Estimating the Preservation of Tooth Structures: Towards a New Scale of Observation

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For a better understanding of the fossilization processes and the palaeoenvironmental records, knowing the state of preservation of fossil structures is essential. This paper presents how the analysis of tooth structures can be improved by using techniques increasing spatial resolution and accuracy, like atomic force microscopy (AFM). Micro- and nanostructural changes of the fresh and fossil dentine and enamel of two Suidae were thus observed with scanning electron (SEM) and atomic force microscopes. AFM and SEM show similar images for enamel and dentine in fresh teeth, whereas discrepancy occurs for fossil teeth. Both techniques show that dentine is modified by taphonomic and diagenetic processes, but only AFM is able to reveal that enamel is also altered, because AFM magnification and resolution are better than SEM ones. The apparent state of tissue preservation depends on the scale of observation and AFM, an analytical tool and a non-destructive/direct technique, allows a better understanding of the evolution of tissues at a nano-scale.

Keywords: DENTINE; ENAMEL; MICROSTUCTURE; NANOSTRUCTURE; SUIDAE

Introduction

Taphonomic studies of bones and teeth are focused on physical changes, such as selective preservation of skeletal elements, abrasion, etc. (Retallack & Leahy, 1986; Andrews, 1990; Behrensmeyer, 1991; Fiorillo, 1991). However, the mineralogy of the secondary fillings in bones is useful in determining the diagenetic history of fossil
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samples (Barker et al., 1997; Karkansas et al., 2000; Clarke, 2004). Numerous studies deal with the geochemical composition of fossil tissues, but the main part does not take into account the microstructural preservation of the samples. So, few studies are devoted to the relationships between microstructural alterations and geochemical diagenetic changes. However, it has been shown that structural and chemical alterations of rodent bones and teeth are dependant of the predators in modern samples (Dauphin et al., 1989; Denys et al., 1992; Dauphin et al., 2003). The identification of the predators in fossil sites is more difficult, because of the double origin of the alterations: digestion and diagenesis. Nevertheless, some case studies of integrated analyses (combining taphonomic, microstructural and chemical analyses) have provided new data for a better reconstruction of the history of the fossil sites (Dauphin & Denys, 1992; Dauphin et al., 1994, 1999; Denys et al., 1996).

Palaeoenvironmental reconstructions also use chemical and/or isotopic compositions (DeNiro & Epstein, 1983; Katzenberg, 1992; Cerling & Sharp, 1996; Ambrose & Krigbaum, 2003), and most of them consider that fossil bone and dentine did not preserve a “good” palaeoclimate record. Abundance of organic matrices in these tissues is said to be the main reason for this susceptibility to diagenesis. It has been shown that the amount of chemical and physical modifications is not uniform within a given assemblage, or within a skeleton (Dauphin & Denys, 1992; David et al., 1999; Price et al., 1992). Conversely, enamel is usually considered as exempt from diagenesis. From this hypothesis, most palaeoenvironmental studies use fossil enamel without any control of the state of preservation of the samples. However, changes in microstructure and chemical composition have been described in fossil enamels (Dauphin & Badrè, 1997; Hoppe et al., 2003; Dauphin & Williams, 2004). According to Sponheimer & Lee-Thorp (1999), a significant change in the proportion of carbonate ions occurs, and the alteration varies significantly within and between sites.

Thus, the relationships between the preservation of the microstructures and chemical compositions are not yet deciphered, and the stability of enamel is now questioned (Sponheimer & Lee-Thorp, 2006). It can be argued that such topographical relationships can be elucidated by using electron microprobe analysis and scanning electron microscopy. Such in situ microscale analyses allow one to see simultaneously the state of preservation of the tissue and the composition. However, some enamels show a discrepancy between “intact” microstructures and altered chemical compositions (Dauphin et al., 1988, 1989, 1999). So, analysis of structures of tooth tissues must be improved by use of techniques that increase spatial resolution and accuracy.

