ArcheoSciences

Revue d'archéométrie

35 | 2011

Varia / International ArBoCo Workshop

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Electronic version

URL: <https://journals.openedition.org/archeosciences/3075>

DOI: 10.4000/archeosciences.3075

ISBN: 978-2-7535-1849-0

ISSN: 2104-3728

Publisher

Presses universitaires de Rennes

Printed version

Date of publication: 30 April 2011

Number of pages: 143-158

ISBN: 978-2-7535-1847-6

ISSN: 1960-1360

Electronic reference

Ina Reiche, Céline Chadefaux, Katharina Müller and Aurélien Gourrier, "Towards a Better Understanding of Alteration Phenomena of Archaeological Bone by a Closer Look at the Organic/Mineral Association at Micro- and Nanoscale. Preliminary Results on Neolithic Samples from Chalain Lake Site 19, Jura, France", *ArcheoSciences* [Online], 35 | 2011, Online since 30 April 2013, connection on 01 March 2022. URL: <http://journals.openedition.org/archeosciences/3075> ; DOI: <https://doi.org/10.4000/archeosciences.3075>

Towards a Better Understanding of Alteration Phenomena of Archaeological Bone by a Closer Look at the Organic/Mineral Association at Micro- and Nanoscale.

Preliminary Results on Neolithic Samples from Chalain Lake Site 19, Jura, France

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Abstract: In this paper we present the extension of existing analytical schemes for the precise evaluation of archaeological bone preservation states. The new methodological developments concerning the study of the morphological and structural features of archaeological bones at micro- and nanoscale are emphasized in order to elucidate fine diagenetic modifications and to better understand the underlying alteration mechanisms. A combination of synchrotron X-ray microtomography, infrared micro-spectroscopy imaging and quantitative scanning small-angle X-ray scattering (sSAXS) imaging allowed studying micromorphological changes and the distribution of the organic and mineral components of bone at a histological level. Transmission electron microscopy gives precise information on changes of apatite crystal dimensions, orientations, periodicity of collagen fiber arrangement and crystal distributions within the fibers in archaeological bone. It allows establishing criteria to evaluate more precisely the preservation state of archaeological bone material with respect to modern references. The potential of these methods is highlighted on the basis of the study of some macroscopically well preserved archaeological bone samples from the Chalain lake station 19, Neolithic period, Jura, France. The investigations allowed a differentiation in preservation state between these samples and the postulation of an alteration sequence in such humid chalk-rich burial environments.

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Résumé : Dans cet article nous présentons une extension de méthodes analytiques existantes pour évaluer précisément l'état de conservation des os archéologiques. Les nouveaux développements méthodologiques concernent particulièrement l'étude des caractéristiques structurales et morphologiques des ossements archéologiques aux échelles micro- et nanoscopiques afin de pouvoir élucider des modifications diagénétiques fines et de mieux comprendre les mécanismes d'altération sous-jacents. Une combinaison de microtomographie X, de micro-spectroscopie infrarouge et de micro-imagerie SAXS à balayage au synchrotron a permis d'étudier les altérations micromorphologiques et la répartition spatiale des composés organiques et minéraux dans les os à l'échelle histologiques. La microscopie électronique à transmission donne des informations précises sur les modifications de la taille des cristaux, leur orientation, la périodicité des arrangements des fibres collagéniques ainsi que la répartition des cristaux au sein des fibres dans l'os archéologique. Ces observations ont permis d'établir des paramètres permettant l'évaluation fine des modifications diagénétiques de la structure des os archéologiques en comparaison aux références modernes. Le potentiel de ces méthodes est illustré grâce à l'étude d'os archéologiques qui sont bien préservés à l'échelle macroscopique provenant du site néolithique de Chalain 19, Jura, France. Cette investigation a permis de montrer des différences dans l'état de conservation de ces os et permet de proposer une séquence d'altération pour les os enfouis dans des environnements humides et riches en craie.

Keywords: archaeological bone, diagenesis, synchrotron microCT, synchrotron microFTIR, TEM, scanning-SAXS imaging.

Mots clés : os archéologique, diagenèse, microCT au synchrotron, imagerie microFTIR au synchrotron, MET, micro-imagerie SAXS.

1. INTRODUCTION

Bones or objects made of bone material are an important part of the archaeological witnesses and contribute largely to the understanding of ancient societies. In archaeological studies they are, among others, used to estimate the number of individuals that lived at a given archaeological site, [1] and to get information on the exploitation of animal troops by humans of past societies (mode of use, treating with primary materials, fabrication techniques) [2-5]. As biological materials they also register in their chemical and isotopic composition a wealth of information on the life style of individuals [6-10]. More recently ancient DNA studies have also been used to extract information on past civilizations [11, 12].

