

Nanoscale heterogeneity promotes energy dissipation in bone

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Nanomechanical heterogeneity is expected to influence elasticity, damage, fracture and remodelling of bone. Here, the spatial distribution of nanomechanical properties of bone is quantified at the length scale of individual collagen fibrils. Our results show elaborate patterns of stiffness ranging from ~ 2 to 30 GPa, which do not correlate directly with topographical features and hence are attributed to underlying local structural and compositional variations. We propose a new energy-dissipation mechanism arising from nanomechanical heterogeneity, which offers a means for ductility enhancement, damage evolution and toughening. This hypothesis is supported by computational simulations that incorporate the nanoscale experimental results. These simulations predict that non-uniform inelastic deformation over larger areas and increased energy dissipation arising from nanoscale heterogeneity lead to markedly different biomechanical properties compared with a uniform material. The fundamental concepts discovered here are applicable to a broad class of biological materials and may serve as a design consideration for biologically inspired materials technologies.

Bone, like many natural materials, is inherently structurally, and hence mechanically, heterogeneous owing to spatial distributions in the shape, size and composition of its constituent building blocks. Heterogeneity also arises from a multitude of different types of biomacromolecule, pores containing fluids of variable viscosities and numerous interfaces (for example solid–solid, solid–fluid, damage based)^{1,2}. As a consequence of the hierarchical nature of bone³, mechanical heterogeneity is expected to exist at multiple length scales. Macroscopically, significant variations in mechanical properties have been observed for different anatomical locations⁴, as well as for regions within a particular anatomical location⁵, and are thought to develop in response to distinct loading requirements⁶. At the microscopic level, instrumented indentation has further identified differences in moduli and hardness for specific features such as trabeculae, interstitial lamellae and thick and thin lamellae in osteons, which have been attributed to collagen fibril orientation and anisotropy, as well as variations in mineral content^{7,8}. Heterogeneity at this length scale is also expected to arise from the continual cellular remodelling processes resulting in a mixture of ‘old’ and ‘new’ bone at any given time. For example, osteonal bone typically undergoes substantial remodelling, whereas older interstitial bone⁹ has a higher degree of mineralization¹⁰, leading to increased stiffness⁸. Atomic force microscopy (AFM)-based nanoindentation has been used to distinguish mechanically heterogeneous microscale regions in bone tissue from genetically modified mice with various levels of transforming growth factor, TGF- β , which correlate with compositional heterogeneity measured by Raman microspectroscopy, and additionally has been shown to possess the capability for quantifying nanoscale heterogeneity¹¹. All of these studies have ignited speculation on the role of heterogeneity in strain concentration, fracture risk, adaptation and damage accumulation^{8,11–16}. They also raise

important issues as to whether heterogeneity is advantageous or disadvantageous to the mechanical function of bone². Detailed and quantitative studies of the consequences of heterogeneity, in particular at the nanoscale, on the structural integrity and proper function of the tissue are largely unknown.

In this paper, we first quantify mechanical heterogeneity at nanometre length scales. Here, the mechanical response arises from an individual mineralized collagen fibril within the bone extracellular matrix, which is composed of platelike carbonated apatite mineralites (\sim tens of nanometres in length and width, and 3–5 nm in thickness¹) that permeate in and around type-I collagen in an overlapping manner¹⁷, as well as a small concentration of ~ 200 different types of non-collagenous protein (< 10 wt% of total protein)¹. It has become increasingly evident that the unique nanoscale properties of bone play a key role in its macroscopic biomechanical function^{17–22}, as this is where inelastic deformation and fracture must first initiate. Furthermore, because osteocytic processes have dimensions that are of the nanometre length scale²³, nanoscale heterogeneities in material properties of the surrounding extracellular matrix would be expected to modulate local stresses, thereby potentially influencing processes such as remodelling, migration and adhesion.

Nanoindentation using a spatially controlled AFM-based instrument was carried out with a sharp tetrahedral silicon probe tip (end radius ~ 15 nm, as estimated by high-resolution scanning electron microscopy imaging and AFM imaging of a gold-nanoparticle calibration standard) in ambient conditions as shown in Fig. 1 (see the Methods section). This instrument has a fully three-dimensional (3D) closed-loop piezoelectric displacement system that ensures positional accuracies in three dimensions of < 1 nm. Grids of indentations over a $2 \mu\text{m} \times 2 \mu\text{m}$ area were carried out on adult bovine tibial cortical bone (prepared with no alcohol

