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Experiment Report Form

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Deadlines for submission of Experimental Reports

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- 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:** Nanoscale mechanics of cartilage collagen under physiologically realistic dynamic loading conditions	**Experiment number**: SC 4061
Beamline: ID02	**Date of experiment**: from: 26 June 2015 to: 29 June 2015	**Date of report**: 08/09/2015
Shifts: 9	**Local contact(s)**: Sylvain Prevost (email: sylvain.prevost@esrf.fr)	*Received at ESRF:*

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Report:

Understanding and correcting the biophysical and cellular mechanisms responsible for articular cartilage (AC) deterioration in degenerative joint conditions such as osteoarthritis (OA) is a medical priority area world-wide [1,2]. Many studies point to the importance of physical loading in the aetiology of arthritis. The mechanical functionality of articular cartilage is dependent on the interaction between the collagen and proteoglycan which comprise the bulk of the extracellular matrix (ECM) [3, 4]. In particular, the prestressed collagen fibrils restrain the swelling of the hydrated negatively charged proteoglycans providing a complex, anisotropic graded viscoelastic fibre composite. However, little is known about the fundamental nanomechanics of the collagen fibrils in both healthy and osteoarthritic cartilage.

In **Experiment SC4061** at ID02 (ESRF), we use high brilliance synchrotron radiation to measure the dynamic mechanical properties of the collagen fibrils in articular cartilage using *in situ* compressive loading with time-resolved SAXS. Shifts in the fibrillar D-period, calculated from the meridional SAXS pattern, were used as a measure of fibril strain. Osteoarthritic degradation was mimicked in bovine cartilage explants using the cytokine Interleukin-1 beta. The initial results show that there is a overall fibrillar relaxation following a relatively low number of cycles during dynamic loading, that fibril orientation These results suggest that the fibrils are responding dynamically to the changes in the surrounding matrix i.e. as the bulk tissue swelling pressure reduces via fluid release under compression, the 'pre-strained' fibrils are able to relax by around 0.1%.

Experimental details:

Bovine explants were harvested from the flattest portion of the proximal surface of the metacarpophalangeal joints. Joints were taken from 18-24 month old adult steers that were freshly slaughtered and obtained from a

local abattoir. The 2 mm explants were then incubated in Interluekin-1 beta (IL-1β) (Sigma Aldrich, UK) at 5ng.ml for 7 days at 37°C. The samples were then dynamically compressed in a customized micromechanical stage (20 N load rating) [5, 6, 7]. Small-angle X-ray scattering (SAXS) measurements were carried out using a 15 µm diameter beam at a wavelength of λ=0.995Å and a Frelon detector. The tissue was maintained in a physiologically hydrated state in phosphate buffered saline during testing.

The n=5th order meridional diffraction peak arising from the 65-67 nm D-stagger in the collagen fibril [5] was used to determine the fibril D-period and the degree and direction of orientation. An example SAXS spectrum from cartilage is shown in **Figure 1A**. By fitting 3 radially integrated narrow sectors around the 3rd order peak, we were able to subtract the contribution of the diffuse scattering to identify the SAXD signal due to the collagen fibril alone. The peak position (in angles) gave the predominant orientation of the fibrils [6].

During the experiment, a line scan through from the superficial to deep zone was performed followed by
a) ***cyclic loading*** to 20% strain at either 0.3 or 1Hz for 200 cycles, located in the deep zone. At the peak and trough of each loading cycle, a SAXS pattern was acquired (**Figure 1B**). To reduce effects of radiation damage, the sample stage was continously moved in a small range of a few tens of microns to allow measurements to be taken across this range, with reduced X-ray exposure for any particular tissue volume in this range, over multiple cycles.
b) ***stress relaxation*** following 20% loading. The SAXS spectra were taken with 0.5 s exposure times continously from the start of the relaxation segment.