Generally, archaeological materials are buried in soils or sediments and feature complex alteration phenomena. It is indispensable to study these modifications in order to better assess their original state, and thus to gather as much information as possible from them. They are particularly important for prehistoric times as there are no written sources and all information obtained on the past basically needs to be deduced from the material discovered and its spatial distribution on an archaeological site. Many studies have already been devoted to the assessment and the understanding of the evolution of bone materials in different environments because of the importance of osseous remains in archaeology and paleontology [13-23]. These studies allowed defining key “diagenetic parameters” (% collagen, mineral crystallinity index, $\text{CO}_3^{2-}/\text{PO}_4^{3-}$ ratio, Oxford Histological index (OHI), Cracking index, ‘s’, ‘m’ ‘l’ porosity, bulk density, skeletal density, amino acid

contents) through microscopic observations and physico-chemical measurements and, as a consequence, the estimation of the bone's conservation state. Some “diagenetic trajectories” and even long term prediction models for the bone's evolution in special burial conditions could be postulated. General tendencies of alteration processes could be established and categorized as a function of the soil chemistry and the state of the bone remains before burial by means of a statistical approach [20, 21]. Important parameters to be considered for alteration phenomena are: taphonomic and hydrological conditions, soil composition, biological activity and temperature. This approach is powerful to generalize the alteration phenomena but does not take into account the intrinsic particularities of each site. In particular, trace element concentrations, isotopic ratios, ancient DNA preservation, crystallinity state and the presence of special crystalline phases and their distributions in the bone material might be highly variable as a function of the biogeochemical conditions for each site, and even for different stratigraphical units. Some cases of exceptional bone conservation, especially of its organic content, have been reported [24, 25]. Therefore, it seems appropriate to study the conservation state of bone remains for each archaeological context and stratigraphical unit, where physico-chemical analyses are performed such as stable isotopic or carbon-14 dating studies etc. This allows estimating more reliably their informative potential.

Bone properties are essentially determined by its hierarchical structure and the strong linkage of the organic and mineral phase at the nanoscale [26]. Consequently, it seems important to study the modifications induced by alteration phenomena of archaeological bone on the micro- and nano-

level and to link these observations to those made on higher hierarchical levels.

Using a case study of archaeological bone fragments coming from a Neolithic site with moderate climatic environments typical for Northern European archaeological sites, it is intended, in this article, to highlight the potential of a methodology established in the framework of the French ArBoCo ANR research program to evaluate the conservation state of bone material with a spatial resolution in the micrometer and even in the nanometer regime. These methods (Fig. 1) allow evidencing very subtle changes in archaeological samples which seem well preserved and show no significant differences at the macroscopic level.

2. NEW ANALYTICAL APPROACH AND EXPERIMENTAL CONDITIONS

In this paper, we focus on a new methodological approach comprising several complementary analytical techniques to study the morphological and structural features of archaeological bones at micro- and nanoscale. The chemical composition of major, minor and trace element level was also measured by means of SEM-EDX analyses and micro-Proton-Induced X-ray and gamma-ray emission spectroscopy (micro-PIXE-PIGE). The micro-PIXE-PIGE analyses were

conducted at the external micro-beam line at the 2 MV tandem particle accelerator AGLAE at the LC2RME, Paris [27]. These spatially resolved investigations of the chemical composition have already been discussed in former publications [23], [28, 29] and are therefore not presented here.

Micro-morphological analyses

The morphology of the bone samples is usually evaluated by observing thick sections under the optical (OM) and the scanning electron microscope (SEM). In this study, synchrotron radiation microtomography (microCT) was used to gather the 3D microstructure of bone material and to image directly porosity changes. These experiments have been performed in collaboration with the Federal Institute of materials research and testing (BAM) at the BAMline at the synchrotron source BESSY II. Although different contrast modes such as phase contrast [30] or refraction-enhanced tomography [31, 32] are available to study in detail the bone micromorphology, synchrotron microCT measurements were performed in a classical absorption mode using monochromatic radiation at 14 keV, which provides best spatial resolution. For one CT scan, images at 1600 angles per 180° were measured with an individual exposure time of 2 s. The CT scan of one sample lasted for about 1 h. Samples were fixed in a Kapton® tube with

Figure 1: Scheme summarizing the analytical methods that have been developed in the ArBoCo programme and the information obtained on bone structure and composition at micro- and nanoscale.

Figure 1 : Schéma de synthèse des méthodes analytiques développées au cours du programme de recherche ArBoCo et informations obtenues sur la structure osseuse à micro- et nanoéchelle.

a diameter of 1 mm adapted to the size of the bone fragment or object under investigation. The set-up is explained in detail in the following references [33, 34]. The voxel data were reconstructed with a filtered-backprojection algorithm and visualised using the software VGStudio Max 2.1 (VolumeGraphics GmbH, Heidelberg/Germany). The reconstructed volumes have a voxel size of about 0.4 μm [35, 36]. Generally, virtual sections (2D images) of the samples are represented in this paper.