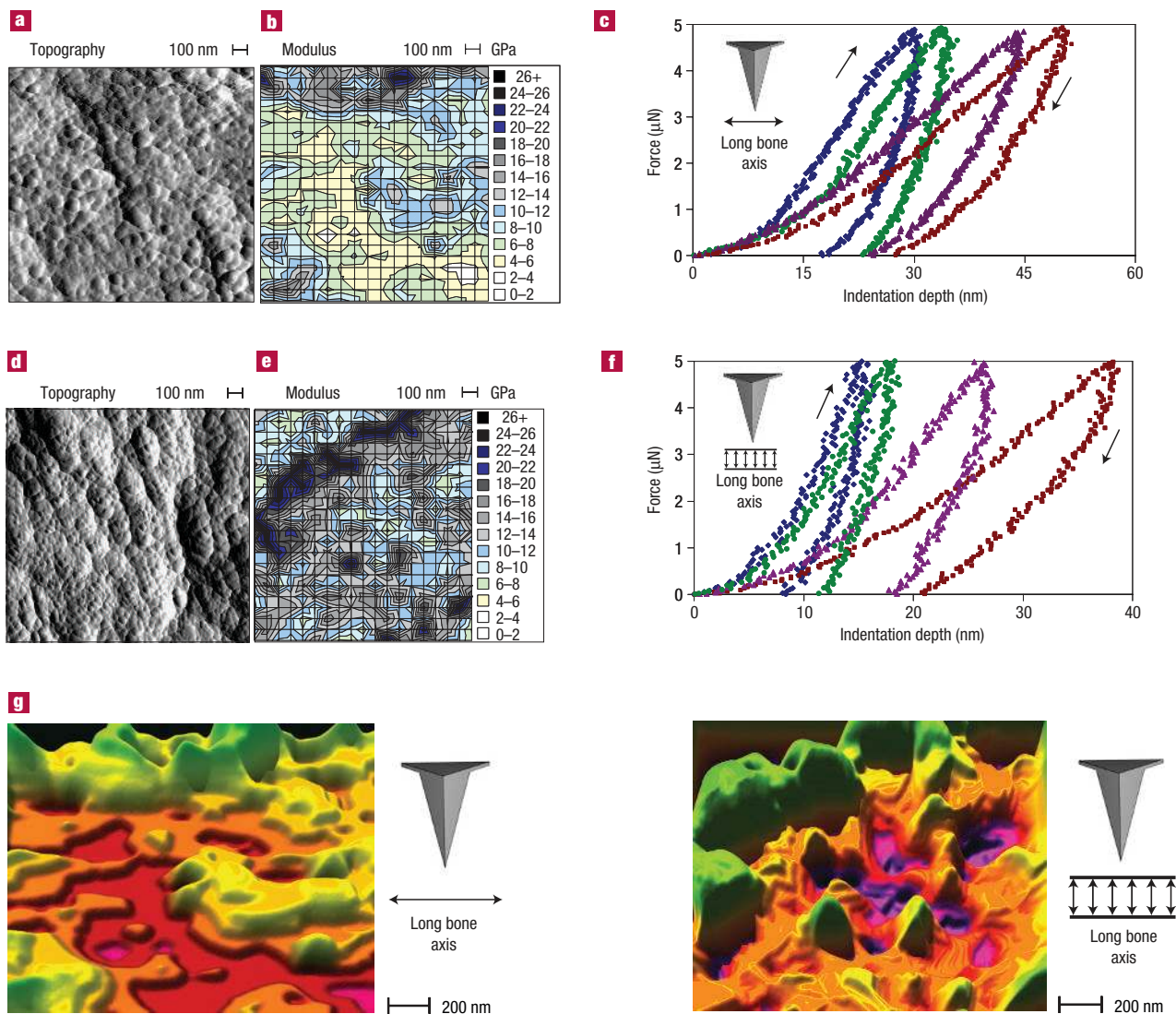


Figure 2 The ultrastructure and nanomechanical spatial heterogeneity of bone stiffness. **a**, An a.c. intermittent contact-mode AFM amplitude image viewed perpendicular to the long bone axis. **b**, 2D contour map of modulus reduced from nanoindentation data²⁵ for the area shown in **a** with loading axis perpendicular to the long bone axis. **c**, Individual nanoindentation curves at different locations within the modulus map **b**; moduli (GPa) blue diamonds 12.5, green circles 10.5, purple triangles 5.4, brown squares 4.2. **d**, An a.c. intermittent contact-mode AFM amplitude image parallel to the long bone axis. The average maximum lateral dimension measured from corresponding AFM height images was determined to be 64.9 ± 26.4 nm, which is consistent with the known dimensions of mineral particles in bone and other calcified tissues, as measured by scanning electron microscopy⁵⁰, transmission electron microscopy⁵¹ and small-angle X-ray scattering⁵². These values are somewhat smaller than those previously observed on similar samples²⁰ owing to optimization of the experimental imaging protocol to achieve higher resolutions. **e**, 2D contour map of modulus for the area shown in **d** with loading axis parallel to the long bone axis. **f**, Individual nanoindentation curves at different locations within the modulus map **e**; moduli (GPa) blue diamonds 24.2, green circles 18.1, purple triangles 15.8, brown squares 5.7. **g**, 3D graphic illustrations of the modulus maps shown in **b** (left), where loading was carried out perpendicular to the long bone axis, and **e** (right), where loading carried out parallel to the long bone axis. The vertical axis corresponds to stiffness, where the highest vertical topography corresponds to the maximum value and the lowest topography corresponds to the minimum value. For **b** and **e**, in the 26+ bin 1.5% of the data points showed moduli > 28 GPa.

nano- to microscale organic–inorganic compositional or structural variations (for example, different sizes, shapes and/or spacing between nanoscale constituents). Compositional fluctuations are known to exist down to $\sim 1\ \mu\text{m}$, as revealed by micro-Raman spectroscopy²⁸.

