Experiment Report Form

The double page inside this form is to be filled in by all users or groups of users who have had access to beam time for measurements at the ESRF.

Once completed, the report should be submitted electronically to the User Office via the User Portal:

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Reports supporting requests for additional beam time

Reports can be submitted independently of new proposals – it is necessary simply to indicate the number of the report(s) supporting a new proposal on the proposal form.

The Review Committees reserve the right to reject new proposals from groups who have not reported on the use of beam time allocated previously.

Reports on experiments relating to long term projects

Proposers awarded beam time for a long term project are required to submit an interim report at the end of each year, irrespective of the number of shifts of beam time they have used.

Published papers

All users must give proper credit to ESRF staff members and proper mention to ESRF facilities which were essential for the results described in any ensuing publication. Further, they are obliged to send to the Joint ESRF/ ILL library the complete reference and the abstract of all papers appearing in print, and resulting from the use of the ESRF.

Should you wish to make more general comments on the experiment, please note them on the User Evaluation Form, and send both the Report and the Evaluation Form to the User Office.

Deadlines for submission of Experimental Reports

- 1st March for experiments carried out up until June of the previous year;
- 1st September for experiments carried out up until January of the same year.

Instructions for preparing your Report

- fill in a separate form for each project or series of measurements.
- type your report, in English.
- include the reference number of the proposal to which the report refers.
- make sure that the text, tables and figures fit into the space available.
- if your work is published or is in press, you may prefer to paste in the abstract, and add full reference details. If the abstract is in a language other than English, please include an English translation.

ESRF	**Experiment title:** **Combining micropillar testing and SAXS/WAXD measurements to identify the mechanical behaviour of mineralised collagen fibril assemblies**	**Experiment number**: ME 1415
Beamline:	**Date of experiment**: from: 25/08/2016 to: 29/08/2016	**Date of report**: 29/08/2016
Shifts:	**Local contact(s)**: Michael Sztucki, Manfred Burghammer	*Received at ESRF:*

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Report:

Scientific background:

Osteoporosis is characterised by a loss of structural integrity and mechanical competence of whole bones. Bone mechanical properties are different on different length scales making up a hierarchy that allows nature to generate a remarkable strong and ductile self-healing macroscopic material that is absolutely unique. The fundamental building block of this hierarchy is the mineralised collagen fibril (Ø ~ 100 nm) consisting of carbonated hydroxyapatite crystals (~5 x 50 x 100 nm^3) impregnating a dense packing of extended type I collagen molecules. This fibril is assembled in mineralised collagen fibres making up the extra-cellular matrix (ECM).

While the mechanical behaviour of bone is currently well characterised at the upper level of tissue organisation (e.g. Wolfram and Schwiedrzik (in press) and references therein), the mechanical behaviour of mineralised collagen fibril assemblies, i.e. the fibre, remains unclear. This is due to the fact that experimental techniques were missing with which displacement controlled uniaxial tests could be performed directly on individual mineralised collagen fibril assemblies so that the results are not biased by large heterogeneities at the observational level such as porosities, lamellar organisation, cement lines, cracks etc. To exclude those heterogeneities such experiments need to be performed on micron-sized volumes of ECM, ideally on individual mineralised collagen fibres (MCF).

Micropillar compression tests on dry bone ECM have been conducted recently (Schwiedrzik et al. 2014, Luczynski et al. 2015) revealing unexpected high strength and an absence of damage. Currently it is the only experimental method that allows to conduct uniaxial stress-strain tests on volumes small enough to test individual MCFs. If micropillar testing could be combined with SAXS and WAXD measurements it would be possible to complement stiffness and strength measurements on individual MCFs with measurements of the deformations in the collagen network (SAXS) and the mineral platelets (SAXS/WAXD). Unlike earlier studies on mineralised tissue where mechanical tests on macroscopic specimens (several structural units) and

SAXS/WAXD measurements were combined (e.g. Gupta et al. 2006) such an approach would circumvent the confounding effects of existing interfaces.

Therefore, we aimed at performing simultaneous uniaxial strength tests and structural measurements on micron-sized volumes of ECM to identify the strain distribution in individual MCFs to better understand their mechanical behaviour and the scale transition of bone mechanical properties.

Results

Sixteen instead of 20 micropillars were produced from mineralised turkey leg tendon, a mineralised tissue featuring a uniaxial setup of MCFs that in turn are made of uniaxially aligned mineralised collagen fibrils. The tissue features a cirumferential compartment surrounding osteon-like structures with MCFs of 3-5 µm diameter and an interstitial compartment with MCFs of 5-10 µm diameter (Spiesz et al., 2012). The latter compartment represents an ideal model system for uniaxial compression tests on individual MCFs. Micropillars were prepared using a combination of laser ablation and focussed ion beam milling and centred on a single MCF (Figure 1c).

