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Research paper

Influence of SEM vacuum on bone micromechanics using in situ AFM

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ABSTRACT

The mechanical properties of rat bone at micron length scales have been evaluated as a function of environmental conditions using an in situ atomic force microscope (AFM) setup while observing using scanning electron microscopy (SEM). Focused ion beam fabricated rat bone cantilever samples were tested in both low and high vacuum conditions in the SEM as well as wet in air using the AFM to measure their elastic modulus. The elastic modulus of rat bone at micron length scales is shown to be independent of the environmental testing conditions and indicates water is bound to bone material even under relatively high vacuum conditions. Our work therefore shows how in situ mechanical testing of bone while observing using high resolution SEM can provide results similar to testing wet in air.

1. Introduction

Bone is a complex biological composite material that performs a variety of mechanical functions, most notably load bearing. Bone achieves the ability to perform its mechanical functions both through the use of a variety of component phases and organization of these phases across a range of different length scales. A solid mechanics approach can define bone components predominantly as a soft collagen organic matrix incorporating a stiffer mineral phase. The mechanical properties can therefore be directly related to the amount of mineral phase within the bone (Currey, 1999b). For example, deer antler is a form of bone with a relatively low volume fraction of mineral, resulting in a low elastic modulus, whereas larger mineral volume fractions in equine femur (Currey, 2002) provide correspondingly higher elastic modulus values.

Extensive studies indicate that the compositional changes, described in terms of increasing mineral volume fraction, lead to an increase in the elastic modulus but a decrease in the ultimate stress and strain, and therefore work to failure of bone material (Currey, 2002). The relationship between bone composition and mechanical functions has been investigated by a series of experiments evaluating the mechanical properties of bone components, especially at relatively small length scales where the structural hierarchy of bone can be largely ignored. Examples include nanoindentation (Tai et al., 2007) and tensile testing of individual mineralized collagen fibrils (Hang and Barber, 2011), as well as elucidation of

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micromechanics from larger bone specimen deformation by X-ray scattering (Krauss et al., 2009) and observation of fracture (Koester et al., 2008). Critically, mechanical testing of bone material while observing deformation and failure using scanning electron microscopy (SEM) has been shown to be particularly effective for elucidating bone structure–function behaviour (Hang and Barber, 2011; Koester et al., 2008). Despite the potential benefits of this in situ mechanical testing methodology, the effects of the SEM vacuum conditions on bone mechanics are at this time unclear. Indeed, any material that is at least partially hydrated may become modified when introduced into the vacuum chamber of an SEM, resulting in a change in its mechanical properties relative to the hydrated state. As bone is physiologically in a hydrated state, a number of studies have attempted to define the effects of water on the mechanical properties of bone. First investigations of bone mechanical behaviour under different hydrated conditions revealed an increase in strength but no change in elastic modulus associated with drying the samples in air after one hour, with a significant increase in elastic modulus observed only after drying in an incubator at 105 °C for a week (Sedlin and Hirsch, 1966). Currey later performed mechanical investigations to assess the effect of rehydrating bone after holding in air for 25 days. Currey observed little change in bone mechanical performance between the initial hydrated state and the rehydrated state (Currey, 1988). Recent work has indicated an increase in the elastic modulus and strength, with a decrease in failure strain, of dry deer antler bone relative to its hydrated state using bending mechanical testing (Currey et al., 2009). Morais et al. have indicated similar increases in elastic modulus but decrease in work of fracture for bovine cortical bone on dehydration (Morais et al., 2010). Further studies on elk antler bone also highlight the increase in strength and decrease in failure strain upon dehydrating, although the elastic modulus of the bone showed little dependence on the hydration state (Chen et al., 2009). These previous works examined the effects of dehydration on bone mechanics by critically evaluating samples with relatively large length scales of the order of millimetres. Mechanical testing of bone at smaller length scales is particularly advantageous when local property measurements defining the properties of the bone material are required. Nanoindentation has provided to be the most widely used technique for probing mechanical properties of bone at micron to sub-micron length scales with a comprehensive review of nanoindentation (Lewis and Nyman, 2008) highlighting local mechanical property measurements and effects of dehydration. In particular, nanoindentation at numerous positions across an osteon within human cortical bone revealed a lower elastic modulus of the wet bone relative to the dehydrated state (Hoffler et al., 2005). Individual bone trabeculae from cancellous human vertebrae also showed a raised elastic modulus with dehydration (Wolfram et al., 2010). The general trend in both large and small scale mechanical testing reveals an increase of the elastic modulus of bone with dehydration and a corresponding lowering of the work of fracture. This observation is irrespective of the testing method used, with bending tests employed extensively at macroscopic sample length scales whereas indentation was employed at micron length scales. The lack of scaling effects in mechanical property changes in bone with dehydration suggests that water content affects the mechanical properties, as indicated by Nyman et al. (Nyman et al., 2006), and does not depend on bone structural hierarchy.