Micro-structural investigations

Fourier-transform Infrared Spectroscopy (FT-IR) analysis and X-ray Diffraction (XRD) generally allow the structural analysis at the atomic level of the main components present in the bone mineral. These techniques permit the determination of a special index indicating the degree of mineral alteration in bone described by the “splitting factor” (SF) and the “crystallinity index” (CI), respectively [14, 37]. The principal organic phase of bone, collagen, can also be analyzed at the molecular level by FT-IR by means of the detection of the amid absorption bands. These bands reflect amid polypeptide groups and the lateral chains of amino acids. The secondary protein structure can also be analyzed. Thus, it is possible to detect organic residues in ancient bones and to evaluate the integrity of the preserved organic phase [38].

Within the ArBoCo research programme, we developed a method in order to obtain spatially resolved information of both, the bone organic and mineral phases. For this issue, synchrotron microFT-IR seemed to be well suited for studying the heterogeneity of the preservation state of archaeological bones at microscale. The experiments were performed at the IRIS beamline (BESSY II, HZB, Berlin, Germany) on a Thermo Nicolet ContinuumTM microscope equipped with a MCT detector. The sample was mounted on a 1 mm thick BaF₂ pellet on a motorized microscope stage and raster scanned through the synchrotron beam with a diameter of 10 μm collecting a grid-like pattern of

IR spectra with increments of 7 μm . Measurements were performed in transmission mode at a magnification of x32 using confocal objectives. Infrared spectra have been registered between 4000 and 650 cm^{-1} with a spectral resolution of 8 cm^{-1} . For each measuring point, 128 scans were accumulated. Background spectra were collected under identical conditions in constant intervals. The spectrum acquisition has been performed by using the OMNIC AtlysTM software [39]. Spectral data analysis was performed using the OMNIC 7.4 (Thermo) and OPUS (Bruker Optic GmbH) software.

The spectra were baseline corrected (by means of the concave rubberband correction) in the spectral region between 2000-800 cm^{-1} . Subsequently, the contribution of the impregnation resin (indicator = vibration band at around 1730 cm^{-1}) was subtracted as indicated in [40]. The table 1 shows the IR parameters used for imaging. The visualization of IR parameter distributions in the scanned zone was performed with the help of MATLAB 7.7 software (MathsWorks).

Scanning-SAXS measurements were performed at MySpot beamline at BESSY II, Berlin, Germany. SAXS patterns were obtained with a 30x30 μm^2 X-ray microbeam on 40 μm thick sections cut in the central part of the archaeological bone specimens [41]. Experimental conditions are analogous to those described in [41, 43]. A W/Si (311) multilayer monochromator was used to monochromatize X-rays to an energy of 15keV ($\lambda=0.8265$ nm). The SAXS patterns were recorded with a 16 bit CCD area detector (MarMosaic 225, Rayonix, Evanston, USA) with a 225 x 225 mm^2 square converter screen. The images were recorded with a readout time of ~ 2.5 s at full resolution (3072 x 3072 pixels with a pixel size of 73.24 x 73.24 μm^2) and the sample-detector distance as well as the detector tilt and center were determined by measuring a silver behenate standard.

Prior to the SAXS measurements a transmission scan of the sample is performed using a photodiode.

IR parameters	Intensity ratio of vibration bands	Baseline / cm^{-1}
Collagen/ PO_4	$I_{(\text{max } 1670-1630)} / I_{1095}$	1800-1300 / 1175-900
CO_3/PO_4	$I_{1415 (\text{max } 1428-1398)} / I_{1095}$	1800-1300 / 1175-900
Crystallinity	I_{1030} / I_{1020}	1175-900
HPO_4/PO_4	I_{1106} / I_{1095}	1175-900
Collagen integrity	I_{1690} / I_{1660}	1800-1300
Random coil / α -Helix	I_{1645} / I_{1660}	1800-1300

Table 1: Selected IR parameters for imaging.
Tableau 1 : Paramètres IR définis pour l'imagerie.

The SAXS data analysis included the spherical integration along the azimuthal direction of the two-dimensional SAXS patterns using the FIT2D software package [42]. The one-dimensional profiles were subsequently analyzed using a dedicated SAXS analysis library written in Python language by A. Gourrier optimized for large data sets [43, 44]. The structural parameters relating to the mineral nanoparticle thickness can thus be determined following procedures established by Fratzl *et al.* for bone studies (see, e.g. [45]). The average chord length (so-called T parameter) can be considered as a standard parameter in the SAXS analysis of bone from archaeological contexts [46].

Nanostructural investigations

Transmission Electron Microscopy coupled with an Energy-Dispersive X-ray System (TEM-EDX) was used to characterize specific apatite crystal shapes and sizes as well as the localization of the crystals within the collagen fiber matrix [47]. Transmission Electron Microscopy gives localized information whereas 2D scanning Small-Angle X-ray Scattering (sSAXS) imaging provides a more global view on the texture and the crystal size distribution in the bone material.

Sample observation using TEM, giving direct evidence of the preservation state of the organic and mineral bone phases, was carried out by means of a Philips EM208 microscope at 80 kV at the Common centre of electron microscopy (CCME) UMR 8080 CNRS, Orsay, France.

