Tissue viscoelasticity is related to tissue composition but may not fully predict the apparent-level viscoelasticity in human trabecular bone - An experimental and finite element study

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Tissue viscoelasticity is related to tissue composition but may not fully predict the apparent-level viscoelasticity in human trabecular bone - an experimental and finite element study


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Running title: Viscoelasticity of trabecular bone

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Abstract

Trabecular bone is viscoelastic under dynamic loading. However, it is unclear how tissue viscoelasticity controls viscoelasticity at the apparent-level. In this study, viscoelasticity of cylindrical human trabecular bone samples (n=11, male, age 18-78 years) from 11 proximal femurs were characterized using dynamic and stress-relaxation testing at the apparent-level and with creep nanoindentation at the tissue-level. In addition, bone tissue elasticity was determined using scanning acoustic microscope (SAM). Tissue composition and collagen crosslinks were assessed using Raman micro-spectroscopy and high performance liquid chromatography (HPLC), respectively. Values of material parameters were obtained from finite element (FE) models by optimizing tissue-level creep and apparent-level stress-relaxation to experimental nanoindentation and unconfined compression testing values, respectively, utilizing the second order Prony series to depict viscoelasticity. FE simulations showed that tissue-level equilibrium elastic modulus ($E_{eq}$) increased with increasing crystallinity ($r=0.730$, $p=0.011$) while at the apparent-level it increased with increasing hydroxylysyl pyridinoline content ($r=0.718$, $p=0.019$). In addition, the normalized shear modulus $g_1$ ($r=-0.780$, $p=0.005$) decreased with increasing collagen ratio (amide III/CH$_2$) at the tissue-level, but increased ($r=0.696$, $p=0.025$) with increasing collagen ratio at the apparent-level. No significant relations were found between the measured or simulated viscoelastic parameters at the tissue- and apparent-levels nor were the parameters related to tissue elasticity determined with SAM. However, only $E_{eq}$, $g_2$ and relaxation time $\tau_1$ from simulated viscoelastic values were statistically different between tissue- and apparent-levels ($p<0.01$). These findings indicate that bone tissue viscoelasticity is affected by tissue composition but may not fully predict the macroscale viscoelasticity in human trabecular bone.
Introduction

Bone tissue consists of organic and inorganic material arranged in highly hierarchical structure. The organic material is mainly type-I collagen while the inorganic material includes essentially hydroxyapatite (HA) crystals and water (McNamara, 2011). Bone exhibits viscoelastic behavior that enables dissipation of energy under mechanical loading (Dong et al., 2004; Isaksson et al., 2010b; Linde et al., 1988; Yamashita et al., 2002). However, repeated loading over time may cause cumulative damage or micro cracks in the bone (Pattin et al., 1996). The tissue-level damages are mostly repaired through the bone’s regenerative capability (Parfitt, 2010) but with age and osteoporosis there is a substantial decrease in the regenerative properties of bone and an increase in fracture risk can be seen (Keaveny et al., 2001; Seeman, 2009). At the tissue level, mechanical properties of trabecular bone, independent of microstructure and porosity, are governed by the ultrastructure of collagen infused crystals, including collagen-crosslinking, degree of mineralization, crystallinity and bound water (Eberhardsteiner et al., 2014; Rho et al., 1998; Sukhodub et al., 2004).

At the apparent-level, stiffness and strength are highly dependent on the bone mineral content, apparent density and microstructure (Hodgskinson and Currey, 1990; Keller, 1994) while the organic matrix controls the bone toughness (Wang et al., 2001). Both the organic and inorganic phases, including water, contribute to the viscoelasticity of bone (Eberhardsteiner et al., 2014; Wang and Feng, 2005; Yamashita et al., 2002). The mineral phase reduces the motion of collagen chains and this, in turn, reduces the viscosity of the bone (Wang and Feng, 2005; Yamashita et al., 2001). When the HA crystals become larger, bone viscoelastic property seems to increase (Ojanen et al., 2015) while viscosity of the bone matrix seems to decrease with increasing number of mature collagen crosslinks (Isaksson et al., 2010a). As the bone is exposed daily to dynamic loading, its viscoelastic properties and their changes with age or disease, may critically contribute to e.g. stress fractures or biocompatibility of biomimetic bone materials (Adharapurapu et al., 2006; Huang et al., 2016).
During aging, the changes in bone structure and collagen network make the bone more brittle (Raisz and Seeman, 2001; Seeman, 2009; Wang et al., 2002). Therefore, mechanical integrity of the whole bone is closely related to tissue mass and structure (Hoffler et al., 2000). In addition, with age there is an accumulation of advanced glycation end-products (AGEs) from non-enzymatic glycation (Tang et al., 2007; Wang et al., 2002). The accumulation of AGEs has been shown to make collagen network more brittle as indicated by post-yield strain energy and damage fraction of demineralized collagen of trabecular bone at the apparent-level under unconfined compression (Tang et al., 2007). However, AGEs were shown not to affect the stiffness of the bone at the apparent-level although it increased the stiffness of individual trabeculae (Tang et al., 2007). Bone tissue mechanical properties, however, are highly related to tissue age instead of the donor age (Nyman et al., 2016; Ojanen et al., 2015). Using finite element (FE) modeling, material properties have been incorporated to 3D bone structure, as imaged using μCT, to predict apparent-level mechanical response of bone (Chen et al., 2017; Hambli, 2013; Müller and Rüegsegger, 1995; Sandino et al., 2015). Based on the FE simulations, the tissue-elastic properties are closely related to the elastic properties of the trabecular bone at the apparent-level when heterogeneity of the sample is taken into account (Harrison et al., 2008; Wolfram et al., 2010). Therefore, the apparent level viscoelastic properties of trabecular bone maybe influenced by the bone’s tissue viscoelastic properties. Thus, the examination of bone viscoelasticity at different hierarchical levels is needed.