Indentation-modulus data taken with the loading axis parallel to the long bone axis on the same sample show less heterogeneity (coefficient of variation of 0.37), with values ranging from 5 (light yellow) to 31 (black) GPa and increased mean stiffness ($\sim 12.9 \pm 4.8$ GPa) (Fig. 2d–f) compared with the perpendicular orientation

(by a factor of ~ 1.5). This trend is consistent with macroscopic tissue-level data⁵ and collagen fibril anisotropy (increased stiffness along the fibril axis compared with transverse directions)²⁷. A large curved region is observed with increased stiffness (thickness ~ 250 nm) in the upper left hand side of the map, as well as the lower right hand side (distance between high stiffness regions $\sim 1.5\ \mu\text{m}$). Similar experiments were carried out on five different osteonal regions of the same bone sample. Nanoscale mechanical heterogeneity was observed in all of these maps and showed COV values ranging from 0.37 to 0.43 (data not shown). Again, the fine

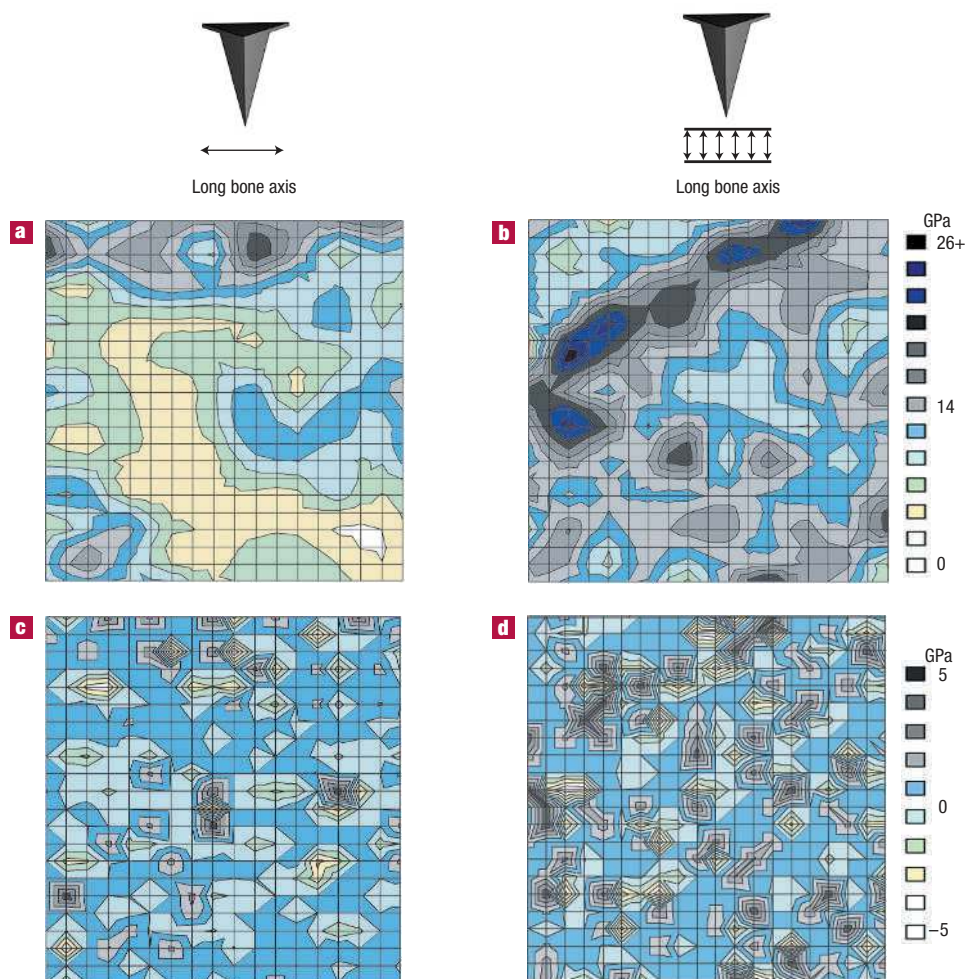


Figure 3 Quantitative analysis of nanomechanical-property maps using discrete wavelet transform. **a**, Level-1 approximation for modulus map given in Fig. 2b (loading axis perpendicular to the long bone axis). **b**, Level-1 approximation for modulus map given in Fig. 2e (loading axis parallel to the long bone axis). **c**, First-level detail corresponding to **a**. **d**, First-level detail corresponding to **b**.

patterns of each stiffness map varied among different regions and the mean stiffness values ranged from 11.8 ± 3.6 to 14.1 ± 5.3 GPa. Figure 2g provides a 3D visualization of the nanoscale stiffness data, with the vertical direction corresponding to the stiffness magnitude, comparing the parallel and perpendicular orientation directions. Although the COV for the contour map perpendicular to the long bone axis is greater, the differences in peak-to-valley modulus values are more apparent for the map from indents taken parallel to the long bone axis in the 3D visualizations. Assuming the spiral model for collagen fibril orientation²⁹, this occurrence may arise from off-axis loading of collagen fibrils, as opposed to in-plane loading in the perpendicular orientation. If the heterogeneity observed (for loading parallel to the long bone axis) was solely due to collagen fibril orientation, a more-or-less-continuous stiffness gradient would be observed, which does not seem to be the case here. Thick and thin lamellae have been measured to be approximately ~ 5 – 7 and $1 \mu\text{m}$ in width, respectively³⁰, with the thin lamellae being $\sim 10\%$ softer than the thick ones⁸. No distinct patterns were observed that could be conclusively associated with such features. Hence, the fluctuations observed must partially originate from smaller-length-scale features.

The modulus maps were analysed quantitatively using the discrete wavelet transform³¹, where decomposition of spatial

frequencies creates a coarser approximation of the image (see the Methods section). Details can be associated with a ‘pseudofrequency’ that identifies the length scales of the patterns removed at spatially specific regions. Figure 3a,b depicts the first-level approximations of the original images corresponding to indents perpendicular and parallel to the long bone axis, respectively. The details for these corresponding maps were removed to obtain the coarser image (Fig. 3c,d). The detail images show largely random features that prevail at the high spatial frequencies. However, they also capture specific patterns associated with the high-modulus areas. One can observe high-modulus peaks that coincide with the larger high-modulus domains (more apparent with the image parallel to the bone axis, Fig. 3d). The first level of decomposition thus seems to remove the local high-modulus peaks, leaving broader regions of stiffness. The characteristic length scale given by the pseudo(-spatial-)frequency analysis, identified as $\sim 156 \pm 28$ nm, corresponds closely to the experimentally measured mean diameter of an individual collagen fibril²⁴.