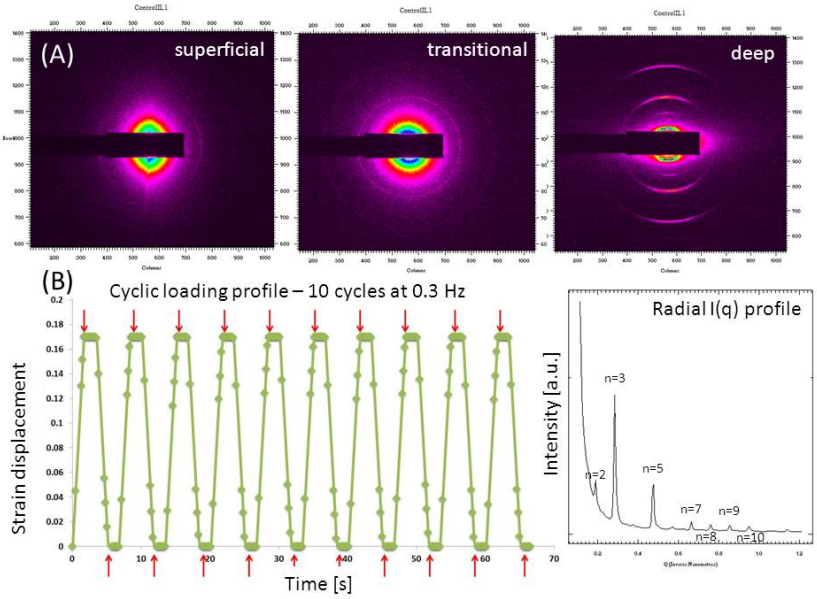

Figure 1: *(A) Example 2D SAXS images from the different zones of cartilage. (B) Cyclic loading protocol used during tests of bovine cartilage during in situ SAXS; samples were compressed to 20% strain each cycle. (C) Example radially integrated intensity profile I(q) showing the different orders (n) of the meridional peaks.*

Fibril strain and D period: A single Gaussian with a linear background was used to fit the azimuthally integrated intensity around the 5th order peak via $I(q) = a+bq + I_0 \exp(-½ ((q-q_0)^2/\Delta q_0)^2))$. The D period was calculated from the peak position q_0 via $D = 5 \times 2\pi/D$. Fibril strain was calculated from percentage shifts in the D period relative to the unstressed values.
Fibril orientation distribution: A fit function consisting of two Gaussians of identical shape and separated by 180° ($I(\chi) = I_0(\exp(-½ ((\chi-\chi_0)^2/\Delta\chi_0)^2)) + \exp(-½ ((\chi-\pi-\chi_0)^2/\Delta\chi_0)^2))$) was used to fit the data.

A total of 40 samples were measured. Cyclic loading measurements were carried out at 0.3 Hz and 1 Hz loading frequencies, and both control and IL-1 treated bovine cartilage samples were measured. For stress relaxation, a single strain level (20%) was used and an equal number of control and IL-1 samples were measured.

Initial Results:

While data analysis is still ongoing, we show here four examples of initial results regarding fibril strain and reorientation:

1) **Fibril D-period behaviour over initial cycles during onset of cyclic loading**: We plot the obtained D-period over the initial 4 cycles in **Figure 2**. It is seen that external load induces a periodic compression in the fibril D-period (compressive fibril strain), and that the second loading cycle compresses the fibril to a lower D-period than the first, indicating that there is not perfect elastic coupling between the external force field and the collagen fibril mesh, possibly due to viscoelastic effects in the extrafibrillar matrix.

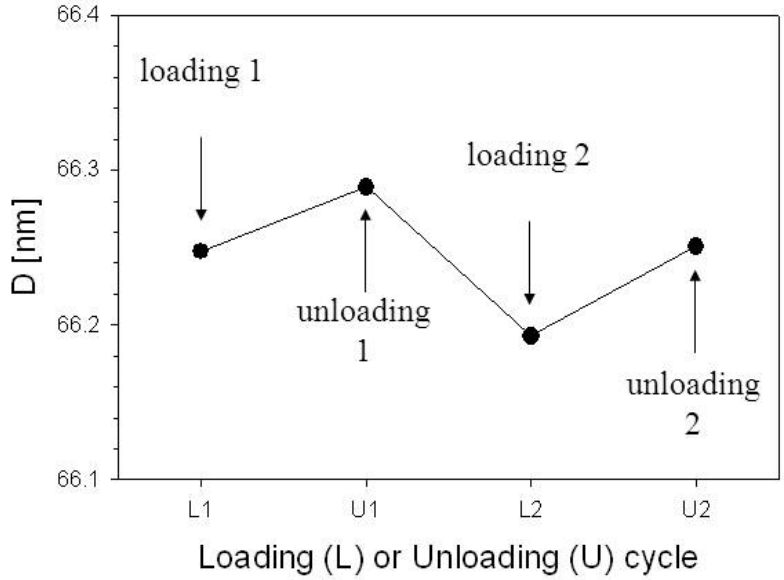

Figure 2: *D-period variation over the first 4 loading cycles in a loading protocol of the type shown in **Figure 1B**.*