A mechanism to describe the effects of water loss during dehydration on bone mechanical properties was proposed by Nyman et al. for human cortical bone (Nyman et al., 2006). Bone samples held at room temperature showed similar elastic moduli to hydrated bone, slight increases in bending strength and loss of toughness. Samples dried at higher temperatures show an increase in the elastic modulus compared to hydrated samples, with increasing temperature accelerating losses in toughness but significantly showing decreases in strength. Nyman et al. proposed a mechanism where water loss in collagen causes a lowering of bone toughness but water loss at mineral surfaces decreases both bone toughness and strength. An energy-based approach explained how binding of water to mineral is stronger than water–collagen binding. Thus, higher temperatures remove water in collagen and at mineral surfaces but lower temperatures only remove water from the collagen. NMR studies provided further support for this model by defining three states of water within bone (Nyman et al., 2006; Wilson et al., 2006). The first state is free water found in bone pores such as Haversian and Volkmann's canals, canaliculi and lacunae (Yan et al., 2008). This water is expected to play only a minor role in the mechanical properties of bone and its removal during dehydration is not accounted for by Nyman et al. (Nyman et al., 2006). The second state is water loosely bound to collagen via hydrogen bonds; this water fraction can be removed at room temperature and acts as a plasticizer, which protects the collagen by reducing the stress transferred between collagen fibrils in bone when an external load is applied (Wilson et al., 2006). Reducing the stress transfer in bone allows the components, in this case the collagen and the mineral, to slide between one another and avoid failure. Conversely, high stress transfer in bone indicates strong binding between components, which would be effective in transferring the loads throughout the bone. Bone toughness is known to be critically dependent on the stress transfer between components, defined by the hydration state (Nyman et al., 2006; Yan et al., 2008). Loss of the second water state in bone ensures increased stress transfer in components but removes bone plasticization as the components are bound too tightly to slide. Thus, the removal of bone plasticization provides lower overall bone toughness. Finally, mineral-bound structural water has been shown to exist in the imperfect carbonated apatite crystal lattice, providing stability via hydrogen bonding with neighbouring ions and preventing the crystallites from collapsing or rearranging. The removal of this water occurs only at higher temperatures and has been shown to destabilize the mineral and cause significant strength reductions of the bone (Nyman et al., 2006; Wilson et al., 2006; Yan et al., 2008). Similarly the triple helix of tropocollagen would also lose stability if the water molecules forming a highly ordered network with the tropocollagen were removed. The stability of the tropocollagen molecule arises from the formation of additional water-mediated hydrogen bonds in the remaining backbone peptide groups, which would not exist without water due to spatial constraints (Beck and Brodsky, 1998; Bella et al., 1995; Wilson et al., 2006; Yan et al., 2008). Further dehydration models

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Table 1 – Dehydration of bone using increasing concentrations of ethanol in water.

% Ethanol	Time (min)
70%	Storage
90%	60
95%	30
100%	30
100%	30

have been suggested based on the amount of mineral present within the bone material (Currey, 1999a; Currey et al., 2009; Wilson et al., 2006). As biomineralization in bone occurs by replacing water, bone with low mineral content will contain more water than highly mineralized bone. Dehydration processes will therefore cause more structural, and thus mechanical, changes in bone with relatively low mineral content (Currey, 1999a; Utku et al., 2008).

