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Bone mineral change during experimental heating: an X-ray scattering investigation

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Abstract

The effects of heating and burning on bone mineral have previously been studied using techniques such as X-ray diffraction (XRD) with the aim of discerning a characteristic signature of crystal change. This would enable a better understanding of alteration to bone mineral during heating, which would in turn impact on the preparation and use of natural bone hydroxyapatite as a biomaterial resource. In addition, this knowledge could prove invaluable in the investigation of burned human remains from forensic and archaeological contexts in cremation and funerary practice. Here we describe a complementary method, small-angle X-ray scattering (SAXS), to determine more accurately the changes to bone crystallite size and shape during an experimental heating regimen. Samples were subjected to controlled heating at 500°C, 700°C, or 900°C for 15 or 45 min. Our results show bone crystallites begin to alter in the first 15 min of heating to 500°C or above. They then appear to stabilise to a temperature-specific thickness and shape with prolonged heating. While the samples heated to lower temperatures or for shorter periods produce XRD traces showing little alteration to the apatite, corresponding information obtained from SAXS shows an early, subtle change in crystal parameters.

Keywords: XRD; Microstructure; Crystal growth; Bone; Hydroxyapatite

1. Introduction

Bone mineral is an important biomaterial resource. Accurate measurement of bone crystal alteration, both in structure and composition, has been a focus of biomaterial research for several years (e.g. [1–6]). Heat treatment has been used to deproteinate bone mineral for use in osteoimplantation, since natural hydroxyapatite with the organic matrix removed is potentially a better basis for bone grafting than synthetic materials [1]. The usefulness of this material, however, relies on the retention of biogenic crystallite characteristics throughout the preimplantation treatment process. Changes to the biogenic composition and structure of bone mineral following heat treatment at different temperatures could affect its efficacy in these procedures. It would be valuable to know the temperature at which crystallites begin to change, and how rapid the alteration can be. This would allow for an optimisation of the heat treatment process to maximise the removal of the organic material in bone with the minimum of disruption to the mineral.

In addition, the ability to identify burning and burned bone in the forensic and archaeological records has long been an important and contentious issue. Several techniques to determine burning or heating regimen used in archaeological contexts have been derived, with varying levels of success [7–10]. Determination of the temperature and duration of burning, as well as the background noise of potential diagenetic effects [11] would shed light on cooking practices, the early use of fire, cremation as a burial rite, and other archaeological and paleoanthropological puzzles. Further, the effects of burning on bone specimens and the determination of the techniques used are crucial in the resolution of forensic cases where cremation or other fire damage to remains is present (e.g. [3,12–15]).

Previously, X-ray diffraction (XRD) and Fourier-transform infrared spectroscopy (FTIR) have been used

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to determine changes to the mineral phase of bone during heating or burning (e.g. [3,4,7,10,16,17]). Results have shown that there is a generalised trend toward a more 'perfect' or 'crystalline' phase of hydroxyapatite at temperatures up to 1000°C; above this, the emergence of different mineral phases can sometimes be discerned. Fine-scale changes to bone ultrastructure at temperatures below 1000°C can be difficult to detect using XRD, however [5,6], and a complementary measure of crystal change would be useful in these contexts.

In the last few years, advances in technology have contributed to a resurgence in the use of small-angle X-ray scattering (SAXS) to examine crystallite nanostructure in a range of materials. This technique allows for the accurate determination of crystal size, shape, and orientation within bone independent of crystal lattice perfection [18–20]. SAXS has been shown to provide information regarding crystallite structure that is complementary to other techniques [21] and recently has been used to characterise diagenetic change in archaeological bone [22,23].

This study aims to elucidate more clearly the changes to bone mineral during burning using a combination of XRD (or wide angle-X-ray scattering, WAXS) and SAXS techniques. We aim to test the hypothesis that changes to crystallite size and shape during early stages of burning and at lower temperatures may be more readily visible using SAXS, thus opening up a new route into the investigation of the effects of heat treatment on bone mineral in biomaterials research as well as archaeological and forensic contexts.