Relationships between tissue composition, especially collagen content and cross-linking, of trabecular bone with its viscoelastic properties at tissue- and apparent-level are not well known. In this study, mechanical tests and finite element analyses were conducted for the trabecular bone samples at the tissue- and apparent-levels. Bone composition and collagen crosslinks were determined using Raman micro-spectroscopy and biochemical analysis. The microstructure of the
bone sample was extracted using µCT. Using these methods, we investigated associations between bone composition and collagen crosslinks, and bone viscoelasticity at different length scales.

Methods
The study overview is depicted in Figure 1.

Sample preparation
Trabecular bone cylinders (diameter=10 mm) were extracted from the femoral neck from human male cadavers (n=11, age=46±20 years, range from 18 to 78 years) at Kuopio University Hospital, Kuopio, Finland. The cadavers had no reported history of any metabolic bone disease. The use of human tissue was approved by the National Authority for Medicolegal Affairs (5783/04/044/07) and the ethical committee of North-Savo hospital district (161/2006). The extraction process has been described previously (Ojanen et al., 2015). One of the flat ends of the cylindrical samples was first polished (FORCIPOL IV, Metkon Instruments Ltd., Bursa, Turkey) with progressively finer silicon carbide (SiC) paper (grit P1000 and P2500) for scanning acoustic microscope (SAM) measurements. Subsequently, the other flat end of the sample was polished using only grit P1000 SiC paper to shorten the sample height to 6.5 mm for macroscale mechanical testing.

Nanoindentation and Raman micro-spectroscopy
In our previous study, tissue-level mechanical properties and composition using nanoindentation and Raman micro-spectroscopy respectively were determined from the end-surface of same sample cylinders (Ojanen et al., 2015). Briefly, 10 nanoindentations (five per trabecula) per sample were performed with a Berkovich diamond tip using a ramp loading with a loading rate of 5mN/s to the maximum load of 30mN (NanoTest, Micro Materials Ltd, Wrexham, United Kingdom). The maximum load was maintained for 60s. Displacement over time was recorded for creep analysis.
and a four element Burger’s model (Fisher-Cripps, 2004) was fit to the data using the Nelder-Mead simplex algorithm (Matlab 7.6.0, MathWorks, Inc., MA), where \( E_1 \) and \( E_2 \) are the elastic moduli, \( \eta_1 \) is the long-term creep viscosity and \( \tau (= \eta_2/E_2) \) is the creep time constant. Raman microspectroscopy measurements were matched to the indented trabeculae for each sample. The measurements were done using a dispersive Raman microscope (Senterra 200LX, Bruker Optics GmbH, Ettlingen, Germany) (see Supplementary material A1). Collagen composition (amide III peak (\( ~1180-1345 \text{cm}^{-1} \))/CH\(_2\) wag (\( ~1454–1461 \text{cm}^{-1} \))) (Ager et al., 2005; Goodyear et al., 2009), mineral-to-matrix ratio (mineralization, phosphate \( \nu_1 \) at \( ~958 \text{cm}^{-1}\)/CH\(_2\) at \( ~1447 \text{cm}^{-1} \)) and carbonate-to-phosphate ratio (carbonate substitution, type-B carbonate at \( ~1070 \text{cm}^{-1}\)/phosphate \( \nu_1 \) at \( ~958 \text{cm}^{-1} \) ) and crystallinity (1/FWHM of phosphate \( \nu_1 \) peak) were determined (Ojanen et al., 2015).

