Granke et al 2011, Lefèvre et al 2015). Recently, resonant ultrasound spectroscopy (RUS) was developed to measure bone (Bernard et al 2013, 2015). The technique is in principle more precise and less limited by the small sample size than the time-of-flight method. RUS can also be used to measure viscoelastic constants.

Anisotropy ratios of cortical bone range between 1.2 and 2.5 depending on the composition and porosity of the specimen. Large relative variations of the tissue elastic moduli, typically of the order of 100%, are observed across anatomical locations and between subjects due to variations in density (porosity, mineral content) and preferential orientation of collagen fibers.

A remarkable property of cortical bone is that the yield strength in compression (~ 200 Mpa in the direction of the bone axis) is larger than that in traction (~ 110 MPa in the direction of the bone axis) (Reilly and Burstein 1975).

As regards post-yield properties, most authors describe a quasi-brittle behavior, i.e., with a small plastic region. Introduction to the subject may be found in Cowin (2001), and Gupta and Zioupos (2008). Identification of constitutive laws is still an open subject. Recent papers on the subject for cortical bone are Nyman et al (2009), Li et al (2013), Schwiedrzik et al (2014), and Mirzaali et al (2015). For the identification of constitutive laws in trabecular bone, recent relevant papers are Bayraktar et al (2004), Cowin and He (2005), Rincón-Kohli and Zysset (2009), Wolfram et al (2012), Ridha and Thurner (2013), and Schwiedrzik et al (2013).

Note also that a large body of the current work on bone mechanics is devoted to understanding and quantifying the fracture process through the measurement of toughness (Zioupos and Currey 1998; Peterlik et al 2006; Nalla et al 2003, 2005; Granke et al 2015).

6. MACROSCOPIC MECHANICAL CHARACTERIZATION

Mechanical testing of whole bones (typically, femurs, vertebrae, tibia and radius) have essentially been performed with the objective to determine critical loads leading to macroscopic fracture (see, e.g., for the femur, Juszczyk et al (2001) and Bousson et al (2006)) and elucidate the structural and material determinants of whole bone strength. Some studies were devoted to the validation of numerical models used for in sillico prediction of fracture strength based on clinical imaging data (Zysset et al 2013). See, e.g., for the femur Viceconti et al (2006), Duchemin et al (2008), Schileo et al (2008), Enns-bray et al (2014) and for the radius Varga et al (2010), and Zapata et al (2015). In many studies, the authors tried to replicate boundary conditions mimicking falls or physiological loadings.

7. BONE POROSITIES (IN VITRO ASSESSMENT)

The cavities in bone at the different hierarchical levels are nowadays best investigated in three-dimensions with X-ray computed tomography (CT). The terms μCT and SR-μCT usually refer to micro-CT and synchrotron radiation micro-CT. The latter is the gold standard for the measurement of porosity (Bousson et al 2004; Cooper et al 2007).

The highest level of porosity, sometimes referred to as vascular porosity (Haversian and Volkman channels) is now conveniently measured with desktop (conventional) μCT (see i.e., Jorgenson 2015). Images of cell lacunae and canaliculi can be obtained with SR-μCT, although the latter is still challenging (Mader et al 2013; Dong et al 2014). Note that nuclear magnetic resonance can in principle distinguish porosity (free water) from the water bound to the collagen-mineral compound (Granke et al 2015b). Two-dimensional techniques like optical microscopy or scanning acoustic microscopy (SAM) may be used to assess porosity but significant artifacts can be expected due to the three-dimensional nature of the pores.

8. CHARACTERIZATION OF BONE MECHANICAL PROPERTIES FOR DIAGNOSIS

Mechanical properties of bones can be partially recovered in vivo. X-rays are widely used to image bone. Mechanical information can hardly be derived from standard two-dimensional X-ray, primarily because it is not quantitative and the bone is a highly three-dimensional structure. Much of the knowledge on the mechanical behavior of whole bones was derived from Finite element (FE) models based on X-ray Quantitative computed tomography (QCT). QCT is widely used in basic and clinical research to image bone, but is seldom used in clinical practice (Kang et al 2003, Bousson et al 2006, Bouxsein and Seeman 2009). The typical resolution of in vivo QCT is a little below 1 mm. High resolution QCT exists for peripheral sites (forearm, leg) where a resolution of about 80 μm can be achieved which allows the imaging of large vascular pores (Jorgenson et al 2015). FE models of whole bones based on QCT may be used for the evaluation of fracture risk (Viceconti 2006. Keaveny et al 2008). A key issue for the constructions of such models is the translation of X-ray gray values (apparent mineral density) into elastic coefficients. (Duchemin et al 2007 ; Helgason et al 2008). In the whole bone FE models, bone tissue is most often considered to be isotropic, with constant Poisson's ratio, for practical reasons.

In vivo techniques using ultrasound are attractive because they are intrinsically sensitive to stiffness (Laugier 2008). Recent developments have focused on cortical bone at the radius or tibia (Talmant et al 2009, Moilanen et al 2013, Foiret et al 2014, Egorov et al 2014) and the rib (Mitton et al 2014), which can be conveniently be probed by guided elastoacoustic waves. When combined with a model describing the local bone geometry, typically a plate, ultrasound can yield the speed of sound of bulk waves (Minonzio et al 2010).

The reference point indentation (RPI) technique has been recently developed to assess locally the apparent stiffness and post-yield properties of bone in vivo (Diez-Perez et al 2010, Granke et al

2015a). This technique performs an indentation probing bone properties at the scale of about 100 μm. The technique can be used in vivo after local anesthesia. One major interest of the technique is to measure mechanical quantities which might be strongly related to the propensity of cracks to propagate.

9. CONCLUSIONS

Although a large body of literature exists on the mechanical characterization of bone, several challenges remain to be addressed: (1) There is no well accepted constitutive law(s) for bone material accounting for anisotropic elastic and post-yield behaviors. Such laws may incorporate the effects of damage, strain rate, loading mode and loading history. Ideally, the constitutive laws would be parametrized (e.g., for porosity, pre-existing damage, etc.) such that they can be made specific to each patient. (2) Because bone properties vary with anatomical site, subject age, pathology, etc. there is a wide need for in vitro sample characterization in order to achieve a broad picture of bone properties and define mechanical biomarkers which can be probed in vivo. However, mechanical characterization of bone is often performed on small numbers of samples due to the complexity of sample preparation and of measurement techniques. (3) The variability of published mechanical properties measured for bone is in part due to the measurement and sample preparation protocols. Standardized measurement protocols are emerging but are not the rule.

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