A number of high quality studies have been performed on modern tooth microstructures, first with transmission electron microscopy (TEM), then with scanning electron microscopy (SEM). Because of the size of the crystalline units of both enamel and dentine, detailed observations were done on replica and ultra-thin sections of only small surfaces with TEM. These methods are somewhat destructive, so that few fossil teeth have been studied. These disadvantages can now be partly avoided when using no-destructive scanning probe microscopy methods such as atomic force microscopy (AFM), which
allows resolution on the scale of TEM on the actual sample.

This paper deals with the comparison of micro- and nanostructures of enamel and dentine of molars and premolars of a modern Suidae, *Sus scrofa*, and a fossil one: *Kubanochoerus massai* collected in the Miocene levels from Libya.

**Material and methods**

**Material**

Modern and fossil samples were selected among the Suidae (Artiodactyla), because of the large size of the teeth, and because they are abundant in numerous fossil sites.

*Sus scrofa* Linnaeus 1758 originally occurred in Europe, Asia, North Africa, and the Malay Archipelago. The studied modern sample was about 5 years old (Fig. 1A), and comes from the South Western region of Paris. Fragments of isolated teeth of *Kubanochoerus massai* (Arambourg 1961) come from Miocene (set III) near Gebel Zelten of Sirte basin (Cirenaica, Libya) (Fig. 1D-F). The Marada Formation shows a series of facies which suggest the presence of large rivers. The locality has yield abundant fossil vertebrates (mammals, crocodiles, aves) (Llinas Agrasar, 2004).

**Teeth preparation**

Molars and premolars of *Sus scrofa* were extracted from the lower jaw (Fig. 1A), and coarsely crushed to expose fresh surfaces of enamel and dentine (Fig. 1B, C). Fossil samples were broken to observe fresh fractures, exempt of secondary modifications.

![Figure 1. A, The lower jaw of *Sus scrofa*. B, Fractured fresh tooth, showing enamel (e) and dentine (d). C, Closer view of the enamel with the prismatic structure of the enamel (e) and the inner dentine (d). D–F, Fragments of teeth of *Kubanochoerus massai*.](image)
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such as weathering, surface dissolution, etc. In both cases, samples were cleaned with Milli-Q water, then air-dried at room temperature.

**Scanning electron microscope and atomic force microscope**

Scanning electron microscope (SEM) observations were done with Philips 505 and XL30 SEM on gold coated fragments. Samples were also studied using an atomic force microscope (AFM) multi-mode scanning probe microscope operating in tapping mode.

The atomic force microscope (AFM) maps the topography of a surface. Height images are similar to the secondary electron images of SEM, but the actual surface of the sample is observed, no conductive coating being required. In phase imaging, a variant of tapping mode, the phase lag of the cantilever oscillation relative to the signal sent to the cantilever's piezo driver is used as a basis for image generation. Phase images can be generated as a consequence of variations in material properties such as friction, adhesion, etc. AFM observations were conducted with a Digital Instruments (Veeco) Nanoscope III Dimension 3100 housed at the Laboratoire de géologie, Université d'Orsay, at room temperature and in air. The probe consisted of a cantilever with an integrated silicon nitride tip. Images were acquired using tapping mode. The tapping mode AFM utilises an oscillating tip; since the tip is not in permanent contact with the sample surface during the scanning motion, unwanted alterations such as etchings can be avoided to a large extent. The resolution of tapping mode AFM is in the order of a few nanometers.

Untreated natural and broken surfaces were observed with SEM and AFM. Small pieces of teeth were polished through a series of grinding and polishing disks. The possible remains of polishing products (diamond pastes) were removed with a short ultra-sonication. Polished surfaces were then etched with H₃PO₄ 5% for 30 sec. The detailed procedures of the sample preparations are given in the figure legends.

**Results**

*Sus scrofa*

**Enamel**

The complex structure of the thick enamel layer is clearly visible (Fig. 1C, 2A-C). The main part is composed of Hunter-Schreger bands, with prism (or rod) and inter-prismatic (=inter-rod) enamels. The diameter of a prism is about 4 μm, and each prism is composed of small crystallites (Fig. 2B, D). From SEM images, the length of a crystallite is smaller than 1 μm. The exact shape and orientation of crystallites are not easy to determine in fractures. However, crystallites in prisms and inter-prismatic zones are arranged in different directions, but in each structure the crystallites are ordered.