According to a study by Rubin *et al.* (2003) [48] in longitudinal ultrathin sections of normal and osteoporotic human bone the apatite crystals are oriented parallel following the collagen fibers. To estimate the degree of disorientation and alteration we decided to prepare ultrathin longitudinal sections for the direct observation of the mineralized collagen.

Parameters have been established to characterize at the nanoscale the state of preservation of the organic-mineral arrangement in the archaeological material: L_s represents the width of the deposited crystals at the surface of the fibers; L_f the width of collagen fibers; Λ the spatial periodicity of the crystals in the fibers; e the thickness of the crystallites and l the width of the crystals (fig. 2a and b).

Preparation of archaeological bone sections and thin sections

It has to be pointed out that the sample preparation procedures were crucial steps for all analyses at the micro-

Figure 2: a) Electron micrographs on a micrograph of an ultrathin section of a modern antler MA. b) Indications of characteristic parameters (L_s : width of deposited crystals at the surface of the fibers, L_f : width of collagen fibers, Λ : spatial periodicity of the crystals in the fibers, e : thickness of the crystallites, l : width of the crystals).

Figure 2 : a) Micrographie électronique d'une coupe ultrafine de l'os bovin moderne de référence MA. b) Indications des paramètres caractéristiques (L_s : largeur des cristaux déposés à la surface des fibres, L_f : largeur des fibres de collagène, Λ : périodicité spatiale des cristaux dans les fibres, e : épaisseur des cristallites, l : largeur des cristaux) sur une micrographie électronique d'une coupe ultrafine d'un bois de renne moderne.

and the nanoscale. Indeed, the preparation procedure comprised partly embedding, ultramicrotomy and polishing in order to keep all necessary information on the structure at micro- and nanoscale and on the texture of the material. It is important that the procedure does not change the features of the samples and does not introduce artefacts into the sample.

Fragments of two archaeological cortical bones (AB_CH19nb1 and nb2) were prepared for the analyses. First, thick bone sections were cut with a water-cooled diamond saw. Sections were subsequently ultrasonically rinsed with water, distilled water and ethanol, before they were dried in air. For synchrotron microFTIR, transverse thin sections with a thickness of less than 2 μm were realized on in polymethyl methacrylate (PMMA) embedded bone material using a ReichertTM ultramicrotome equipped with a glass knife set (at CCME, Orsay) according to the procedure described in [49].

Ultrathin bone sections of approximately 70 nm thickness could be directly cut without embedding the bone fragment with a diamond knife on a Reichert Ultracut E Ultramicrotome at CCME, Orsay and picked up on 200-mesh copper grids coated with a membrane of carbon Formvar for TEM observations. The cut orientation was chosen to be longitudinal to better visualize the texture of mineralized collagen fibrils in bone. Three grids were prepared for the bone sample.

For SAXS measurements, bone fragments of about 1 mm thickness were cut and subsequently machine-ground using SiC grained paper of the fineness of 800, 1200 and 4000 before being rinsed with distilled water. The sections were glued with superglue on a glass slide and polished using a LAM PLAN M.M.8027S machine with SiC in water as polishing liquid down to a thickness of about 40 μm . The final polishing was obtained by diamond paste polishing to a precision of 0.25 μm .

Possible effects of the sample preparation

Sample preparation is a key step in the analyses of slight changes in the preservation state of bone materials. Indeed, it is fundamental to avoid any changes of these fragile and precious remains induced by sample preparation procedures in order to conserve the state of the material. However, most of archaeological bone materials are altered and therefore brittle. Measurements conducted here show that impregnation treatment can be avoided in some cases if the mineral and the organic parts are still conserved in the considered bone materials to provide cohesion of the bone structure but generally more reliable results can be obtained on

PMMA embedded samples. However, the main drawback of the embedding procedure is that the analysis needs to take into account the resin contribution in the spectra.

3. MATERIAL

The Neolithic bone material originates from the lacustrine sites of the Chalain lake, Jura, France. At this lake situated in the Franche-Comté region, over thirty archaeological sites were discovered during the excavations and prospection carried out by Pierre Pétrequin and his team [50]. Seven Neolithic villages (3850 to 2650 BC) have been excavated there within the past thirty years. Multidisciplinary research projects have been undertaken in order to take advantage of the archaeological, biological, chemical, and geological information contained in the objects found at these sites (see e.g. [51]). Research on diagenetic modifications of bone remains are integrated in the context of these investigations. At Chalain, the bones are generally found to be well preserved by optical observations. According to archaeological observations, the bone fragments originating from the preparation of meals (butchering and cooking) were discarded in the dumps in front of the only entrance to the houses on pilings, built on flood prone ground. This refuse (including the bone remains) therefore fell onto humid soil or into shallow water. They were quickly covered by the vegetal litter brought in by humans to stabilize and reclaim the exterior soils during low water periods. After the villages were abandoned, the lake's level rose again and lake chalk was deposited.