The mechanical heterogeneity described above for the AFM-based nanoindentation is much more pronounced compared with depth-sensing microindentation carried out on similar specimens (see the Supplementary Information). The COVs for microscale

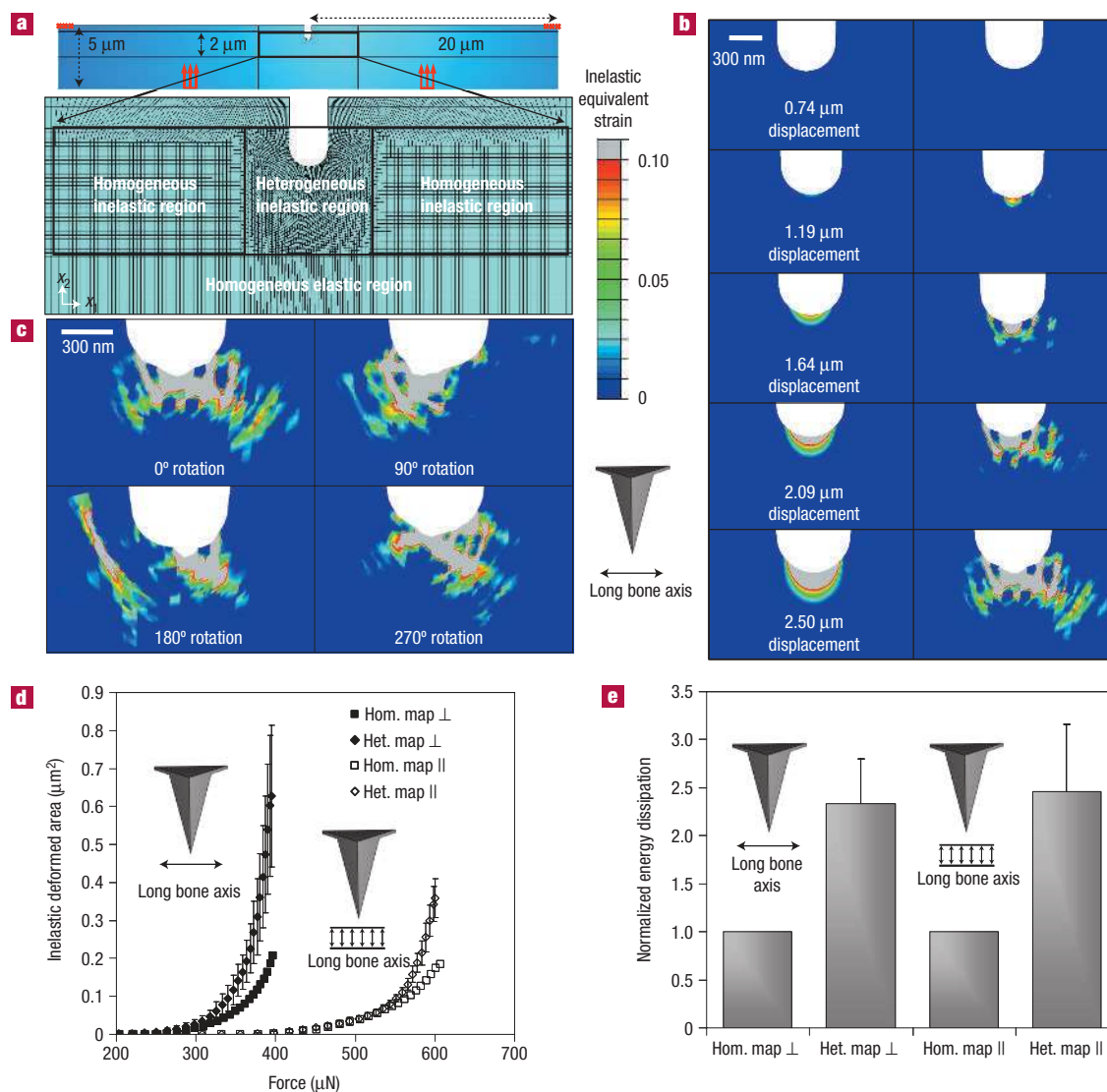


Figure 4 FEA simulations of the effect of nanomechanical spatial heterogeneity on larger-scale biomechanical properties. **a**, 2D notched four-point bend FEA model geometry showing dimensions and mesh. Modulus maps for adult bovine cortical bone were superimposed in a $2\ \mu\text{m} \times 2\ \mu\text{m}$ heterogeneous region ahead of the notch. The simulation was displacement controlled with the bottom two boundary conditions (spaced $20\ \mu\text{m}$ apart) displaced $2.5\ \mu\text{m}$ upwards from the bottom whereas the two fixed regions are at the end. Four-node bilinear plane-strain quadrilateral elements (CPE4) were used in this study and the mesh was highly refined in the heterogeneous mapped region. There were 9,424 elements in the $600\ \text{nm}$ notched specimen; 3,116 of these elements were within the heterogeneous region. The number of yielded elements was tracked through the equivalent inelastic strain and the reaction force in the vertical direction was measured at the fixed points. Energy dissipation was calculated by integrating the area between the loading and unloading force–displacement curves up to $2.5\ \mu\text{m}$ displacement. **b**, Snapshots of equivalent inelastic strain for the four-point bend FEA virtual simulations comparing the homogeneous to the heterogeneous case for loading up to $2.5\ \mu\text{m}$. This particular dataset used heterogeneous data with the loading axis perpendicular to the long bone axis (Fig. 2b). **c**, Equivalent inelastic strain for the four-point bend FEA virtual simulations, which incorporated the heterogeneous nanoindentation data taken with the loading axis perpendicular to the long bone axis (Fig. 2b), where the $2\ \mu\text{m} \times 2\ \mu\text{m}$ map is rotated in the plane of the long bone axis (the plane of the paper) by 0° , 90° , 180° and 270° . **d**, The inelastically deformed area is plotted as a function of force