Comparison of human and bovine dental enamel by TEM and t-EBSD investigations

A. Koblischka-Veneva¹,², M. R. Koblischka¹,², J. Schmauch¹, M. Hannig³

¹ Experimental Physics, Saarland University, P.O.Box 151150, 66041 Saarbrücken, Germany
² Superconducting Materials Laboratory, Department of Materials Science and Engineering, Shibaura Institute of Technology, Tokyo 135-8548, Japan
³ Clinic of Operative Dentistry, Periodontology and Preventive Dentistry, Saarland University, Building 73, 66421 Homburg/Saar, Germany
E-mail: anjela@shibaura-it.ac.jp

Abstract. The microstructures of human dental enamel and bovine enamel are compared to each other. To obtain samples for transmission electron microscopy (TEM), focused ion-beam (FIB) milling is used. The preparation of such TEM-slices is found to be very effective when operating the FIB with adapted parameters. After the milling process, the TEM-slices are then thinned by means of the ion beam to achieve samples being transparent for the electron beam. With a home-built sample holder, the electron backscatter diffraction (EBSD) can be operated in the scanning electron microscope (SEM) in transmission mode called t-EBSD. This technique enables the crystallographic orientation measurement on nanometer-sized, non-conducting enamel grains with a reasonable quality. Both TEM and t-EBSD images reveal a similar arrangement of the apatite crystals within the enamel, but it is obvious that the nanostructure of human enamel follows a more complex construction principle. The grain sizes of bovine enamel are much larger, and it is difficult to recognize the chain arrangement as found previously in the human enamel. As a result of the comparison, one can state that the nanostructure of human enamel is clearly more complicated than the bovine counterpart.

1. Introduction

The hierarchal micro- and nanostructure of tooth enamel can be considered as a masterpiece of natural nanotechnology [1]. The tooth enamel is an important natural biomaterial with unique properties, including hardness and life-long durability [2–4]. The enamel is built from calcium hydroxyapatite, \([\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]\) (HAP). On the microscale, the enamel contains highly organized prisms, forming a pillar-like structure. While this structural arrangement was investigated with many different imaging techniques, there is only little knowledge about the HAP arrangement on the nanoscale. Recently, good quality TEM images could be obtained from TEM slices prepared by focused ion-beam milling [5]. Moreover, these TEM slices could be employed for transmission electron backscatter diffraction (t-EBSD) investigations enabling the measurement of HAP grain orientations with a resolution in the 20 nm range. From these observations, it became possible to deduce important principles of how the nanostructure is built up.

The electron backscatter diffraction (EBSD) technique has proven to be a very useful tool mostly on metallic samples, and more recently, also on various ceramic sample types.
However, the standard EBSD technique suffers from distinct problems concerning the observation of nanometer-sized grains. Therefore, the t-EBSD technique was very recently developed by several authors [10–13] with the goal to improve the spatial resolution of EBSD on such materials. As result, the spatial resolution could be improved from several tens of nanometers to ∼5-10 nm, depending on the material to be studied [14, 15]. Furthermore, the effects of charging in non-conducting samples are considerably reduced, which is a great advantage for ceramic or even biomaterials [5]. All these improvements make t-EBSD the method of choice to analyze the crystallographic orientations and grain boundary misorientations of tooth enamel.

The processes in nature are very complex, so it is only understandable that the microstructure of tooth enamel differs for different species. In this contribution, a comparison of human tooth enamel with bovine tooth enamel is performed using TEM and t-EBSD. The bovine enamel is often used in the laboratory as a model system for the human one.

2. Experimental procedures

2.1. Electron microscopy

Figure 1 gives a schematic drawing of the arrangements for conventional EBSD (= reflection mode) and t-EBSD (transmission mode). The primary electron beam hits the sample placed with an inclination towards the beam. After entering the sample, an electron cone is formed due to Bragg reflection at the crystal layers [16]. This electron cone is intercepted by a phosphor screen, where the Kikuchi patterns are recorded. Transmission EBSD works with a transmitted electron beam, and the cone is formed on the backside of the sample. Therefore, an electron-
Figure 2. (a), (c), (e): TEM images of the human enamel sample in different magnifications 10000× (a), 25000× (c) and 80000× (d), sample HS2 from Ref. [5]. (b), (d), (f): TEM images of the bovine enamel sample at the same magnifications (sample S8) for comparison.

transparent sample is required for t-EBSD.