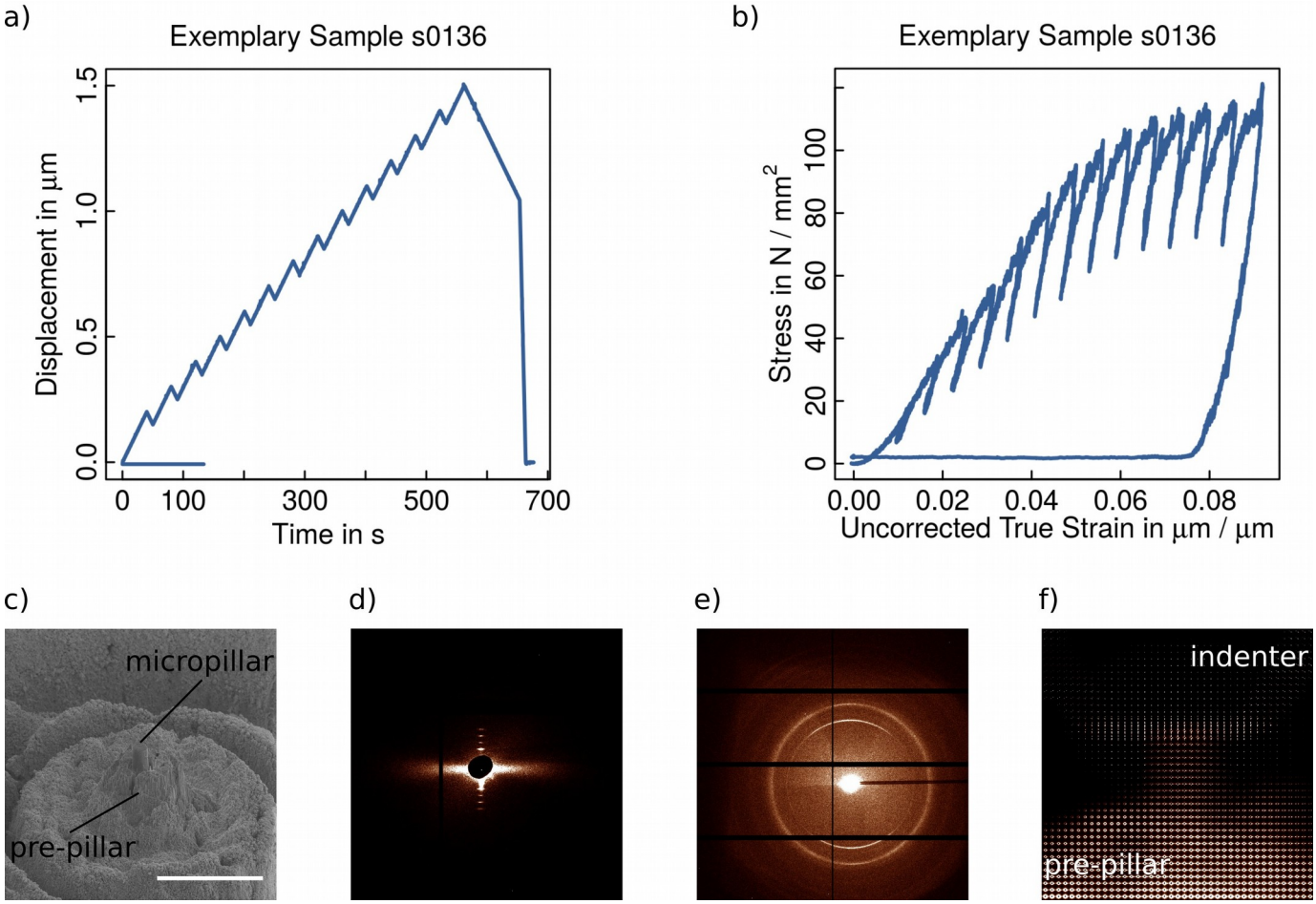

Figure 1: Early results of an exemplary sample. (a) shows the monotonic loading with partial unloading steps of 200 nm and (b) the corresponding loading path. (c) shows a SEM image of a micropillar (scale bar is 50 µm) and (d) and (e) SAXS and WAXD patterns under load. (f) depicts a SAXS scanning of the whole setup that reveals the diamond indenter on top, the micropillar in between and the pre-pillar at the bottom.

We successfully tested 14 out of 16 micropillars. Unlike planned in the proposal, SAXS and WAXD measurements were performed in two groups (n = 6) rather than on the same specimen. This was due to the long time necessary to switch between both setups and the additional radiation dose that would have been taken up by the specimens during a total of two measurements.

We performed radiation damage tested on one pre-pillar with 1000 exposures for SAXS and WAXD using an exposure time of 25 ms and 200 ms, respectively. In the SAXS radiation test, damage was observable as pattern degradation after 500 frames leaving a time window of 12.5 s for total exposure.