for each set of tests, both perpendicular and parallel to the long bone axis, comparing the homogeneous and heterogeneous cases. The heterogeneous plots are averages of the rotated and shifted contour-mapped regions, indicated by standard error bars (‘shifted’ simulations involved moving the notch $300\ \text{nm}$ vertically upwards and downwards; see Supplementary Information). **e**, Energy-dissipation bar plots for each set of simulations, both perpendicular and parallel to the long bone axis, comparing the homogeneous and heterogeneous cases where the values are normalized to the homogeneous case. The heterogeneous plots are averages of the rotated and shifted contour-mapped regions, indicated by standard error bars. A unit thickness of $1\ \mu\text{m}$ in the 2D model is used to compute the energy dissipation.

indentations (Berkovich geometry at $500\ \mu\text{N}$ maximum load) perpendicular and parallel to the long bone axis were found to be 0.25 and 0.26, respectively (methods are provided in our previous publication²⁴). This is markedly less than the nanoscale data presented here, suggesting a scale-dependent homogenization

effect. Other studies have reported even smaller microscale COV values of 0.07–0.15 (refs 7,8,32).

To better understand how nanoscale mechanical heterogeneity influences larger-length-scale biomechanical properties, finite-element simulations were carried out using the experimentally

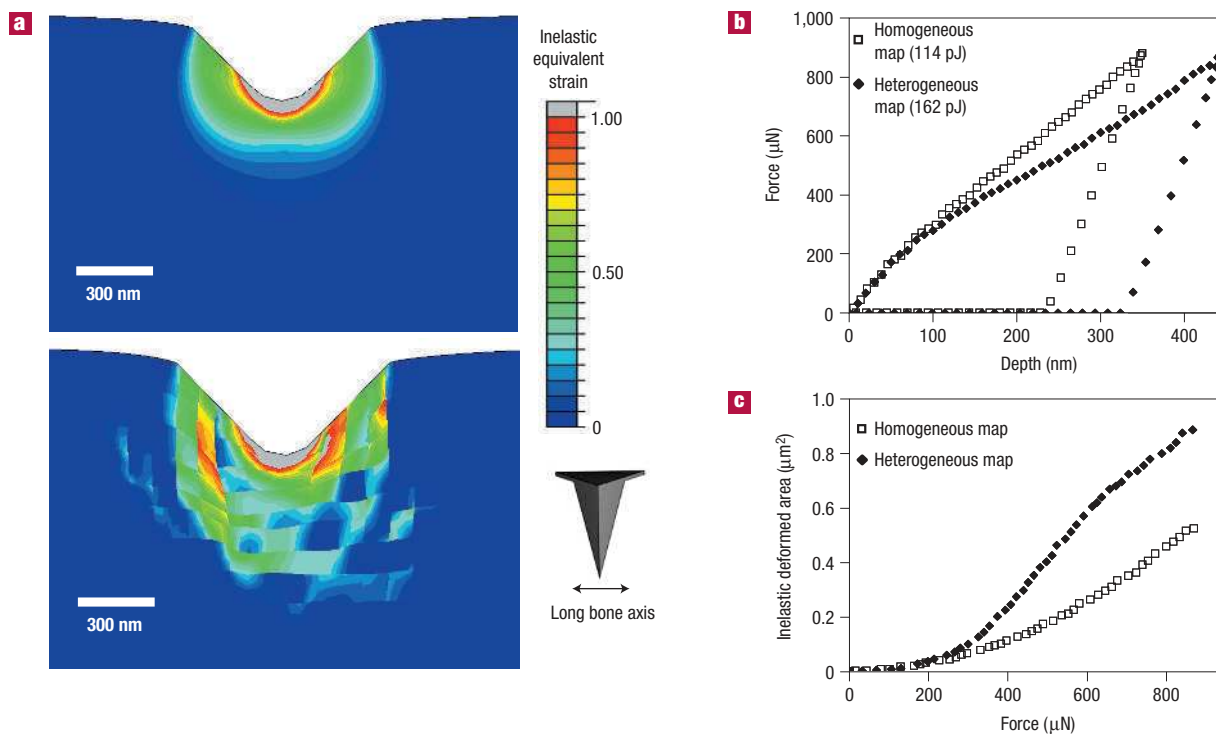


Figure 5 FEA simulations of the effect of nanomechanical spatial heterogeneity on larger-scale compressive loading. **a**, 2D indentation FEA model showing snapshots of equivalent inelastic strain for an indentation virtual simulation comparing the homogeneous (top) with the heterogeneous (bottom) case for loading up to ~ 850 μN . This particular dataset used heterogeneous data with the loading axis perpendicular to the long bone axis (Fig. 2b). A unit thickness of 1 μm in the 2D model is used to compute the energy dissipation. **b**, The force–depth curves for the homogeneous and heterogeneous cases. **c**, The inelastically deformed area as a function of force, comparing the homogeneous and heterogeneous cases.

