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Compressive behaviour of uniaxially aligned individual mineralised collagen fibres at the micro- and nanoscale

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Abstract

The increasing incidence of osteoporotic bone fractures makes fracture risk prediction an important clinical challenge. Computational models can be utilised to facilitate such analyses. However, they critically depend on bone’s underlying hierarchical material description. To understand bone’s irreversible behaviour at the micro- and nanoscale, we developed an in situ testing protocol that allows us to directly relate the experimental data to the mechanical behaviour of individual mineralised collagen fibres and its main constitutive phases, the mineralised collagen fibrils and the mineral nanocrystals, by combining micropillar compression of single fibres with small angle X-ray scattering (SAXS) and X-ray diffraction (XRD). Failure modes were assessed by SEM. Strain ratios in the elastic region at fibre, fibril and mineral levels were found to be approximately 22:5:2 with strain ratios at the point of compressive strength of 0.23 $\pm$ 0.11 for fibril-to-fibre and 0.07 $\pm$ 0.01 for mineral-to-fibre levels. Mineral-to-fibre levels showed highest strain ratios around the apparent yield point, fibril-to-fibre around apparent strength. The mineralised collagen fibrils showed a delayed mechanical response, contrary to the mineral phase, which points towards preceding deformations of mineral nanocrystals in the extrafibrillar matrix. No damage was measured at the level of the mineralised collagen fibre which indicates an incomplete separation of the mineral and collagen, and an extrafibrillar interface failure. The formation of kink bands and the gradual recruitment of fibrils upon compressive loading presumably led to localised strains. Our results from a well-controlled fibrillar architecture provide valuable input for micromechanical models and computational non-linear bone strength analyses that may provide further insights for personalised diagnosis and treatment as well as bio-inspired implants for patients with bone diseases.

Keywords: Mineralised collagen fibre, In situ micromechanical testing, Nanoscale deformation mechanisms, SAXS/XRD, Failure analysis, Compressive strength
Statement of significance

Musculoskeletal diseases such as osteoporosis, osteoarthritis or bone cancer significantly challenge health care systems and make fracture risk prediction and treatment optimisation important clinical goals. Computational methods such as finite element models have the potential to optimise analyses but highly depend on underlying material descriptions. We developed an in situ testing set-up to directly relate experimental data to the mechanical behaviour of bone’s fundamental building block, the individual mineralised collagen fibre and its main constituents. Low multilevel strain ratios suggest high deformations in the extracellular matrix and energy dissipation at the interfaces, the absence of damage indicates both an incomplete separation between mineral and collagen and an extracellular interface failure. The formation of kink bands in the fibril-reinforced composite presumably led to localised strains. The deformation behaviour of a well-controlled fibrillar architecture provides valuable input for non-linear bone strength analyses.
1 Introduction and Motivation

Musculoskeletal diseases such as osteoporosis, osteoarthritis or bone cancer cause serious personal and socio-economic burdens. Osteoporosis, for instance, is characterised by a decrease in bone density and structural integrity which lead to a reduction of bone strength [1, 2]. It affects 200 million women and is the cause of 9 million fractures per year worldwide. Those fractures are the most common cause of accident-related deaths in older people [3] and are expected to rise with an increasing average age of the population. Therefore, fracture risk prediction is a major clinical objective and computational finite element modelling has the potential to optimise treatment by providing a convenient way to calculate fracture risk under multiple loading conditions [4–7]. To develop realistic constitutive and quantitative predictive models for bone stiffness and failure, a profound understanding of the underlying material description and structure-mechanics relationships is essential [8–10]. In addition, bone replacements and scaffold materials in tissue engineering critically depend on realistic bio-inspired materials whose development relies, in turn, on experimental data gathered from tests at the material’s different hierarchical levels [11–13].

Bone is a hierarchical material and is organised along multiple length scales. It has three main constituents, collagen, mineral and water. At the nanoscale, mineralised collagen fibrils comprise type I collagen molecules and mineral platelets, primarily carbonated hydroxyapatite [14, 15]. The mineralised collagen fibrils are embedded in an extracellular matrix and bundles of parallel fibrils combine into fibril arrays forming the mineralised collagen fibre at the microscale [16–19]. The extracellular matrix act as a glue layer between the fibrils and consist of non-collagenous proteins, proteoglycans, extracellular minerals and water [20–24]. Mineralised collagen fibrils and fibres represent characteristic elementary units of bone, the latter being bone’s fundamental building block at the microscale [17, 25–27]. Bone shows a complex mechanical behaviour with different apparent mechanical properties at various length scales. As a result, the governing deformation mechanisms need to be investigated at multiple hierarchical levels [16, 17, 19, 25, 28, 29]. While the mechanical behaviour is well understood at the upper levels of tissue organisation, there is still limited knowledge on the irreversible mechanical behaviour at the lower levels [30], partially based on the lack of experimental data that directly relate to the mechanical behaviour at the lower length scales. Morphological and mechanical data can further be used to calculate the macroscopic yield behaviour based on homogenisation approaches [8, 31–33]. For an accurate representation as well as prediction of bone’s apparent mechanical behaviour under different loading conditions, detailed information from lower length scales is needed [28, 34, 35].

We use a model system for bone, the mineralised turkey leg tendon, which also consists of mineralised collagen fibres but has the advantage of a uniaxial fibre arrangement. At the level of the mineralised collagen fibre, it shows the same hierarchical structure as bone [21, 36–40]. With respect to osteonal bone, the fibre arrays have their equivalence in bone’s sub-lamellae where the mineralised collagen fibres show a slightly larger mean diameter
than in bone tissue [41]. The collagen molecules within the mineralised collagen fibrils are staggered axially by a shift with respect to each other, referred to as the D-period representing a periodic electron density profile along the fibril’s axis. The mineral particles are located within the gap zones of the collagen molecules and thus show the same staggered arrangement along the fibrils’ longitudinal axis [14, 15, 36, 39]. The highly-ordered arrangement of this calcified collagen in the axial direction gives rise to well oriented diffraction patterns in small-angle X-ray scattering (SAXS) [36] and wide-angle X-ray diffraction (XRD, WAXD) measurements with additional mineral in the extrafibrillar matrix contributing to the signal [24, 36, 42]. Both morphological regions of the mineralised tendon, the circumferential zone and the interstitial zone [40, 43] consist of unidirectionally aligned mineralised collagen fibres. Compared to the circumferential zone, the interstitial zone shows a higher microporosity and its fibres a larger diameter. For the current study, mineralised collagen fibres from the interstitial region were chosen due to their larger diameter and thus the possibility to centre the sample preparation process on a single fibre.

Micropillar compression is a micromechanical testing technique which allows a straightforward interpretation of the results at the level of the extracellular matrix [29, 35, 44–46]. To obtain deformations in the calcified collagen network (mineralised collagen composite), i.e. of the mineralised collagen fibrils, as well as the mineral nanocrystals, mechanical testing can be combined with structural measurements by means of SAXS and XRD, respectively. Fibril strains can be assessed based on the radial shift of the axial peaks of the SAXS pattern reflecting changes of the D-period, initially in the range of 65 nm and 67 nm [15, 36, 47–62]. In addition, the analysis of the radial shift of XRD peaks allows assessing changes in the lattice spacings within the carbonated hydroxyapatite nanocrystals providing the mineral strains [47, 52, 53, 55, 56, 58, 61, 63]. The combination of SAXS and XRD thus allows to quantify the deformation behaviour of the fibre’s two constitutive phases, the mineralised collagen fibril representing the mineralised collagen composite (calcified collagen phase), and the mineral nanocrystals (mineral phase).

The referenced studies on SAXS and XRD were based on probing macroscopic bone samples with X-rays under tensile and compressive loading at the millimetre length scale but not at the extracellular matrix level and below. Apart from Karunaratne et al. [62] who used a beamsize of 10 µm x 12 µm, these studies report beamsizes of 50 µm to a few 100 µm. None of these studies tested a mineralised collagen fibre individually to investigate the fibre’s apparent mechanical behaviour and its constitutive phases directly.

Ascenzi et al. [64–66] tested individual osteons with dimensions of a few hundred µm under tension and compression including a combined set-up with X-ray diffraction measurements. The authors report cyclic testing of cylindrical samples of individual osteons of 400 µm in length and 230 µm in diameter and an X-ray beam of 3 mm x 1 mm. Exposure times from 1 to 24 hours were reported.