**Micro computed tomography (\( \mu \)CT)**

Microstructure and tissue mineral density (TMD) were determined with a high-resolution \( \mu \)CT scanner (Skyscan 1172, Bruker, Belgium), using isotropic voxel size of 14\( \mu \)m, tube voltage of 100 kV and a 0.5mm aluminum filter. The rotation step was 0.7\(^{\circ}\) and five frames were averaged for each projection. The fresh samples were imaged immersed in PBS. For determination of sample TMD, three hydroxyapatite phantoms (0.75g/cm\(^3\), 1.00g/cm\(^3\) and 1.25g/cm\(^3\)) were imaged. Images were processed in CTAn software (v. 1.15.4, Bruker, Belgium). First, images were binarized to separate trabecular bone and bone fragments. A global thresholding was applied and TMD over 0.36g/cm\(^3\) was considered as bone. Fragments were removed with sweep process and 3D Gaussian smoothing was applied to provide smoother segmentation for FEM mesh. The bone microstructural parameters were calculated and expressed in accordance with the ASBMR guidelines (Bouxsein et al., 2010).

**Scanning acoustic microscopy**
To determine the spatial distribution of trabecular bone tissue elasticity, the acoustic impedances of fresh trabecular bone surfaces were recorded following our previously established protocols using a scanning acoustic microscope (SAM) (Ojanen et al., 2016). A custom SAM (Männicke et al., 2016) equipped with a focused 100 MHz transducer (69MHz to 128MHz, -6dB, 30µm spot size, 12.6mm focal length, V3194, Panametrics Inc., Waltham, MA) was used. The scan size was 10mm² and the scan step size in both x- and y-directions was 10µm. The samples were measured in PBS (37°C). Prior to the bone scans, materials with known acoustic properties were measured and used as references (Ojanen et al., 2016). From the ultrasound images, the calcified tissue was segmented from PBS (Ojanen et al., 2016). Elastic coefficient values were estimated from the acoustic impedances of trabecular bone surfaces using a previously established relation (Preininger et al., 2011). From the measurement pool of calcified tissue of a bone scan, measurement points were picked randomly and the average elastic coefficient of the selected measurement points were compared to the average elastic coefficient of all the pixels representing bone (Eq.(1)). The absolute relative error of each sample was plotted against the number of measurement points picked.

\[
|\text{Absolute relative error}| = \left| \frac{\bar{C}_r - \bar{C}_{\text{tot}}}{\bar{C}_{\text{tot}}} \right| \times 100\%.
\]  

where \(\bar{C}_r\) is the average elastic coefficient of randomly selected bone pixels and \(\bar{C}_{\text{tot}}\) is the average elastic coefficient of all bone pixels.

**Compression testing at macroscale**

The bone samples were subjected to mechanical compression using a bi-axial servohydraulic testing device (Instron 8874; Instron Co, Canton, MA) equipped with a 1kN loading cell and an extensometer (Dynamic Extensometer 2620-603, Instron Co, Canton, MA). The samples were tested nondestructively using stress-relaxation and dynamic loading protocols in the elastic region to characterize macroscale mechanical properties. First, the hydrated cylindrical core
(diameter=10mm and height 6.5mm) was compressed in unconfined geometry between two smooth and low friction metallic plates with a 9 N (0.11MPa) pre-load (Linde et al., 1989). The sample was then compressed to 0.6% strain with a strain rate of 0.01s\(^{-1}\) (Linde et al., 1989). The strain was maintained for 10min before the start of cyclic loading. During the 10min period the sample was allowed to relax and subsequently, the strain was reduced to 0.37% and the sample was compressed sinusoidally (1Hz) for 20 cycles with a displacement amplitude of 15µm (strain amplitude=0.23%, maximum total strain=0.6%).

Under sinusoidal compression, the trabecular bone core exhibits viscoelastic behavior by displaying a phase shift (\(\delta\)) between strain and stress (Dong et al., 2004; Lakes et al., 1979). In addition to the loss tangent (\(\tan (\delta)\)), storage modulus (\(E'\)), loss modulus (\(E''\)), and dissipated energy (\(\Delta E\)) per cycle per unit volume was determined (Findley et al., 1989; Hayes and Bodine, 1978).

**Biochemical analysis**

The samples were rinsed and lipidic substances were removed in three changes of acetone, one of equal parts of acetone and diethylether, and finally one of diethylether. The samples were then dried in vacuum to constant weight and crushed. A third of the powder was dried in high vacuum, and then acid hydrolyzed at 110°C for 24h using a vapor phase technique (Cohen et al., 1986). The resulting hydrolysates were evaporated to complete dryness and dissolved in 15µl of 2M hydrochloric acid per 1mg (dry weight) of bone tissue. Enzymatic mature crosslinks hydroxylysyl pyridinoline (HP) and lysyl pyridinoline (LP) and non-enzymatic advanced glycation end product pentosidine (PEN) were separated using a high performance liquid chromatography (HPLC) instrument (Waters, Milford, MA) equipped with a fluorescence detector (model 474). The protocol mainly followed previously established methods (Bank et al., 1997), with inclusion of pyridoxamine as internal standard (Colwell et al., 1993) and with the eluent time profile optimized.
for the presently used column (Kinetex 2.6 μm C18 100A 100 x 4.6 mm, Phenomenex, Torrance, CA) (see Supplementary material A2). The concentrations of HP, LP, and PEN were expressed as moles per mole of collagen type I (Eyre et al., 1984; Eyre et al., 1988).