TEM investigations were performed using a JEOL JSM-2011 transmission electron microscope operating at 200 KV with a LaB₆ cathode. The EBSD analysis was performed in a JEOL 7000F SEM microscope equipped with a TSL (TexSEM Labs, UT [17]) analysis unit. The Kikuchi patterns were generated at an acceleration voltage of 15 KV, and were recorded by means of a DigiView camera system. To produce a crystallographic orientation map, the electron beam was scanned over a selected surface area and the resulting Kikuchi patterns were indexed and analyzed automatically, represents the common EBSD method working in reflection mode. Automated EBSD scans were performed with a step size down to 50 nm. Although also traditional methods of producing samples for TEM investigations could be used also for t-EBSD, the FIB milling of TEM slices is the method of choice as it enables a proper selection of the area for analysis. Furthermore, the subsequent treatment of the prepared slice with the Ga-ion beam allows larger areas to be analyzed by t-EBSD as compared to the traditional TEM sample preparation methods. For t-EBSD, the TEM-slices were mounted in the SEM on a specially fabricated sample holder allowing for the correct 70° inclination of the sample required for EBSD. The stage with the sample holder is inclined to an angle of -20°, which enables together with the sample mounting the same detector position to be used for the EBSD detector as in the standard configuration. Here, the electron beam is passing through the sample (transmission mode) and the electron cone is formed on the backside of the sample. The electron beam operates at 30 KV, and the working distance is set to 5 mm. The t-EBSD stepsize was chosen to be 5 nm [5,18,19]. An image of the entire arrangement within the SEM chamber is presented in Fig. 1. To improve the imaging quality even further, the TEM slices were treated by additional low-angle argon ion-
Figure 3. t-EBSD Inverse pole figures (IPF) maps in [001] (a) and [100]-direction (b) of the human enamel sample. The color code for the maps is given in the stereographic triangle. In (a), the orientation of the apatite unit cells is indicated, and in (b) the apatite grains are encircled by ellipses to view the arrangement and the grain shape aspect ratio. (c) represents the central result from Ref. [5], where a building principle of the apatite nanostructure could be deduced by formation of chains of grains with similar orientation starting from nodal points (blue-filled grains). The plots (d) give the EBSD-determined grain size distribution and the distribution of the grain aspect ratio.

polishing (5 KeV, 5 min) to increase the image quality (IQ) of the resulting Kikuchi patterns as described in [20] for the investigation of ferrite samples.

2.2. Surface preparation and TEM slices
For the microstructure analysis, the sample surfaces were polished mechanically using various 3M lapping films (100 µm down to 0.05 µm), and the final treatment was carried out employing Struers SiO$_2$ (OP-S) suspension with the respective polishing cloth on an automatic polishing machine at a speed of 200 rpm. This procedure yields a total roughness of several nanometers as determined by atomic force microscopy. Further details of the polishing procedure are described in Refs. [5,9,21]. No additional carbon layers to enhance the electric conductivity were applied.
Figure 4. (a,b): IPF maps in [001] and [100]-directions of the bovine enamel sample. The enamel grains are considerably larger as in the human case, but the principal grain orientation is similar. The plots (c) give the EBSD-determined grain size distribution and the distribution of the grain aspect ratio.

The TEM slices for the t-EBSD measurements were produced by focused-ion beam (FIB) milling in a dual-beam FIB workstation (FEI) using a routine allowing for reduced surface damage. The polished sample surface is covered by Pt to reduce the influence of charging effects. After lifting-off the TEM slice from the sample with the micromanipulator, the surface is ion-polished in a separate step by 2 KeV Ga-ions to a thickness of about 80 nm. This step serves to further reduce the preparation damage of the surface area and for a further thinning of the sample to be transparent to the electron beam. Figure 1 (c) presents a typical TEM slice fabricated from a human tooth sample after the completed ion-beam thinning process in a surface view (upper image) and the cross section (lower image). In Fig. 1 (d), the TEM slice is shown ready for t-EBSD investigations in the SEM chamber mounted on the sample holder for t-EBSD.