A nano-mechanical tensile testing set-up for mineralised collagen fibrils and fibrils partially embedded in a fibril bundle was developed by Hang and colleagues [67, 68] based on a combination of atomic force microscopy (AFM) and scanning electron microscopy (SEM).
Antler bone samples of mm size were fractured and exposed fibrils were stretched with an AFM tip on which a glue was deposited. The authors state that the AFM probe was positioned perpendicular to the fracture plane and along the principal axis of the fibril during the experiments. Nevertheless, a deviation from this perpendicular alignment is highly likely based on using randomly distributed fibrils from a fractured surface. In addition, the set-up does not allow for a simultaneous quantification and interpretation of deformations of individual mineralised collagen fibres and its constituents.

The ultrastructural features of bone can be assessed by means of synchrotron X-ray phase nano-tomography [69–72], polarised Raman microscopy [72–75], electron diffraction and STEM tomography [76]. However, to uncover the mechanical behaviour at the length scale of the individual mineralised collagen fibre and its constituents, an integrative approach is needed combining mechanical testing and structural measurements in situ. The combined experimental testing of micropillars from individual mineralised collagen fibres allows us to bring the set-up down to a length scale where uncertainties can be minimised and data can be directly related to the mechanical behaviour of bone’s fundamental building block and the deformation behaviour of its constitutive phases.

Therefore, we aimed at (i) extracting micropillars from individual mineralised collagen fibres, (ii) combining micropillar compression tests with simultaneous SAXS or XRD measurements using a beam size of a few micrometres and (iii) quantifying the tissue’s multiscale mechanical response at the very level of the mineralised collagen fibre. The approach allows us to identify the fibre’s apparent mechanical behaviour as well as the mechanical response of its constituents at various stages of compressive loading including the post-yield region. The fibre’s failure patterns were investigated by means of a SEM-based failure mode analysis.

2 Materials and Methods

2.1 Extraction of micropillars from individual mineralised collagen fibres

Ten turkey legs were obtained from a local abattoir and kept frozen at -22°C until dissection. After thawing, surrounding tissues were removed and a bundle of flexor tendons of the tarsometatarsus (part of the lower leg) was dissected. From this bundle, a highly mineralised tendon [21, 77] with about 100.0 mm in length and about 4.0 mm in width was separated.

The separated tendon was the same for every specimen and further cut with a diamond band saw (Exakt, Norderstedt, Reichert-Jung) and a scalpel in transverse and longitudinal direction. Resulting tendon pieces of about 1.5 mm in diameter and 10.0 mm in length were dried at ambient room temperature for 24 h before being glued into a cylindrical aluminium sample holder using a 2-component epoxy resin adhesive (Schnellfest, UHU, Germany) so that a 2.0 mm tendon piece was exposed for further machining. The free end was polished with an ultramiller (Polycut E, Reichert-Jung, Germany) to create a plane surface for the compression tests (Figure 1).

Ultra-short pulsed laser ablation [78] was used to cut pre-pillars of approximately 32-34 µm
in diameter and 50-55 µm in height (TruMicro 5250-3C, Trumpf, Germany) centred around a single mineralised collagen fibre. Fibres were chosen at the edge of the tendon piece and surrounding material was removed as to allow for simultaneous micropillar compression and X-ray scattering/diffraction measurements. For the current study, mineralised collagen fibres were selected from the tendon’s interstitial region to facilitate the fabrication of micropillars from single mineralised collagen fibres. The beam-spot diameter was 20-25 µm, the pulse duration 6 ps and the pulse repetition frequency (PRF) 1 kHz, operating at a wavelength of $\lambda = 515$ nm. For the laser scanning pattern, a spiral inbound anticlockwise hatch style was used at a scanning speed of 2 mm/s. Separate rectangular and circular scanning patterns were programmed for the removal of the surrounding material and the fabrication of the actual pre-pillar, respectively. The used laser parameters corresponded to a fluence of 3.86 J/cm$^2$ and a beam overlap of 89.90%. This led to a plasma mediated ablation process and a Coulomb explosion minimising the thermal impact and the heat affected zone [79, 80]. The heights of the pre-pillars were quantified by means of three-dimensional surface topography profiles using non-destructive focus-variation microscopy [81]. Regions of interests (ROIs) were identified with a light microscope preceding the ablation process (Alicona Infinite Focus, Austria) (Figure 1).

Focused ion beam milling (FIB) (dual FIB-SEM Quanta 3D FEG, FEI, USA), operated at $E = 30$ keV, was used to cut the final micropillars. Since the diameter of the beam correlates to the beam current, it was reduced along three successive steps. A coarse milling step at $I = 7.0$ nA machined a pillar of 10-12 µm in diameter and 12-15 µm in height, a second step at $I = 0.5$ nA milled down the pillar to 7.5-8.5 µm in diameter and a third step at $I = 0.3$ nA polished the pillar to the final diameter of 6.0-7.5 µm. The diameter, height and aspect ratio (mean ± s.d.) of the micropillars were determined from SEM images ($E = 5$ keV and 52° specimen tilt): diameter = 6.08 ± 0.83 µm, height = 12.44 ± 1.78 µm, aspect ratio = 2.12 ± 0.23 (Figure 1). To minimise the drift, samples were sputtered with gold and a silver paste was applied next to the tendon piece to close the gap between the inner edge of the aluminium cylinder. Occurring beam shifts were corrected manually.

To assess possible effects of the FIB induced Gallium implantation on the fibre’s mechanical behaviour, Monte Carlo simulations of ion-solid interactions were performed for two different incident angles accounting for the different milling steps using the software SRIM [82]. The results show that the layer influenced by the Gallium ions is confined to a mean depth of 29.44 ± 0.03 nm in x-direction at an incident angle $\theta = 0^\circ$, 27.84 ± 0.03 nm in y-direction at an incident angle $\theta = 70^\circ$ and layers below 0.1 nm in remaining directions. All layers can thus be considered negligibly thin compared to the micropillars’ average diameter [83, 84] (see supplementary material).

2.2 Combined micropillar compression and SAXS/XRD

A portable custom-built microindenter (Aleminis AG, Switzerland) with a diamond flat punch (Synthon MDP, Switzerland) was aligned with the experimental set-up at the microfocus beamline ID13 of the European Synchrotron Radiation Facility (ESRF) to allow
Figure 1: The overview illustrates the extraction of individual mineralised collagen fibres (MCFs) by means of dissection, ultra-milling, ultra-short pulsed laser ablation and focused ion beam milling. The SEM image shows the final micropillar. The extracted mineralised collagen fibre was tested mechanically until failure by means of micropillar compression while small angle X-ray scattering (SAXS) or X-ray diffraction (XRD) measurements were acquired. ROI = region of interest.
transmitted beam, was optimised to allow measuring SAXS at sufficiently low angles while allowing moving the detector sufficiently close to the sample for large angle measurements. The samples were compressed uniaxially in a displacement-controlled test to 1.5 µm deformation at 5 nm/s including 50 nm partial unloading steps every 150 nm while load, displacement and time data were recorded throughout the measurements.

Two groups of samples were used to measure either SAXS or XRD to obtain the deformation of the mineralised collagen network (fibril strain; strain in the calcified collagen phase) and mineral nanocrystals (mineral strain; strain in the mineral phase), respectively. A single acquisition was taken every 5 s at E = 13.3 keV. The exposure time per acquisition was 75 ms for SAXS and 185 ms for XRD for a total of 120 acquisitions per sample where the samples were not exposed to radiation between consecutive acquisitions. The beamsize was focused to ≃ 5.5 µm in vertical direction and ≃ 7.0 µm in horizontal direction using a combination of Be lenses and a transfocator. The samples were positioned at the focal spot at a location within the micropillar which was chosen to be the same for every sample. To reduce the exposure time, the scattering data were acquired using a single-photon counting detector (Eiger, Dectris, Switzerland). Selected exposure times for both the SAXS and the XRD regime were chosen in order to minimise effects of X-ray radiation on the deformation and fracture behaviour of the tissue [85, 86] while ensuring a sufficient X-ray scattering signal intensity for the data analysis. The influence of radiation on the mechanical behaviour of the samples was further assessed by relaxation tests in both the SAXS and XRD groups. For this, a constant displacement of 0.3 µm was applied while X-ray acquisitions were taken.
according to the measurement protocol in the SAXS regime and without intermediate time steps in the XRD regime. The relaxation characteristics in the irradiated and non-irradiated state of the micropillar were compared based on fitting an exponential function via a non-linear regression model to the relaxation data using R [87] (see supplementary material). Any influence of irradiation was further quantified based the continuously recorded load and displacement data for the mineralised collagen fibres during the actual compression test. This also included the analysis of the stress-strain curves and the stiffness evolution in the elastic and plastic regions based on the unloading segments. A beam induced deterioration of the material can be quantified at the fibre level with the micropillar testing (see 2.3).