determined fine-scale heterogeneity and local mechanical-property variations extracted from AFM-based indentation experiments. One previous study reported finite-element analysis of a perfectly elastic heterogeneous microstructure on the basis of mean tissue-level experimental data. In that work, the indentation elastic modulus decreased nonlinearly with increasing COV (artificially induced in the simulation). In addition, a marked increase in the number of failed elements (determined by an elastic-limit-strain criterion) was observed with increasing heterogeneity¹⁶. Here, we directly incorporate the experimentally measured heterogeneous nanoscale-stiffness maps into a highly refined region of finite elements just ahead of a notched four-point bend mesh (Fig. 4a), so as to capture the localization of strain in a region with nanoscale structural non-uniformity. Inelasticity was also incorporated into the four-point bend mesh as follows. Inelastic deformation was first assessed during individual indentation experiments by constructing an axisymmetric 2D FEA simulation with an equivalent tip apex angle of 23.5° , that is, the same indentation volume versus depth as for the AFM probe tip, that assumed a perfect von Mises inelastic constitutive law, as well as a modulus evaluated from the unloading slope²⁵. For a number of force-versus-depth indentation curves, various trial yield-stress (σ_Y) values were chosen to determine the optimal value at which the simulated data fitted the experimental curve. Each individual best-fit value (with an average σ_Y value of ~ 395 MPa) was then used to calculate the corresponding equivalent (elastic) limit strain at the yield point as given by $\bar{\epsilon}_Y = (2(1 + \nu)\sigma_Y)/3E$, where ν is Poisson's ratio and E is the elastic modulus (ν was taken to be 0.25). The $\bar{\epsilon}_Y$ values were found to be $\sim 0.04 \pm 0.02$ for

specimens tested parallel to the long bone axis and $\sim 0.044 \pm 0.02$ for specimens tested perpendicular to the long bone axis. These observations suggest that a constant-elastic-limit-strain (inelastic-yield-strain) criterion is a reasonable first-order approximation. Therefore, taking the above average value of $\bar{\epsilon}_Y$, the local yield-stress value at each position within the heterogeneous map, σ_Y , can be obtained conversely using $\sigma_Y = (3E\bar{\epsilon}_Y)/2(1 + \nu)$. The energy-dissipative inelastic deformation is incorporated here because a finite residual depth is observed after unloading of the indentation curves (Fig. 2c,f).

Strain-controlled micro- and macro-scale failure of bone has also been studied experimentally using four-point bend specimens with rounded notches³³. Damage preceding failure was observed to occur immediately at the notch tip (where maximum strain occurs) as opposed to further ahead of the notch tip (where maximum stress occurs), supporting the constant-failure-strain hypothesis. The tensile yield behaviour of bone has also been characterized by critical strain³⁴. As noted in earlier work³³, the inelastic constitutive response of bone is at present not fully understood. In compression, nanogranular friction and intraorganic cohesion are thought to be major contributing factors to the inelastic resistance of bone initially²⁰. Other mechanisms probably come into play at higher strains owing to the effects of collagen fibril shear¹⁹ and denaturation²⁴, crack initiation/propagation³³ and sacrificial bond rupture¹⁷. Although bone is known to show significant tension–compression asymmetry, plasticity models involving the von Mises yield criterion are typically used to capture, at least approximately, the stress/strain fields in bone undergoing inelastic deformation. In the spirit of developing detailed quantitative trends

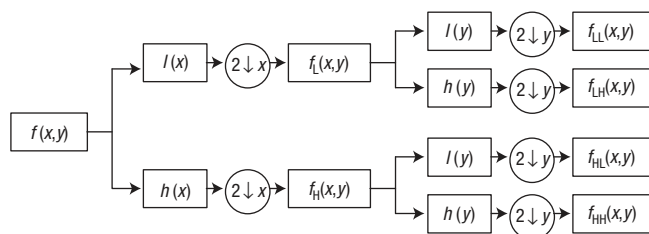


Figure 6 Wavelet decomposition of a 2D image⁴⁷. $f(x, y)$ is the 2D image, $h(x)$ the high-pass filter in the x direction, $l(x)$ the low-pass filter in the x direction, $f_L(x, y)$ the 2D image following the low-pass filter, $f_H(x, y)$ the 2D image following the high-pass filter, $f_{LL}(x, y)$ the 2D image following the low-pass/low-pass filter combination, resulting in the smoothed approximation, $f_{LH}(x, y)$ the 2D image following the low-pass/high-pass filter combination, resulting in the horizontal detail, $f_{HL}(x, y)$ the 2D image following the high-pass/low-pass filter combination, resulting in the vertical detail, $f_{HH}(x, y)$ the 2D image following the high-pass/high-pass filter combination, resulting in the diagonal detail, and $2(\downarrow)x$ the downsampling in the x direction.

for relative assessments of the role of structural heterogeneity from the nano- to the microscale, we invoke, as in earlier studies³³, plasticity analysis (described below) in our finite-element model, with the full realization that comprehensive constitutive models for inelastic deformation of bone have not yet been developed. These simulations, however, are seen to capture key trends of the inelastic deformation of bone when compared with our parallel computational studies that invoke anisotropy and pressure sensitivity of deformation (as noted below).