**Finite element analysis**

FE-models were created to simulate experimental nanoindentation creep \((n=11)\) and whole sample stress-relaxation tests \((n=10)\). One of the stress-relaxation simulations failed to optimize. In the following, nanoindentation creep simulations will be referred to as tissue-level simulations and the whole sample stress-relaxation test simulations as apparent-level simulations. For tissue-level simulations, a single trabecula was modeled as hyper-viscoelastic material using two term Prony series. A geometry representing a part of a single trabecula was modeled using 21 248 hexahedral elements (axisymmetric element CAX4, Abaqus v6.14, Dassault Systemes). The geometry was assumed as axisymmetric (Lichinchi et al., 1998) and the diamond Berkovich indenter was modeled as a rigid cone. Contact between the indenter tip and bone surface was considered frictionless. No lateral displacement was allowed along the axis of symmetry of the trabecula while the bottom of the trabecula was constrained in the axial direction. The loading corresponded the experimental nanoindentation creep testing.

For apparent-level simulations, the sample geometry was retrieved by segmentation of the μCT images. The obtained triangulated geometry was meshed with about 700 000 tetrahedral elements (Hypermesh v.14.0) (Ulrich et al., 1998) and imported into Abaqus (element type=C3D4) (see Supplementary material B1). The experimental stress-relaxation loading protocol was applied to the top nodes of the sample while the bottom nodes were fixed in the axial direction. The material model for whole sample was the same as for the single trabeculae. Possible localized damage
caused by low strain compression (0.2-0.63%) (Morgan et al., 2005; Nagaraja et al., 2005) was not included in the material model.

For both tissue- and apparent-level FE analyses, trabecular bone was modeled as hyper-viscoelastic material where the nonlinear elastic mechanical response was modeled using a Neo-Hookean strain energy function (see Supplementary material B2). The viscoelastic material behavior was defined in terms of Prony series expansion. In all FE simulations in both length scales, unique material parameters were obtained for each sample using a multidimensional unconstrained nonlinear minimization routine (fminsearch) in Matlab (v. 2014a, Mathworks Inc., USA) (see Supplementary material B3). During the optimization, the Poisson’s ratio was assumed to be constant ($\nu=0.325$) (Cowin, 1999) and the dimensionless (normalized) bulk relaxation moduli (bulk Prony parameter) were set equal to shear relaxation moduli (Shear Prony parameter) i.e., $k_1=g_1$ and $k_2=g_2$ (Sandino et al., 2015) (see Supplementary material B2). Thus, the optimized material parameters from creep and stress-relaxation curves in the hyper-viscoelastic model included the equilibrium elastic modulus $E_{eq}$, the dimensionless (normalized) shear relaxation moduli $g_1$ and $g_2$ and viscoelastic relaxation times $\tau_1$ (~short relaxation) and $\tau_2$ (~long relaxation). The optimized material parameters from tissue- and apparent-level simulations were compared to each other. In addition, a parametric analysis was conducted to quantify the effect of variation in the properties on the apparent-level response. This was done by varying values of each optimized creep material parameter by ±50%.

Statistics

Tissue- and apparent-level viscoelasticity relationships in association with structure, composition and collagen crosslinking were analyzed using Pearson’s linear correlation after confirming normality using Shapiro-Wilk test. The simulated viscoelastic parameters at tissue- and apparent-
level were compared using paired t-test. SPSS program (v. 21, SPSS Inc., Chicago, IL) was used for statistical analyses.

**Results**

The mean and standard deviations (SD) of experimental tissue- and apparent-level viscoelastic and microstructural properties, composition and collagen crosslink content are presented in Table 1.