2.3 Integrated mechanical and structural analysis

Custom codes written in Python were used to analyse the mechanical data at the fibre level (Python Software Foundation, Python Language Reference, version 2.7), primarily based on the SciPy [88] and NumPy [89] packages. Base- [90, 91] and frame-compliance corrected engineering strains $\varepsilon_{eng}$ in the tendon’s axial direction were determined based on the initial unloaded condition and geometric dimensions of the micropillars, compliance-corrected engineering stresses in the tendon’s axial direction $\sigma_{eng}(\varepsilon_3 \otimes \varepsilon_3)$ based on the micropillar’s surface area. Assuming constant volume (i.e. the Jacobian of the deformation gradient $J(X,t) = det (F(X,t)) = 1$ in $\sigma = J^{-1} P F^T$), the data were transferred to true stress-strain values, $\sigma_{33}$ and $\ln(U_{33})$ (referred to as $\varepsilon_{33}$ within the text), according to the following relations [35, 92]:

$$\sigma_{33} = \sigma_{eng}(1 + \varepsilon_{eng})$$

(1)

$$\ln(U_{33}) = \ln(1 + \varepsilon_{eng})$$

(2)

Stress-strain data were fitted with a natural cubic spline with ten degrees of freedom (df) using the inflection points of loading and (partial) unloading segments as nodes. This envelope curve was used to extract yield stress $\sigma_{33}^{yield}$ and yield strain $\varepsilon_{33}^{yield}$ which were determined based on the 0.2% - offset criterion (Figure 1). Strength $\sigma_{33}^{str}$ and ultimate strain $\varepsilon_{33}^{ult}$ were identified via the maximum of the envelope. Apparent moduli $E_{app}$ were calculated via a least squares regression as the slopes of the (partial) unloading segments in the elastic and post-yield region with the fibre’s apparent Young’s modulus $E_{app}^{fibre}$ identified as the last stiffness value before the yield point (Figure 1). Plastic strains $\ln(U_{33}^p)$ were calculated based on the intersection of the 0.2% - offset criterion used to calculate the yield point and the apparent moduli values identified in the post-yield region [35] according to:

$$\ln(U_{33}^p) = \ln(U_{33}) - \frac{\sigma_{33}}{E_{app}}.$$

(3)

To estimate damage as stiffness reduction, apparent moduli in the plastic region were normalised to the fibre’s apparent Young’s modulus value. A least squares regression was used to assess the stiffness evolution.
Since no direct measurements of stresses were accessible in the mineral phase and the calcified collagen phase, with the latter representing the cooperative deformation of the mineralised collagen fibrils, apparent pseudo-stiffness values in the mineral phase $E_{\text{mineral}}^{\text{app}}$ and mineralised collagen fibrils $E_{\text{fibril}}^{\text{app}}$ were identified as slopes of the least squares regression of apparent true stresses plotted against mineral and fibril strains in the elastic region, respectively. Although only based on the applied apparent stress, the comparison of the apparent pseudo-stiffness at the different levels provides a mechanistic insight on how load is transferred between the phases. In this respect, protocols from the literature [56, 58, 93–95] were followed, to also allow for a direct comparison with reported values and to avoid the assumption of non-measured experimental data such as density values, mineral volume fractions and aspect ratios of the constituents that influence their moduli [34].

Fibril and mineral strains were analysed based on SAXS and XRD patterns recorded at different stages of the loading and (partial) unloading segments. The sharp Bragg peaks in the SAXS pattern (meridional reflections) can be attributed to the periodicity gaps in the axial arrangement of the intrafibrillar collagen molecules, at least partially filled by mineral nanocrystals [15, 49, 96–98]. Fibril strains were determined by analysing changes of the D-period along the loading protocol with an unloading state as the reference. The SAXS pattern thus represents the deformation behaviour of the mineralised collagen fibrils in the used X-ray scan window. In detail, the calculation of strain was based on the initial X-ray acquisition for both the SAXS and the XRD regime where the sample was still un unloaded, i.e. no contact was made between the flat punch of the microindenter and the micropillar’s top surface. To check the initial values of the D period spacing of the mineralised collagen fibrils and to estimate possible residual strains, the D values were calculated for the samples based on the SAXS patterns and the relation $d = \frac{2\pi}{q}$ where $q$ denotes the wave vector identified via the integration of the radial profile (Figure 3) and $n$ the order of the SAXS Bragg reflection. The broad diffuse scattering in the middle part of the SAXS pattern perpendicular to the axial direction can be attributed to the lateral packing of the collagen molecules, which is known to be less ordered than in the axial direction [48, 49, 99] (Figure 3). The XRD pattern represents the diffraction peaks of the mineral particles of carbonated hydroxyapatite characterised by a hexagonal lattice structure. Since both the SAXS meridional reflections and the c-axis of the mineral nanocrystals are predominantly aligned along the collagen fibril axis, the (002) peak of the XRD pattern allows to analyse the mineral strains along the axial direction. This was done based on radial profiles of the Bragg peak intensities, thus tracking the changes of the lattice spacing during loading (Figure 3) [36, 53, 54, 100]. Mineral strain values were then calculated based on the initial acquisition of the unloaded condition of the sample and engineering strain values were transferred to logarithmic strains as described before. The preferred initial orientations of both the mineralised collagen fibrils and the mineral nanocrystals were quantified based on the maximum azimuthal intensity over an integration range from 0 to $2\pi$ of the initial X-ray acquisition in the unloaded state of the samples (see Figure S3 in the supplementary material). More specifically, the orientation of the carbonated hydroxyapatite crystals was assessed via the azimuthal distribution.
of the integrated intensity along the Debye-Scherrer ring of the 002 reflection and the orientation of the mineralised collagen fibrils along the 5th order SAXS Bragg peak. Data analysis was done with two software packages, a Python based custom-written software called “pySXIm” (courtesy of Aurélien Gourrier, Université Grenoble Alpes, CNRS, LIPhy, France), and the ESRF-developed software Fit2d [101]. The calibration to determine the beam centre, sample-to-detector distance and tilt angle was achieved using silver behenate (SAXS) and dialuminium dioxide (XRD). To allow an overall comparison between strain data at mineral, fibril and fibre levels, the data were fitted with a natural cubic spline with ten degrees of freedom (Figure 6). Strain ratios between fibril and fibre levels as well as mineral and fibre levels were calculated at the yield point and the point of compressive strength based on these cubic splines. Strain ratios in the elastic region were further calculated as the slope of the least squares regression in the elastic region of fibre and fibril strains as well as fibre and mineral strains. To study the deformation behaviour between the constitutive phases, the calcified collagen phase and the mineral phase, during different stages of compressive loading, the quotients of these strain ratios at yield point and compressive strength were calculated for each sample. This allowed to estimate possible shifts in the contribution and the proportion of strain taken up by the constitutive phases. Microindenter- and X-ray data were synchronised based on time stamps in the corresponding data log files to account for the offset between the X-ray acquisitions and mechanical loading data. To compare data from different hierarchical levels, data pairs were thus identified based on the times when the sample was exposed to X-rays (see supplementary material).

Figure 3: SAXS- and XRD- data-analysis procedure. Left: Close-up view of a representative SAXS-pattern of mineralised collagen fibrils (mineralised collagen composite). The analysis was based on the 3rd and 5th order of the Bragg reflections. In this image, the azimuthal integration sector is shown for the 5th order. The diffuse scattering from the microindenter imposed the choice of a small beamsize in the vertical direction as well as short exposure times. Right: Close-up of a representative XRD-pattern. The analysis was based on the 002-reflection representing the c-axis of the mineral nanocrystals and the axial direction of the mineralised collagen fibre. Azimuthal integration sectors were chosen to track the corresponding peaks during the loading cycle. White areas denote regions with zero intensity due to both the shading of the beam stop and “dead zones” of the detector. These areas were omitted from the data analysis. Both patterns, SAXS and XRD, show a preferred azimuthal orientation along the longitudinal direction of the fibre and fibril.
2.4 SEM-based failure mode analysis

Failure modes and failure patterns of the compressed micropillars were assessed based on SEM images (Quanta 3D FEG, FEI, USA, backscattered electrons, $E = 5$ keV, tilt angle = $52^\circ$) and failure modes reported in the literature. A classification scheme was developed accordingly. Based on the interfibrillar matrix and the mineralised collagen fibril of the mineralised collagen fibre, the fibre was considered as a unidirectional fibril reinforced composite [33]. The classification categories were derived from compressive and tensile failure modes as well as toughening mechanisms reported for fibre reinforced composites [102–116] and bone tissue [23, 29, 35, 117–122] where more than one failure mode could be present in a single specimen at the same time [108]. All micropillars were assigned to one or a combination of these failure modes and corresponding mean values and standard deviations for yield stress, strength, yield strain, ultimate strain and Young’s modulus at the fibre level were related to the failure modes (see section 3.3).