The internal structure of a prism is clearly visible with AFM images. Each prism is composed of parallel elongated crystallites, and synchronous transverse growth lines are present within a prism, as shown by AFM height (Fig. 2E) and phase (Fig. 2F) images. The surface of a prism is not smooth, probably because in fractures, small crystallites of the adjacent prisms
Figure 2. Sus scrofa: enamel. A, Polished and etched surface showing the prismatic pattern and the decussation of enamel. H$_3$PO$_4$ 5% for 30 seconds. B, Fracture showing the change in the crystallite orientation in adjacent prismatic units. Untreated sample. C, Polished and etched oblique section showing the mineralization varies widely. H$_3$PO$_4$ 5% for 30 seconds. D, Fracture showing adjacent prisms composed of elongated parallel crystallites (arrow) within a prism. Untreated sample. E, Untreated fracture showing the elongated crystallites (arrow) within a prism. AFM height image. F, AFM phase image of Fig. 2E, emphasizing the presence of small aligned granules. G, Untreated fracture showing compositional heterogeneities with crystallites (arrows). AFM phase image. H, Untreated fracture showing the elongated crystallites within a prism. AFM height image. Compare with Warshawsky, 1989, Fig. 17. I, AFM amplitude image of Fig. H, emphasizing the shape and boundaries of the crystallites.
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Figure 3. Sus scrofa: dentine. A, Untreated fracture showing the parallel and regular tubules in the orthodentine. B, Untreated oblique fracture showing tubules (t) and peritubular dentine (pt). C, Untreated cross section showing the circular shape of a tubule (t) surrounded by peritubular dentine (pt). AFM height image. D, AFM phase image of the same tubule (t), showing that peritubular dentine is composed of several layer (pt), and that the intertubular dentine is heterogeneous. E, Regular structure lining a longitudinal section of a tubule. Untreated sample, AFM phase image. F, Similar structure showing "growth layers" in each horizontal unit lining a tubule: peritubular dentine? Untreated sample, AFM phase image. G, oblique fracture in the intertubular dentine showing a complex structure. Untreated sample, AFM phase image. H, Detail of the same. Each round crystallite is surrounded by a thin dark envelope, composed of organic matrix.
remain. Enamel crystallites are elongated, with a diameter of about 50 nm. In natural fractures without any preferred orientation, several prisms are broken so that crystallites show various shapes and sizes (Fig. 2G). Phase images are sensitive to differences in composition, hardness, etc., so that the dark zones within a crystallite suggest some heterogeneity (Fig. 2G, arrows). In some fractures, the elongated shape of enamel crystallites is clearly apparent (Fig. 2H, I).

The thick prismatic enamel of *Sus scrofa* shows the decussation pattern of mammal teeth, and that the prisms are composed of small elongated crystallites

**Dentine**

Sections show numerous parallel empty tubules with a diameter of 1-1.5 m (Fig. 3A). Oblique sections confirm the regular distribution of tubules within the dentine, and the presence of a thick peritubular zone (Fig. 3B, pt). AFM images also show tubules and peritubular dentine (Fig. 3C, D), and a thin layer is visible between the tubule and the peritubular dentine (Fig. 3D). Some longitudinal or transverse sections show columns of elongated crystallites, with a width of about 50 nm (Fig. 3E, F). Height images do not show specific details, but transverse bands are visible in phase images on these crystallites. Such parallel elements are present along the tubules, and it is suggested that they compose the peritubular dentine. Intertubular dentine shows an interwoven pattern of small crystallites (Fig. 3G). Larger magnifications show that each crystallite is surrounded by a thin layer (Fig. 3H, arrows) only visible in phase images. Thus, it may be suggested that these thin layers are organic.

The dentine of *Sus scrofa* is a mammalian orthodentine, with peritubular tissue.