2. Methods

2.1. Samples

Samples of fresh cortical bone removed from sheep long bones were defleshed and heated experimentally for either 15 or 45 min. Pairs of samples were subjected to the same heating regimen, and 14 samples, including two controls, were analysed in all. Table 1 shows the details of the samples used, including heating times and temperatures, dimensions, and percent weight loss following heating. Whole long bone samples were heated in pairs in an electric muffle furnace. The samples were placed on heatproof ceramic trays in order to aid retrieval after heating. Each pair of long bones was placed into the furnace once the temperature had reached 200°C and allowed to heat up to the designated temperature. This was deemed more akin to natural burning or heating situations where soft tissue insulates the bone surface in the early stages of heating. Preheating at 200°C removes the potential impact of extremely rapid heating as an influence on hard tissue microstructure [24]. Once the chosen temperature had been reached, the samples remained at that temperature for 15 or 45 min. After this time they were removed from the furnace and allowed to cool naturally before being handled again. Fig. 1 illustrates the heating rate of the furnace used.

Following heating and subsequent cooling, the samples were ground by hand into a fine powder using an agate mortar and pestle, and stored in micro test tubes. For scattering measurements, the powdered samples were loaded into a sample carriage between two mica sheets and mounted in the vacuum chamber of the NanoSTAR (Bruker AXS, Karlsruhe) X-ray facility at the University of Stirling. The data collection procedure used followed that described in detail in [22]. Scattering profiles were taken over 3 or 9 h exposures using sample to detector distances of 22.5 cm (WAXS) or 1.25 m (SAXS). Collected data was corrected for camera distortions, a background image was subtracted, and images were analysed using in-house software. The two-dimensional detector output was converted into spherically averaged one-dimensional profiles. Values for crystal thickness (T), as well

Table 1
Details of experimental heating samples

Sample code	Temperature (°C)	Time (min)	Weight (g)	Length (mm)	Width (mm)	Loss (%)
3-0002-05	500	15	64.30	218.40	45.10	30.79
3-0002-07	500	15	55.50	153.00	44.60	32.97
3-0003-02	700	15	39.50	147.00	45.80	34.43
3-0003-06	700	15	47.70	181.00	48.90	35.01
3-0004-03	900	15	74.20	211.00	45.60	50.67
3-0004-05	900	15	69.00	149.00	47.00	48.13
3-0005-01	500	45	105.30	184.00	49.60	55.94
3-0005-05	500	45	93.70	186.00	52.20	51.01
3-0006-01	700	45	23.90	160.00	37.70	38.08
3-0006-02	700	45	14.50	120.00	30.30	38.62
3-0007-02	900	45	65.70	186.00	52.10	51.88
3-0007-10	900	45	51.40	207.00	42.80	44.16
Control 1	N/A	N/A	64.30	218.40	45.10	N/A
Control 2	N/A	N/A	55.50	153.00	44.60	N/A

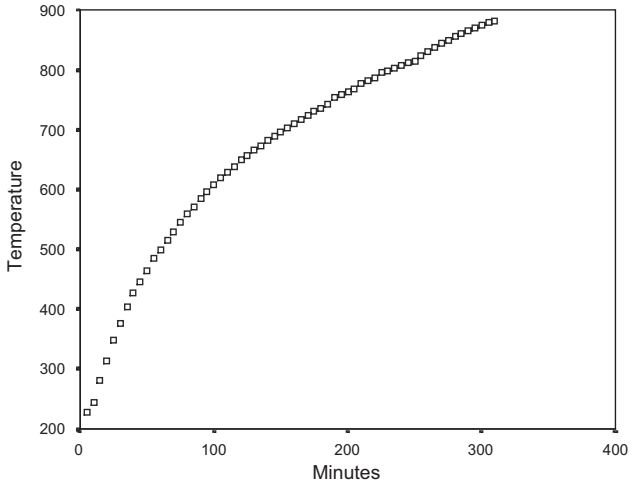

Fig. 1. Trace showing the heating rate of the furnace used, from the starting point of 200°C to the endpoints of 500°C, 700°C, or 900°C.

as curves describing crystallite morphology, were determined for each sample using the SAXS data. A detailed procedure for these calculations, including the determination of T from the surface to volume ratio of the mineral fraction of bone, is presented in [19]. Briefly, T as defined here is the thickness of the smallest dimension of the crystallite, for example, the cross-sectional diameter of a needle-like crystallite. WAXS data was converted to one-dimensional plots for comparison with previous XRD work on heated bone.

