Collagen Fibers in Crocodile Skin and Teeth: A Morphological Comparison Using Light and Scanning Electron Microscopy

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Abstract

Collagen is one of the most versatile tissues of living organisms that comes in many shapes and sizes, providing functions ranging from tissue matrix through, ligament formation up to enabling mineralization in teeth. The detailed light microscopy and Scanning Electron Microscopy (SEM) observations conducted in this study, allowed us to investigate morphology, sizes and crimp patterns of collagen fibers observed in crocodile skin and teeth. Moreover, the microscopy study revealed that although two completely different tissues were investigated, many similarities in their structure based on collagen fibers were observed. Collagen type I is present in crocodile skin and teeth, showing the flexibility in naturally constructed tissues to obtain various functions. The crimp size investigation of collagen fibers confirmed experimentally the theoretical 67 nm D-periodicity expected for collagen type I. The collagen in teeth provides a matrix for crystal growth and in the skin provides flexibility and is a precursor for corneous scales. Importantly, these observations of the collagen in the skin and tooth structure in crocodiles play an important role in designing biomimetic materials with similar functions and properties.

Keywords: crocodile skin, collagen, crimp, fiber, crocodile tooth, SEM

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1 Introduction

Nature is an excellent source of designs that were developed and perfected over millions of years. Fibers in nature have many functions such as reinforcement or insulation with the most common two proteins being collagen and keratin. Hairs made out of keratin provide insulation and protection from the environment^[1]. Spiders use silk fibers build shelter or webs for hunting and collecting water^[2,3]. Collagen is a building block of life and as such is the most abundant protein in animals^[4]. In its many forms, collagen is responsible for a variety of roles such as: organizing extracellular matrix (ECM), providing a tensile strength in tissues, elasticity in the skin, scaffolding for tissue formation and as a matrix allowing mineralization in teeth^[5,6].

Crocodiles belong to an animal family that inspired many biomimetic inventions such as flexible body armor, haptic feedback systems, and advanced domed micro-sensors^[7–9]. Even though crocodile skin is well known and respected in the clothing and leather industry, not

many researchers focused on crocodile skin from a materials engineering point of view. Crocodile skin is a layered structure responsible for protection from desiccation and abrasion^[10]. It can be described as a complex laminate comprising a corneous plate-like epidermis supported by an intertwined meshwork of collagen type I dermis^[10,11]. This natural composite provides armor-like properties connecting a hard corneous tissue with the flexibility of the collagen matrix giving unparalleled protection from a rough environment. In crocodile teeth, collagen type I is the main constituent of dentin surrounding the pulp^[12]. Dentin is a composite of flexible collagen fibrils interspersed with carbonate-rich apatite mineral that provides rigidity and strength.

In this study, we take a closer look at crocodile skin and teeth to investigate different collagen fibers structure in this most extraordinary predators from a family that dates back more than 200 million years and survived end-Triassic extinction^[13–15]. We focused on Cuban crocodile (*Crocodylus rhombifer*) that in the wild can be found only in Cuba's Zapata Swamp and the Isle of

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Youth making it the smallest known distribution of any extant crocodilian. The Cuban crocodiles are medium-sized, adult males being $197 \text{ cm} \pm 8.1 \text{ cm}$ long on average. Cuban crocodile's natural habitat is mostly restricted to freshwater reservoirs, however, thanks to its comparatively long and robust legs it is one of the most terrestrial crocodilians. Due to that, its diet is highly terrestrial comprising of mammals, birds, tortoises, crustaceans, and fish. The Cuban crocodile is a critically endangered species with a population estimated up to 6000 individuals^[16]. There are many reports on crocodile skin based on histology, ultrastructure, biochemistry, molecular biology and gene expression^[17–21]. However, in this work we provide new viewpoint using high resolution imaging to correlate the morphology with the desired function of crocodile soft and hard tissues that has not been directly compared in such details.

Using Light (LM) and Scanning Electron Microscopy (SEM), two vastly different tissues that contain collagen fibers were investigated to understand nature engineered tissues with different mechanical performances. Investigated collagen differed in size and geometrical arrangement of collagen fibrils depending on the structure. Collagen in skin had basket-weave structure, providing flexibility. In teeth, collagen fibers were aligned in one direction and act as a teeth precursor and provide mineralization matrix of future enamel. Interestingly, the crimp size investigation using high-resolution SEM imaging confirmed the presence of collagen Type-I in both tissues. This fundamental study could bring new nature-inspired designs in fiber-based materials and especially for composites.