2.5 Statistical analysis

The statistical analysis was done in R [87] and Python with a statistical significance level of $p=0.05$. To test statistical differences, a two-tailed t-test was used. Quantile-quantile plots and Shapiro-Wilk tests [123] were done to verify that the data were normally distributed and the mean ± standard deviation were calculated. In addition, raw data of the samples by means of distribution independent median as well as minimum and maximum values are presented.

3 Results

3.1 Stress and strain analysis at fibre level

A total of 17 micropillars were fabricated from which two samples were used for pre-tests and alignment, two samples were lost due to operator error and one sample each was used for separate irradiation tests in the SAXS and the XRD group. The remaining micropillars (N=11) were used for the actual testing and the analysis. The stress relaxation tests in the SAXS regime did not show any change in the relaxation characteristic between the irradiated and non-irradiated state of the micropillar. The tests in the XRD regime showed a non-negligible change in the relaxation characteristic when all 120 sans were performed without time steps between X-ray acquisitions (see supplementary material). The decrease in load of about 0.70 mN to 0.75 mN during the relaxation test was found to be comparable in both the SAXS and the XRD regime. No impact of the X-ray beam was detectable based on the load and displacement data as well as the stress-strain curves for the apparent mechanical behaviour of the micropillars. No beam induced deterioration of the material was found based on the stiffness evolution in the elastic and plastic regions. No significant difference in $\sigma_{33}^{\text{yield}}$, $\epsilon_{\text{33}}^{\text{yield}}$, $\sigma_{33}^{\text{ult}}$, $\epsilon_{\text{33}}^{\text{ult}}$ and $E_{\text{fibre app}}$ between the SAXS group (N=6) and XRD group (N=5) were found and samples from both groups were pooled for the overall mechanical analysis.
at fibre level with median values shown in Figure 4. In addition, mean values and standard deviation were calculated to be $\sigma_{33}^{\text{yield}} = 0.154 \pm 0.051$ GPa, $\epsilon_{33}^{\text{yield}} = 0.040 \pm 0.011 \, \mu$m/$\mu$m, $\sigma_{33}^{\text{str}} = 0.180 \pm 0.042$ GPa, $\epsilon_{33}^{\text{ult}} = 0.060 \pm 0.016 \, \mu$m/$\mu$m and $E_{\text{fibre app}} = 16.47 \pm 3.40$ GPa. The Shapiro-Wilk tests showed that the data were normally distributed. No significant increase in damage, i.e. reduction in stiffness, was detectable for an accumulating plastic strain of up to 9.0% (Figure 5).

![Figure 4: Overview of the mechanical properties at the fibre level. (a,b) Stress-strain curves after sink-in and frame compliance correction for all samples of both the SAXS group (left, N=6) and XRD group (right, N=5) at the fibre level with respect to the calculated logarithmic strain in axial direction of the fibre. (c,d) Median values and range for the yield stress, strength, yield strain and ultimate strain at the fibre level for both the samples measured in the SAXS group (N=6) and the XRD group (N=5). Since no significant difference between both experimental groups were found (statistical significance threshold $p=0.05$), both groups were pooled (N=11). Additional mean and standard deviation values calculated for the fibre level are reported in the text.](image)

### 3.2 Fibril and mineral level and comparison to fibre level

The analysis of the maximum azimuthal intensity over an integration range of 0 to $2\pi$ revealed the preferential in-plane orientation of both the mineralised collagen fibrils and the mineral nanocrystals to be around the azimuthal angles $\eta = 90^\circ$ and $\eta = 270^\circ$ which
Figure 5: Median values of apparent Young’s modulus and estimation of damage as stiffness evolution based on the identified apparent modulus values determined from the unloading segments in the elastic and plastic region. (a) Median and range values of the apparent Young’s modulus at fibre level, identified as the slope of the last unloading segment before the yield point. (b) Stiffness evolution, i.e. normalised apparent modulus vs. plastic strain. The stiffness was not reduced after overloading up to a plastic strain of 9.0% indicating an absence of damage.

represent the longitudinal direction of the mineralised collagen fibre during the experiment.

The initial difference to the longitudinal arrangement was calculated to be $5.76 \pm 4.99^\circ$ for the SAXS patterns and $4.78 \pm 3.81^\circ$ for the XRD patterns (Figure 3 and supplementary material). The analysis of the initial D period spacing was calculated to be $66.10 \pm 0.33$ nm showing a very small distribution of the initial configuration of the collagen molecules’ axial stagger within the mineralised collagen fibrils.

Mean apparent pseudo-stiffness values were $12.99 \pm 7.66$ GPa ($R^2_{\text{adjusted}} = 0.71$) for the mineralised collagen fibrils and $44.46 \pm 10.16$ GPa ($R^2_{\text{adjusted}} = 0.92$) for the mineral phase resulting in corresponding ratios between fibre-fibril-mineral of approximately 5:4:12.

The analysis showed higher strain values at the fibre level compared to the fibril and mineral levels. We found ratios of strain at the apparent yield point at fibre, fibril and mineral levels of approximately 22:5:2 based on the linear regression of true fibril strain versus true fibre strain ($R^2_{\text{adjusted}} = 0.71$) and true mineral strain versus true fibre strain ($R^2_{\text{adjusted}} = 0.95$) in the elastic region. The mean strain ratios at the yield point based on the cubic spline fit were $0.20 \pm 0.17$ for fibril-to-fibre levels and $0.09 \pm 0.02$ for mineral-to-fibre levels.

Based on the cubic spline fit, the mean strain ratio of the fibril-to-fibre level at the point of compressive strength was calculated to be $0.23 \pm 0.11$ and the corresponding ratio for the mineral-to-fibre level $0.07 \pm 0.01$. In addition, multilevel strain ratios were calculated along the entire loading cycle. The results show a descending trend of the strain values from fibre to fibril to mineral level which is represented by the strain ratios (Figure 6).

A comparison of the cubic spline fits for the strain ratio data with the fibre’s apparent mechanical behaviour showed that the maximum for the calcified collagen phase occurs towards the point of apparent compressive strength while the maximum of the mineral phase towards the yield point. Ratios of the strain at yield point and compressive strength for each sample resulted in $0.84 \pm 0.37$ for fibril-to-fibre level and $1.45 \pm 0.61$ for mineral-
to-fibre level. The corresponding values for the ratio between the point of compressive strength and yield point were $1.41 \pm 0.63$ and $0.77 \pm 0.23$, respectively.

Different initial strain responses to the onset of compression were observed for the different length scales. The mineralised collagen fibril showed a toe region where the first onset of strain was detected after an apparent strain of about 2% was reached. In the mineral phase, this delayed strain response is not visible (Figure 7). In addition, data for the fibril strain did show a higher dispersion towards the end of the loading cycle (Figure 6).

**Figure 6:** Comparison of fibre level with fibril and mineral levels. Data were fitted with a natural cubic spline with ten degrees of freedom (df). The top row shows overlays of fibre and fibril strain (left) and fibre and mineral strain (right). The data illustrate the higher strain values at fibre level compared to the fibril and mineral levels during compression. Time series were synchronised. The bottom row shows strain ratios for the fibril and mineral levels with respect to the apparent fibre strain. Based on the cubic spline fit, a connection to the fibre’s apparent mechanical behaviour was drawn with the calcified collagen phase showing the highest strain ratio (middle grey dashed line) after the yield point and towards the compressive strength and the mineral phase before or around the yield point, respectively (compare with Figure 4 for mechanical properties at fibre level).
Figure 7: Mechanical response on different hierarchical levels for two exemplary samples. Left side: Synchronised data of fibre and fibril levels. Right side: Synchronised data of fibre and mineral levels. The shaded areas pinpoint to the strain onset at different hierarchical levels illustrating that the linear increase of fibril strain starts at a fibre strain of roughly 2%. This delayed response is not visible in the mineral phase where the strain of the mineral nanocrystals lacks a distinct toe region.

3.3 Failure patterns and modes

10 out of 11 tested micropillars were used to assess the occurring failure modes in the compressed micropillars. Based on the reviewed literature (Section 2.4) and identified features in the post-test SEM images, a classification scheme of failure patterns was developed which served as a basis for the failure analysis (Figure 8) where a combination of failure mechanisms can lead to a global loss of stability of the fibril reinforced composite. In addition, schematic drawings for three of the failure patterns are presented. Identified failure modes are listed in Table 1 with descending order of frequency complemented by the corresponding apparent mechanical properties yield stress $\sigma_{33}^{\text{yield}}$, yield strain $\varepsilon_{33}^{\text{yield}}$, strength $\sigma_{33}^{\text{str}}$, ultimate strain $\varepsilon_{33}^{\text{ult}}$ and apparent Young’s modulus $E_{\text{app}}$. 