While the studies detailed above examine the dehydration of bone and the corresponding effects on its mechanical behaviour, little work has been done to examine the effects of vacuum conditions on bone structure. Observation of water loss from various regions resulting in dimensional contractions in bone has been directly observed by environmental scanning electron microscopy (Utku et al., 2008) but the corresponding effects on mechanical behaviour have been lacking. The evaluation of bone mechanics using techniques involving SEM has distinct advantages compared to previous works. Principally, observation and manipulation of relatively small volumes of bone can be achieved using the SEM, potentially in conjunction with other testing methods such as in situ mechanical testing (Hang and Barber, 2011; Koester et al., 2008), so that the properties of the bone material can be evaluated. The mechanical testing of these relatively small volumes is advantageous when compared to larger scale testing as structural hierarchy effects can be potentially ignored or simplified. Such simplification allows the study of the material properties of bone and not whole bone behaviour. Discrete bone volume mechanical testing can also be used in order to assess the effects of the SEM vacuum chamber on potential structural changes in bone due to water removal.

Previous literature has highlighted the use of focused ion beam (FIB) microscopy to isolate micron-sized cantilevers from teeth for subsequent bending tests (Chan et al., 2009). The dual beam system setup allows FIB technology to be combined with the structural analysis of a scanning electron microscope (SEM) (Utku et al., 2008). The capacity to isolate discrete bone volumes within a dual beam system using the FIB and observe structural changes using SEM is therefore persuasive but mechanical testing capability within the SEM is required. Critically, dual beam instruments operate in partial vacuum that have the capacity to dehydrate bone material. Recent work has used an atomic force microscope (AFM) within a dual beam system to mechanically test individual a range of biological and synthetic nanofibres within a vacuum environment (Hang and Barber, 2011; Hang et al., 2011). Collagen fibrils from antler bone mechanically tested using AFM within the chamber of the SEM was critically shown to fail heterogeneously (Hang and Barber, 2011), displaying similar behaviour to fully hydrated antler bone evaluated using small angle X-ray scattering (Krauss et al., 2009). The similar mechanical behaviour of collagen fibrils in the SEM and a fully hydrated sample indicates that dehydration of bone in a vacuum chamber does not have an effect on the mechanical properties at nanometre length scales. This paper extends mechanical testing of bone material further by using a combination of AFM and dual beam system to bend micro cantilevers of bone. In particular, the effects of different environmental conditions on the mechanical properties of bone at micron length scales using AFM while observing using in situ SEM is investigated.

2. Materials and methods

Femora from 8 month old sprague dawley rats were used in all of our investigations. The mechanical properties of individual micron-sized units of bone were evaluated by isolating individual beams from the parent material using a multi-stage process. The diaphysis from the extracted rat femur was first isolated using a water cooled diamond blade slow speed circular saw (Buehler, USA). Beams of cortical bone with dimensions of $12\times1\times1$ mm were produced and stored in 70% ethanol. Bone samples were further dehydrated by submerging in a series of water/ethanol solutions, summarized in Table 1, prior to FIB milling. The dehydrated bone was gold coated and, using a two part epoxy glue (Poxipol, Arg.), fixed to the sample stage of a dual beam microscope (Quanta 3D, FEI, USA/EU) incorporating both SEM and FIB. Samples are required to be dehydrated and gold coated in the dual beam instrument to avoid charging effects that could interfere with the FIB milling process and to prevent drying cracks occurring in the sample from the vacuum pumping system. FIB milling was performed by a succession of processes summarized in Fig. 1. Briefly, FIB was used to remove material in order to produce discrete beam volumes by first polishing a bone sample edge using a high current ion beam of 65 nA and accelerating voltage of 30 kV. Flattening of the bone edges allows further removal of smaller bone volumes using smaller ion beam currents down to 0.1 nA. The smaller ion beam currents avoid observable ion beam damage. In addition, the FIB milling is always performed parallel to the produced sample faces to reduce embedding the impinging gallium ions from the FIB within the discrete beam volumes produced. The resultant FIB process allowed the fabrication of micro cantilever beams with dimensions $10\times2\times2$ μm as shown in Fig. 2, with the long axis of the cantilever beam parallel to the long axis of the rat femur.

