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

Piotr Krzysztof Szewczyk, Urszula Stachewicz*
Metals Engineering and Industrial Computer Science and International Centre of Electron Microscopy for Materials Science, AGH University of Science and Technology, Kraków 30-059, Al. Mickiewicza 30, Poland

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

Copyright © The author(s) 2020.

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 microsensors[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...
Youth making it the smallest known distribution of any extant crocodilian. The Cuban crocodiles are medium-sized, adult males being 197 cm ± 8.1 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\cite{16}. There are many reports on crocodile skin based on histology, ultrastructure, biochemistry, molecular biology and gene expression\cite{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 nitrogen 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\cite{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)\cite{23–27}. Crocodile epidermis (Figs. 2a – 2c) is built from multiple layers of corneous scales, consisting mainly of β-proteins\cite{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\cite{28} and importantly in reptiles\cite{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\cite{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\cite{18}. Our investigation of layers present in crocodilian skin agrees with structures previously reported by Dubansky and Close on alligator skin\cite{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\cite{11}.

Crocodile teeth are typically cone-shaped and sometimes curved with hollow roots, a typical tooth is shown in Fig. 3\cite{31}. Their size depends on the age and size of an individual animal. Bone is a hierarchically organized bio-composites possessing remarkable mechanical properties\cite{32–34}. Dentin is comprised mostly of collagen whereas enamel is mostly mineral\cite{35}. In Fig. 4a, we show a clear transition in outer layers of crocodile tooth starting with enamel trough dentin-enamel junction into dentin. This finding falls in line with previous research\cite{31}. Crocodile enamel (Fig. 4b) has been reported as a phase consisting of densely packed mineral crystallites rich in calcium, sodium, and phosphates\cite{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\cite{34}. Confirming previous observations, no collagen fibers were observed in enamel sections of teeth as shown in Fig. 4b\cite{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\cite{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\cite{38}. Importantly, collagen in crocodile teeth act as a template for apatite initiation and elongation to strengthen the tissue\cite{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 nm ± 31.1 nm and 92.3 nm ± 25.2 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
previous reports showing that the diameter of collagen type I is in the range of 50 nm – 200 nm\textsuperscript{[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\textsuperscript{[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\cite{43}. Therefore, slight variations from the theoretical 67 nm D-periodicity were observed\cite{41,42,43}. 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\cite{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 nm ± 25.2 nm and 118 nm ± 31.1 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.

Acknowledgment

The authors thank Adam Hryniewicz from Warsaw Zoo for crocodile skin and teeth samples used in this study. This study was conducted as part of the “Nano-fiber-based sponges for atopic skin treatment” project, which is carried out within the First TEAM programme of the Foundation for Polish Science co-financed by the European Union under the European Regional Development Fund, Project No. POIR. 04.04.00-00-4571/18-00. This study was supported by the infrastructure at the International Centre of Electron Microscopy for Materials Science (IC-EM) at AGH University of Science and Technology.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made.

The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

[1] Metwally S, Martinez Comesaña S, Zarzyka M, Szewczyk P K, Karbowniczek J E, Stachewicz U. Thermal insulation design bioinspired by microstructure study of penguin feather and polar bear hair. Acta Biomaterialia, 2019, 91, 270–283.
[2] Römer L, Scheibel T. The elaborate structure of spider silk: Structure and function of a natural high performance fiber. Prion, 2008, 2, 154–161.
[3] Szewczyk P K, Knapczyk-Korczak J, Ura D P, Metwally S, Gruszcyński A, Stachewicz U. Biomimicking wetting properties of spider web from Linothele megatheloides with electrospun fibers. Materials Letters, 2018, 233, 211–214.
[4] Kadler K E, Holmes D F, Trotter J A, Chapman J A. Collagen fibril formation. Biochemical Journal, 1996, 316, 1–11.
[5] Canty E G, Kadler K E. Procollagen trafficking, processing
and fibrillogenesis. *Journal of Cell Science*, 2005, 118, 1341–1353.

[6] Fratzl P, Misof K, Zizak I, Rapp G, Amenitsch H, Bernstoffer S. Fibrillar structure and mechanical properties of collagen. *Journal of Structural Biology*, 1998, 122, 119–122.