2 Materials and methods

Skin tissue was obtained post-mortem from 11 years old Cuban crocodile (*Crocodylus rhombifer*) that was living in captivity (Warsaw Zoo in Poland). The skin was cut out from the upper body region using a scalpel and separated from soft-tissues during an autopsy of the animal immediately after confirmation of its death. The tissues were then dried to prevent damage to the material without chemical treatment. Teeth used in this study were picked after shedding in crocodile habitat. To expose the inner structure and cross-section of crocodile skin the tissues were immersed in liquid ni-

trogen for 5 min and cracked using a scalpel. Crocodile teeth were placed in a vice and cut open using hacksaw along the longitudinal axis. For light microscopy, digital microscope Dino-lite AM31131 (AnMo Electronics, Taiwan) was used prior to sample preparation for SEM.

All tissues used for SEM imaging were coated with a 5 nm gold layer using a rotary-pump sputter coater (Q150RS, Quorum Technologies, UK). Gold-coated samples were then fixed to carbon tape mounted on the SEM stubs. SEM (Merlin Gemini II, Zeiss, Germany) investigation was carried out with an accelerating voltage of 3 kV, 150 pA current, keeping the working distance of 3 mm – 6 mm. Obtained SEM micrographs were analyzed with ImageJ (J1.46r, Fiji, USA) to determine collagen fibers diameters and crimp size. Statistical measurements of average fiber diameter and crimp size were calculated using OriginPro (2019b, OriginLab, USA) using ANOVA with a T-test using a significance level of 0.05.

3 Results and discussion

3.1 Morphology investigation of skin and teeth

Crocodile skin is a layered structure with a scaly epidermis (Fig. 1a) and pale dermal tissue forming a meshwork (Fig. 1b). As observed by light microscopy, scales have a large outer surface, rectangular shape and single scales are separated. The connection between two plates, also known as a hinge^[22] is an overlay of two scales shown in the cross-section image (Fig. 1c). In hinges present in crocodile skin we can observe plates parallel to the skin surface filling the gap providing the continuity of plating (Fig. 1d). In contrast to the rest of the scale, these narrow hinge regions are comprised of thin plates built from mostly α -keratin with reduced amounts of corneous β -proteins (formerly termed as β -keratins)^[23–27]. Crocodile epidermis (Figs. 2a – 2c) is built from multiple layers of corneous scales, consisting mainly of β -proteins^[11]. Densely packed and highly organized collagen bundles can be observed under the corneous epidermis (Figs. 2d and 2e). In our case, a typical basket-weave meshwork of collagen type I (banded) was observed. Such an arrangement can be found in the healthy skin of most of the organisms in the animal kingdom such as mice^[28] and importantly in reptiles^[29]. The basket-weave arrangement provides

Fig. 1 Light microscopy images of crocodile skin from (a) epidermis and (b) dermis side. SEM micrographs showing of (c) cross-section of two overlaying scales and (d) intersection of scales image taken from epidermis side of crocodile skin. Selected areas marked with numbers 1 and 2 are regions of overlapping plates from epidermis and dermis respectively.

Fig. 2 Layers in crocodile skin (a) cross-section from the normal plane of view, (b) top-down view (c) plates in the epidermis, (d) collagenous dermis with a marked region for crimps studies, (e) close up of dermis with visible crimps on collagen fibers.

high mechanical strength in all directions for the skin^[30]. Possible early stages of plate formation can be observed near the epidermis layer (Fig. 2c) suggesting that cornification happened on a collagen matrix, the cellular mechanism of crocodilian scale growth was previously proposed by Alibardi^[18]. Our investigation of layers present in crocodilian skin agrees with structures previously reported by Dubansky and Close on alligator skin^[11]. However, our analysis is mainly based on SEM observation rather than histological techniques providing a high-resolution images, thus giving many more details and totally different perspective on the tissue's morphology. The layered structure of Cuban crocodile's skin provides its high mechanical performance and resilience to abrasion while preserving wide range of motion necessary for animals' survival^[11].

