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Precision of nanoindentation protocols for measurement of viscoelasticity in cortical and trabecular bone

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ABSTRACT

Nanoindentation has recently gained attention as a characterization technique for mechanical properties of biological tissues, such as bone, on the sub-micron level. However, optimal methods to characterize viscoelastic properties of bones are yet to be established. This study aimed to compare the time-dependent viscoelastic properties of bone tissue obtained with different nanoindentation methods. Bovine cortical and trabecular bone samples ($n=8$) from the distal femur and proximal tibia were dehydrated, embedded and polished. The material properties determined using nanoindentation were hardness and reduced modulus, as well as time-dependent parameters based on creep, loading-rate, dissipated energy and semi-dynamic testing under load control. Each loading protocol was repeated 160 times and the reproducibility was assessed based on the coefficient of variation (CV). Additionally, three well-characterized polymers were tested and CV values were calculated for reference.

The employed methods were able to characterize time-dependent viscoelastic properties of bone. However, their reproducibility varied highly (CV 9–40%). The creep constant increased with increasing dwell time. The reproducibility was best with a 30 s creep period (CV 18%). The dissipated energy was stable after three repeated load cycles, and the reproducibility improved with each cycle (CV 23%). The viscoelastic properties determined with semi-dynamic test increased with increase in frequency. These measurements were most reproducible at high frequencies (CV 9–10%). Our results indicate that several methods are feasible for the determination of viscoelastic properties of bone material. The high frequency semi-dynamic test showed the highest precision within the tested nanoindentation protocols.

1. Introduction

The mineral phase in bone controls its stiffness, whereas the collagen contributes to the ultimate strength and toughness of the bone (Boivin et al., 2008; Wang et al., 2001). Bone becomes more brittle with age (Burr, 2002). Most likely, this is due to decreased ductility, which may be related to viscoelasticity of the collagen fibers (Les et al., 2004, 2005). Hence, the changes in the collagen phase can contribute significantly to increased fragility of bone (Banse et al., 2002; Knott and Bailey, 1998; Wang et al., 2002). Optimal methods should be determined to sensitively evaluate the time-dependent properties of bone, and to assess effectively the effects of experimental bone interventions.

Although nanoindentation is widely used in material science, it is a relatively new tool for testing biological tissues (Lewis and Nyman, 2008; Oyen and Cook, 2009). The obtained pertinent parameters, which have previously been quantified in bone (Hengsberger et al., 2002; Oyen et al., 2008; Rho et al., 1997; Roy et al., 1999; Zysset et al., 1999), include hardness and reduced elastic modulus. In contrast, determination of the viscoelastic properties of bone or mineralized tissues by nanoindentation is less covered. The creep behavior of human enamel (He and Swain, 2009) and bone (Bembey et al., 2006b) and the determination of dissipated energy in cortical bone using repeated loading (Fan and Rho, 2003), are the only subjects investigated so far. Also, the viscoelastic properties of a collagen scaffold were quantified with quasi-static indentation (Chaudhry et al., 2009). The aforementioned studies used different indentation protocols to characterize different physical parameters on various types of tissues. A systematic extensive characterization of bone viscoelastic properties and the reliability and precision of the different methods using nanoindentation have not yet been presented.

Bone is an inhomogeneous, anisotropic and viscoelastic material. Hence, measurements on the nanoscale add new aspects

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to the inter-individual biological variation and require precise measurement protocols. This study aimed to characterize how different nanoindentation methods quantify the mechanical properties of bovine cortical and trabecular bone tissue; specifically, which of the following methods can be used to measure the viscoelastic mechanical properties with the highest precision, i.e. with the lowest intra-method variation: creep, effect of loading-rate, dissipated energy, or semi-dynamic testing.