*Experimental viscoelasticity parameters*

In the present set of 11 human trabecular samples, no significant correlations were observed between microstructure and experimental tissue-level viscoelasticity ($E_1$, $E_2$, $\eta_1$, $\eta_2$ and $\tau$). No significant correlations were found between the tissue-level viscoelasticity and tissue composition or collagen crosslink content. In addition, elastic coefficient determined using SAM was not significantly related to trabecular bone microstructure, tissue composition, collagen crosslink or to other tissue- or apparent-level mechanical parameters. At the apparent-level $E'$ was significantly correlated with bone mineral density (BMD) ($r=0.922$, $p<0.001$), bone volume fraction (BV/TV) ($r=0.912$, $p<0.001$), structural mode index (SMI) ($r=-0.760$, $p=0.011$), trabecular bone thickness (Tb.Th) ($r=0.878$, $p<0.001$) and trabecular bone number (Tb.N) ($r=0.677$, $p=0.022$). $E''$ and $\Delta E$ were significantly correlated with Tb.N ($E'': r=0.623$, $p=0.041$ and $\Delta E$: $r=0.624$, $p=0.040$), trabecular separation (Tb.Sp.) ($E'': r=-0.679$, $p=0.022$ and $\Delta E$: $r=-0.683$, $p=0.021$), and degree of anisotropy (DA) ($E'': r=0.658$ $p=0.028$ and $\Delta E$: $r=0.627$, $p=0.039$). No significant correlations were observed between apparent-level mechanical properties and tissue composition or collagen crosslinks. Similarly, correlations between the experimental tissue- and apparent-level viscoelastic parameters were not significant.

*Finite element modeling*
The mean values for optimized material properties from nanoindentation creep and unconfined compression stress-relaxation curves are presented in Table 2. When examining the material properties optimized from tissue-level simulations, $E_{eq}$ and $\tau_2$ were significantly correlated with crystallinity ($E_{eq}$: $r=0.730$, $p=0.011$ and $\tau_2$: $r=-0.613$, $p=0.045$) (Table 3) (Figure 2). In addition, $g_1$ and $\tau_1$ correlated with collagen content derived with Raman spectroscopy ($g_1$: $r=-0.780$, $p=0.005$ and $\tau_1$: $r=0.783$, $p=0.004$) and HPLC ($g_1$: $r=-0.659$, $p = 0.027$ and $\tau_1$: $r=0.793$, $p=0.004$). Further, $g_2$ was negatively correlated with HP content ($r=-0.615$, $p=0.044$). When examining the material properties from apparent-level simulations, $E_{eq}$ was positively correlated with HP content ($r=0.718$, $p=0.019$) and $g_1$ was positively correlated with collagen content determined with Raman spectroscopy ($r=0.696$, $p=0.025$) (Figure 3). Furthermore, $g_2$ was significantly correlated with $E''$ ($r=0.838$, $p=0.002$) and $\Delta E$ ($r=0.825$, $p=0.003$). In addition, $\tau_2$ was found to correlate with $\tan (\delta)$ ($r=-0.696$, $p=0.025$). No correlations were found between apparent- and tissue-level simulation parameters $E_{eq}$, $g_1$, $g_2$, $\tau_1$, and $\tau_2$.

The parametric analyses illustrated how local variations in viscoelastic material parameters affect viscoelastic behavior of the trabecular bone at the macroscale (Figure 4). The mechanical response of the cylindrical trabecular bone sample was strongly dependent on $E_{eq}$. $g_2$ was the secondary major contributor by affecting the long-term load response, while the other material parameters ($g_1$, $\tau_1$ and $\tau_2$) had a minor effect the viscoelastic behavior of trabecular bone at the apparent-level. $g_1$ affected the short-term load response at the apparent-level. Paired t-test showed a significant difference between the simulated tissue- and apparent-level viscoelastic material parameters $E_{eq}$, $g_2$ and $\tau_1$ (Table 2) (see Supplementary material Table B1).

**Spatial distribution of tissue elasticity**

The variation of elastic coefficient of fresh trabecular bone sample surfaces was between 27 and 37% (Table 4). The average number of measurement points from one bone surface scan was about
133000 (ranged 82 000 to 192 000). Figure 5 depicts the number of measurement points needed to get a representative average of elastic coefficient value for whole sample surface with an absolute relative error below a wanted error percentage.

**Discussion**

In the present study, we examined the relationships between the tissue- and apparent-level viscoelasticity in association with the tissue structure and composition of human trabecular bone, using experimental and computational approaches. We applied a hyper-viscoelastic FE model utilizing Prony series expansion to include tissue viscoelasticity. In earlier studies on bone tissue-level viscoelasticity using Burger model, the creep parameters were not related to collagen content or collagen crosslinks (Isaksson et al., 2010a; Ojanen et al., 2015; Wu et al., 2012). However, in the present study, the FE model was able to show a decrease in $g_1$ and an increase in $\tau_1$ with increasing collagen content. In addition, the FE model showed a decrease in $g_2$ with increasing HP content. Further, with increasing crystallinity, there was a significant increase in $E_{eq}$ but a significant decrease in $\tau_2$. These findings suggest that increasing crystallinity increases the stiffness of the bone tissue while increasing mature crosslink content reduces its viscoelasticity. However, increasing collagen content seems to simultaneously stiffen and prolong the short-term load response. Especially, the present FE model may be sensitive to possible changes in collagen in trabecular bone at the microscale.