Figure 4b shows the progression of equivalent inelastic strain (defined as the time integral of $\sqrt{2/3}$ multiplied by the magnitude of the inelastic strain rate) during two virtual four-point bend FEA simulations (displacement controlled). The first (right column) incorporated the heterogeneously mapped data from Fig. 2b (loading axis perpendicular to the long bone axis). The second (left column) was a control, which was an elastic–perfectly plastic simulation with purely homogeneous mechanical properties (set to the volume-averaged mean value of modulus measured experimentally in Fig. 2b and the volume-averaged mean value of yield stress obtained from the strain-based yield criterion). The measured inelastic strain in the vicinity of the notch is continuously greater for the heterogeneous case than the homogeneous from 1.5 to 2.5 μm displacement. The inelastic strain develops irregularly ahead of the notch and extends about twice as far for the heterogeneous material compared with the homogeneous one at 2.5 μm displacement. Non-uniform notch opening and blunting took place in the heterogeneous case, as compared with a more uniform shape change for the homogeneous case. Figure 4c shows the inelastic equivalent strain for the four-point bend FEA simulations at 2.5 μm displacement for the same nanoindentation data (Fig. 2b), where the heterogeneous map ahead of the notch is rotated by 0°, 90°, 180° and 270° in the plane defined by the long bone axis (in the plane of the paper). Although the fine details of the inelasticity pathways are different for different rotations, all simulations show characteristic heterogeneous diffuse deformation over a much greater area compared with the homogeneous material. There is less than 10% variation in the inelastically deformed area for a complete rotation over 360°. The inelastically deformed area as a function of applied force is given (Fig. 4d) for cases both perpendicular and parallel to the long bone axis for both heterogeneous and homogeneous cases. For tests perpendicular to the long bone axis, the inelastically deformed area was about

three times as great for the heterogeneous than the homogeneous case at 390 μN maximum force. For tests parallel to the osteonal axis, the inelastic deformation area was about twice as great for the heterogeneous case at 610 μN maximum force. In general, for a 2.5 μm displacement, the inelastic strain region corresponded to an increased applied force for stiffer modulus values, on average. A comparison between maps taken with different loading orientations showed similar energy dissipation, which was about 2.4 times greater for the heterogeneous map compared with the homogeneous case (Fig. 4e). Also, as the notch diameter was varied between 300 and 1200 nm, there was a slight increase ($\sim 10\%$) in the inelastic strain region for heterogeneous and homogeneous cases in both orientations (data not shown). However, the relative ratio of inelastically strained areas between heterogeneous and homogeneous cases remained similar.

Further FEA simulations were carried out incorporating anisotropy (using elastic constants from ultrasonic velocity tests³⁵) and, separately, pressure sensitivity²⁰. These additional simulations (see the Supplementary Information) showed the same overall trends as the foregoing elastoplastic simulations, of increased inelastically deformed area (about threefold) and energy dissipation (about twofold) for the heterogeneous system compared with the homogeneous case. Therefore, although the specific extent of damage evolution and energy dissipation could be somewhat influenced by the particular constitutive model, material anisotropy and pressure sensitivity, it is evident that the overall conclusions extracted from the present computational simulations hold irrespective of the particular choice of material model. Another important issue pertains to the accuracy with which the 2D plane strain model captures the behaviour of the 3D microstructure. Indeed, significant quantitative differences would be expected in the inelastic strain distributions predicted by the 3D model as compared with the 2D case^{36,37}. Nevertheless, statistically, inelastic strain and stress (for example maximum principal stress) distributions from a plane-strain model are in general quite similar to those from a full 3D model, with the heterogeneity slightly underestimated by the plane-strain model³⁶. These results^{36,37} suggest that the plane-strain model used here should be sufficiently accurate to distinguish statistically the relative difference between the heterogeneous case and the homogeneous case.

An additional 2D FEA nanoindentation model was also built with compression as the chief loading mechanism (Fig. 5a). Here, similar to the bending case, the modulus map was superimposed onto a fine mesh with a 90° included-angle indentation geometry to demonstrate that the same trends are observed as with the four-point bend simulations. Figure 5b shows that the simulated force-versus-indentation-depth curves for the homogeneous stiffness map show a smaller deformation for a given load compared with the heterogeneous material (Fig. 2b) for loads larger than $\sim 250 \mu\text{N}$. The energy dissipations given by the curves were 114 and 162 pJ for the homogeneous and heterogeneous cases, respectively (for 850 μN maximum load). The inelastically deformed area for the heterogeneous case was markedly greater than that for the homogeneous case (Fig. 5c), consistent with the results obtained from the four-point bending simulations. This result further suggests that, even though heterogeneity at the micro- and macro-scale may have deleterious effects, leading to fracture and interfacial instabilities³⁸, inelastic heterogeneity at the nanoscale offers an advantageous mechanism for ductile energy dissipation.

In summary, we propose a new energy dissipation mechanism for bone arising from nanomechanical heterogeneity, by recourse to experimental results that provide high-resolution, spatially specific nanomechanical tests in conjunction with detailed computational simulations of locally inelastic deformation. These results provide compelling evidence for enhanced ductility and

energy dissipation through nanoscale structural heterogeneity. This concept is generally applicable to a broad class of natural materials because nanomechanical heterogeneity is expected to be ubiquitously present. In addition, because osteocytes are continually sampling their mechanical environment³⁹, deformation spread out over greater spatial areas owing to nanomechanical heterogeneity may facilitate damage detection in the extracellular matrix and improved remodelling responses. The heterogeneous nanomechanical patterns measured experimentally would in turn cause corresponding local heterogeneous strains when loaded macroscopically. Such strains are expected to be amplified by the softer surrounding pericellular matrix of osteocytes⁴⁰, further affecting cellular processes, possibly acting as ‘nanomechanical messages’. Lastly, heterogeneous local strains are also expected to influence interstitial fluid flow, which has been shown to be critical to the proper maintenance of metabolic activity⁴¹.