2. Materials and methods

2.1. Sample preparation

Bovine knee joints were obtained from the local slaughter house (Atria Oyj, Kuopio, Finland). Trabecular bone samples were taken from the proximal tibia and cortical bone samples from the distal femur from 18-month-old animals ($n=8$). From both locations, one 10 mm thick bone disc was cut transversely with a high-speed band saw (Fig. 1a). Cylindrical trabecular bone samples ($\phi=14$ mm) were drilled under PBS irrigation, and cortical bone samples were cut with the band saw from the medial side of the bone disc (Fig. 1b). The samples were dehydrated in a series of alcohol solutions and embedded in polymethylmetacrylate (PMMA). The samples were cut to a final height of 5 mm using a diamond saw and were then polished using silicon carbide paper of decreasing grid size (500, 800, 1000, 1200 and 4000 grid) under deionized water. Each polishing step was performed for 2 min, followed by washing in deionized water to remove debris. The result was evaluated using an optical microscope. The average surface roughness of the measurement grid area was 55 nm.

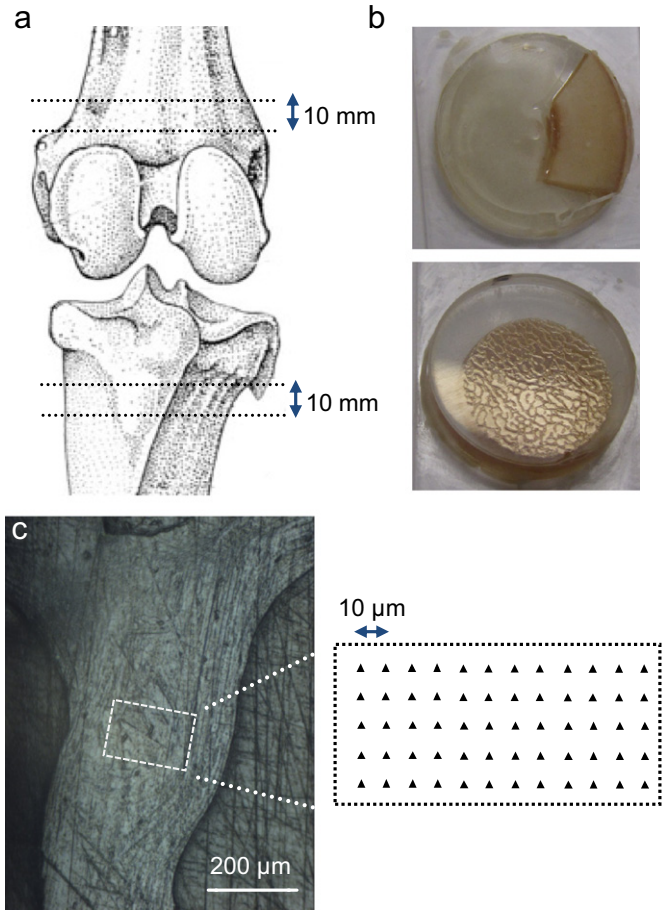

Fig. 1. The samples were harvested from bovine distal femur (cortical) and proximal tibia (trabecular) (a). The samples were cut to 5 mm, glued onto glass plates and polished (b). Nanoindentations were performed in the longitudinal direction of the trabeculae, and in the circumferential direction of the cortical samples. Twelve methods were repeated five times at each location, and at four locations per sample. The distance between each measurement point in the grid was 10 µm (c).

In addition, three reference samples were prepared. Samples of low-density polyethylene (LDPE) and polycarbonate (PC) were cut from 1 mm-thick sheets (Goodfellow Cambridge Ltd., Huntingdon, England) with perfectly smooth surfaces, and PMMA was mixed, cut and polished with the same protocol as the bone samples.

Nanoindentation measurements were performed with a Hysitron TriboIndenter equipped with a cube corner diamond tip (radius 40 nm). High resolution positioning of the tip was achieved using a piezoelectric scanner. Corrections were made for thermal drift of this element. The temperature was controlled (± 0.1 °C), and a custom built damping system was used to minimize vibration. Calibration of the tip area function was conducted according to standard protocols by performing a number of independent indentations on fused silica with different applied forces and fitting the data to 5th order polynomial function (Oliver and Pharr, 1992). The calibrated tip area function had three non-zero coefficients.