$tan (\delta)$, $E''$ and $\Delta E$ determined using macroscale dynamic testing were found to relate to the viscoelastic material parameters $\tau_2$, $g_2$ and $g_2$ simulated at the apparent-level, respectively. The relations were in line with the theoretical relations between viscoelastic expression using Prony series and complex modulus (Findley et al., 1989). The material properties optimized from microscale creep and macroscale stress-relaxation curves were of the same order. However, there
were significant differences in some of the material parameters \((E_{eq}, g_2\) and \(\tau_1\)) as well as in their associations with composition. Based on the parametric analyses, \(E_{eq}\) and \(g_2\) were the most sensitive macroscale viscoelastic material parameters to changes at the microscale. These findings suggest that the viscoelasticity at apparent-level is not fully represented by the viscoelasticity at the tissue-level. The change in bone tissue stiffness is related to crystallinity at the tissue-level while at the apparent-level it is related to the changes in mature crosslink content. In addition, while trabecular bone tissue-level viscoelasticity seemed to decrease with increasing collagen content, the apparent viscoelasticity of trabecular bone was found to increase with collagen content.

The experimental apparent-level viscoelastic parameter values (Table 1) were in range of previously reported values for hydrated trabecular bone samples (Manda et al., 2016; Töyräs et al., 2002) and were found to be related to bone microstructure. In addition, with age, an accumulation of AGEs has been observed with increase in bone brittleness (Tang et al., 2007; Wang et al., 2002). The accumulation of AGEs, as indicated by increase in PEN content, was seen in the present study as well (results not presented). However, in the present study apparent-level experimental viscoelastic properties were not significantly related to collagen crosslink content. This could be due to the fact that the current study limited the compression of mineralized trabecular bone cores at the elastic region of the sample while previous studies’ observations were based on post-yield behavior.

In the present study, the viscoelastic parameters measured at tissue-level were not significantly related to the viscoelastic parameters measured at the apparent-level. As the femoral trabecular bone is heterogenic at the tissue-level (Norman et al., 2008), significant uncertainty arises from the fact that we conducted only five indentations on two trabeculae, numbers too small for representation of the viscoelastic properties at the tissue-level for the whole sample. In this study, the coefficient of variation of trabecular bone tissue elasticity assessed using SAM was found to be between 27 and
37%. This is consistent with our previous findings using human trabecular bone embedded in PMMA (Ojanen et al., 2016) suggesting that the variation in tissue elasticity is due to material property and not sample preparation. The SAM data of the current study also showed that, the absolute relative error rises fast when less than 400 measurements are used for calculating the mean elastic coefficient. This means that more than 0.3% of the calcified tissue surface from different parts of the sample needs to be measured to get the measurement error under 3%. Thus, heterogeneity of the trabecular bone tissue needs to be accounted for when implementing material parameters into the FE model (Harrison et al., 2008). In addition, the load applied in mechanical tests at the tissue- and apparent-level were only compressional and from the same orientation and therefore the effects of anisotropy on trabecular bone viscoelasticity at different hierarchal levels were not examined in the current study. This may also be a factor in having lack of correlation between tissue- and apparent-level viscoelasticity. Furthermore, current study used Berkovich indenter that deformed the bone material beyond the elastic region while the macroscale compression stayed in the elastic region, which may have contributed to the values of correlation between the determined tissue- and apparent-level properties. Finally, the differences in experimental mechanical methodology (dynamic response vs. creep response) may have also contributed to the lack of correlation between tissue- and apparent-level viscoelastic parameters. These are obvious explanations, related to experimental methodology, for the discrepancy between determined tissue- and apparent-level mechanical properties. However, the apparent-level viscoelasticity, as measured in unconfined compression, may partly be due to different mechanisms as tissue-level viscoelasticity, such as the fluid flow in pores and interactions between microstructures (Lakes and Katz, 1979). These mechanisms could be more accented in the trabecular bone, compared to cortical bone (Shepherd et al., 2011).
To our knowledge, this is the first study to assess viscoelasticity at different length scales in association with composition and collagen crosslinks in the human trabecular bone. The strength of our study is the use of hydrated samples and inclusion of sample specific FE models. However, the sample size in this study was limited ($n=11$). In addition, in the FE models, local strain maximum at tension and compression were about 0.7% and 0.1%, respectively. At these strains, there is possibility for local micro damage on the trabecula (Morgan et al., 2005; Nagaraja et al., 2005) that may affect the mechanical behavior of the apparent-level simulations. However, this was not included in the material model. Nevertheless, this study showed that at the tissue-level, bone tissue viscoelasticity is complexly affected by changes in the tissue composition and that viscoelasticity at the tissue-level may not fully predict the viscoelasticity at the apparent-level.