Table 1: Identified failure modes and apparent mechanical properties.
Figure 8: Failure modes and patterns of compressed micropillars based on the developed classification scheme. Three failure modes are illustrated as schematic drawings on the right with thick black lines representing fibrils, thin black lines filaments and darker areas failure zones. The number of observed failure modes in the tested micropillars and corresponding mechanical properties at fibre level are presented in Table 1.

4 Discussion

4.1 Apparent micropillar behaviour at the fibre level

Stress and strain curves at the fibre level show the same characteristic development as reported for micropillar compression tests on osteonal bone [29, 35]. Compared to dry axial micropillar compression tests on ovine bone [35] and bovine bone [45], the compressive strength is three to four times smaller but the ultimate strain 2.5 times larger. Differences in these absolute values can be associated with the different structural set-up of the fibrils [70] in bone tissue which is tailored towards compression whereas tendon tissue is tailored towards tension. In addition, different degrees of mineralisation of both tissues need to be
Table 1: Failure patterns of uniaxially compressed micropillars with corresponding apparent mechanical properties. The mineralised collagen fibre is considered as a unidirectional fibril-reinforced composite. A total of 10 micropillars were included in the failure analysis where a combination of failure mechanisms can be present in one specimen [108].

<table>
<thead>
<tr>
<th>Failure mode</th>
<th>N</th>
<th>$\sigma_{33}^{\text{yield}}$ in GPa</th>
<th>$\epsilon_{33}^{\text{yield}}$ in %</th>
<th>$\sigma_{33}^{\text{str}}$ in GPa</th>
<th>$\epsilon_{33}^{\text{ult}}$ in %</th>
<th>$E_{\text{fibre}}^{\text{app}}$ in GPa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interfibrillar matrix fracture</td>
<td>10</td>
<td>0.155 ± 0.053</td>
<td>3.996 ± 1.144</td>
<td>0.184 ± 0.042</td>
<td>6.191 ± 1.585</td>
<td>16.725 ± 3.476</td>
</tr>
<tr>
<td>Fibril-matrix interface failure</td>
<td>9</td>
<td>0.155 ± 0.057</td>
<td>3.915 ± 1.183</td>
<td>0.186 ± 0.044</td>
<td>6.330 ± 1.614</td>
<td>17.200 ± 3.325</td>
</tr>
<tr>
<td>(debonding, 'Bulging')</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>w/ fibril microbuckling (matrix yielding)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kink band formation</td>
<td>8</td>
<td>0.152 ± 0.060</td>
<td>4.035 ± 1.204</td>
<td>0.185 ± 0.047</td>
<td>6.377 ± 1.719</td>
<td>17.169 ± 3.553</td>
</tr>
<tr>
<td>(localised shear plane)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibril fracture (with buckling)</td>
<td>7</td>
<td>0.164 ± 0.058</td>
<td>4.129 ± 1.254</td>
<td>0.193 ± 0.047</td>
<td>6.371 ± 1.309</td>
<td>17.519 ± 3.767</td>
</tr>
<tr>
<td>Fibril microbuckling (matrix elastic)</td>
<td>5</td>
<td>0.139 ± 0.044</td>
<td>4.150 ± 0.858</td>
<td>0.165 ± 0.027</td>
<td>6.874 ± 1.888</td>
<td>15.995 ± 2.093</td>
</tr>
<tr>
<td>Longitudinal cracking</td>
<td>5</td>
<td>0.159 ± 0.049</td>
<td>4.031 ± 0.840</td>
<td>0.185 ± 0.041</td>
<td>5.779 ± 1.701</td>
<td>15.279 ± 2.021</td>
</tr>
<tr>
<td>Filament based crack bridging</td>
<td>2</td>
<td>0.122 ± 0.054</td>
<td>3.165 ± 0.294</td>
<td>0.161 ± 0.027</td>
<td>6.190 ± 0.137</td>
<td>16.086 ± 0.266</td>
</tr>
</tbody>
</table>

N = 10, several failure modes may combine in any one specimen. As reported for ovine bone for plastic strains up to 8% [35], we found an absence of damage for up to 9% of plastic strain in the post-yield region. Damage was estimated as the relative change in stiffness after yielding. As proposed by Schwiedrzik et al. [35], this lack of a significant change in the apparent modulus after overloading might point towards a lack of diffuse crack opening until failure [124] of the micropillar. It furthermore implicates that no complete intrafibrillar separation between the mineral and collagen takes place, also in the post-strength region [111]. A slip at the interfaces [111], nevertheless, is possible and our data shows (Figure 6) that individual mineralised fibrils, if not fractured, stay intact while a movement between individual fibrils is still possible. This is visible in the mineral unloading (Figure 6). The high apparent ductility at the microscale observed in the mechanical data can be attributed to the interaction between the mineral and collagen that governs the fibre’s mechanical compressive behaviour. The fibre’s mean apparent Young’s modulus fits to results reported on mineralised turkey leg tendon measured by nanoindentation [125] and is larger than corresponding values from microindentation [125] which is expected, considering that the influence of microporosity is minimised when probing at lower length scales. In addition, the scale and structural size effects for the tissue’s mechan-
ical properties need to be considered [44, 126–129]. Compared to micropillar compression
tests on ovine and bovine bone [35, 45], our fibre’s mean apparent Young’s modulus is ap-
proximately half of what is reported in the axial direction. Again, this can most likely be
attributed to the different degree of mineralisation [40].

4.2 Multilevel moduli and strain ratios

4.2.1 Multilevel moduli and interaction of hierarchical levels

A comparison of the apparent mean Young’s modulus at the fibre level as well as the
pseudo-stiffness values at the level of the mineralised collagen fibril (calcified collagen
phase) and the mineral nanocrystals (mineral phase) allows an estimation of the load trans-
fer between the constitutive phases.

The pseudo-stiffness value for the mineralised collagen fibril is comparable to the literature
with respect to uniaxial compression tests on bovine dentine samples of a few millimetres
[61]. Due to deformation mechanisms between the fibre’s constituents and the presence of
mineral, the modulus of pure collagen is expected to be smaller than the apparent modulus
in the mineralised fibril composite [95]. This is supported by our findings when the usually
reported range of values for the Young’s modulus of pure collagen between 1 and 9 GPa is
taken as a reference [8, 25, 54, 130, 131].

The mean apparent pseudo-stiffness for the mineral phase was 10-17% higher on average
but comparable with reported results from the literature [56, 95] who analysed XRD pat-
terns of millimetre sized canine bone samples under compressive loading and performed
simulations of elastic properties for mineralised collagen fibrils. The authors reported a
value for the apparent modulus of the mineral phase of 38.2 ± 0.5 GPa based on the 004-
reflection which represents the same crystal plane as the 002-Bragg peak used in our study.
Their values were calculated via estimated internal stresses derived from calculations pro-
posed by He and Smith [132] for a biaxial strain model and the Kröner-Eshelby model [133]
for the actual approximation. This approach was not accessible in our study due the weak
intensities of the XRD patterns of relevant reflections for the transverse deformation. This
was mainly a result of the short exposure time that was chosen to minimise the influence of
irradiation. In general, a higher apparent modulus value can be expected when comparing
our test at the level of the individual mineralised collagen fibre with macroscopic compres-
sion tests, most probably due to the smaller amount of porosities and interfacial volume
between the constitutive phases and along the length scales. Similar explanations were
made by Deymier-Black et al. [61, 94] who found low apparent modulus values for their
hydrated macroscopic bovine dentine samples in the mineral phase (applied stress versus
measured mineral strain) of about 18 ± 2 GPa and 26.5 ± 7.2 GPa, respectively. They at-
tributed differences to the model predictions of 44 GPa [94] partially to the high fraction of
interfacial volume. A comparison with hydrated samples is viable assuming a negligible
effect of water on the mechanical properties in the mineral phase. A higher apparent mod-
ulus value for the mineral phase is also related to a weakening of the interfacial bonding
which leads to a decrease in the load transfer as discussed for raloxifene that supposedly
modifies the interface between the collagen and the mineral nanocrystals [134].