3. Results

Upon heat exposure, the samples lost 30–55% of their initial weight. Since the samples received no pre-treatment other than defleshing, it is likely that this weight loss reflects dehydration and extraction of the lipids present in bone as well as alteration to and loss of the protein present in bone. The composition of adult faunal bone has been reported as 45% water, 25% ash (mineral), 20% protein (collagen and non-collagenous proteins) and 10% lipid [25]; these proportions seem to support the loss of water and lipid in these samples.

3.1. Wide-angle X-ray scattering

Figs. 2–4 show results of WAXS techniques. The two control samples (Fig. 2) contain prominent broad peaks that correspond to the major apatite lattice reflections [4,6,7]. The 2.9 nm^{-1} peak corresponds to the (0 0 2) reflection and the 3.7 nm^{-1} peak to the (3 0 0) reflection of carbonate hydroxyapatite. These main peaks are present in all the WAXS results. In Fig. 4, a new peak is visible as a shoulder at 3.8 nm^{-1}; this corresponds to the (2 0 2) reflection of carbonate hydroxyapatite. Reflections were obtained from PDF file 19-0272 (PCPDFWIN version 2.1, ICDD, 2000).

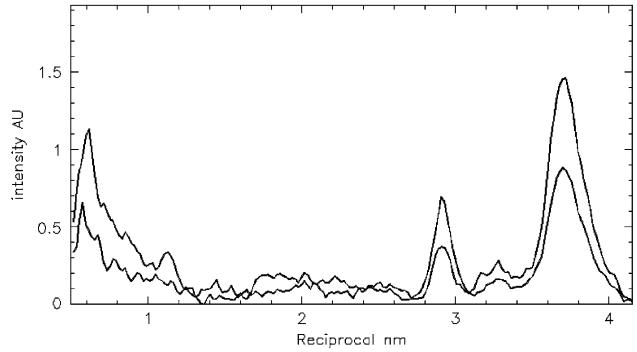

Fig. 2. Wide-angle XRD traces of two control (unheated) samples. X-axis in nm^{-1}.

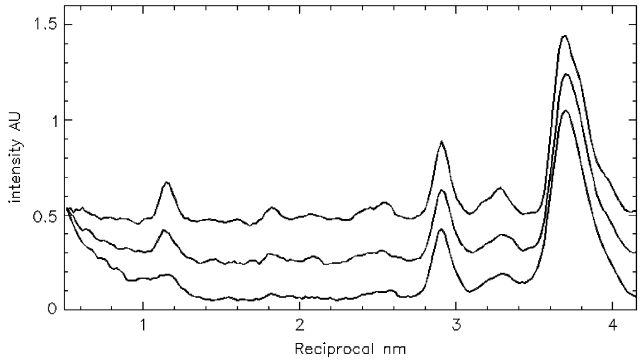

Fig. 3. XRD traces of samples heated for 15 min. Uppermost trace, 900°C; middle trace, 700°C; lowermost trace, 500°C. X-axis in nm^{-1}.

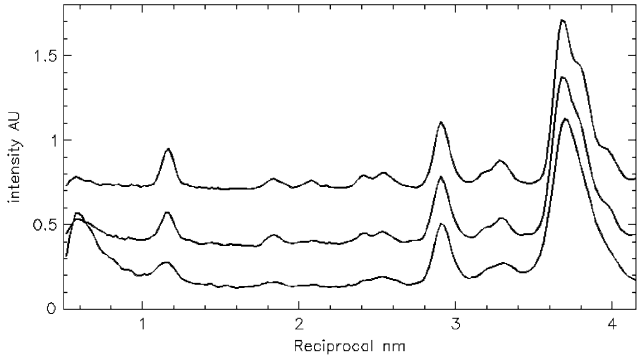

Fig. 4. XRD traces of samples heated for 45 min. Uppermost trace, 900°C; middle trace, 700°C; lowermost trace, 500°C. X-axis in nm^{-1}.

Following heating for 15 min (Fig. 3), the samples still show broad, single peaks at locations similar to those seen in the control; in particular, very little change is seen in the samples heated for 15 min at 500°C. Samples heated to 700°C or 900°C show evidence of a slight shoulder on the 3.6–3.8 nm^{-1} peak, which also appears to have narrowed slightly. Otherwise, no marked difference is apparent, and the traces still correspond with those for unheated, poorly crystalline hydroxyapatite [4].