**Kubanochoerus massai**

**Enamel**

Old fractures of teeth do not clearly show the microstructure of the enamel. On the contrary, fresh fractures of the same sample show the prismatic structure of the enamel (Fig. 4A, B). According to the orientation of the broken surfaces, prisms are seen in transverse sections (Fig. 4B, C) or show the usual plywood pattern (Fig. 4D) (von Koenigswald, 2004). The diameter of a prism is 2-3 m. In such sections, the changes of orientation of the prisms in two adjacent layers are apparent. In each layer, the prismatic units are parallel, and within each prism, the crystallites are parallel.

AFM height images of old fractures show the corrugated surface of the prismatic units (Fig. 5A). Phase images confirm this aspect (Fig. 5B, C). In some places, elongated crystallites are visible (Fig. 5D), but in the main part of the enamel, the shape and arrangement of these crystallites are irregular, and the elongated crystallites are not seen (Fig. 5E, F).

**Dentine**

In fresh fractures, the regular parallel tubules of the orthodentine are visible, but most of them are filled with secondary deposits so that the pattern is faint (Fig. 6A). Larger magnifications show that the density of tubules is low, and some tubules seem surrounded by peritubular dentine.
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Discussion

A discussion of the results can be divided into two main subcategories: (1) The comparison of the microstructures and nanostructures of the enamel and dentine in a modern sample allows us to estimate the potential use of the AFM for the study of skeletal tissues on unpolished samples. (2) Then, the comparison at the micro- and

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Sus scrofa micro- and nanostructures

The structure of prismatic mammal enamel has been well-known since the pioneering work of Korvenkontio (1934), and various patterns have been described (von Koenigswald, 1997a, b). The two-layered enamel of pigs has been described (von Koenigswald, 1997a; Kierdorf et al., 2000; Popowics et al., 2004): an outer radial enamel, and a thick inner enamel with Hunter-Schreger bands. Similar changes in orientation between prisms and interprisms have been described in various mammal taxa, with SEM and TEM (Compare Fig. 2B and Boyde, 1989, Fig. 25). Variability in the prism cross sections was described in molars (von Koenigswald, 1997b). However, the main part of what is known about the inner arrangement of the crystallites within a prism is deduced from human and rodent teeth studied with TEM, and there is no consensus on the shape and size of the enamel crystallites. AFM images show that the orientation and shape of crystallites within a prism of Sus scrofa are similar to those of human enamel (compare Fig. 2A and 2D with Boyde, 1989, Fig. 20 and Fig. 25). Crystallites are flat hexagons with diameters in the range 50-100 nm; the length varies from 160 nm to 1 mm. For human enamel, the average size is 160 nm in length and 20 nm in width (Sakae et al., 1997). Nanostructures of the enamel of non-erupted teeth have been described, focusing on the amelogenins (Fincham et al., 1995; Moradian-Oldak, 2001), so that few details are available on the mineral component.

SEM images of the enamel of Sus scrofa show the usual patterns observed in large mammal teeth. The arrangement, the shape and size of crystallites within a prism correspond to what has been described in other mammal teeth with TEM. The elongated crystallites shown in Fig. 2H-I are similar to the carbon replicas of rat incisors observed with TEM (Warshawsky, 1989, Fig. 17).

Orthodentine is the normal tubular dentine of mammals, with parallel tubules, peritubular dentine (the “walls” of tubules) and intertubular dentine. Kierdorf & Kierdorf (1992) have shown that in pigs, the presence of peritubular dentine has an unequal distribution within a tooth. There is no data about the size of crystallites and the organic matrices. Again, the main data comes from human and rodent tissues. The size of the crystallites is about 10-80 nm length, and 4-6 nm thick (Nakahara, 1982; Nakahara & Kakei, 1984). Decalcified dentine shows organic fibrils. However, the banding pattern of the collagen was not described in these matrices (Breschi et al., 2002). The intertubular dentine pattern does not show regular orientations (Frank & Nalbandian, 1989). Occasionally, the tubule is surrounded by a thin layer of “hyaline peripheral material”, then by the peritubular dentine (Frank & Nalbandian, 1989, Fig. 45). Peritubular dentine is hypercalcified (Frank & Nalbandian, 1989). A similar structure is present in Sus scrofa. In both fresh and etched dentine, columns of flat crystallites have been observed along the tubules. No similar structure seems to be known from TEM observations. AFM phase images show that the dentine crystallites are surrounded by a thin dark envelope (Fig. 3H). From what is known of the composition of the dentine (mineral and