[7] Chintapalli R K, Mirhalaf M, Dastjerdi A K, Barthelat F. Fabrication, testing and modeling of a new flexible armor inspired from natural fish scales and osteoderms. *Bioinspiration & Biomimetics*, 2014, 9, 036005.

[8] Berth J E, Ho V A, Liu H B. Morphological computation in haptic sensation and interaction: From nature to robotics. *Advanced Robotics*, 2018, 32, 340–362.

[9] Kanhere E, Wang N, Kottapalli A G P, Asadnia M, Subramaniam V, Miao J, Triantafyllou M. Crocodile-inspired dome-shaped pressure receptors for passive hydrodynamic sensing. *Bioinspiration & Biomimetics*, 2016, 11, 056007.

[10] Elkan E, Cooper J E. Skin biology of reptiles and amphibians. *Proceedings of the Royal Society of Edinburgh, Section B: Biological Sciences*, 1980, 79, 115–126.

[11] Dubansky B H, Close M. A review of alligator and snake skin morphology and histotechnical preparations. *Journal of Histotechnology*, 2019, 42, 31–51.

[12] Lin C P, Douglas W H, Erlandsen S L. Scanning electron microscopy of type I collagen at the dentin-enamel junction of human teeth. *Journal of Histochemistry & Cytochemistry*, 1993, 41, 381–388.

[13] Erickson G M, Brochu C A. How the ‘terror crocodile’ grew so big. *Nature*, 1999, 398, 205–206.

[14] Sennikov A G. The first ctenosauriscid (Reptilia: Archosauroomorpha) from the lower triassic of eastern europe. *Paleontological Journal*, 2012, 46, 499–511.

[15] Drymala S M, Zanno L E. Osteology of carnufex carolinensis (archosauira: pseudosuchia) from the pekin formation of north carolina and its implications for early crocodylomorph evolution. *PLOS ONE*, 2016, 11, e0157528.

[16] Webb G J W, Manolis S C, Brien M L. *Crocodiles: Status Survey and Conservation Action Plan*, 3rd ed, Crocodile Specialist Group: Darwin, Darwin, Australia, 2010.

[17] Alibardi L. Keratinization in crocodilian scales and avian epidermis: Evolutionary implications for the region of avian apertic epidermis. *Belgian Journal of Zoology*, 2005, 135, 9–20.

[18] Alibardi L. Histology, ultrastructure, and pigmentation in the horny scales of growing crocodilians. *Acta Zoologica*, 2011, 92, 187–200.

[19] Holthaus K B, Strasser B, Lachner J, Sukseere S, Sipos W, Weissenbacher A, Tschachler E, Alibardi L, Eckhart L. Comparative analysis of epidermal differentiation genes of crocodilians suggests new models for the evolutionary origin of avian feather proteins. *Genome Biology and Evolution*, 2018, 10, 694–704.

[20] Pressinotti L N, Borges R M, Alves De Lima A P, Aleixo V M, Iunes R S, Borges J C S, Cogliati B, Cunha Da Silva J R M. Low temperatures reduce skin healing in the Jacare do Pantanal (*Caiman yacare*, Daudin 1802). *Biology Open*, 2013, 2, 1171–1178.

[21] Dalla Valle L, Nardi A, Gelmi C, Toni M, Emera D, Alibardi L. β-keratins of the crocodilian epidermis: Composition, structure, and phylogenetic relationships. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 2009, 312B, 42–57.

[22] Alibardi L, Toni M. Cytochemical, biochemical and molecular aspects of the process of keratinization in the epidermis of reptilian scales. *Progress in Histochemistry and Cytochemistry*, 2006, 40, 73–134.

[23] Alibardi L, Thompson M B. Keratinization and ultrastructure of the epidermis of late embryonic stages in the alligator (*Alligator mississippiensis*). *Journal of Anatomy*, 2002, 201, 71–84.

[24] Alibardi L. Adaptation to the land: The skin of reptiles in comparison to that of amphibians and endotherm amniotes. *Journal of Experimental Zoology*, 2003, 298B, 12–41.