Two samples were chosen for the present study: The human enamel sample HS2 (Ref. [5]), and the bovine enamel sample S8. Both samples were cut perpendicular to the original tooth surface in an identical way to allow for direct comparison.

3. Results and discussion

Figure 2 presents TEM images at various magnifications (10000×, 25000× and 80000×) from human and bovine enamel. For comparison, the same magnification steps were used in (a), (c), (e) and in (b), (d), (f). The TEM images reveal the dense packing of the HAP grains with no voids between them. All grain boundaries are well defined and clearly resolved in the images. It is further visible that the HAP grains have commonly a long extension. The comparison directly
reveals that the bovine HAP grains are larger than the ones of the human enamel sample.

In Fig. 3, the results of the t-EBSD orientation mapping on the human tooth enamel sample HS2 in [001]-direction (a) and [100]-direction (b) are shown. The color code for the HAP orientations is given in the stereographic triangle. The maps reveal the polycrystalline structure of the enamel with many high-angle grain boundaries. The HAP grains are, however, not fully randomly oriented as green and violet orientations are dominating in (a), and the orientation in [001]-direction (red) in (b). Figure 3 (c) represents the main result of Ref. [5]: From the EBSD orientation mappings in [100]-direction, it was deduced that HAP grains of similar orientation form chains which begin at some nodal points from which several chains branch away. This arrangement may provide the necessary strength of the entire nanostructure. As a result, the human enamel structure is a pretty complex one. Finally, Fig. 3 (d) gives the EBSD-determined grain size distribution and the distribution of the grain shape aspect ratio. To determine the latter one, the HAP grains are encircled by ellipses with a major \(a\) and minor \(b\) axis. The grain shape aspect ratio is then defined as \(g_{ar} = b/a\), which ranges between 0 and 1 [17].

Figures 4 (a) and (b) give the t-EBSD mapping of the orientations in [001]- and [100]-directions of the bovine enamel sample S8. The color code for the HAP orientations is given in the stereographic triangle. The orientation mapping reveals the polycrystalline grain arrangement with many high-angle grain boundaries, similar to the human enamel. Also here the HAP grains are not oriented fully randomly, even though due the larger grain size the present mapping contains only a small number of HAP grains. Furthermore, on the bovine sample S8 it was not possible to observe a chain formation like in the human enamel sample, nor some nodal points. Figure 4 (c) presents the plots of the grain size distribution and the distribution of the grain shape aspect ratio of the bovine enamel.

The comparison of the plots of Fig. 3 (d) and 4 (c) enable to draw more interesting conclusions: The bovine enamel sample exhibits only a narrow range of the grain shape aspect ratio with values of \(~0.5\), whereas the human enamel shows a much wider range spanning of \(g_{ar}\) between 0.35 and 0.85. This result was obtained on TEM slices of various human tooth samples, so this wider range of \(g_{ar}\) may indicate an adaptation of the human teeth for a different use (dietary adaptations omnivore vs. herbivore, [3]).

Using the t-EBSD technique on focused ion-beam prepared TEM slices of enamel stemming from human and bovine teeth, the mapping of the HAP crystallite orientations becomes possible with a spatial resolution in the 10-20 nm range. The measurements clearly reveal the common grain arrangements of the HAP grains in the enamel, but also obvious differences between the two types of teeth are revealed. The grain size, the grain size distribution, and the distribution of the grain shape aspect ratio are different in the two types of teeth. Furthermore, the missing chain formation of the enamel grains in the bovine enamel may be responsible for a different resulting strength of the enamel structure. These observations suggest that the different use of the teeth is reflected in the building scheme of the enamel. This demonstrates that the knowledge about the HAP crystal orientation and shape is essential for the understanding of biomaterials [22].

4. Conclusions

Human enamel is found to be different from bovine enamel (the samples are prepared in the same orientation to enable a comparison). The overall HAP grain orientation is found to be similar for human and bovine enamel in both [001] and [100] orientations. The bovine enamel exhibits only a narrow range of the grain shape aspect ratio at \(~0.5\), whereas the human enamel shows a much wider range. The grain size of bovine enamel is larger than the human one, and the grain size distribution of the bovine enamel is much more narrow. Furthermore, it was not possible to observe the characteristic chain formation of the enamel grains in the bovine enamel sample. Future work will consider the comparison of the enamel grain orientations of various animals.
(carnivores, herbivores) with the human enamel, and also the differences between human baby tooth enamel and that of adults.