Samples were rehydrated before mechanical testing by removing from the dual beam chamber and placing for two hours in a closed vessel containing a high vapour concentration of Hank's buffer solution. We note that equine bone samples have been observed to require only 45 min for rehydration (Utku et al., 2008). Such a hydration process was considered more suitable than submerging the sample in water, which in our experience results in the surface tension of the water deforming the relatively fragile micro cantilever beams sufficiently to cause fracture. Mechanical testing of the individual bone beams, as shown in Fig. 2(b), was performed using a custom build AFM (Attocube GmbH, Ger.) incorporated within the dual beam chamber. Mechanical testing was facilitated by moving the AFM probe into contact with the FIB milled micro cantilever beams in three different

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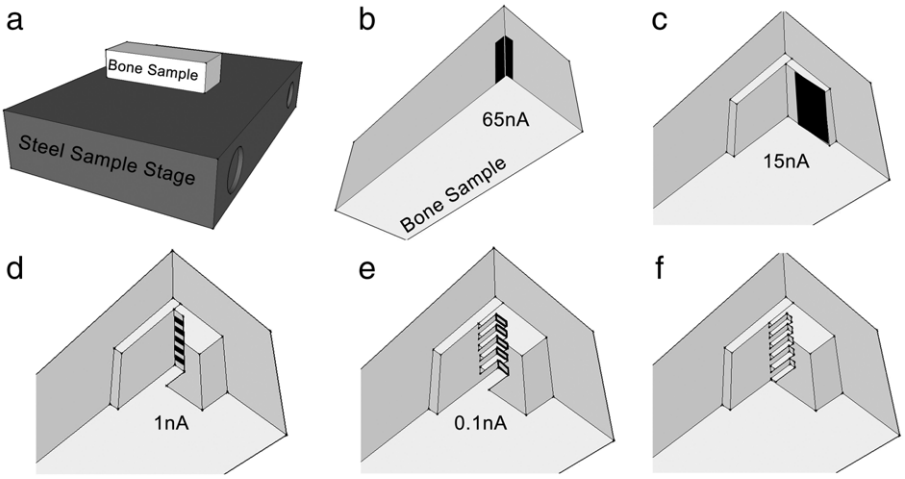

Fig. 1 – Schematic of the Focused Ion Beam (FIB) milling process. The black rectangles mark the FIB milled area. (a) bone sample mounted on AFM steel sample stage, (b) initial edge cleaning cut using a current of 65 nA, (c) separation of bulk from edge using 15 nA, (d) isolation of beams using 1 nA, (e) fine cutting and shaping of beams using 0.1 nA, (f) finalized sample showing 8 beams across. All cuts performed with an acceleration voltage of 30 kV.

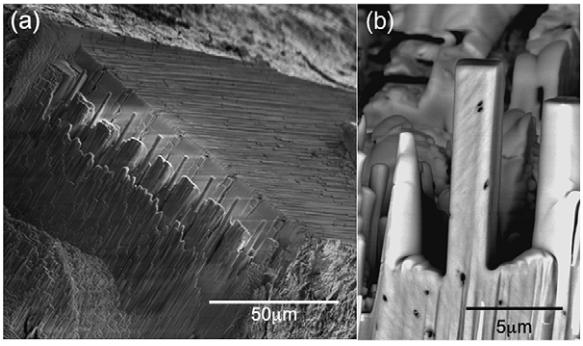

Fig. 2 – SEM micrographs indicating (a) a series of beams fabricated from the parent rat bone sample and (b) higher magnification image showing an individual bone micro cantilever beam.

environments: (i) high vacuum (5.25×10^{-4} Pa) in the chamber of the dual beam system, (ii) low vacuum (120 Pa pressure provided by water vapour) in the chamber of the dual beam system and (iii) removal of the sample from the vacuum chamber for beam bending wet, in air. This testing in air environment consisted of placing wet tissue around the bone sample and covering in order to provide a high humidity environment for sample rehydration. Samples were kept in the closed vessel for two hours to ensure the samples were fully hydrated prior to testing in each of the vacuum and air conditions. Fig. 3 shows an SEM image of the AFM bending tests on an individual bone micro cantilever beam. The bone micro cantilever beam was deformed using a FIB flattened AFM probe, to avoid AFM probe indentation into the sample, with a AFM cantilever spring constant of 28 N m^{-1} operating at an approximate testing rate of 0.2 μm s^{-1}. AFM beam bending was performed for two hours in each of the three different environments. Three small cantilever beams were tested in all three environments. The testing procedure took a total of 12 h for each beam: two hours of testing in each environment with intervals of two hours inside the closed vessel with a high vapour concentration of Hank's buffer solution in order to rehydrate the samples before each test.