[25] Baden H P, Maderson P F. Morphological and biophysical identification of fibrous proteins in the amniote epidermis. *Journal of Experimental Zoology*, 1970, 174, 225–232.

[26] Alibardi L. Sauropsids cornification is based on cornuous beta-proteins, a special type of keratin-associated corneous proteins of the epidermis. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 2016, 326, 338–351.

[27] Holthaus K B, Eckhart L, Dalla Valle L, Alibardi L. Review: Evolution and diversification of cornuous beta-proteins, the characteristic epidermal proteins of reptiles and birds. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 2019, 330, 438–453.

[28] Berthod F, Germain L, Li H, Xu W, Damour O, Auger F A. Collagen fibril network and elastic system remodeling in a reconstructed skin transplanted on nude mice. *Matrix Biology*, 2001, 20, 463–473.

[29] Matoltsy A G, Huszar T. Keratinization of the reptilian epidermis: An ultrastructural study of the turtle skin. *Journal of Ultrastructure Research*, 1972, 38, 87–101.
Collagen: Applications of a natural polymer in regenerative medicine and tissue engineering, in: Cells and Biomaterials, InTech, London, UK, 2011, 13, 287–300.

Enax J, Fabritius H O, Rack A, Prymak O, Raabe D, Epple M. Characterization of crocodile teeth: Correlation of composition, microstructure, and hardness. Journal of Structural Biology, 2013, 184, 155–163.

Weiner S, Wagner H D. The material bone: Structure-mechanical function relations. Annual Review of Materials Science, 1998, 28, 271–298.

Gupta H S, Stachewicz U, Wagermaier W, Roschger P, Wagner H D, Fratzl P. Mechanical modulation at the lamellar level in osteonal bone. Journal of Materials Research, 2006, 21, 1913–1921.

Dutta P, Vyas V, Dhara S, Chowdhury, A R, Barui, A. Anisotropy properties of tissues: A basis for fabrication of biomimetic anisotropic scaffolds for tissue engineering. Journal of Bionic Engineering, 2019, 16, 842–868.

De Leeuw N H, Rabone J A L. Molecular dynamics simulations of the interaction of citric acid with the hydroxyapatite (0001) and (011′0) surfaces in an aqueous environment. CrystEngComm, 2007, 9, 1178–1186.

Boskey A L. Mineralization of bones and teeth. Elements, 2007, 3, 385–391.

Erickson G M, Gignac P M, Steppan S J, Lappin A K, Vliet K A, Brueggen J D, Inouye B D, Kledger D, Webb G J W. Insights into the ecology and evolutionary success of crocodilians revealed through bite-force and tooth-pressure experimentation. PLOS ONE, 2012, 7, e31781.

He G, George A. Dentin matrix protein 1 immobilized on type I collagen fibrils facilitates apatite deposition in vitro. Journal of Biological Chemistry, 2004, 279, 11649–11656.

Lodish H F, Berk A, Zipursky S L, Matsudaira P, Baltimore D, Darnell J. Molecular Cell Biology. W. H. Freeman, New York, USA, 2000, 1084.

Parry D A D, Barnes G R G, Craig A S. A comparison of the size distribution of collagen fibrils in connective tissues as a function of age and a possible relation between fibril size distribution and mechanical properties. Proceedings of the Royal Society of London, Series B, Biological Sciences, 1978, 203, 305–321.

Franchi M, Raspanti M, Dell’Orbo C, Quaranta M, De Pasquale V, Ottani V, Ruggeri A. Different crimp patterns in collagen fibrils relate to the subfibrillar arrangement. Connective Tissue Research, 2008, 49, 85–91.

Franchi M, Fini M, Quaranta M, De Pasquale V, Raspanti M, Giavaresi G, Ottani V, Ruggeri A. Crimp morphology in relaxed and stretched rat Achilles tendon. Journal of Anatomy, 2007, 210, 1–7.

Raspanti M, Manelli A, Franchi M, Ruggeri A. The 3D structure of crimps in the rat Achilles tendon. Matrix Biology, 2005, 24, 503–507.