During the excavation campaign conducted in 1998, some fragments of burned and unburned bones from the emerged station 19 were entrusted to us for analysis [28]. Selected results obtained by the new analytical strategy on two compact bone fragments AB_CH19nb1 and nb2, probably *Bos taurus* [52] (fig. 3) are presented here in comparison to those obtained on a modern bovine reference sample

Figure 3: (See colour plate) Archaeological bone samples from lacustrine Chalain site 19.

Figure 3 : (Voir planche couleur) échantillons d'os archéologiques du site lacustre 19 de Chalain.

MBB. It is of importance to provide a modern bone control as close as possible to the archaeological bone samples under investigation. Therefore, a bovine bone reference was chosen in this study, which has been submitted to the same type of thorough investigations as the archaeological specimens in order to differentiate variations in the defined parameters within the modern sample from possible diagenetic changes at micro- and nanoscale in the archaeological ones. The archaeological samples have been selected because they show not only a very good preservation state at macroscale but also little differences at the histological level as evidenced by SEM observations on cross sections (fig. 4). The newly established approach should provide a better description of the samples at micro- and nanoscale in order to better assess their preservation state, which is relevant for the understanding of the underlying alteration phenomena and for a better evaluation of their informative potential in archaeological studies. The archaeological samples (AB_CH19nb1 and nb2) originate from layers H and K of station 19 of Chalain, and are dated to 3033-2886 calBC (86.8 %) and 3141-3002 calBC (41.4%), respectively [53]. The layers H and K correspond to remains of villages with raised floor houses with sediments of anthropic dung that contain a small percentage of calcium carbonates deposited by the lake.

4. RESULTS

Determination of the micromorphological changes using synchrotron microtomography

The study of the preservation state of the micromorphology could be performed on one archaeological sample AB_CH19nb1 using synchrotron microCT (fig. 5). These investigations allowed evidencing the micromorphological structure of this sample. The information is equivalent to

those that can be obtained on cross sections by SEM (fig. 4) but with a tridimensional resolution so that virtual section can be observed in several depths of the sample. A typical bone structure with secondary osteons can be clearly observed in fig. 5a.

The regular structure of a Haversian system can be seen as well as other intrinsic bone porosities in another representation evidencing bone porosities (fig. 5b). They seem smaller in comparison to the modern bone reference measured. The porosity estimates based on the image analysis for the volumes selected are summarized in the table 2. The volume p of open pores is calculated as a ratio with respect to the total volume according to the following formula. These calculations show smaller values for the archaeological sample compared to the modern one.

Spatially resolved analysis of the organic and mineral content at microscale using synchrotron microFTIR in transmission mode

The IR maps and the corresponding optical images of the archaeological bone thin sections AB_CH19nb1 and nb2 are reported in [39] and represented in the fig. 6a-g, respectively. The figure 6b represents the distribution of the collagen content in the bone thin section of the sample AB_

Sample	Porosity estimation (%)
MBB	7.4 ± 0.5
AB_CH19nb1	4.9 ± 0.5

Table 2: Porosity estimated by image analysis of the archaeological bone sample AB_CH19nb1 compared to that of the measured reference AB_CH19nb1 and MBB.

Tableau 2 : Porosité estimée par analyse d'image de l'échantillon d'os archéologique AB_CH19nb1 comparé à celle de la référence moderne mesurée MBB.

Figure 4: Electron micrographs in secondary electron mode of the archaeological bone thick sections of a) AB_CH19nb1 and b) AB_CH19nb2.

Figure 4 : Micrographies électroniques en mode d'électrons secondaires des coupes épaisses des os archéologiques : a) AB_CH19nb1 et b) AB_CH19nb2.

Figure 5: (See colour plate) a) Virtual slice through the sample AB_CH19nb1 and b) tridimensional representation of the sample porosities of a selected part around a Haversian channel.

Figure 5 : (Voir planche couleur) a) Coupe virtuelle à travers l'échantillon AB_CH19nb1 et b) représentation tridimensionnelle des porosités d'une zone sélectionnée autour du canal d'Havers.

CH19nb2. The osteon in the lower central part of the optical image (fig. 6a) can also be revealed using the chemical image of the collagen content (fig. 6b). The collagen content is homogeneously enriched around the Haversian channel of the osteon. A second osteon-like structure can be seen in the lower left part of the image which shows the same collagen distribution as in the other osteon structure. Above these osteon structures no characteristic collagen distribution can be found. Fig. 6c represents the amount of reducible to non-reducible collagen cross links and stands therefore as an image of the distribution of the collagen integrity (high values mean poor preservation of collagen). It is homogeneous except of two hot spots next to the Haversian channels indicating an alteration of the collagen quality in these spots compared to the other parts. Fig. 6d shows an image of the random coil to α -helix ratio indicative of the distribution of changes in collagen secondary structure. It is homogeneously distributed except of two hot spots. This confirms the observation made by the other parameter representative of the collagen quality.

Fig. 6e shows the carbonate to phosphate ratio distribution. It basically shows several carbonate enriched or phosphate depleted zones that correspond to the same areas as those with altered collagen (see fig. 6c and d).