**Conflict of Interest Statement**

The authors have no conflicts of interest.

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**References**


Figure 1. An overview of the methods used in the present study. After sample extraction, experimental tests were conducted (in the order of nanoindentation, Raman spectroscopy, µCT imaging, scanning acoustic microscope (SAM), unconfined compression and biochemical analysis). Then, sample specific finite element models constructed from µCT images were used to extract material parameters by optimizing FE simulations to experimental nanoindentation creep and unconfined compression stress-relaxation. The image in red box denotes the magnification of the trabecular mesh under the indenter.

Figure 2. Linear regressions of simulated tissue-level viscoelastic properties vs. tissue composition and collagen crosslink content (n=11). *p<0.05 and **p<0.01. $E_{eq}$=equilibrium elastic modulus, $g_1$ and $g_2$ are the dimensionless (normalized) shear relaxation moduli, $\tau_1$=short relaxation time, $\tau_2$=long relaxation time, Amide III/CH2=collagen ratio and HP= hydroxylysyl pyridinoline.

Figure 3. Linear regressions of simulated apparent-level viscoelastic properties vs. tissue composition and collagen crosslink content (n=10). *p<0.05. $E_{eq}$=equilibrium elastic modulus, Amide III/CH2=collagen ratio and HP= hydroxylysyl pyridinoline.

Figure 4. Stress-relaxation simulations of a typical trabecular sample after altering the values of viscoelastic material parameters ($E_{eq}$, $g_1$, $g_2$, $\tau_1$ or $\tau_2$, as indicated on top of the subfigures). The solid black line depicts the stress-relaxation curve estimated using the original value of viscoelastic parameter and the gray area is the result of varying the parameter in question by ±50%. Right column zooms the response on the left (dashed lines).
Figure 5. Relative error in estimated elastic coefficient value as a function of number of measurement points used in estimate (total number of measurement points varied between 82 000 and 192 000).
Tables

Table 1. Mean ±SD (n=11) of measured tissue- and apparent-level mechanical and microstructural properties (µCT), composition (Raman) and collagen crosslink content (HPLC) in human trabecular bone samples.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tissue-level mechanical properties</strong></td>
<td></td>
</tr>
<tr>
<td>$E_1$ (GPa)*</td>
<td>6.89 ± 1.79</td>
</tr>
<tr>
<td>$E_2$ (GPa)*</td>
<td>57 ± 15</td>
</tr>
<tr>
<td>$\eta_1$ (GPas)*</td>
<td>6016 ± 1745</td>
</tr>
<tr>
<td>$\eta_2$ (GPas)*</td>
<td>248 ± 67</td>
</tr>
<tr>
<td>$\tau$ (s)*</td>
<td>4.38 ± 0.12</td>
</tr>
<tr>
<td>Elastic coefficient (SAM) (GPa)</td>
<td>8.49 ± 1.22</td>
</tr>
<tr>
<td><strong>Apparent-level mechanical properties</strong></td>
<td></td>
</tr>
<tr>
<td>tan ($\delta$) (-)</td>
<td>0.09 ± 0.06</td>
</tr>
<tr>
<td>$E'$ (MPa)</td>
<td>594 ± 538</td>
</tr>
<tr>
<td>$E''$ (MPa)</td>
<td>36 ± 19</td>
</tr>
<tr>
<td>$\Delta E$ (J/m$^3$)</td>
<td>542 ± 297</td>
</tr>
<tr>
<td><strong>Microstructural properties</strong></td>
<td></td>
</tr>
<tr>
<td>TMD (g/cm$^3$)</td>
<td>1.08 ± 0.04</td>
</tr>
<tr>
<td>BMD (g/cm$^3$)</td>
<td>0.20 ± 0.07</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>19 ± 7</td>
</tr>
<tr>
<td>SMI (-)</td>
<td>1.02 ± 0.33</td>
</tr>
<tr>
<td>Tb.Th (µm)</td>
<td>200 ± 40</td>
</tr>
<tr>
<td>Tb.N (1/mm)</td>
<td>0.92 ± 0.16</td>
</tr>
<tr>
<td>Tb.Sp (µm)</td>
<td>760 ± 11</td>
</tr>
<tr>
<td>DA (-)</td>
<td>0.55 ± 0.07</td>
</tr>
<tr>
<td><strong>Composition</strong></td>
<td></td>
</tr>
<tr>
<td>Collagen composition (AmideII/CH$_2$) (-)</td>
<td>0.93 ± 0.12</td>
</tr>
<tr>
<td>Mineralization (-)</td>
<td>7.45 ± 1.09</td>
</tr>
<tr>
<td>Carbonate substitution (-)</td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td>Crystallinity (-)</td>
<td>0.037 ± 0.002</td>
</tr>
<tr>
<td><strong>Collagen crosslink content</strong></td>
<td></td>
</tr>
<tr>
<td>Collagen (nmol)</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td>HP (mol/mol of collagen)</td>
<td>0.14 ± 0.03</td>
</tr>
<tr>
<td>LP (mol/mol of collagen)</td>
<td>0.10 ± 0.05</td>
</tr>
<tr>
<td>PEN (mol/mol of collagen)</td>
<td>0.011 ± 0.004</td>
</tr>
</tbody>
</table>