2) **Fibril orientation changes with loading**: The angular intensity distribution I(χ) changes on application and release of load. We show in **Figure 3** I(χ) plots before and after loading on IL-1 treated bovine cartilage.We see that on compressive loading, the cartilage fibrillar distribution widens considerably, indicating that the vertically oriented fibrils in the deep zone are forced into off-axis positions on application of load. Such fibrillar reorientation will also be a significant energy dissipating mechanism due to the fibrillar sliding in the viscous matrix.

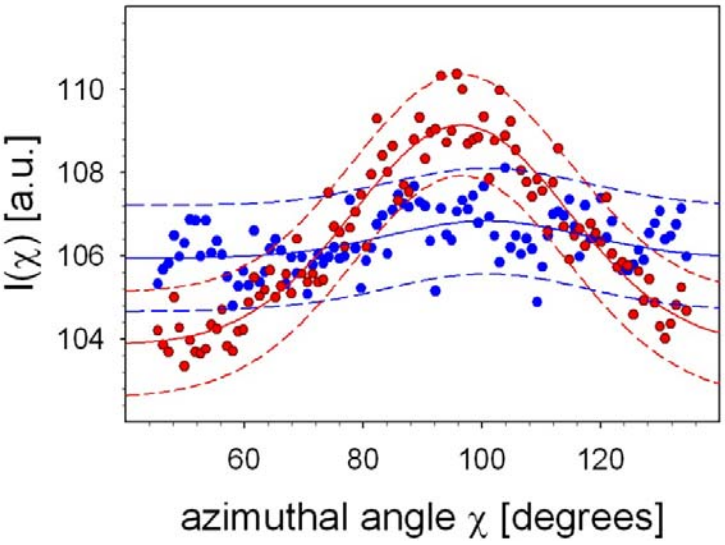

Figure 3: *Angular intensity profile I(χ) measured before (red) and after (blue) loading. The solid lines are best fits to Gaussian functions and the dashed lines are 95% prediction intervals.*

3) **Long-term decrease of fibrillar-D period with cyclic loading**: We find that on loading up to 200 cycles, a progressive decrease in the D-period of the cartilage at peak load. As seen in **Figure 4**, the D-period decreases by ~ 0.15% over 200 cycles. Such a phenomenon indicates a progressive compression of the fibrils under repeated loading. We postulate that the origins of this relatively slow process is due to progressive diffusion of water in the interfibrillar space away from the loaded zone. We base this idea on the fact that the hydration of the interfibrillar matrix constituents, specially proteoglycans, leads to a swelling pressure in cartilage. In unloaded cartilage, these forces are resisted by the fibrils, which have an increased D-period as a result (which we have shown previously). When water is

pushed out of the interfibrillar matrix, the swelling pressure reduces, as a result of which the fibrillar pre-strain and the D-period reduces.

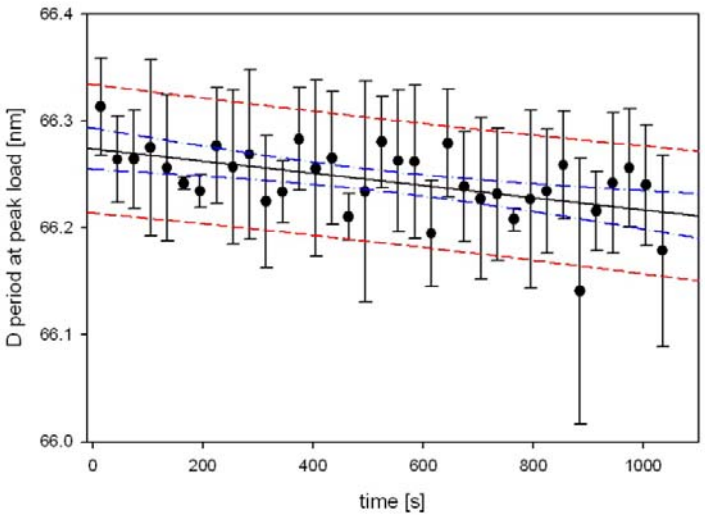

Figure 4: *Progressive reduction of D period at peak load over 200 cycles. The data is binned for clarity. The solid line is a linear regression, and dashed lines are 95% prediction intervals. Linear regression D(n) = D0 + dDdN * n, where D0 = 66.27 nm, and dDdN = -5.71E-005 nm/cycle (p = 0.0009 for the fit; R^2 = 0.2875)*