The parameter characterizing the apatite crystallinity is represented in fig. 6f. The osteonic structure in the lower part in the middle is recognizable by a higher crystallinity in the centre and enhanced values around the Haversian channel in comparison to the other parts of the sample. An inverse behavior was observed for the other osteon structure in the lower left part of the image. This difference within one sample is difficult to explain. Fig. 6g shows high acid phosphate content in the areas of the Haversian channels and its distribution is anti-correlated to that of carbonate as well as to the advanced alteration state of the collagen structure in this thin section.

When comparing these chemical maps with those obtained on the other archaeological bone sample, AB_CH19nb1 described in [39], a good agreement of the ratio value variations as well as correlation in the distribution of the parameters can be found, except for that of the carbonate content and the crystallinity enhancement of the Haversian channel in the lower middle of the AB_CH19nb2 map (fig. 6f). No comparison is available for the acid phosphate to phosphate ratio distribution. All values observed for the archaeological samples lie above those observed in modern references [49].

Figure 6: a) (See colour plate) Optical image of the zone selected on the archaeological bone thin section AB_CH19nb2 from the Chalain Lake site corresponding to an area with visible osteons. Image obtained from the microscope Continuum™ (Nicolet) at the IRIS Beamline (BESSY II, Berlin) and showing the points analyzed. Represented area corresponds to $120 \times 220 \mu\text{m}^2$. SR microFTIR maps of the same zone b) of the amide I/phosphate ratio (ca. 1660/1095), Chalain Lake site, c) the collagen cross-links represented by the 1690/1660 ratio corresponding to reducible to non-reducible cross-links, d) the ratio of random coils to α -helix subbands (1645/1660), e) the carbonate/phosphate ratio (ca. 1415/1095), f) the apatite crystallinity (1030/1020) and g) the acid phosphate to phosphate ratio (1106/1095). A false colour scale from red (high intensity) to blue (low intensity) was adopted for all images.

Figure 6 : (Voir planche couleur) a) Image optique d'une zone sélectionnée d'une coupe fine de l'os archéologique AB_CH19nb2 du lac de Chalain correspondant à une zone avec des osteons visibles. Image obtenue grâce au microscope Continuum™ (Nicolet) à la ligne IRIS (BESSY II, Berlin) montrant les points analysés. La zone représentée correspond à $120 \times 220 \mu\text{m}^2$. Des cartographies en microIRTF de la même zone b) du rapport amide I/phosphate (ca. 1660/1095), c) des cross-links du collagène représentés par le rapport 1690/1660 correspondant au rapport entre les cross-links réductibles et non-réductibles, d) du rapport (1645/1660), e) du rapport carbonate sur phosphate (ca. 1415/1095), f) de la cristallinité de l'apatite (1030/1020) et g) du rapport hydrophosphate au phosphate (1106/1095). Une échelle en fausses couleurs allant du rouge (intensité forte) au bleu (intensité faible) a été adoptée pour toutes les images.

Statistical view of crystalline structure of the nanoparticles by means of scanning-SAXS imaging

The SAXS/WAXS pattern shown in fig. 7a was obtained in the central part of sample AB_CH19nb2 and contains typical features for bone X-ray scattering, i.e. an intense elliptical signal in the centre, which corresponds to the SAXS signal from the mineral nanoparticles, and a number of diffraction rings related to the internal crystalline structure of the nanoparticles. Changes in shape and intensity of the SAXS region can be observed in fig. 7b as a function of scan position pointing towards structural fluctuations within the sample. Those fluctuations are found to be related to typical histological features which are observable in modern cortical bone, such as e.g., sample porosity and interface between remodeling units (i.e. cement lines) as further shown in the image of the integrated SAXS intensity displayed in fig. 7c. The image of T (fig. 7d) reveals nanoscale differences in mineral particle structure at the tissue level, while the histogram of the image, so-called T-distribution (fig. 7e), can be used to quantify the statistical dispersion of this characteristic length for the nanoparticles. The results show that both archaeological samples, AB_CH19nb1 and nb2 are statistically different from a modern bovine reference in terms of mineral nanoparticle structure as revealed by the

Sample	T_{MAX}	T_{FWHM}
MBB	3.2	0.9
AB_CH19nb1	3.8	0.5
AB_CH19nb2	3.6	0.5

Table 3: Characteristic parameters of the T-distribution in fig 7e: T_{MAX} , the position of the peak maxima and T_{FWHM} , the full width at half-maximum.

Tableau 3 : Valeurs caractéristiques de la répartition du paramètre T de la fig 7e : T_{MAX} est la position des maxima des pics et T_{FWHM} correspond à la largeur à mi-hauteur.