Based on this and the long time to switch between SAXS and WAXD setup, we decided against the time lapsed loading protocol initially proposed. These exposure times and the sample grouping in a SAXS and

WAXD group allowed to drive a monotonic loading protocol with partial unloading steps (Figure 1a and b) to measure continuum damage which is much more suitable to study the mechanical behaviour of MCFs. We loaded the micropillars with 5 nm/min to 1.5 µm total deformation. Unloading and reloading steps of 50 nm were carried out every 150 nm of loading. We sampled the load displacement curve with a SAXS or WAXD measurement every 5 s so that 120 measurements including an unloaded reference were taken. The shutter was closed for 4.91 s and 4.8 s for SAXS and WAXD, respectively.

Based on the results of the radiation damage tests we changed the exposure time for the SAXS measurements to 90 ms yielding a total exposure during the investigation of the collagen network of 10.8 s which was smaller than the 12.5 s identified in the radiation damage test. In case of WAXD the total exposure time was 24 s. However, we consider the dose uptake less detrimental for the mineral phase especially at the later stages of the experiment.

As an additional check we performed radiation tests for the final SAXS and WAXD protocol. To do so, we performed relaxation tests for one specimen in SAXS and one specimen in WAXD configuration. We loaded the specimens up to 300 nm and kept the deformation constant for 10 minutes. Subsequently, we conducted the imaging protocol used during the micropillar experiments. No change in the load-displacement curves was observable due to the radiation. Furthermore, the degrading diffraction patters were observable for the WAXD measurements at the end of the test so that the radiation damage unfolds its detrimental effects at a later stage of the compression test after passing the ultimate point. In addition Barth et al. (2010) found minimal radiation damage during similar SAXS and WAXD measurements of fracture mechanics samples.

The SAXS and WAXD data showed the expected diffraction order (Figures 1d and e). The scans were located 7 µm below the interface between diamond indenter and micropillar to minimise artefacts. These were visible as vertical streaks in the SAXS measurements beneath the beamstop (Figure 1d) and are observable in Figure 1e as bright region at the bottom of the indenter. Despite this artefact the important 3rd order in the SAXS image is brightly visible just above the beamstop and below the streak artefact (Figure 1d). This allows an efficient displacement measurement in the collagen network based on changes of the D-spacing of the collagen fibrils which is initially 65-67 nm (Gupta et al., 2006) and, thus, allows identifying the strain in the mineralised collagen fibrils of the MCF. The WAXD images were much less affected by artefacts and the primary order to identify platelet strain is well visible. However, a conic shadow of the diamond indenter is visible in Figure 1e. The gathered data is currently evaluated and we are confident that, after detailed analyses, we will be able to compute strains in the collagen network and the mineral platelets to complement apparent stiffness and strength measurements.

Early evaluation of the mechanical data indicates a two times higher yield strength of the micropillar compared to yield stresses of the whole tendon (Gupta et al. 2006) . This fits well to the scale effect observed in lamellar bone (Schwiedrzik et al. 2014, Luczynski et al. 2015). Furthermore, no continuum damage of the micropillar was observable which is also in agreement with findings for lamellar bone (Schwiedrzik et al. 2014). Finally, early assessments of fibril and mineral strain after passing the yield point of the micropillar indicate ~1.5 % and 0.2 % in the collagen and mineral phase, respectively which corresponds to what could be expected assuming a parallel springs setup of mineralised collagen fibrils.

Discussion and Conclusion

The early results presented here are only indicative as the data is currently processed and analysed. The integration of a mechanical device to combine compression tests on micron sized volumes of ECM with SAXS and WAXD seems to be robust and reliable. Specifically, the alignment of the indenter, the beam and the micropillars was less difficult than expected and we were able to test all but two micropillars. These two micropillars were lost on day four due to operator fatigue.

One micropillar of the WAXD group was, by accident, imaged in a SAXS setting so that we have a SAXS specimen with longer exposure time. This sample will be of use to reconfirm that the chosen exposure time of 90 s was long enough. An early analysis of this sample did not show any advantages of the longer exposure.

We deliberately chose to perform this experiment in dry condition since combining micropillar testing and SAXS/WAXD based strain measurements was challenging by itself. Tests on dry specimens, however, are problematic since the ECM is tested in an unphysiologic state. It is known that the hydration state influences the elastic mechanical properties of MCF assemblies (Wolfram et al., 2010) but its impact on the ultimate

strength of the ECM remains unclear. Early results on rehydrated FIB milled micropillars, tested in another experiment, suggest that the anisotropy ratio of the yield strength changes from 1.6 (dry) to 1.3 (rehydrated) which compares well to the anisotropy ratio of microindentation hardness, a yield strength surrogate, which changes from 1.5 (dry) to 1.2 (fully hydrated tissue) (Mirzaali et al. in press). This implies that tests on dry tissue may, indeed, not be representative of the mechanical behaviour under physiologic conditions but it also implies that this tissue condition may be re-established by rehydration. Given this and the success of the scanning campaign reported here, we believe that we now have a unique experimental tool-box at hand that allows us to realise the same measurement on re-hydrated ECM and probe individual MCFs under quasi-physiologic conditions.

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