4) **Multistage fibrillar relaxation in response to stress relaxation**: On stress relaxation following loading to 20%, we see that the fibrils initially compress (as expected), but do not immediately return to the unloaded state in the strain holding segment. Rather, they continue to compress (stage A in **Figure 5**). After ~200 seconds, the D-period starts to increase again, but surprisingly after ~500 seconds the D-period again starts decreasing. Measurements in both the deep and transitional zone of cartilage showed this effect. While we have to analyze this phenomenon more closely and test for statistical significance of these trends, it is likely that these changes over relatively long periods ~1 minute or more are driven also by stress induced fluid flow gradients across the tissue.

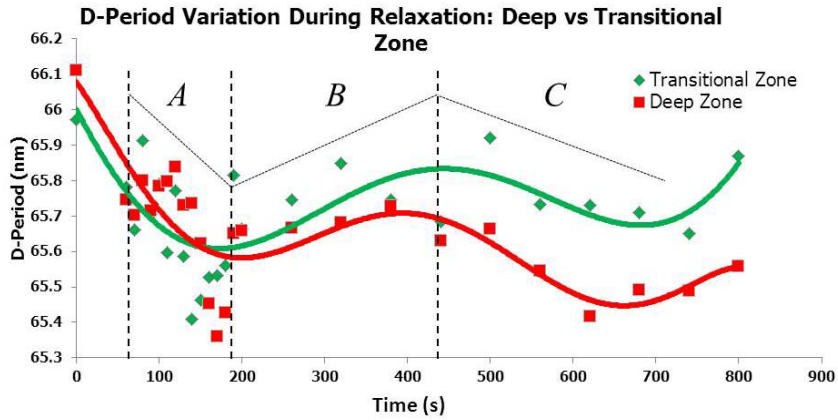

Figure 5: *Multistage changes in fibril D-period in deep (red) and transitional (green) zones of cartilage. Following loading to 20% strain, the three stages of the fibrillar deformation are indicated as A, B and C with dashed lines separating the zones as guides to the eye, and dotted slope lines indicating the trend of increasing or decreasing D.*

Issues encountered during the beamtime:

The beamtime ran very smoothly largely due to the excellent support of the beamline scientists, especially Dr. Sylvain Prevost and Dr. T. Narayan.

One minor issue we encountered was that in order to synchronize the acquisition of frames with the peaks and troughs of the loading cycle, we attempted to use a USB-based pulse generator connected to our mechanical test control software. Due to technical issues relating to pulse width, it was not possible to automate the synchronization in this beamtime, However, we received very useful guidance from the beamline scientists in how to modify our control software to allow the synchronization to work in subsequent beamtimes.

Summary and Outlook:

Our initial analysis of nanoscale deformation of bovine cartilage during cyclic and stress-relaxation protocols is very promising, showing indications of quantifiable fibrillar deformation kinetics involving both fibril compression and reorientation. Currently we are analyzing the obtained data in more detail, with measures including angular dependent fibril strains, modelling the stress relaxation at the fibrillar level as a multi-scale viscoelastic process and developing a model for the collapse, compression and reorientation of cartilage fibril "arcades" under loading. We will complement these measures of nanoscale fibril strain with microscale strain measurements using epifluorescence microscopy, in order to factor out the effect of microscale strain gradients. In the medium term, we wish to apply a similar protocol to human cartilage explants in order to begin to translate our findings about nanoscale cartilage mechanics to clinically relevant conditions like osteoarthritis and joint degeneration.

References:

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[2] Thomas, E., et al., Pain, (2004)

[3] A. M. Bhosale et al, Br. Med. Bull. (2008)

[4] T. Aigner, Adv. Drug Deliv. Rev., (2003)

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[6] A. Karunaratne et al, *Bone* **52**, 689 (2013)

[7] A. Karunaratne et al, *15th Intl. Conf. Biomed. Engg.* (2014)