Sample	Crystal thickness e (nm)	Crystal width l (nm)	Spatial periodicity of the crystal distribution Λ (nm)	Width of deposited crystals at the fiber surface Λ_s (nm)	Width of collagen fibers Λ_f (nm)
MBB	5 ± 1	30 ± 5	60 ± 5	70 ± 20	150 ± 20
AB_CH19nb1	5 ± 1	25 ± 5	disordered	80 ± 10	120 ± 30
AB_CH19nb2	5 ± 1	25 ± 5	70 ± 5	70 ± 10	120 ± 30

Table 4: Characteristic parameters determined by TEM on ultrathin sections of the modern reference MBB compared to that of the archaeological samples

Tableau 4 : Paramètres caractéristiques déterminés par MET sur des coupes ultrafines d'os moderne de référence, MBB comparés à ceux des os archéologiques.

position of the maxima and the width of the T-distributions which correspond respectively to the average particle size and dimensional dispersion to a good approximation. Those parameters are summarized in table 3 for the three samples. Furthermore, it can be observed that the sample AB_CH19nb2 is closer to the structure of modern bone, which is consistent with the other observations.

Observation of the crystal structure and the organic – mineral arrangement at nanoscale by TEM

Table 4 summarizes the parameters determined on the electron micrographs of the archaeological bone ultrathin sections (fig. 8a-d). It needs to be emphasized that the determination of bone crystal dimensions by TEM is not as precise as the values obtained by sSAXS showing real variations at the nanoscale. Therefore, observed crystals show only slight differences in their dimensions in the archaeological samples compared to the modern reference but significant variations in fiber and apatite crystal organization are observed. As expected a privileged apatite crystal orientation following the collagen c axis was found in the micrographs of the archaeological samples (Fig. 8a and c). The sample AB_CH19nb1 shows a loss of periodicity of the fiber arrangement and the crystal distribution whereas they stay periodic for the sample AB_CH19nb2. Bragg reflections can be observed on the (002) ring of AB_CH19nb1 (Fig. 9a) while that of the MBB sample and that of AB_CH19nb2 (Fig. 9b) stays continuous. This reveals that the sample AB_CH19nb1 is better crystallized than MBB and AB_CH19nb2.

The observed thickness and width of the crystals are found to be similar in archaeological samples to those in the modern reference. The width of the collagen fibers is smaller in the archaeological samples compared to the modern one. This can be due on one hand to alteration phenomena

Figure 7: Scanning-SAXS imaging results of AB_CH19nb2. a) SAXS/WAXS pattern obtained with a $30 \times 30 \mu\text{m}^2$ X-ray microbeam in the central part of AB_CH19nb2; b) composite image of the SAXS region indicated by a rectangle in a); c), d), images of the integrated SAXS intensity and T, respectively; e) T-distribution of the modern bovine bone reference (full line) and of the specimens AB_CH19nb1 (dotted line) and AB_CH19nb2 (dashed line). Note that the dotted line corresponds to the histogram of the image in d).

Figure 7 : Résultats en imagerie SAXS à balayage de l'échantillon AB_CH19nb2. a) données SAXS/WAXS obtenues avec un microfaisceau X de $30 \times 30 \mu\text{m}^2$ dans la partie centrale de l'échantillon AB_CH19nb2; b) image composite de la région SAXS indiquée par le rectangle dans a); c), d), images de l'intensité SAXS intégrée et T, respectivement; e) répartition du paramètre T de la référence de l'os bovin moderne (ligne continue) et du spécimen archéologique AB_CH19nb1 (ligne en pointillés larges) et AB_CH19nb2 (ligne en pointillés fins). Il est à noter que la ligne en pointillés correspond à l'histogramme de l'image en d).

or to a difference in bone type with respect to the modern reference.

5. DISCUSSION AND CONCLUSIONS

The newly developed analytical strategy represents an extension of existing analytical schemes for the study of bone diagenesis to finer structural hierarchical scales. It allows a precise evaluation of the preservation state of archaeological bone with respect to an appropriate modern reference. Information is obtained on the microscale on morphological

changes by microCT and the distribution of organic and mineral components by means of synchrotron microFTIR and scanning-SAXS imaging. At the nanolevel, it was possible to study precisely crystal dimensions and orientations, the periodicity of the collagen fiber arrangement and the crystal distribution variations in the fibers on a localized scale by means of TEM. The high potential of new scanning-SAXS imaging results obtained on archaeological bone was highlighted allowing a more statistical view of nanoscale differences in mineral particle structure and sizes at the tissue level. Therefore, we dispose today of efficient tools for characterizing fine changes of the mineral and organic

AB_CH19nb1

AB_CH19nb2

Figure 8: Electron micrographs performed on ultrathin sections of the archaeological bone samples investigated: a) bone AB_CH19nb1 and b) bone AB_CH19nb2.

Figure 8 : Micrographies électroniques des sections ultrafines des échantillons d'os archéologique étudiés : a) os AB_CH19nb1 et b) os AB_CH19nb2.

Figure 9: Selected area electron diffraction performed on the archaeological bone samples a) AB_CH19nb1 and b) AB_CH19nb2.

Figure 9 : Diffraction électronique sur zone choisie réalisée sur les échantillons d'os archéologique : a) AB_CH19nb1 et b) AB_CH19nb2.

parts as well as their arrangements in the archaeological bone structure with molecular information.