$E_1$ and $E_2$ are the elastic moduli of Burger’s creep model, $\eta_1$=long-term creep viscosity, $\tau$ (= $\eta_2/E_2$)=creep time constant, tan ($\delta$)=loss tangent, $E'$=storage modulus, $E''$=loss modulus, $\Delta E$=dissipated energy, TMD=tissue mineral density, BMD=bone mineral density, BV/TV=bone volume fraction,
SMI=structural mode index \((n = 10, \text{one sample gave a negative value and was eliminated})\),


Table 2. Mean ±SD of viscoelastic material parameters optimized from tissue-level creep and apparent-level stress-relaxation experiments using hyper-viscoelastic FE models.

<table>
<thead>
<tr>
<th>FE optimized material parameters</th>
<th><strong>E_{eq}</strong> (GPa)</th>
<th>g_1</th>
<th><strong>g_2</strong></th>
<th><strong>τ_1</strong> (s)</th>
<th>τ_2 (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue-level creep</td>
<td>2.69 ± 0.94</td>
<td>0.25 ± 0.03</td>
<td>0.20 ± 0.03</td>
<td>3.40 ± 1.05</td>
<td>181 + 33</td>
</tr>
<tr>
<td>Apparent-level stress-relaxation</td>
<td>5.28 ± 1.74</td>
<td>0.22 ± 0.05</td>
<td>0.05 ± 0.02</td>
<td>6.92 ± 2.50</td>
<td>163 ± 63</td>
</tr>
</tbody>
</table>

* **p<0.01 between tissue- and apparent-level values.

Table 2. Pearson’s correlations between tissue composition and tissue-level \((n=11)\) and apparent-level viscoelastic parameters \((n=10)\).

<table>
<thead>
<tr>
<th>FE optimized material parameters</th>
<th>Tissue-level creep</th>
<th>Apparent-level stress-relaxation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E_{eq}</strong> (GPa)</td>
<td>g_1</td>
<td>g_2</td>
</tr>
<tr>
<td>Amidi III/CH</td>
<td>.131</td>
<td>-.780**</td>
</tr>
<tr>
<td>Mineralization</td>
<td>.084</td>
<td>-.450</td>
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<tr>
<td>Carbonate substitution</td>
<td>-.312</td>
<td>.074</td>
</tr>
<tr>
<td>Crystallinity</td>
<td>.730*</td>
<td>.257</td>
</tr>
<tr>
<td>Collagen content (nmol)</td>
<td>-.051</td>
<td>-.659*</td>
</tr>
<tr>
<td>HP</td>
<td>.513</td>
<td>-.096</td>
</tr>
<tr>
<td>LP</td>
<td>.523</td>
<td>.172</td>
</tr>
<tr>
<td>PEN</td>
<td>.328</td>
<td>.133</td>
</tr>
</tbody>
</table>

* **p<0.01 and *p<0.05
Table 4. The mean ±SD and coefficient of variation (CV) of elastic coefficient of individual trabecular sample SAM scans.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Elastic coefficient (GPa)</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.32</td>
<td>2.56</td>
<td>34.9</td>
</tr>
<tr>
<td>2</td>
<td>8.37</td>
<td>2.80</td>
<td>33.5</td>
</tr>
<tr>
<td>3</td>
<td>6.80</td>
<td>1.97</td>
<td>28.9</td>
</tr>
<tr>
<td>4</td>
<td>8.11</td>
<td>2.96</td>
<td>36.5</td>
</tr>
<tr>
<td>5</td>
<td>10.48</td>
<td>3.63</td>
<td>34.7</td>
</tr>
<tr>
<td>6</td>
<td>9.43</td>
<td>3.32</td>
<td>35.2</td>
</tr>
<tr>
<td>7</td>
<td>8.21</td>
<td>2.63</td>
<td>32.0</td>
</tr>
<tr>
<td>8</td>
<td>7.50</td>
<td>2.07</td>
<td>27.6</td>
</tr>
<tr>
<td>9</td>
<td>8.40</td>
<td>3.06</td>
<td>36.4</td>
</tr>
<tr>
<td>10</td>
<td>9.16</td>
<td>3.05</td>
<td>33.3</td>
</tr>
<tr>
<td>11</td>
<td>9.29</td>
<td>2.91</td>
<td>31.3</td>
</tr>
</tbody>
</table>