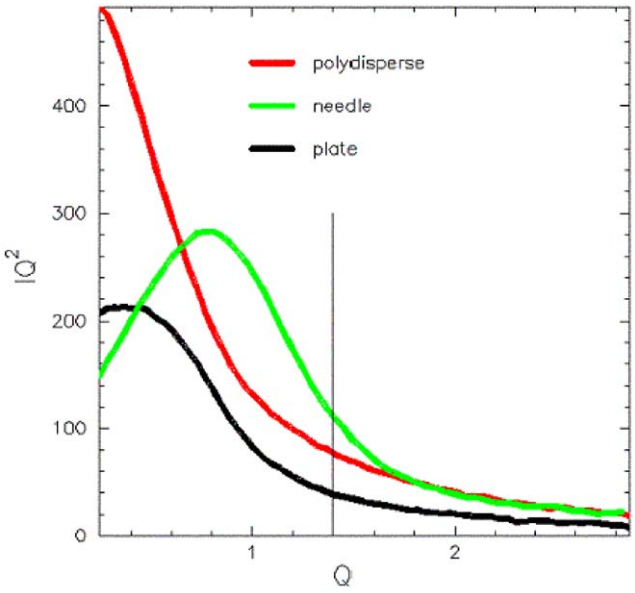

Fig. 5. An example of a Kratky plot showing the three morphologies of crystallites commonly found in bone. After Wess et al. (2001).

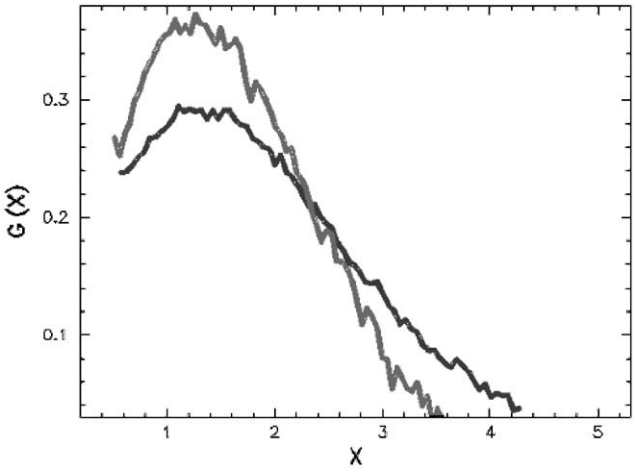

Fig. 6. Thickness-corrected plots illustrating needle-like morphology in both control (unheated) samples.

In Fig. 4, evidence of increasingly crystalline hydroxyapatite is evident in narrower peaks and some peak separation, particularly in the samples heated to 900°C for 45 min. A much more prominent peak at 1.15 nm^{-1} becomes apparent in the samples heated to 700°C or 900°C, and one of the samples heated to 500°C shows a new peak at 2.2 nm^{-1}. In all, however, the traces show increasingly crystalline hydroxyapatite at high temperatures. Increased heating times led to greater crystal perfection, evident in the increased level of peak splitting and narrowing in the higher temperature samples. Overall, no new mineral phases were evident, reinforcing previous results that heating to these temperatures leads to an increase in crystal perfection, but a retention of hydroxyapatite as the predominant mineral phase [4–7].

Table 2
Crystal thickness values from SAXS profiles

Sample code	Temperature (°C)	Time (min)	T (nm)
3-0002-05	500	15	5.24
3-0002-07	500	15	5.65
3-0003-02	700	15	10.37
3-0003-06	700	15	14.09
3-0004-03	900	15	17.49
3-0004-05	900	15	22.59
3-0005-01	500	45	7.81
3-0005-05	500	45	6.71
3-0006-01	700	45	16.11
3-0006-02	700	45	15.60
3-0007-02	900	45	31.26
3-0007-10	900	45	26.66
Control 1	N/A	N/A	2.79
Control 2	N/A	N/A	2.36

3.2. Small-angle X-ray scattering

Initial SAXS measurements were run for 3-h exposure times. Thickness values for the heated and control samples are provided in Table 2.