To assess the rehydration processes used in this paper, the weight of whole 2 mm thick cross-sections of diaphysis of rat femora were measured following the same preparation process as described for the micro-beam samples above. Table 2 records the weight loss measured for three rat femora samples subjected to the various environments used in the experimental preparation. Bone samples were initially held in Hank's buffer solution for 2 h. The bone samples were removed from the solution and, after removing excess surface water with filter paper, weighed using an electronic analytical microbalance (Sartorius, Germany) with μg accuracy. The weights of these bone samples were taken as fully hydrated bone weight. Further preparation processes were recorded by the percentage of weight lost by the bone samples relative to this fully hydrated bone weight as shown in Table 2. The bone samples were first exposed to ethanol treatments, as indicated in Table 1, and resulted in a bone weight loss of approximately 3%. Exposure to the vacuum conditions of the SEM chamber used for FIB milling caused a further 9% weight loss in the bone samples. The total weight loss from the bone samples during the ethanol and vacuum exposure was 12.56 ± 0.97%. Previous literature indicates that up to 12% weight loss during dehydration of bone is due to the removal of free water from pores such as Haversian and Volkmann's canals, canaliculi and lacunae (Nyman et al., 2006; Yan et al., 2008). The initial bone weight loss of 3% during ethanol treatment therefore indicates a partial removal of the free water in bone whereas exposure to the SEM vacuum removes the remaining free water. Rehydration of bone samples for two hours in a high vapour concentration of Hank's buffer solution recovers some of the free water and reduces the weight loss of bone to 3.63 ± 0.49% relative to the fully hydrated bone weight. Repeating the bone exposure to vacuum conditions removes all of the contained free water, resulting in the total bone weight loss of 12%. Repeating the rehydration of bone and subsequent vacuum exposure

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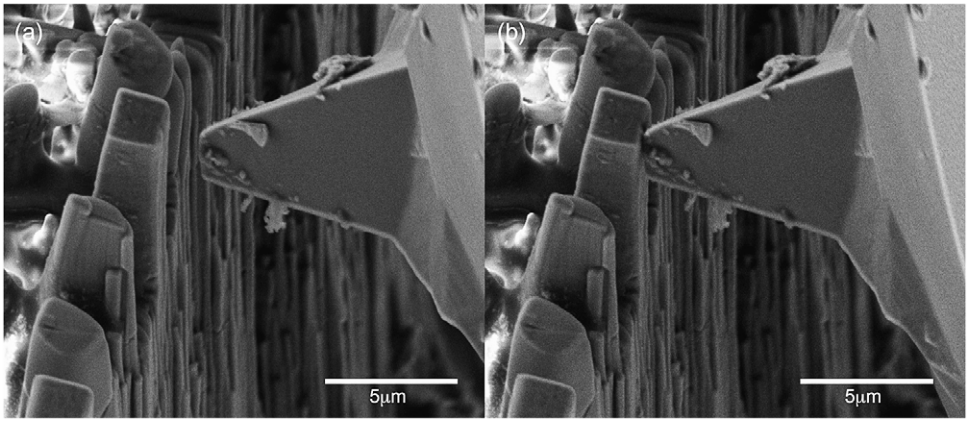

Fig. 3 – SEM micrographs showing (a) the AFM probe before contact with an individual rat bone beam and (b) contact of the probe with the bone beam for mechanical bending tests.

Table 2 – Change in weight, as a percentage, of whole rat bone femur cross sections with exposure to various environmental conditions. The exposure conditions mimic those of the mechanically tested bone micro cantilevers. A total of three samples were tested and the weight difference shown relative to bone rehydrated in Hank's buffer solution.

	Time	Loss in bone weight relative to hydrated in Hank's buffer solution (%)
Soaked in Hank's buffer solution	2 h	0
Dehydrated (see Table 1)	3.5 h	3.29 ± 0.5
Vacuum dried (High Vacuum 1.51×10^{-3} Pa)	2 h	9.27 ± 0.47
Rehydrated in closed vessel with high vapour concentration of Hank's buffer solution	2 h	3.63 ± 0.49
Vacuum dried (High Vacuum 1.51×10^{-3} Pa)	2 h	9.97 ± 0.55
Rehydrated in closed vessel with high vapour concentration of Hank's buffer solution	2 h	3.70 ± 0.27

produced repeatable weight loss values, as would be expected if the rehydrating conditions replacing water and the vacuum conditions removing the water were consistent. We therefore conclude that the rehydration and dehydration processes remove free water only and not the bound states of water in the bone.