For experimental and numerical studies on mineralised tissue such as bone and mineralised turkey leg tendon, a Young's modulus value of hydroxyapatite in the range of 85 to 120 GPa is usually reported [8, 33, 55, 135]. Values for hydroxyapatite with an amorphous layer between 75 and 107 GPa have been reported as well [136] and some authors [16, 33, 135, 137] discuss that the high number of 85 - 120 GPa might be overestimated for biomaterials since it refers to synthetic apatite crystals whereas hydroxyapatite in mineralised tissue show impurities and a non perfect crystalline structure [16, 33, 135, 137]. Differences between our values and those reported for hydroxyapatite can be attributed to the fact that our values are not obtained from a single crystal but from a mineral phase comprising several nanocrystalline platelets impregnating a fibril network as a powder glue mixture as a result of its biosynthesis. Due to deformation mechanisms between the fibre's constituents and along the length scales, the modulus of pure hydroxyapatite is expected to be higher than the apparent one in the mineral phase corresponding to a decrease in the stiffness values of the hydroxyapatite nanocrystals in the bulk [94, 128]. Hydroxyapatite crystals found in mineralised turkey leg tendon further show a smaller thickness, about a factor of 0.5 to 0.67, compared to bone [21, 37, 95]. As mentioned earlier, Almer and Stock [53, 56] did base their results on internal stresses in the mineral crystal. They report stress ratios between the mineral phase and the tissue level which, together with findings from Borsato and Sasaki [138], leads to an average ratio of 2.5. By multiplying this value with our mean apparent pseudo-stiffness in the mineral phase, a value of about 110 GPa can be calculated which is comparable to values reported for pure hydroxyapatite crystals.

The discussed explanations did not consider any differences in the fibril and mineral volume fraction of the tissue which are discriminating factors for the mechanical properties, with the latter especially dominant for the elastic stiffness [8, 15, 61, 95]. These parameters were not assessed in this part of the data analysis of the study.

4.2.2 Multilevel strain ratios and interaction of hierarchical levels

Strain values at the level of the fibre, fibril and mineral showed a decreasing trend. Strain ratios at the point of compressive strength for fibril to fibre levels and for mineral to fibre levels are lower than comparable values reported for macroscopic tensile tests on millimetre sized bone samples of 0.41 ± 0.02 for fibril to tissue levels and 0.24 ± 0.02 for mineral to tissue levels [55]. While differences in the post-yield region might be attributed to the different testing regimes, macroscopic uniaxial compression tests on bovine cortical bone also showed higher strain ratios between the macroscopic strain of millimetre sized samples and fibril and mineral strains [139] where ratios higher than approximately 0.4 can be derived from the presented data. In all reported cases, tensile as well compressive, the mean fibril strain shows higher values than the mineral strain [55, 61, 139, 140] which is expected and in accordance to our findings. The differences in the strain ratios might have different origins and be attributed to a structural reorganisation at the nanoscale that manifest itself in extrafibrillar and intrafibrillar gliding mechanisms [54, 141, 142], the latter associated
with mineral platelet gliding. A structural reorganisation is supported by the development of our fibril strains which show a higher dispersion (Figure 7), most pronounced after the point of compressive strength, partly attributed to a decrease in the SAXS signal intensity.

As reported for tensile tests on bone [141–143], the gradual disruption of the ordering of the calcified collagen phase leads to decreased intensities of the SAXS meridional Bragg peaks as this is related to the destruction of the long range order in the collagen network and an intrafibrillar gliding [144]. The deviation from a perfectly ordered system also results in an increase in peak widths while the scattered intensity by the most disordered part appears as a diffuse background [100]. This reduction of the intensity of Bragg peaks was visible in most of the analysed SAXS patterns but not present in the sample that was used for the irradiation test during stress relaxation. This suggests that the decreasing intensity is essentially related to a structural disorder. The low fraction of apparent strain that is taken up by the mineral phase as well as the calcified collagen phase might therefore be explained by shear deformations and frictional sliding at the intra- and interfibrillar interfaces that lead to energy dissipation [24]. Furthermore, a large amount of strain might also be taken up by the interfibrillar matrix what would suggest that a higher portion of the mineral nanocrystals is located within the fibrils.

The small strain ratio between the fibril- and the fibre-levels might, furthermore, be related to a gradual recruitment of the mineralised collagen fibrils upon the onset of compressive loading. In this case, not all fibrils are loaded initially upon the start of the compression leaving a small number of fibrils to bear a large amount of load. This situation leads to a stress concentration which induces a strain localisation. Since the SAXS signal represents the result of an integration over the radiated area, this localisation might affect the measured strain values.

Based on the strain ratio and the corresponding plots (Figure 6) as well as the failure mode analysis (Figure 8), the low strain ratios may result from a rigid body movement as well as transverse strains that lead to a structural displacement which dominates the apparent deformation. This structural displacement is not picked up by the constituents of the fibre but leads to increasing apparent strains at the fibre level.

This explanation is accompanied by the localisation of strain and work in the failure zone, more specifically by the localised formation of kink bands. The SEM based failure analysis revealed that the formation of kink bands was one of the most prominent failure modes where the compressed micropillars were considered as a unidirectional fibril reinforced composite (see section 4.3). Kink bands are one of the primary failure mechanism in unidirectional fibre reinforced composites and are characterised by a cooperative fibre kinking in a narrow band when fibre microbuckling occurs [106, 116, 145]. It is often associated with the anisotropy of the material [105]. The deformation localises into the inclined bands [105, 110] and surrounding fibrils which have not undergone failure, unload. The localised strain might then lead to the development of both a plastic region concentrated in the failure area and an elastic region in the surroundings as a result of stress relaxation. While the apparent strain increases, the SAXS and XRD measurements do not pick up this localisation.
of strain which is one explanation for the high apparent strain compared to the small fibril
and mineral strains. In addition to localised shear bands, localised compressive buckling
is another dominant failure mode in aligned-fibre polymeric matrix composites, followed
by fibre crushing [106]. The former is associated with a pronounced bulging in our tested
micropillars, often accompanied with an axial split [114] (Figure 8). As for kink bands, this
leads to a plastic region within the actual failure zone and an elastic region nearby, accom-
panied by a corresponding redistribution and energy release [127]. These failure modes as
well as longitudinal cracking of the micropilar are also not picked up by the mineral and
fibril strain measurements.

The onset of the described kink band formation can be attributed to an initial fibril mis-
alignment [106] or a matrix failure [146]. In both cases, a shear component is triggering the
onset of instability in aligned composites [107]. In our fibril reinforced composite, this shear
might originate in the extrafibrillar matrix manifested by interfibrillar sliding but based on
the absence of damage not as a result of an intrafibrillar decoupling of mineral and collagen
as proposed as a mechanism in macroscopic tensile tests [141, 142, 147]. It rather supports
the assumption that the failure onset can be related to the interface between the fibril and
the extrafibrillar matrix [141, 148]. We suggest that the mineral and the collagen do not sep-
arate but the impregnation can be split to allow the system to accommodate movement. As
a result of the related mineral platelet sliding, the associated friction can serve as an expla-
nation for the plasticity [149] in the mineralised collagen fibres as reported for bone [150].

When the multilevel strain ratios are related to the apparent mechanical data, results show
that on average the strain ratio between the fibril and fibre levels is highest towards the
point of compressive strength and that of the mineral and fibre levels before or around the
yield point, respectively (Figures 6 and 4). Strain ratios between the mineral phase and the
apparent strain are approximately 1.4 times higher at the yield point than at the point of
compressive strength. For the calcified collagen phase, i.e. the mineralised collagen fibrils,
a corresponding factor of 1.5 was found vice versa. This means that the contribution of the
mineral phase to the apparent fibre strain decreased from the apparent yield point towards
the compressive strength and the contribution of the calcified collagen phase increases. A
direct comparison of both constitutive phases regarding the ratio to the fibre level showed
an increase in contribution between the calcified collagen phase and the mineral phase of
about 50% from the yield point to the compressive strength though fibril strains are higher
throughout, apart from an initial toe region. The shift in the constituents’ contributions can
be based on the interaction between a phase with a high specific stiffness and strength and
a phase with ductility. In our case these are represented by the mineral nanocrystals and the
mineralised collagen composite, respectively, which allows load partitioning and load shar-
ing [58, 93]. Similar to the load partitioning mechanism reported by Carter and Bourke [151]
who studied the deformation behaviour of Beryllium-Aluminium composites by means of
neutron diffraction, our data show a load partitioning between a stiff reinforcement and
a ductile matrix allowing for a brittle and ductile behaviour combination. An increasing
proportion of load is being picked up by the calcified collagen phase towards the appar-
ent elasto-plastic transition. Lower strains placed on the mineral phase can also lead to a
greater overall deformation prior to failure, as discussed by Gallant et al. [134] regarding
the effect of raloxifene on the load transfer between the collagen matrix and the mineral
nanocrystals. In addition, the behaviour can be based on an initial co-loaded state of both
the mineral and the calcified collagen phase and an onset of mineral platelet sliding which
forces the calcified collagen phase to deform to a greater extent. An increase in the fibril
strain in later stages of compressive loading has been reported on bovine plexiform bone
[58]. The prolonged ductility in the overall mechanical behaviour [151] of the fibre is ac-
commodated by the calcified collagen phase. The ductile behaviour complies with results
from micropillar compression tests on dry ovine bone [35]. Data suggests that the mineral
crystals thus initially prevent the mineralised collagen fibrils to fracture, before the calcified
collagen phase governs the ductile behaviour.