The two control samples have crystallite thicknesses of approximately 2.3–2.8 nm, which fall slightly below the average values for crystallites in mature faunal bone [19,22]. With heating, the crystallites grow substantially in size, from just over 5 nm in the samples heated at 500°C for only 15 min to over 30 nm in samples heated at 900°C for 45 min. The thickness values increase for longer heating times, but a substantial change has been wrought during the first 15 min at high temperature. With prolonged heating, the difference between the samples within a pair is reduced compared to the differences between pairs, indicating there may be a stable crystal size for a specific temperature. Samples heated to 900°C displayed such high levels of alteration, which resulted in crystal thickness increases of up to tenfold, that these were rerun for 9-h exposures. The 3-hour runs produced very weak data for these four samples. It appears that with increasing crystal size, the scattering profiles become weaker and less informative as the necessary incident angle for sufficient X-ray scattering becomes smaller. Thickness values from the nine-hour runs are provided in Table 3. From the longer runs similar, although slightly larger, thickness values were obtained for the samples heated for 45 min. The samples heated for 15 min produced anomalous results due to the limitations of the technique: SAXS can potentially determine crystal thicknesses of up to 50 nm [19], but large crystallites become increasingly difficult to measure accurately.

Table 3
Crystal thicknesses measured over 9 h for samples heated at 900°C

Sample code	Temperature (°C)	Time (min)	T (nm)
3-0004-03	900	15	29.39
3-0004-05	900	15	74.04
3-0007-02	900	45	31.83
3-0007-10	900	45	32.90

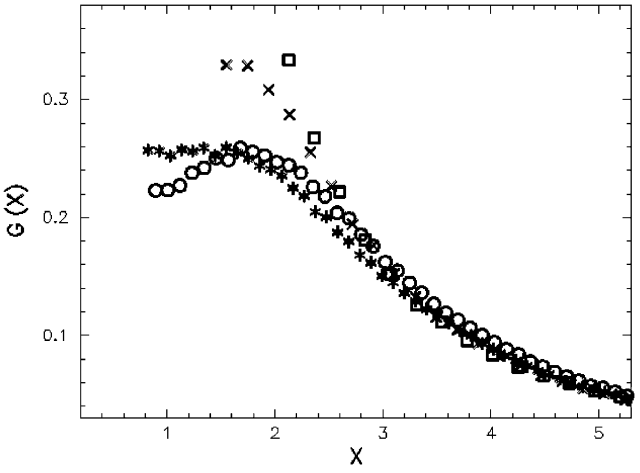

Fig. 7. Thickness-corrected plots for pairs of samples heated for 15 min at 500°C (circles and asterisks) or 700°C (squares and crosses). The development of a more plate-like habit at the lower temperature, progressing to polydisperse crystals as the heat increases, is evident.

Crystal shape profiles were also determined for the 14 samples, and are shown in Figs. 7 and 8 (see also Figs. 6–8). Following [19] and [22,23], crystal shape profiles were derived from Kratky plots generated via radial averaging of the scattering intensity for each sample. An example of a Kratky plot illustrating the three shape parameters—needle-like crystals, plate-like crystals, and polydisperse crystals—is shown in Fig. 5. In Figs. 6–8, the plots were corrected for thickness variation following a procedure detailed in [19], allowing a more direct comparison of crystallite habit alone. Fig. 6 displays the even needle morphology seen in the unheated control samples. In Fig. 7, shape changes after 15 min of heating are evident. The pair of samples heated to 500°C shows a more plate-like shape, whereas the considerably larger crystals in the samples heated to 700°C have a polydisperse morphology. This may reflect the difficulty in calculating the shape of crystals so large using this method. After 45 min of heating (Fig. 8), the shapes of the samples heated to 500°C are still plate-like, although the two curves are much more similar to each other. The samples heated to 700°C show a similar trend: still polydisperse, but more alike. The samples heated to 900°C are not shown; because of their large size and weak scattering trait, the crystal habit could not be determined.

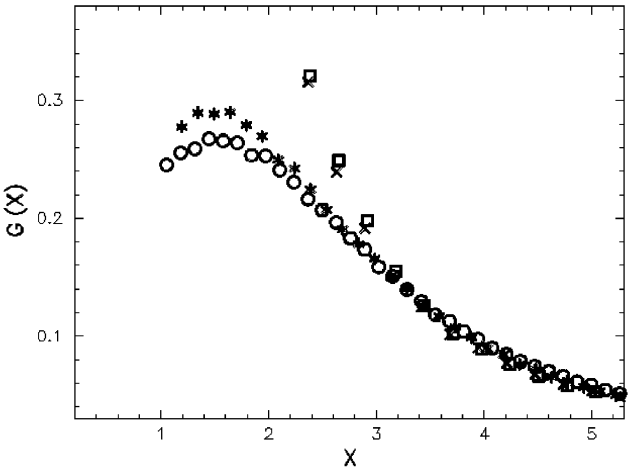

Fig. 8. Thickness-corrected plots for pairs of samples heated for 45 min at 500°C (circles and asterisks) or 700°C (squares and crosses). The curves again show plate-like crystals in the lower temperature and polydisperse crystals in the higher temperature samples.