3. Results and discussion

The force applied by the AFM causes a corresponding deflection in the bone beam during the mechanical bending tests. The AFM data is gathered from an interferometer which measure the changes in the distance between the AFM probe and the optical fibre as the AFM probe bends. Instead of a linear curve as produced by a typical mechanical testing machine, the AFM system outputs a sinusoidal curve representing the change in distance in terms of stage movement versus the change of laser intensity caused by the change in distance due to the bending of the AFM cantilever. The sinusoidal curve is then translated into a force–displacement curve. Further details of the AFM mechanical testing have been published previous and shown to be effective in measuring even subtle changes in the mechanical performance of a range of materials sensitive to hydration effects (Hang et al., 2011).

The force–displacement curves for one of three rat bone beams tested in bending in high vacuum, low vacuum and wet in air after 10 min of exposure to each environment are shown in Fig. 4. The bending tests were conducted up to beam displacement of ~1.5 μm, and show a linear force–displacement relationship. The gradient of the linear region ($df/d\delta$) of the force–displacement curves in Fig. 4 can be used to calculate an effective elastic modulus of the rat bone beam, E, using:

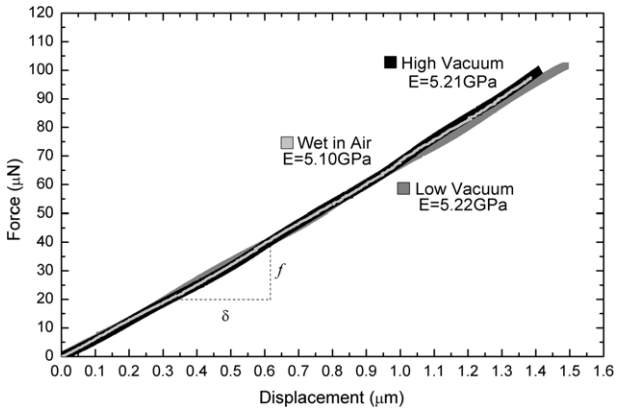

Fig. 4 – Force–displacement plot for AFM mechanical bending of one of the three rat bone beams tested under high vacuum, low vacuum and wet in air environments.

$$E = \frac{12l^3}{3bh^3} \cdot \frac{f}{\delta} \quad (1)$$

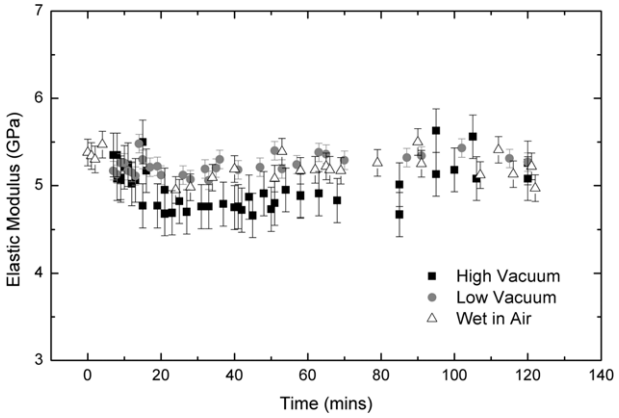

Fig. 5 – Plot of calculated elastic modulus, determined from AFM bending of one of the three rat bone beams, with time under high vacuum, low vacuum and wet in air environments.

where l, b and h are the length from the base of the sample to testing contact point, breadth and height of the rat bone beam respectively. Typical geometric values of the rat bone beam are $l = 10$ μm, $b = 2$ μm and $h = 2$ μm.

