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Ultrastructural organization and micromechanical properties of shark tooth enameloid ☆

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ABSTRACT

The outer part of shark teeth is formed by the hard and mineral-rich enameloid that has excellent mechanical properties, which makes it a very interesting model system for the development of new bio-inspired dental materials. We characterized the microstructure, chemical composition and resulting local mechanical properties of the enameloid from teeth of *Isurus oxyrinchus* (shortfin mako shark) by performing an in-depth analysis using various high-resolution analytical techniques, including scanning electron microscopy, qualitative energy-dispersive X-ray spectroscopy and nanoindentation. Shark tooth enameloid reveals an intricate hierarchical arrangement of thin (50–80 nm) and long (>1 μm) crystallites of fluorapatite with a high degree of structural anisotropy, which leads to exceptional mechanical properties. Both stiffness and hardness are surprisingly homogeneous in the shiny layer as well as in the enameloid: although both tooth phases differ in structure and composition, they show almost no orientation dependence with respect to the loading direction of the enameloid crystallites. The results were used to determine the structural hierarchy of shark teeth, which can be used as a base for establishing design criteria for synthetic bio-inspired and biomimetic dental composites.

1. Introduction

Shark teeth have different macroscopic geometries which are evolutionarily optimized for their specific biological function, i.e. the way of hunting and biting. The teeth can be classified by their geometry, e.g. as "tearing-type", "cutting-type" or "cutting-clutching type" [1–4]. The chemical and crystallographic composition of shark teeth of such different shape and function are still very similar [5]. Morphologically, the teeth of sharks consist mainly of dentin that is covered by an outer hard and highly mineralized layer in the crown area [6]. In, for example, reptiles and mammals, including humans, this outermost layer of the teeth is denoted as enamel [7,8]. In sharks, the external tooth layer has no ectodermal enamel and is, therefore, denoted as "enameloid" [9]. The enamel of mammalian teeth, including human teeth, consists of hydroxyapatite ($Ca_5(PO_4)_3OH$), associated with small amounts (~1 wt.%) of an organic matrix composed mainly of the proteins amelogenin and enamelin [10,11]. The mineral phase of shark tooth enameloid consists of fluoroapatite ($Ca_5(PO_4)_3F$) [12–15], with a fluoride content nearly as high as that of geological fluoroapatite crystals (3.1 and 3.64 wt.%, respectively) [5]. The total enameloid contains ~5–8 wt.% of organic matrix consisting of collagens and enamelins [5,16–18]. In mammalian and human tooth enamel, the hydroxyapatite forms needle-like crystallites organized in bundles ("enamel prisms") that originate at the dentin–enamel junction and are oriented perpendicular to the tooth surface [19–21]. The outermost layer ("prismless layer") of human enamel consists of parallel oriented needle-like crystallites [22]. The fluoroapatite in shark tooth enameloid is also present in the form of elongated crystallites that constitute layers with different structural organization. These layers have been classified by the structural analysis of surface etched sectional samples [23]. Three layers of the enameloid were identified and denoted as "shiny-layered enameloid (SLE)", "parallel-bundled enameloid (PBE)" and "tangled-bundled enameloid (TBE)". Since the PBE and TBE show a gradual transition, the enameloid of sharks is generally thought to be organized into two main structural building blocks: a superficial layer (shiny layer) and an inner layer consisting of crystallite bundles with changing degrees of structural organization from distal to proximal [24]. From exterior to interior, the well-organized crystallite bundles of the PBE change to a less ordered TBE. Reaching the dentin–enameloid junction, no defined bundles are visible in the TBE, but randomly arranged crystallites [24]. The formation of

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shark tooth enameloid can be subdivided into three phases of development: first, the formation of the matrix; second, the mineralization of the enameloid; and finally, the maturation of the enameloid [25–27]. The individual fluoroapatite crystallites nucleate and grow in tubular vesicles formed by the odontoblasts. During the mineralization stage, the vesicles disappear and individual crystallites in different states of organization can be observed entangled between organic, presumably collagen molecules [9]. However, little is known about the structural organization of matured shark tooth enameloid at a scale smaller than the crystallite bundles and whether and how the structure and composition influence the local mechanical properties of the material.

Recently, the correlation of structure, composition and mechanical properties in biological hard tissues [28,29] has become an important field of research that aims at the development of novel biomimetic and bio-inspired materials [30,31]. The mechanical properties of shark tooth enameloid have been investigated using mainly micro- and nanoindentation techniques [5,32]. However, this has not been performed with a resolution sufficient to analyze the local mechanical properties of small building units such as individual crystallite bundles. Therefore, we have chosen the teeth of the recent shark species *Isurus oxyrinchus* (shortfin mako shark) to study the ultrastructure and elemental distribution of the enameloid using high-resolution scanning electron microscopy (SEM) and qualitative energy-dispersive X-ray spectroscopy (EDX). We have correlated these results with the local mechanical properties obtained by nanoindentation for the shiny layer and fluoroapatite crystallite bundles indented in different directions on both axial and orthogonal planes of the teeth. The results provide valuable information that can be used as inspiration for the design of bio-inspired dental materials which exploit the advantages of natural shark tooth enameloid.