4. Discussion

From the above results, it appears that significant changes in crystallite shape and thickness occur during experimental heating. WAXS measurements confirmed earlier results that found increasingly crystalline hydroxyapatite at the temperatures used here, but detected no new forms of mineral phase. Previous studies of heated bone have found calcium oxide (CaO) formation at temperatures above 700°C [4], but the primary effect of heating is to generate larger and more crystalline hydroxyapatite [7]. It has been suggested that the formation of different mineral phases as a result of heating may be a function of age: CaO has been found in human samples older than 22 years in one study [6] and a link between skeletal maturity and mineral change during heating has been found to be common to several mammalian species [17]. Our results show a slight narrowing of peaks with increasing heat, but contain no clear evidence of new mineral formation, corroborating the earlier conclusions that the existing hydroxyapatite becomes more crystalline with heat.

SAXS results show an increase in thickness and an alteration in crystal morphology with heat, which correlates with earlier electron microscopy investigations into heated bone structure. Raspanti et al. [2], and more recently Quatrehomme et al. [26], using scanning electron microscopy, showed there was little structural change to bone heated to 500°C, but on heating to 700°C or higher, the mineral phase was replaced by large clumps of crystallites. A similar change is reflected in the mineral alteration seen in the SAXS profiles, albeit on a different scale; larger crystallites of indeterminate polydisperse habit appeared upon heating to 700°C, and heating to 900°C made measurement of

crystallite size or shape using SAXS difficult at best. To more accurately determine the characteristics of the crystallites in samples heated past 700°C, an ultra-small angle X-ray scattering method (USAXS) could be used; this may delineate the structure of these large crystallites more clearly, but is beyond the technological capacity available at the time of this study.

Shape changes in crystallites and an initial thickening were evident in the samples examined here in the first 15 min of heating. With increased heating time, the shape alteration remained very similar to that obtained in 15 min, but the differences between the samples in each pair were slightly reduced. Crystals became thicker after 45 min of heating, and the differences between pairs became more pronounced than the differences within pairs. This implies that there is a temperature-specific stable mineral structure that emerges gradually with increased heating time: the first 15 min allow for initial shape change, while after 45 min the thickness increases without much additional shape change. This may be due to a sintering process that produces hydroxyapatite crystals of a particular shape and size following specific heating regimens.

The crystal change apparent in these SAXS measurements is not as easily discernible in XRD traces. Here we have shown corresponding WAXS measurements in which little difference aside from slight peak narrowing and splitting was evident, while the crystallites were growing substantially and changing habit. Unlike XRD, SAXS provides direct measurements of crystallite size and shape that are independent of the perfection of the crystal lattice [19]. The pattern of crystal change, however, from small imperfect crystallites to larger, more perfect ones is reinforced by both sets of measurements, verifying the usefulness of SAXS as a complementary technique to study bone mineral structure and change resulting from heat treatment. With a greater range of samples and longer heating times, it may be possible to discern the precise characteristics of crystals heated to a specific temperature or for a specific time. This additional information would make SAXS valuable in establishing effective screening techniques for the generation of biogenic apatite with minimal alteration to mineral structure for osteoimplantation. It could also act as a simple method, requiring minimal sample preparation, for the tracing of heating regimens or exposures encountered in forensic or archaeological contexts.

5. Conclusions

SAXS provides evidence complementary to that generated by traditional XRD or WAXS in the characterisation of heated bone. Fine-scale changes in crystallite size and shape that are not measured directly using XRD are readily elucidated using SAXS, however, and therefore changes in the crystal structure that may not be readily apparent otherwise become more clear. We are confident that the techniques described here can be honed for use as a more accurate determinant of crystallite change during heating, thus providing an additional means of determining the effects of heat treatment on biogenic hydroxyapatite or tracing burning practices in the forensic and archaeological records.

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