Our data furthermore revealed a delayed mechanical response of the mineralised collagen
fibril compared to the apparent behaviour with an onset of the fibril strain only detected
after about 2% of apparent strain. In the mineral phase, this delayed response is not visible.
This behaviour is in contrast to findings in the literature which reported that strains in the
mineral phase rise less rapidly or equally with applied stress than in the calcified collagen
phase for both tensile testing of bovine bone and compressive loading of canine and bovine
plexiform bone [54–56, 58]. Gao et al. [152], however also state that in bio-composites such
as bone, dentine and nacre, most of the load is carried by the mineral platelets. An explana-
tion that this toe region was found in the SAXS data only, might be associated with the pres-
ence of mineral nanocrystals in the extrafibrillar matrix [20, 22–24]. The XRD data revealed
a direct response of the mineral nanocrystals upon compression and since no immediate
response was seen in the SAXS signal of the mineralised collagen fibril, the observed strain
in the initial region might be attributed to the strain in the extrafibrillar uniaxially aligned
mineral nanocrystals before the strain occurs within the fibrils themselves. Combined with
the identified preferred in-plane orientations of the fibre’s constituents, this would support
the assumption that the intra- and extrafibrillar mineral nanocrystals show a certain degree
of co-alignment. This mechanism might also have been combined with a gradual recruit-
ment of the fibrils upon compressive loading. We suggest that the differences to reported
results in the literature are due to the testing at the fibre level, which reduces the effect of
microporosity and which allowed us to detect the initial recruitments of the fibre’s consti-
tutive phases upon compressive loading.

4.3 Failure modes and patterns

The developed classification scheme was based on considerations from failure and fracture
behaviour mechanisms identified in both fibre reinforced composites and bone tissue. As
individual mineralised collagen fibres have been investigated in this study, the compressed
structure was considered a fibril reinforced composite with collagen fibrils embedded in
an interfibrillar matrix [33]. The failure mode and failure pattern analysis revealed that
several combinations of failure modes are present in a single specimen with the most fre-
quent ones being interfibrillar matrix fracture, fibril-matrix interface failure with debonding including fibril microbuckling and pronounced ‘Bulging’, kink band formation, i.e. formation of a localised shear plane (often with axial splitting), fibril fracture with microbuckling and longitudinal cracking. The influence of the formation of kink bands on the multilevel strain ratios was discussed in section 4.2.2. The combined occurrences fit to findings in the literature [108] stating that different failure mechanisms can be responsible for the global loss of stability in a fibre reinforced composite. In addition, two micropillars, one from the XRD and SAXS group each, showed filament based crack bridging which is discussed as a toughening mechanisms at the micro- and nanoscale of mineralised tissue [35, 119–122] (Figure 8). Local shear deformation in form of kink bands, axial splitting as well as shear cracks were reported for micropillar compression of similar sized samples of osteonal bone [29, 35]. Additional failure modes such as fibril microbuckling accompanied by bulging and fibril fracture are common compressive failure modes of fibre-(and fibril-) reinforced composites and thus also in the uniaxially aligned fibrillar architecture used in our study. Due to the biological variability of the 11 micropillars from 10 different specimens, a statistical distribution of the initial configuration of the micropillars regarding possible defects and pores are to be expected which in turn lead to different failure modes for a single loading mode. Tertuliano and Greer [46] report shearing and brittle failure for their compression tests of nano- and micropillars. Since the reported work only included one female donor, a smaller biological variability is to be expected. In addition, their tested nano- and micropillars had a diameter ranging from 0.25 µm to 3.00 µm. Thus, the maximum size were only half of the samples used in our study. A decrease in sample size is accompanied by a smaller probability that a critical sized defect is present in the material which in turn leads to a higher failure stress [153] and at the same time to less diverse failure modes [152]. Furthermore, Tertuliano and Greer [46] used trabecular bone. With respect to the mechanical properties at the fibre level, the highest mean apparent Young’s modulus, highest mean yield stress and highest mean strength values were found for micropillars whose combined failure pattern included fibril fracture with fibril microbuckling. Those micropillars who showed fibril microbuckling but no fibril fracture had the highest yield and ultimate strain but the second lowest yield stress and strength. The lowest mean apparent Young’s modulus and lowest mean ultimate strain value were found for micropillars whose combined failure pattern included longitudinal cracking.

4.4 Limitations of the study and influence of irradiation

Exposure times for both SAXS and XRD measurements were chosen to be short in order to avoid the influence of irradiation on the tissue’s mechanical behaviour but long enough to provide a reasonable signal for the data analysis. In addition, short exposure times were necessary to minimise the influence of any scattering signal from the diamond flat punch. The relaxation tests revealed no change in the relaxation characteristic for the SAXS regime. Although the irradiation test in the XRD regime showed a non-negligible change in the relaxation characteristic when all 120 X-ray acquisitions were delivered without an inter-
mediate time-step, the decrease in load of about 0.7 mN to 0.75 mN during the relaxation test was found to be comparable in both the SAXS and the XRD regime (see supplementary material). Furthermore, the mechanical properties in the elastic and plastic regions at the fibre level were not significantly different in both regimes and no impact of the X-ray beam on the apparent mechanical behaviour was detectable based on the continuously recorded load and displacement data during the X-ray acquisitions including the analysis of the corresponding stress-strain curves of the compression tests. In addition, we found an absence of damage based on the stiffness evolution so that a deterioration of the material by the beam can be ruled out.

Mineralised turkey leg tendon has been used extensively as a model system for bone in the last decades, mainly due to both the uniaxial fibre arrangement and the identical hierarchical organisation at the level of the mineralised collagen fibre [15, 21, 36–39, 47]. Nevertheless, mineralised turkey leg tendon is not strictly bone tissue and the naturally mineralised tendon tissue is tailored towards tensile not compressive loads. However, at the fibre level the experimental findings give valuable input for numerical models, especially regarding the load sharing between the fibre’s constituents and possible insights into the onset of interface failure.

The tests in this study were limited to dry mineralised tissue. Since it is reported that the hydration state has an influence on the mechanical properties at the material’s different hierarchical levels [29, 154–156], a logical extension of the presented experimental set-up will be the testing under quasi-physiological conditions.

5 Conclusion

We successfully performed simultaneous micromechanical testing and nanostructural analysis of micropillars extracted from individual mineralised collagen fibres by means of combined micropillar compression and SAXS- and XRD-measurements. An extraction of the compressive mechanical behaviour of individual mineralised collagen fibres at the micro- and nanoscale was possible for the first time and accompanied by a SEM-based failure analysis. Testing at the fibre level minimised uncertainties that might arise due to potential effects of structural interfaces and porosities and data could be directly related to the mechanical behaviour and load sharing of the mineralised collagen fibre and its constitutive phases, the mineralised collagen fibrils and the mineral nanocrystals, of a model system for bone. The experimental set-up was able to unveil multilevel strain ratios and load sharing mechanisms among the fibre’s constituents within a well-controlled fibrillar architecture.

The apparent Young’s modulus at the level of the mineralised collagen fibre is comparable with nanoindentation results on mineralised turkey leg tendon [125] and half of what is reported from micropillar compression tests ovine and bovine bone [35, 45], mainly attributed to different degrees of mineralisation [40]. The absence of damage suggests a non-complete separation of the intrafibrillar mineral and collagen after overloading [111]. This indicates that the onset of failure is rather related to the interface between the fibrils and the extrafib-
rillar matrix. This assumption is supported by results from the SEM based failure analysis. The comparison between the fibril and the mineral strains with the apparent micropillar behaviour revealed that only small fractions of the overall strain is taken up by the constitutive phases with ratios between fibre-fibril-mineral of approximately 22:5:2 in the elastic region. At the point of compressive strength, strain ratios between the fibril- and the fibre-level were found to be $0.23 \pm 0.11$ and between the mineral- and the fibre-level $0.07 \pm 0.01$. Ratios are smaller compared to macroscopic tensile tests [55] and compression tests on cortical bone [139]. The small strain ratios might be associated with shear deformation and energy dissipation at the interfaces [24] or due to a very compliant extrafibrillar matrix that takes up a substantial amount of deformation, which would support the assumption that a higher fraction of minerals can be found intrafibrillar. In addition, differences might also be explained by a localisation of strain within the micropillar as a consequence of both the formation of kink bands, identified as a prominent failure mode, and a gradual recruitment of the fibrils upon the onset of compressive loading, both not taken up by the X-ray measurements. The calcified collagen phase and the mineral phase showed maximum contributions to the apparent strain at different points of the compressive loading, with the calcified collagen phase around apparent compressive strength and the mineral phase around the yield point. Results, thus, show a load sharing between these constitutive phases. The mineralised collagen fibrils showed a delayed mechanical response, contrary to the mineral phase. This indicates that the fibre’s initial response to the compressive loading is dominated by the deformation of uniaxially aligned mineral nanocrystals in the extrafibrillar matrix preceding the deformation of the mineralised collagen fibrils themselves. Based on the identified preferred in-plane orientations of the fibre’s constituents, it furthermore suggests a certain degree of co-alignment between intra- and extrafibrillar mineral nanocrystals. This might also be accompanied by a gradual recruitment of the fibrils along the loading cycle.