2. Materials and methods

2.1. Sample preparation and analytical methods

Individual teeth of the recent shark species *I. oxyrinchus* were purchased from commercial sources that obtain their material from a commercial fishery where the teeth were extracted, cleaned and shipped to the retailers. Since the original position of the teeth in the jaw could not be reproduced, we used only specimens with shapes and sizes qualifying them as erupted mature teeth. The taxonomical determination of the shark species was verified with the help of Dr. A. Gillis, Dalhousie University, Canada. All teeth used for the experiments were stored as delivered in dry state at room temperature. We used SEM to study the microstructure, EDX to map the elemental composition and nanoindentation to determine the local mechanical properties of the enameloid and the shiny layer. The used samples were either fractured or embedded and polished in specific orientations with respect to the geometry of the tooth.

The fractured samples were prepared by creating notches at the tip and the cutting edge of two different teeth using a jeweler's saw. A sharp blade was then placed into the notches and splinters of the tooth were produced with careful hammer strokes on the back of the blade. To investigate the structure and distribution of the organic matrix within the enameloid, selected sample pieces from both teeth were superficially etched by immersion in an aqueous ethylenediaminetetraacetic acid (EDTA) solution (0.1 mol l^{-1}; Waldeck, Germany) with 2.5% glutaraldehyde (Merck, Germany) for 2 min, as described by Fabritius et al. [33]. The samples were washed in double-distilled water (1 s) followed by 100% methanol (1 s), dehydrated in an ascending series of acetone–water (30–50–70–90–100 vol.% acetone; 5 min each) and dried in a Bal-Tec CPD 030 critical point dryer. The native and etched tooth pieces were mounted with the exposed internal surface pointing upwards onto standard aluminum SEM holders and rotary-shadowed with a 4 nm thick layer of platinum in a Gatan Precision Etching Coating System (PECS 682). Subsequently, the samples were analyzed in a Zeiss Crossbeam XB1560 FIB-SEM at an acceleration voltage of 5 kV using a 30 μm aperture and an in-lens detector at small working distances. Where necessary, contrast and brightness of the SEM micrographs were adjusted using Photoshop CS2 (Adobe Inc.).

Four different teeth were prepared for EDX mapping and nanoindentation testing by cutting them with a jeweler's saw. Two teeth were axially cut and embedded in a conductive phenolic resin containing carbon fibers (Polyfast, Struers) with their sagittal planes exposed. The resin was cured using a Buehler SimpliMet 3000 heated press (150 bar, 5 min heating time, 180 °C). The other two teeth were orthogonally cut exposing the cross-section of the crown at about half the length of the tooth. These samples were embedded in a one-component UV-curable methyl methacrylate resin (CEM 4000 Lightfix, Cloeren Technology GmbH, Wegburg) that was cured in a Struers UV-Box (3 min using the bottom source only followed by 6 min with bottom and top source together). The exposed sections of all four teeth were polished using a series of abrasive papers with decreasing grit sizes (220, 400, 600, 1000, 2500 and 4000; Hermes) followed by a 3 μm diamond suspension (Struers) and final polishing with a 0.1 μm silica suspension (Buehler; Saphir 320/330 instrument, ATM).

Nanoindentation measurements were conducted with a Hysitron TriboIndenter TI 900 equipped with a top-down closed-loop scanner that maximizes the indent-positioning accuracy. We used a Berkovich indenter (Ti 39-01, tip radius 50 nm) in displacement control mode (100 nm indentation depth) and a triangular load function (10 s–10 s). The area function was determined on a fused quartz standard from Hysitron (0.7–194 nm). On the two axially polished samples, a total number of 2575 indents were set in five rectangular patterns that cover the shiny layer and outer enameloid and were distributed along the tooth margin and one pattern located in the central region of the enameloid. The spacing between the indents was 1 μm for the first and 2 μm for the second sample. On the two orthogonally polished samples, a total number of 435 indents were set in two rectangular patterns placed at the tooth margin that covered the shiny layer and the outer enameloid. The spacing between the indents was 2 μm. The reduced elastic modulus (E_{red}) and the hardness (H) were determined according to the method of Oliver and Pharr [34].

After indentation, the samples were coated with 4 nm of platinum (Gatan PECS 682) and the quality of the indents was inspected using SEM (Zeiss Crossbeam XB1560 FIB-SEM). Elemental maps with a pixel resolution of 512 × 400 were recorded using the built-in Apollo XL Silicon Drift Detector (EDAX) at an acceleration voltage of 10 kV, 120 μm aperture size and a dwell time of 250 μs. The post-processing of the maps, including background subtraction combined with peak deconvolution, was performed with the EDAX Genesis software package.

Subsequently, the indented areas of selected samples were subjected to superficial etching by the application of a droplet of aqueous EDTA solution (0.1 M with 2.5 wt.% glutaraldehyde) for 1.5 h, washed with distilled water and then with ethanol, recoated with 4 nm of platinum and inspected in the SEM using a backscattered electron (BSE) detector for compositional contrast. For each indent pattern, we inspected the position of every individual indentation and assigned the data point either to the shiny layer, to a certain type of crystallite bundle of the enameloid with known orientation or to areas with exposed organic matrix, respectively. Indents that were not placed on a clearly defined structure like defects in the material or on exposed organic envelopes were excluded from

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