The elastic modulus values calculated from Eq. (1) for one of the three rat bone beams tested under different environmental conditions with increasing exposure time are shown in Fig. 5, with the error in E values calculated from the standard deviation of the values for the elastic modulus arising, among other things, from the changes of the contact point during testing. The elastic modulus shows little change either with environment or time of exposure in the SEM chamber. This result is somewhat surprising as an increase in the elastic modulus is expected because of dehydration effects (Currey et al., 2009; Hoffler et al., 2005; Lewis and Nyman, 2008; Morais et al., 2010; Nyman et al., 2006; Wilson et al., 2006). Fig. 5 indicates that the elastic modulus of the rat bone beam in high vacuum is 4.98 ± 0.25 GPa, low vacuum is 5.24 ± 0.11 GPa and wet, in air is 5.22 ± 0.15 GPa and does not vary greatly over the time period examined. Previous mechanical testing on fully hydrated whole rat bone femur using 3-point bending configuration gives an elastic modulus of 5.12 ± 0.77 GPa (Kasra et al., 1997), 8.0 ± 0.4 GPa (Barengolts et al., 1993), 6.88 ± 0.31 GPa (Jorgensen et al., 1991), and 4.9 ± 0.4 GPa (Ejersted et al., 1993) which is similar to the calculated elastic modulus values in our work and indicates that the vacuum of the SEM chamber does not have an effect on elastic modulus of the samples over the time period investigated. Interestingly, the similarity between the elastic modulus of our relatively small bone volumes and whole bone testing suggests an effective transfer of stresses throughout the bone material, indicating that all bone components are not failing or slipping but are tightly bound to one another. We note that the calculated elastic modulus values using Eq. (1) assume shear within the beam is insignificant. Using classic beam bending theory (Blodgett, 1991), the deflection of the beam consists of a deflection due to shear and a deflection due to bending. The total deflection can therefore be written as:

$$\delta = \delta_{\text{bending}} + \delta_{\text{shear}} = \frac{Pl^3}{3EI} + \frac{6Pl}{5AG} \quad (2)$$

where δ is total beam deflection, P is load, l is length from the base to the contact point, E is elastic modulus, I is the moment of inertia, A is the cross sectional area and G is the shear modulus which is calculated theoretically from $G = E/[2(1 + \nu)]$, with $\nu = 0.35$ (Akiva et al., 1998). The beams used in this work have an aspect ratio of 5:1. Therefore, using Eq. (2) above, the shear contribution to the total deflection is ~3.5%. This shear contribution is much smaller than the bending contribution, indicating that the majority of beam mechanical deformation results from pure bending. In addition, possible indentation of the AFM probe with the bone beam sample will cause inaccuracies in the calculated elastic modulus. However, neither direct SEM imaging of the mechanical testing procedure or subsequent SEM examination of the AFM probe–sample contact point showed evidence of indentation on the surface of the cantilever bone beams being tested.

Fig. 5 highlights the lack of environmental influence on the mechanical properties of the bone micro cantilevers. The removal of water using ethanol treatments described in Table 1 and the vacuum conditions used have been shown only to remove the free water in bone samples as shown in Table 2. The mechanical properties of the bone micro cantilevers tested in this work therefore do not change as only free water state is removed during exposure to the different environmental conditions used. Our results correlate with previous work suggesting that the effects of free water on the mechanical properties of bone is minor (Nyman et al., 2006). Previous work indicating changes in the elastic modulus of bone with dehydration time (Currey et al., 2009; Hoffler et al., 2005; Lewis and Nyman, 2008; Morais et al., 2010; Nyman et al., 2006; Wilson et al., 2006) use more aggressive drying conditions, which would be expected to remove not only free water but also the bound water states that influence mechanical behaviour. Our recorded bone elastic modulus values suggest that water removal responsible for mechanical property changes during dehydration is due to removal of the second and third states of water, as defined by Nyman et al. (Nyman et al., 2006), or potentially operate at length scales above those of the micro cantilever beams used in this work. Thus, the second and third states of bound water in collagen and at mineral surfaces in bone respectively are not removed even with the highest vacuum conditions of the SEM. The SEM high vacuum conditions must also be less invasive than the application of the high temperatures (reported in the range of 60–140 °C) used to remove water during complete collagen dehydration (Renugopalakrishnan et al., 1989).

4. Conclusions

Mechanical beam bending of discrete rat cortical bone volumes of the order of 40 μm^3, isolated as micro cantilever beams using FIB, was performed by AFM within the chamber of a dual beam system. The bending behaviour of the rat bone beams was recorded in high and low vacuum SEM environments as well as in air. Calculated elastic modulus values of the rat bone were found to be independent of both the environment used and the time of exposure to each of these environments. Our results suggest that the bone material still remains partially hydrated even in the most potentially dehydrating conditions of high vacuum

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and indicate that a time window of opportunity exists to mechanically test bone material in high vacuum while retaining the mechanical properties of hydrated bone. These results indicate an opportunity to mechanically deform hydrated bone materials in vacuum environments while observing using in situ scanning electron microscopy.

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