Abstract
Bone contains ~2% wt citrate; a concentration ~5-25 times higher than that occurring in most of other biological tissues. This suggests a pivotal role of citrate in collagen mineralization. However, this role has remained elusive to date. Pilot ex situ WAXTS experiments on synthetic collagen fibrils mineralized in the presence of citrate, allowed us to detect in the low angle region the peak originating from the near-neighbour equatorial fibrils distance simultaneously to the appearance of the ACP precursor, visible at wider angles. Hence, simultaneous detection of both peaks allows monitoring in situ the structural evolution of the collagen/mineral system. However, the multiple length scale complexity of this system requires that WAXTS data are complemented with SAXS measurements (sensitive to both the nanoscale mineral size (5-50 nm) and to the longer fibril distances (67-120 nm)). Therefore, we proposed to use in situ time-resolved SAXS/WAXS, at physiological conditions, to follow the structural evolution of collagen/mineral in the presence of citrate to clarify the precise role of the latter in determining the species formed at the early stages and in driving the intrafibrillar collagen mineralization.

Goals
Our overarching goal is to clarify the specific role of citrate in controlling the species formed at the early stages, and in driving the intrafibrillar collagen mineralization. During this beamtime, we have monitored the structural changes of collagen fibrils during their assembly and mineralization in the presence and absence of citrate by in-situ SAXS/WAXS experiments.

Experimental details

Collagen mineralization experiments were carried out in 2 mm glass capillaries. SAXS/WAXS signals of the collagen matrix were collected at different times. Once assembled, collagen fibrils show an axial periodicity, the so-called D-banding. D-bands result from staggered self-assembly of individual collagen molecules into larger fibrils with a periodicity of about 67 nm. This periodicity produces repeated diffraction peaks, the first order (n = 1) appearing at a Q-value of *ca.* 0.0093 $Å^{-1}$. Consequently, we selected a sample-to-SAXS detector distance of 6.2 m, providing a Q-range from 0.002 to 0.07 $Å^{-1}$ for a beam energy of 12.4 KeV.

Experimental results

During this beam-time, the experiments can be divided in the following sections:

a) *Selection of the ideal experimental conditions*. We collected SAXS signals from the collagen matrix prepared in different sample holders, *i.e*, fluid cells provided by the beamline and glass capillaries of different diameters. We obtained optimal SAXS signals for the 2 mm glass capillaries.

b) *Collagen self-assembly*. The scattering curve of collagen shows the first useable data point (Q_{min}) at 0.0026 $Å^{-1}$ corresponding to a d-spacing of 240 nm (Fig. 1). The scattering curve, exhibiting a Q^{-3} like scattering in most of the Q-range, shows peaks well above the noise level. Plotting the *n*-order of these reflections versus the corresponding peak position, one obtains a straight line, with slope equal to the periodic

D-band of collagen fibrils (d = 66 nm, inset of Fig. 1). These data confirm that the assembly of collagen was successfully achieved.

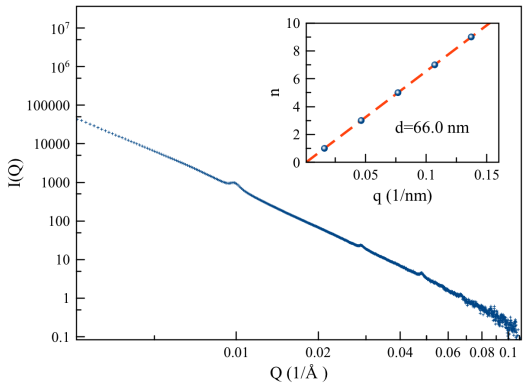

Figure 1. SAXS signal of a collagen matrix in PBS (pH 7.4). The periodic Bragg reflections due to differences in the electron density distribution of the gap and overlap zones of collagen fibrils can be observed. The inset shows the linear plot of the reflection order *n* vs the corresponding peak position (q = 2 sin θ/λ), where the slope is equal to the d-band (d=66 nm).

c) Evaluating the effect of the beam on the collagen structure (beam damage). We are interested in understanding the structural changes induced by the infiltration/incorporation of the mineral phase in the collagen fibrils. Thus, we first investigated the possible impact of the X-ray beam on the collagen d-band. The impact of the X-ray exposure time on the SAXS signal of collagen fibrils is shown in Fig 2. The position of the Bragg reflections due to the d-band is shifted to higher Q-values at increasing exposure times (inset Fig. 2). Therefore, the exposure induces a gradual shrink of the axial periodicity of collagen fibrils. This is clearly observed when plotting the d-band as a function of the exposure time (Fig. 2B). This plot also reveals that the 66-nm d-band can be recovered by collecting data from different sample positions (shifting the irradiated area at the sample), but the same effect emerged again after prolonged exposure (position 2, blue square in Fig 2B). We found that collection times shorter than **20 seconds** do not produce any observable effect on the organization of the collagen fibrils.