The use of complementary methods allows gaining independent insights into the structure and features of bone which makes this approach very robust in order to draw reliable conclusions on diagenetic phenomena as a function of the bone artifact and the burial conditions. The key of the methods is the adequate sample preparation procedure conserving the bone histology and the establishment of appropriate modern bone references. The latter point is probably the one that needs to be developed further in the next future.

New information could be gained on the preservation state of some archaeological bone fragments from the Neolithic station 19 of the Chalain lake site. Generally, the investigated bones seem very well preserved at the macro- and also at the histological scale despite their age of about 5000 years. This can be linked to the favorable burial conditions for organic matter at this site. The archaeological layers of the Chalain lake have been rapidly buried under a chalk-rich sediments with slightly reducing conditions. It seems that microbial activity, an important diagenetic process to consider especially during early diagenesis [54], is absent and therefore chemical processes play the main role in bone degradation. The chalk-rich reducing conditions are very favorable for good collagen preservation, which therefore

prevent the mineral bone phase from important alterations. The established methodology could also evidence differences in the preservation state between different bone fragments from the similar burial environment of the Chalain lake station, thus highlighting the important potential of these methods to reveal very slight modifications in the bone structure. The sample AB_CH19nb2 seems better preserved than the sample AB_CH19nb1 because its features are closer to the modern bone reference. The bone histology of AB_CH19nb1 is preserved although the porosity is decreased according to microCT observations whereas the organic-mineral arrangement at nanoscale is in an advanced state of alteration compared to AB_CH19nb2 and the modern bone reference MBB. The decrease in porosity is unexpected but can be explained by the formation of secondary minerals in the micropores by precipitation induced by interaction with water from the burial environment. Studies using synchrotron microFTIR confirm the local carbonate enrichment and the more heterogeneous state of preservation of the sample AB_CH19nb1 [39]. This is also revealed by TEM observations showing the disorientation of the apatite crystals situated in the collagen fibers of this sample. The mineral phase shows also a more crystalline state than the other bone samples. In further studies it needs to be understood why this bone is less well preserved than the other of a similar age coming from a close environment.

The following alteration sequence can be postulated for the neolithic bone samples from the station 19 of the Chalais lake based on the micro- and nanoscale analyses and observations: 1) disorganization of collagen fibers, 2) loss of apatite crystal texture, 3) dissolution of apatite and hydrolysis of collagen, 4) loss of collagen and increase in apatite crystallinity by dissolution-recrystallization of the mineral phase and 5) uptake and trap of secondary minerals such as calcium carbonates in pores originating from interactions with the burial environment. These processes can take place subsequently but also partly simultaneously. At long term, these processes lead to a complete mineralization of the archaeological bone under such or similar conditions. The informative potential for archaeological studies of the altered bones would be relatively limited because many biomarkers are lost during the diagenetic alteration processes. Further research on these samples is in progress in order to correlate the observed changes of the archaeological bones at micro- and nanoscale to bulk parameters, to isotopic values at the same levels and to DNA preservation.

At the moment, these conclusions are based on the study of quite a few archaeological samples and need to be placed on a more statistical basis in order to get reliable information on diagenetic trajectories as a function of specific burial conditions. Additionally, it is very important to study a larger set of modern references as close as possible to the archaeological samples in order to provide the best suited comparative parameters for the evaluation of the preservation state. It is nevertheless clear that the new methods established allow a closer look at diagenetic modifications indispensable for the understanding of the underlying mechanisms.

Acknowledgements

The authors acknowledge Pierre Pétrequin, UMR 6249 CNRS Besançon, for having provided the archaeological samples and for the detailed explanations on their burial context. Matthieu Lebon, MNHN and C2RMF Paris, is thanked for his advises concerning sample preparation and synchrotron FT-IR measurements. Yvan Coquinot, C2RMF Paris, and Danielle Jaillard, CCME Orsay, are acknowledged for help during thin section sample preparation. We acknowledge the Helmholtz-Zentrum Berlin - Electron storage ring BESSY II for the attribution of access to synchrotron radiation beamtime at the BAMline, IRIS and MySpot beamlines and would like to thank Andreas Staude and Heinrich Riesemeier, BAM Berlin, Ullrich Schade and Michael Gensch, IRIS beamline, Ivo Zizak, BESSY II, Oskar Paris, Chenghao Li and Stefan Siegel, MPICI Potsdam-Golm for assistance during the mea-

surements at BESSY II as well as for the data analyses. Eva-Maria Geigl (Institut Jacques Monod Paris) is thanked for the information concerning the identification of the archaeological bone species and Peter Steier and his group (VERA Vienna) for the C-14 dating of the archaeological samples. The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement n. °226716 through the following EU contracts: R II 3. CT-2004-506008 (BESSY ID.09.1.80792, 08.1.70139, 08.1.70810, 08.2.80355). The French ANR programme ArBoCo ANR-07-JCJC-0149-01 is acknowledged for financial support of this research.

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