Twelve measurement protocols were applied (indentation probing, three creep protocols, three loading-rates, repeated load cycles and semi-dynamic testing with four frequencies). Indentations were performed in a pre-determined grid of 5×12 points (10 µm spacing) at four locations per sample (Fig. 1c). The trabecular bone indentation grids were placed centrally on thick trabeculae (thickness >250 µm) along the main fiber direction to avoid any effects of the embedding media (Mittra et al., 2006). The indentations on the cortical bone were placed on the interstitial lamellar bone.

2.2. Nanoindentation probing

To determine the hardness and reduced modulus of the bone tissue, a load controlled indentation protocol with a maximum load of 100 µN was used. The loading and the unloading rate was 20 µN/s, with a 5 s dwell time. The hardness H and the reduced modulus E_r were calculated from the unloading curve according to Oliver and Pharr (1992). The E_r was related to the modulus of the sample:

$$\frac{1}{E_r}=\frac{(1-\nu_s^2)}{E_s}+\frac{(1-\nu_i^2)}{E_i} \quad (1)$$

where the subscripts s and i refer to the sample and the indenter, respectively, and ν is Poisson's ratio.

2.3. Viscoelastic measurement protocols

2.3.1. Creep

To model the behavior of the viscoelastic solid, a Burger model was applied (Eq. (2)) (Fischer-Cripps, 2004b). According to this model, the penetration depth (h) increases with time:

$$h(t)^2=\frac{\pi}{2}P_0\cot\alpha\left[\frac{1}{E_1}+\frac{1}{E_2}(1-e^{-tE_2/\eta_1})+\frac{1}{\eta_2}t\right] \quad (2)$$

where P_0 is the peak force, α is the equivalent cone semi-angle (42.28° for cube corner indenter), E_1 and E_2 are moduli (GPa), η_2 is the long term creep viscosity (GPas) and η_1/E_2 is the creep time constant (s).

The load–displacement curves were recorded using a loading and unloading rate of 20 µN/s to a maximal load of 100 µN/s. A dwell time of 10, 30 or 60 s was applied (Fig. 2a). E_1, E_2, η_1 and η_2 were determined by minimizing the mean square error between the time–deformation curve and Eq. (2) using the Nelder–Mead-simplex method (Matlab R2006a, Mathworks Inc., USA).

2.3.2. Load-rate dependency

To determine the sensitivity of mechanical parameters to loading rate, indentation tests up to a maximum load of 100 µN were carried out with three different loading rates (10, 50 and 100 µN/s) (Fig. 2b). The area under the force–displacement curve and the reduced modulus during unloading were used as indications of load-rate sensitivity. Additionally, to determine the strain-rate sensitivity for bone, the stress and strain rate at the maximal load was calculated for each indentation. Stress was defined as the ratio between the force and the area at the given depth, and strain rate as the instantaneous displacement rate of the indenter divided by the instantaneous displacement (Fan and Rho, 2003). The stress (i)–strain rate ($\dot{\varepsilon}$) pairs were plotted on a log–log graph to obtain a measure of the strain-rate sensitivity m (the slope of the graph) for each test (Mayo et al., 1990).

2.3.3. Dissipated energy

Repeated loading curves were employed to separate the viscous response from the elastic and plastic deformations (Fan and Rho, 2003). Four loading cycles were applied repeatedly, with loading and unloading rates of 20 µN/s for 4 s and a 1 s dwell period (Fig. 2c). The energy dissipated for irreversible deformation was calculated from each loading cycle by integrating the area bound by the displacement curve. The response appeared mainly viscous when there was no significant change in energy between two consecutive load cycles.