METHODS

SAMPLE PREPARATION AND CHARACTERIZATION

Samples of adult bovine cortical bone from the proximal tibial metaphysis were prepared as previously described²⁴. Sample preparation did not involve chemical treatment, ethanol dehydration or embedding. The mineral content measured by back-scattered electron microscopy was found to be ~58 wt%. The a.c. intermittent contact-mode AFM imaging at ambient temperature and humidity was used with a 3D molecular force probe (MFP-3D, Asylum Research) on hydrated samples of osteonal bone using a silicon etched OMCL-AC160TS (Olympus) rectangular cantilever with a tetrahedral probe tip (cantilever spring constant, $k \sim 56.2 \text{ N m}^{-1}$, probe-tip end radius $\sim 15 \text{ nm}$, Fig. 1) in ambient conditions. The tip radius was measured by a.c.-mode imaging of a gold nanoparticle calibration standard⁴².

NANOINDENTATION

Nanoindentation experiments were conducted in ambient conditions using the MFP-3D and the same probe tip as that used for a.c.-mode AFM imaging. The inverse optical lever sensitivity and cantilever spring constant calibrations were carried out as previously reported⁴³. Displacement-controlled nanoindentation was carried out by loading at a rate of $1 \mu\text{m s}^{-1}$ up to a trigger force of $5 \mu\text{N}$ followed by unloading at the same rate. The loading/unloading rate was varied from 0.05 to $10 \mu\text{m s}^{-1}$ and no statistically significant differences in calculated moduli were observed at loading rates between 0.5 and $5 \mu\text{m s}^{-1}$, indicating that viscoelastic effects were minimized. The tip contact-area function was calibrated with a sample of glycol methacrylate using a 6 GPa modulus from previous indentation data taken on a Hysitron Triboindenter. Values for elastic moduli were calculated from 95 to 20% of the initial unloading curve by fitting these data to contact mechanical theory for an isotropic, elastic half-space²⁵ for each individual curve. Due to the assumptions of this model^{44,45} (for example isotropy) and the hierarchical nanostructure of bone, the extracted modulus is more appropriately defined as an ‘indentation modulus’ in that it reflects relative mechanical stiffness and does not produce absolute quantitative values. However, the relative spatial differences in stiffness can be accurately compared. The 100 nm interindent spacing was chosen to be sufficiently large for minimal interference with a neighbouring residual inelastically deformed zone as well as residual stresses, as verified by 2D elastic–inelastic FEA simulations (described in detail in the FEA methods section). Additionally, experimental histogram distributions of the modulus taken at a much larger interindent spacing of $1 \mu\text{m}$ showed statistically similar modulus distributions ($p < 0.05$). An epoxy control sample (McMaster-Carr) was tested to ensure that the range of stiffness values observed was outside the instrumental scatter and the averaged modulus measured was determined to be 2.9 ± 0.7 with a COV of 0.23.

WAVELET DECOMPOSITION ANALYSIS

Wavelet transform enables localization of both space and frequency, achieved by dilating and translating a finite wavelet function to capture different frequency ranges and their spatial locations⁴⁶. The discrete wavelet transform represents one-dimensional sampled data with a limited number of decomposition levels (frequency scales). A set of low- and high-pass filters is used for decomposing

the data into a coarse approximation and a residual detail. Wavelets also enable particular frequency sub-bands to be isolated within an image, which are treated as 2D signals⁴⁷. Figure 6 depicts a one-level decomposition of a 2D image. Filters are applied in the x -direction and the results are down-sampled by deleting every other column. This yields two images of approximately half the size of the original, one containing high-frequency components of the rows and the other containing low-frequency components. These two images are then each filtered down the columns using the corresponding filters and down-sampling the results along the rows. This results in the smoothed-approximation, the horizontal-detail, the vertical-detail and the diagonal-detail subimages. The process is repeated on the smoothed-approximation subimage to obtain the next level of decomposition.

FINITE-ELEMENT ANALYSIS

The elastic contact radius at maximum load (using a threshold of 0.01 logarithmic strain) and the size of the residual inelastic strain zone (using a threshold of 0.01 inelastic equivalent strain) and residual stress zone (using a threshold of $0.1 \sigma_y$) after unloading were approximated by an elastic–perfectly inelastic 2D axisymmetric-indentation FEA model using the software package ABAQUS (Pawtucket, RI, USA) fitted to individual force–depth curves. The probe-tip geometry was incorporated from direct scanning electron microscopy visualization. The modulus value was fixed to the values obtained from the Oliver–Pharr analysis on the basis of the initial unloading slope²⁵. The yield stress was kept as a free fitting parameter, from which the yield strain could be iteratively determined. More detailed discussions on the computational indentation modelling and mechanical property extraction can be found elsewhere^{48,49}.

A 2D plane-strain four-point bend model ($40 \mu\text{m}$ wide and $5 \mu\text{m}$ high) was built (Fig. 4a). The experimentally measured modulus maps were incorporated within this FEA model for both specimens with indents perpendicular and parallel to the long bone axis by assigning a specific material section to whichever elements spatially coincided with each particular material region. An elastic-limit-strain-based von Mises plasticity constitutive law was used to model the energy-dissipating inelastic deformation and to obtain a local yield-stress value as described in the main text. The elements in the area immediately to the right and left of the $2 \mu\text{m} \times 2 \mu\text{m}$ heterogeneous region were assigned the experimentally measured average homogeneous value of modulus and corresponding yield stress (on the basis of the criterion discussed previously). The elements in the bottom portion were assigned only the purely elastic average homogeneous modulus value.

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Competing financial interests

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