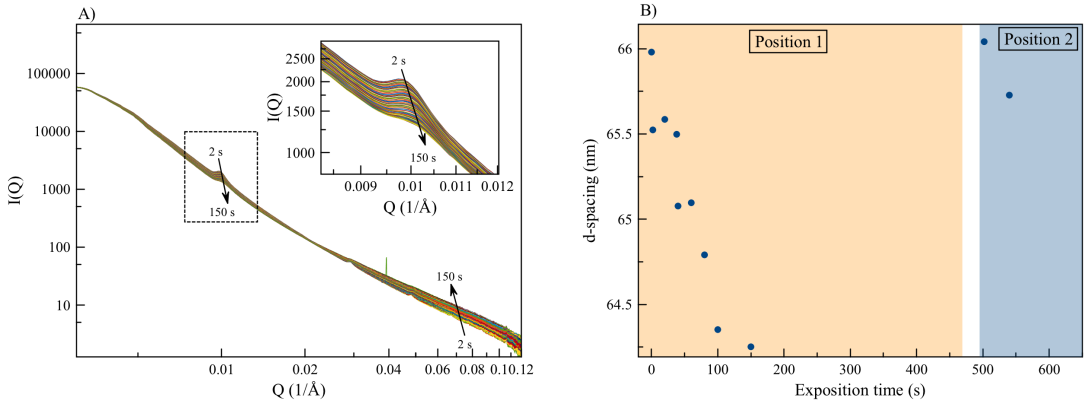

Figure 2. Impact of X-ray exposure time on the SAXS signal of collagen matrix in PBS. (A) Time-dependent SAXS patterns of collagen collected at increasing exposure time ranging from 2 to 150 seconds. The inset shows in more detail the Q-range containing the first order Bragg reflection of collagen fibrils. (B) Plot of the d-band as a

function of the exposure time. SAXS signals from two different positions of the collagen matrix were sequentially collected (position 1: data contained in the orange square; position 2: data contained in the blue square).

d) Collagen mineralization experiments: We monitored the collagen mineralization in the absence and presence of citrate, with different Ca to citrate ratio. Figure 3 shows the SAXS signals of collagen mineralized in the absence of citrate (CP10, yellow) and in the presence of citrate, with $[Cit^{3-}]_0 = 1.5*[Ca^{2+}]_0$ (CP115, green curve) and $[Cit^{3-}]_0 = 2*[Ca^{2+}]_0$ (CP12, red curve). The latter is very similar to the signal of the non-mineralized collagen, both exhibiting a Q^{-3} like scattering in most of the Q-range. It confirms the inhibition of mineral precipitation using this citrate concentration, in agreement with the information from the corresponding WAXS patterns. In contrast, the SAXS signals obtained for reduced citrate concentrations both (CP115 and CP10) exhibit a different dependence attributed to the precipitation of the mineral phase.

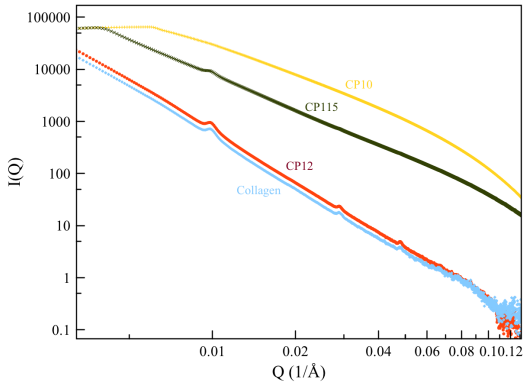

Figure 3. SAXS signals of non-mineralized collagen (light blue), collagen mineralized in the absence of citrate (CP10, yellow curve) and with the following citrate concentrations: $[Cit^{3-}]_0 = 1.5*[Ca^{2+}]_0$, CP115 (dark green); $[Cit^{3-}]_0 = 2*[Ca^{2+}]_0$, CP12 (red curve). The SAXS patterns were collected from the collagen matrix after 53 hours of maturation in contact with the corresponding mineralizing solutions.

We also collected in-situ SAXS/WAXS signals of the CP10 and CP115 series upon maturation. All the SAXS data are being analysed by fitting standard SAXS models using least-squares methods in order to extract the morphological evolution of the mineral phase as well as the structural variations of the organic matrix (d-band). We are paying special attention to the signals collected soon after mixing, in order to identify the species formed during the early mineralization stages.

Potential Publications

Before publication, additional in-situ SAXS and WAXS data are still required to confirm the above observation and fully comprehend the specific role of citrate.