The presented data demonstrated the importance and the benefits when testing mineralised tissue at the length scale of their fundamental mechanical building blocks which gives novel insights into the tissue’s non-linear mechanical behaviour at the level of the mineralised collagen fibre. The mechanical and nanostructural test results and structure-mechanical relations on different hierarchical levels of a well-controlled fibrillar architecture can be used to further develop model representations and constitutive equations for bone at the micro- and nanoscale. The deformation behaviour within an individual mineralised collagen fibre thus provide valuable input for micromechanical models and computational non-linear bone strength analyses optimising approaches for fracture risk prediction. Eventually, the presented results aim to improve personalised diagnosis and treatment solutions as well as bio-inspired implants for people with bone diseases.
6 Acknowledgements

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Figure 1: The overview illustrates the extraction of individual mineralised collagen fibres (MCFs) by means of dissection, ultra-milling, ultra-short pulsed laser ablation and focused ion beam milling. The SEM image shows the final micropillar. The extracted mineralised collagen fibre was tested mechanically until failure by means of micropillar compression while small angle X-ray scattering (SAXS) or X-ray diffraction (XRD) measurements were acquired. ROI = region of interest.

Figure 2: A custom-built microindenter was implemented into the beamline ID13 of the ESRF. The top left image shows a side view of the whole set-up with the detector in XRD position. The microscopic image on the bottom left was taken during the alignment process. The right image shows a close-up of the microindenter and the X-ray set-up.

Figure 3: SAXS- and XRD- data-analysis procedure. Left: Close-up view of a representative SAXS-pattern of mineralised collagen fibrils (mineralised collagen composite). The analysis was based on the 3rd and 5th order of the Bragg reflections. In this image, the azimuthal integration sector is shown for the 5th order. The diffuse scattering from the microindenter imposed the choice of a small beamsize in the vertical direction as well as short exposure times. Right: Close-up of a representative XRD-pattern. The analysis was based on the 002-reflection representing the c-axis of the mineral nanocrystals and the axial direction of the mineralised collagen fibre. Azimuthal integration sectors were chosen to track the corresponding peaks during the loading cycle. White areas denote regions with zero intensity due to both the shading of the beam stop and "dead zones" of the detector. These areas were omitted from the data analysis. Both patterns, SAXS and XRD, show a preferred azimuthal orientation along the longitudinal direction of the fibre and fibril.

Figure 4: Overview of the mechanical properties at the fibre level. (a,b) Stress-strain curves after sink-in and frame compliance correction for all samples of both the SAXS group (left, N=6) and XRD group (right, N=5) at the fibre level with respect to the calculated logarithmic strain in axial direction of the fibre. (c,d) Median values and range for the yield stress, strength, yield strain and ultimate strain at the fibre level for both the samples measured in the SAXS group (N=6) and the XRD group (N=5). Since no significant difference between both experimental groups were found (statistical significance threshold p=0.05), both groups were pooled (N=11). Additional mean and standard deviation values calculated for the fibre level are reported in the text.

Figure 5: Median values of apparent Young’s modulus and estimation of damage as stiffness evolution based on the identified apparent modulus values determined from the unloading segments in the elastic and plastic region. (a) Median and range values of the apparent Young’s modulus at fibre level, identified as the slope of the last unloading segment before the yield point. (b) Stiffness evolution, i.e. normalised apparent modulus vs. plastic strain. The stiffness was not reduced after overloading up to a plastic strain of 9.0%
indicating an absence of damage.

**Figure 6:** Comparison of fibre level with fibril and mineral levels. Data were fitted with a natural cubic spline with ten degrees of freedom (df). The top row shows overlays of fibre and fibril strain (left) and fibre and mineral strain (right). The data illustrate the higher strain values at fibre level compared to the fibril and mineral levels during compression. Time series were synchronised. The bottom row shows strain ratios for the fibril and mineral levels with respect to the apparent fibre strain. Based on the cubic spline fit, a connection to the fibre’s apparent mechanical behaviour was drawn with the calcified collagen phase showing the highest strain ratio (middle grey dashed line) after the yield point and towards the compressive strength and the mineral phase before or around the yield point, respectively (compare with Figure 4 for mechanical properties at fibre level).

**Figure 7:** Mechanical response on different hierarchical levels for two exemplary samples. Left side: Synchronised data of fibre and fibril levels. Right side: Synchronised data of fibre and mineral levels. The shaded areas pinpoint to the strain onset at different hierarchical levels illustrating that the linear increase of fibril strain starts at a fibre strain of roughly 2%. This delayed response is not visible in the mineral phase where the strain of the mineral nanocrystals lacks a distinct toe region.

**Figure 8:** Failure modes and patterns of compressed micropillars based on the developed classification scheme. Three failure modes are illustrated as schematic drawings on the right with thick black lines representing fibrils, thin black lines filaments and darker areas failure zones. The number of observed failure modes in the tested micropillars and corresponding mechanical properties at fibre level are presented in Table 1.

**Figure S1:** Depth of ions and ion trajectories from Monte Carlo simulations in x-direction (direction of ion beam) and y-direction for two different incident angles $\theta$. Top rows: Ion number with respect to the x-direction, i.e. illustrating the ion’s behaviour in the direction of the ion beam. Density distributions based on $N=10^5$ ions with respect to the x-direction illustrate that the depth of the ions is confined to approximately 30 nm at $\theta = 0^\circ$ and to 11 nm at $\theta = 70^\circ$. The grey line represents a normal distribution fitted to the simulation data. Bottom row: Depth of ions in y-direction for both incident angles illustrating the difference in relation to $\theta$.

**Figure S2:** X-ray acquisition points and peak fitting for fibril and mineral strain analysis. Left: Scan points when the micropillars were exposed to X-ray radiation. A total of 120 acquisitions were done covering loading and (partial) unloading segments of the loading protocol. The acquisition points represent the data pairs between fibre level and fibril and mineral levels, respectively, that were identified during the synchronisation procedure. Right: Overlay of radial profiles of the 002-Bragg peak of all 120 acquisition including the
fit of the peak position with a Gaussian function and a fit of the background noise with a first order polynomial function.

**Figure S3:** Overlay of azimuthal profiles of the 002-Bragg peak from the XRD pattern and the 5th Bragg peak of the SAXS pattern for all 120 X-ray-acquisitions illustrating the location of the scattering signal and thus the preferred structural orientation of the mineral nanocrystals and the mineralised collagen fibrils, respectively. Intensity drops to zero and random spikes around 200, 260 and 340 degrees for the left XRD pattern and around 330 degrees for the right SAXS pattern are due to “dead zones” and irregularities of the detector array arrangement but did not influence the data analysis.

**Figure S4:** Time dependent load data during uniaxial relaxation tests for both SAXS and XRD regimes. Red lines denotes an exponential fit based on a non-linear regression model. Regions where the micropillar was exposed to radiation are marked. For both the SAXS and XRD regime 120 X-ray acquisitions were recorded, for the SAXS with an intermediate time in between measurements of 5 s, for the XRD regime without.

**Table 1:** Failure patterns of uniaxially compressed micropillars with corresponding apparent mechanical properties. The mineralised collagen fibre is considered as a unidirectional fibril-reinforced composite. A total of 10 micropillars were included in the failure analysis where a combination of failure mechanisms can be present in one specimen [108].
MICROPILLAR COMPRESSION & SAXS/XRD

FRACTURED MICROPILLAR

20 μm

MICROPILLARS EXTRACTED FROM INDIVIDUAL MINERALISED COLLAGEN FIBRES

Before

2 μm

MICROSCALE DEFORMATION

FIBRE

stress

strain

MECHANICAL BEHAVIOUR OF MINERALISED COLLAGEN FIBRES AS INPUT FOR COMPUTATIONAL BONE STRENGTH ANALYSIS AND FRACTURE RISK PREDICTION

FIBRIL

MINERAL

SAXS

XRD

FIBRIL

MINERAL

SAXS

XRD

NANOSCALE DEFORMATION