nanoscales of the modern and fossil tissues allows us to estimate the preservation of the fossil samples at a new scale of observation.
organic components), and from what is known of the properties of phase images (sensitive to the composition and/or hardness of the sample), it is hypothesized that the dark envelopes are organic matrices. Similar envelopes are present in carbonate biominerals, with a similar interpretation (Dauphin et al., 2003; Dauphin, 2006). No better explanation has yet been suggested.

Despite their different principles, SEM and AFM observations show similar images: SEM secondary electron images and AFM height images give insights of the topography of the sample. However, samples studied with AFM are not carbon or gold coated as they are in the usual SEM mode: AFM observations are done on the actual sample surface, so that reference samples can be used without any damage. The main constraint with AFM is the topography of the sample: a surface with strong pits and knobs is difficult to examine because of the contact between the sample and the tip. In the case of a narrow pit, the tip cannot penetrate and reach the bottom part. Thus, the comparison of broken and polished-etched surfaces can provide helpful data for difficult samples. Similarly, TEM and AFM height images may be compared because resolutions are similar. Nevertheless, neither ultrathin sections nor replicas are used for AFM observations. Small or large pieces can be studied with AFM. Several signals are simultaneously recorded with AFM; among them, phase and amplitude images are “error” signals that are sensitive to the composition of the samples. Because the composition of enamel and dentine are complex, direct interpretations are not easy, but these images suggest heterogeneity.

**Preservation of fossil teeth**

Both modern (Sus scrofa) and fossil (Kubanochoerus massai) teeth of the studied samples show the main characteristics of the enamel and dentine. From SEM studies, the dentine is more modified by the taphonomic and diagenetic processes than the enamel. Many tubules are filled with secondary deposits, so that the apparent density (number of tubules/cm²) of tubules seems lower in the fossil teeth. This is the usual point of view. However, AFM data give the opportunity to discuss this assertion. The prismatic structure of the enamel, the plywood pattern and the crystallites are preserved, as shown by SEM images of fresh fractures of Kubanochoerus massai. Nevertheless, larger magnifications and better resolutions attributable to AFM show that enamel is not exempt of alterations: crystallite shapes are modified, and the arrangement of the crystallites within a prism is not preserved. So there is a discrepancy between the preservation of micro- and nanostructures of enamel. This difference is not so apparent in dentine, because of the tubules: observing the presence of secondary deposits is easy. However, the state of preservation of the intertubular tissue is more difficult to assert. In the studied samples, it seems that the nanostructures of the fossil intertubular dentine are well preserved.

**Conclusion**

For a true taphonomic study, sedimentological parameters have to be known. Kubanochoerus massai has been collected in a sediment indicating a coastal environment, with forests and abundant rivers. However, no
classic taphonomic analysis was done on these samples. Thus, interpretations of the discrepancy between micro- and nanostructure preservation of fossil tissues are not possible.

Control of the state of preservation of fossil tissues is necessary to reconstruct the fossilization processes as well as the paleoenvironments. It was well-known that there is no relation between the preservation of the shape and microstructure in a fossil specimen. Chemical and isotopic compositions are widely used to reconstruct palaeoenvironments or palaeoecologies. The low content in organic matrix of the enamel has lead to the idea that enamel is a stable tissue, usually exempted of diagenetic changes, so that the most studies do not look at enamel microstructure to estimate the possible alterations. This study shows that a microstructural analysis of the enamel structure is not always sufficient to ascertain the preservation of this tissue in fossil samples. The comparison of the modern and fossil samples reveals another problem: the apparent state of preservation of a tissue depends upon the scale of observation and the resolution of the analytical system (here SEM and AFM). Enamel and dentin of *Kubanochoerus massai* evidence there is no evident correlation between the preservation of the micro- and nanostructures within a tooth. Such data explain why some “well-preserved” enamel and dentine are chemically damaged.

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