Crocodile teeth are typically cone-shaped and sometimes curved with hollow roots, a typical tooth is shown in Fig. 3^[31]. Their size depends on the age and size of an individual animal. Bone is a hierarchically organized bio-composites possessing remarkable mechanical properties^[32–34]. Dentin is comprised mostly of collagen whereas enamel is mostly mineral^[35]. In Fig. 4a, we show a clear transition in outer layers of crocodile tooth starting with enamel through dentin-enamel junction into dentin. This finding falls in line with previous research^[31]. Crocodile enamel (Fig. 4b) has been reported as a phase consisting of densely packed mineral crystallites rich in calcium, sodium, and phosphates^[31]. Dentin-enamel junction is an intermediate phase where the mixing of dentin and enamel phases is visible, see

Figs. 4b and 4c. The collagen present in dentin showed in Fig. 4d holds the mineral parts in place in the tissue. As collagen is a main constituent of dentin it can be seen in all of its regions (Fig. 4d). Importantly, we can observe highly anisotropic arrangement of the collagen fibers in dentin as they are stretched in the longitudinal axis of the tooth. This anisotropy is specially design to provide fracture resistance and stopping crack propagation in the tissues of many animal species^[34]. Confirming previous observations, no collagen fibers were observed in enamel sections of teeth as shown in Fig. 4b)^[36]. Crocodile teeth are defined by high deformability compared to their mammalian counterparts. The hardness difference in crocodile teeth comes from a fact that crocodiles use their teeth mostly for grabbing in contrast to chewing and cutting, which results in thinner enamel layer^[31,37]. The periodic crimps found on collagen fibers in dentin, shown in Fig. 4e indicate collagen type I, as previously found in the dentin of other animals^[38]. Importantly, collagen in crocodile teeth act as a template for apatite initiation and elongation to strengthen the tissue^[38].

3.2 Comparison of collagen fibers in skin and teeth

Comparison of collagen fibers in crocodile skin and teeth shown in Fig. 5 revealed that even though the fibers appear similar with an average diameter of $118.2 \text{ nm} \pm 31.1 \text{ nm}$ and $92.3 \text{ nm} \pm 25.2 \text{ nm}$ for skin and teeth respectively the ANOVA test shown that there is a statistically significant difference between diameters at the significance level of 0.05. These results confirmed the

Fig. 3 Light microscopy images of crocodile tooth showing (a) top-down view and (b) into the hollow root. The root of the tooth is marked with the capital letter A.

previous reports showing that the diameter of collagen type I is in the range of 50 nm – 200 nm^[39]. These observations lead to a conclusion that there are variations in diameters depending on function even though we are dealing with the same Collagen type I. As collagen fibril diameter is strongly correlated with its mechanical

properties, higher diameter found in skin indicates a need for high mechanical properties in the tissue as was previously reported^[40].

Crimp size investigation shown in Fig. 5 revealed that collagen in both tissues has virtually the same crimp size of 67.2 nm and 67.1 nm for skin and teeth

Fig. 4 SEM micrographs showing crocodile tooth (a) enamel, (b) cross-section exposing layered structure with indicated dentin-enamel junction, (c) top view of the dentin-enamel junction, (d) dentin, (e) close up on collagen fibers with characteristic crimps.

Fig. 5 SEM micrographs of collagen fibers in crocodile (a), (b) skin, (c), (d) teeth and histograms of collagen fibers (e) diameter and (f) crimp size of fibers.

respectively with no significant statistical difference observed. Similarly to other studies on crimps sizes in collagen fibers, the crimp was marked by a regional deformation ranging from local flattening to limited torsion or more complex phenomena^[41]. Therefore, slight variations from the theoretical 67 nm D-periodicity were observed^[41–43]. As this periodicity is characteristic for collagen type I, it serves as a clear demonstration that both tissues are built by the same material even though their properties and function vary.

4 Conclusion

In conclusion, in this unique work collagen fibers in skin and teeth tissues were extensively investigated and compared at different scales utilizing light and scanning electron microscopy techniques for the first time. We provide easy to follow protocol of investigating soft tissues using SEM technique, which alleviates necessity of using histopathological procedures. Our findings indicate that collagen provides different functions depending on the tissue, such as skin and teeth. The most striking difference was observed in the distribution of collagen fibrils. In the skin, we can observe basket-weave structure which leads to a highly flexible and mechanically tough structure. Such a structure provides natural protection and insulation from the environment. In teeth, we can observe fibers that are mostly aligned in one direction as its main function is to act as a teeth precursor and to provide mineralization matrix of future enamel. Crimp size investigation of collagen fibers confirmed experimentally the theoretical 67 nm D-periodicity expected for collagen type I, therefore confirming that collagen found in both tissues was collagen type I^[42]. The diameters of collagen fibers found in the skin were about 30 nm higher than the diameter of collagen fibers in teeth being $92 \text{ nm} \pm 25.2 \text{ nm}$ and $118 \text{ nm} \pm 31.1 \text{ nm}$ respectively. This study shows that nature in its creativity uses the same material for very different purposes thus providing various functions by playing with geometrical arrangements and sizes of fibers.

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