Acknowledgments
This work was supported by Saarland University (‘Anschubfinanzierung’), which is gratefully acknowledged. We also thank F. Soldera (UdS, Institute of Functional Materials) for the excellent FIB work.

References
[1] Hannig M and Hannig C 2010 Nanomaterials in preventive dentistry. Nat Nanotechnol 5 565
[2] Black J, and Hastings G 1998 Handbook of Biomaterial Properties. Springer Verlag Berlin
[3] Macho G A, Jiang Y and Spear I R 2003 Enamel microstructure: A truly three-dimensional structure. Hum Evol 45 81
[4] Cui F Z and Ge J 2007 New observations of hierarchical structure of human enamel, from nanoscale to microscale. J Tissue Eng Regenerative Med 1 185
[5] Koblishka-Veneva A, Koblishka M R, Schmauch J and Hannig M 2018 Human dental enamel: A natural nanotechnology masterpiece investigated by TEM and t-EBSD. Nano Research 11 3911
[6] Humphreys F J 2004 Characterisation of fine-scale microstructures by electron backscatter diffraction (EBSD). Scr Mater 51 771
[7] Dingley D 2004 Progressive steps in the development of electron backscatter diffraction and orientation imaging microscopy. J Microsc 213 214
[8] Chen D, Kuo J-C and Wu W-T 2011 Effect of microscopic parameters on EBSD spatial resolution. Ultramicroscopy 111 1488
[9] Koblishka M R and Koblishka-Veneva A 2013 Applications of the electron backscatter diffraction technique to ceramic materials Phase Transi 86 651
[10] Trimby P W 2012 Orientation mapping of nanostructured materials using transmission Kikuchi diffraction in the scanning electron microscope. Ultramicroscopy 120 16
[11] Keller R and Geiss R 2012 Transmission EBSD from 10 nm domains in a scanning electron microscope. J Microsc 245 245
[12] Sneddon G C, Trimby P W and Cairney J M 2016 Transmission Kikuchi diffraction in a scanning electron microscope: A review. Mat Sci Eng R 110 1
[13] Suzuki S 2013 Evaluation of transmission-EBSD method and its application to observation of microstructures of metals. J Jpn Inst Met Mater 77 268
[14] Britton B, Holton I, Meaden G and Dingley D 2013 High angular resolution electron backscatter diffraction: Measurement of strain in functional and structural materials. Microsc Anal 27 8
[15] van Bremen R, Ribas Gomes D, de Jeer L T H, Ocelik V and De Hosson J Th M 2016 On the optimum resolution of transmission-electron backscattered diffraction (t-EBSD). Ultramicroscopy 160 256
[16] Reimer L 1985 Scanning Electron Microscopy: Physics of Image Formation and Microanalysis. Springer Science: Berlin Heidelberg
[17] TexSEM Laboratories (TSL). Orientation Imaging Microscopy Software V4.1, User Manual, TexSEM laboratories, (TSL), Draper, UT, 2004.
[18] Koblishka-Veneva A, Koblishka M R, Schmauch J, Inoue K, Muradilhar M, Berger K and Noudem J 2016 EBSD analysis of MgB2 bulk superconductors. Supercond Sci Technol 29 044007
[19] Koblishka-Veneva A, Koblishka M R, Zeng X L, Schmauch J and Hartmann U 2018 TEM and electron backscatter direction analysis (EBSD) on superconducting nanowires. J Phys: Conf Ser 1054 012005
[20] Koblishka-Veneva A, Koblishka M R, Schmauch J, Chen Y and Harris V G 2010 EBSD analysis of the microtexture of Ba-hexaferrite samples. J Phys: Conf Ser 200 082014
[21] Koblishka-Veneva A and Koblishka M R 2004 Surface preparation of high-\text{\textit{Tc}} superconductors for MO imaging. In: MO imaging (eds Johansen T H and Shantsev D V) Kluwer: Dordrecht p 242
[22] Bechtel S, Ang S F and Schneider G A 2010 On the mechanical properties of hierarchically structured biological materials. Biomaterials 31 6378