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Characterization of the nanocomposite laminate structure occurring in fish scales from *Arapaima Gigas*

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Abstract

In the present paper, the nanocomposite laminate structure of scales from the Amazonian fish *Arapaima Gigas* is investigated. The structure and composition of the scales were assessed by means of X-ray diffraction (XRD) and Fourier Transform Infrared spectroscopy (FTIR). The theory of Fickian diffusion is used and discussed in order to rationalize the water absorption and desorption behavior of the scales. Morphology studies and fracture analysis of the native scales were carried out using Transmission Electron Microscopy (TEM), Light Optical Microscopy (LOM) and Scanning Electron Microscopy (SEM). A fibrous layer of collagen and a plywood-like structure were observed. In order to study the mineral phase, the native scales were burned at 600 °C until all the organic components were degraded. The remaining ashes were then observed under the microscope and weighed to determine ratio of organic and inorganic components. The mechanical behavior of dry and wet scales was assessed by tensile tests and the effect of water in mechanical properties is also discussed.

Keywords: Materials; Collagen; Fish scales; *Arapaima Gigas*

1. Introduction

Scales are the skeletal elements that cover and protect the skin of fishes. Basically, they are plywood-like structures of closely packed collagen fiber layers reinforced with a mineral phase of calcium-deficient hydroxyapatite [1].

Fish scales have characteristics that are also found in other structures such as bones, teeth and mineralized tendons. All these materials are mainly formed by an organic component (i.e. collagen), a mineral component (i.e. hydroxyapatite) and water. The proportions of these three components as well as their spatial organization account for the differences among the bone-like materials.

As in bones, type I collagen fibrils are the main organic component in fish scales. Fibrils, which are formed by triple helical molecules of polypeptide chains, are found packed as part of a larger structure known as fiber [2]. Scales are composed of several layers of parallel fiber bundles, with varying fiber orientations in the different layers.

Calcium-deficient hydroxyapatite forms plate-like nanocrystals with thicknesses in the range 1.5–4 nm, according to small angle X-ray measurements [3,4]. These crystals are organized within the collagen fibrils and can be considered as a nano-reinforcement in a collagen matrix [5].

Among the different types of hard tissues that can be found in nature, fish scales have probably received less attention than any of them, inspite of their remarkable characteristics. The only study available to the authors of fish scales using a materials science approach was carried out by Ikoma et al. [1]. They studied fish scales from *Pagrus Major* and found that the mineral phase was calcium-deficient hydroxyapatite containing a small amount of sodium, magnesium and carbonate ions. The tensile strength of the scales was around 90 MPa and the mechanical failure occurred by sliding of the

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lamellae as well as pulling out and fractures of the collagen fibers.

In this study we used fish scales from *Arapaima Gigas*. *A. Gigas* is a fish from the Amazonian region with a rather prehistorical aspect. It is known for its large tough scales which are used in handcrafts and souvenirs. The aim of this paper is to study the underlying structure of these scales using a materials science approach, particularly trying to understand the scales mechanical behavior in the wet state. Different morphological and structural characterization techniques, including optical and electronic microscopy, X-ray diffraction and IR analysis were used to that end. The mechanical behavior of the scales in the dry and wet state is studied using tensile tests. Water absorption and desorption tests are used to assess their transport properties.

2. Experimental

2.1. Materials

Scales from the Amazonian fish *A. Gigas* (body weight 100–150 kg) were removed, washed and stored in standard conditions (20 °C and 80% of relative humidity). They were around 70–75 mm in length and 1 mm thick.

2.2. Characterization techniques

2.2.1. Fourier transform infra red spectroscopy (FTIR)

In order to prepare fish scales for FTIR analysis, they were washed with distilled water, then ground and dried in a dessicator overnight. IR analysis of the samples in KBr was performed in a Perkin-Elmer 1600 Series, FTIR.

2.2.2. X-ray diffraction (XRD)

X-ray diffraction spectra were recorded using a Seifert XRD 3003 TT diffractometer. Ni-filtered CuKα radiation (wavelength of 1.542 Å) was produced at 40 kV and 40 mA.

2.2.3. Water absorption and desorption

For water absorption tests, strips (15 × 5 mm) were cut from the scales and were immersed in water at 20 °C for 4 days. During this process the samples were removed from the bath every hour, blotted dry with a cloth, weighed and immediately placed back in the bath. The same samples were used to perform the desorption tests.

2.2.4. Tensile tests

Tensile tests were performed in a Zwick/Roell tensile testing machine. Rectangular samples (50 mm × 5 mm) were cut from dry scales avoiding the dark region that serves as an attachment to the fish epidermis. The scales were tested in the dry state and after being soaked in distilled water for 4 days. The thickness of the scales ranged from 0.6 to 1.5 mm. The thickness deviation in a single fish scale is around 35%. Therefore, the average thickness of the sample was used to calculate the strength. A crosshead speed of 10 mm/min was used. Tensile strength and Young's modulus values were recorded.

2.2.5. Morphological characterization

Microscopical studies included the use of Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), Light Optical Microscopy (LOM) and stereomicroscopy.

The samples were examined in a Leitz light optical microscopy and a Brunel BMZ trinocular stereomicroscope. Fracture surfaces from tensile testing specimens were studied using an RJ Lee SEM, using voltages in the range 10–20 kV. Specimens were mounted onto stubs and then gold coated as described in a previous report from this laboratory [6].

TEM microscopy was carried out in a Jeol JEM-1010 TEM. The samples were washed with distilled water and immersed 2 h in 0.1 M phosphate buffer and 2% OsO_4 solution at room temperature for fixation. The fixed samples were dehydrated in a series of 50, 60, 79 and 100% of ethanol concentrations, and then embedded in a polar monomer polyhydroxylated acromatic acrylic resin (LR white resin) and cured at 60 °C. A Leica UC6

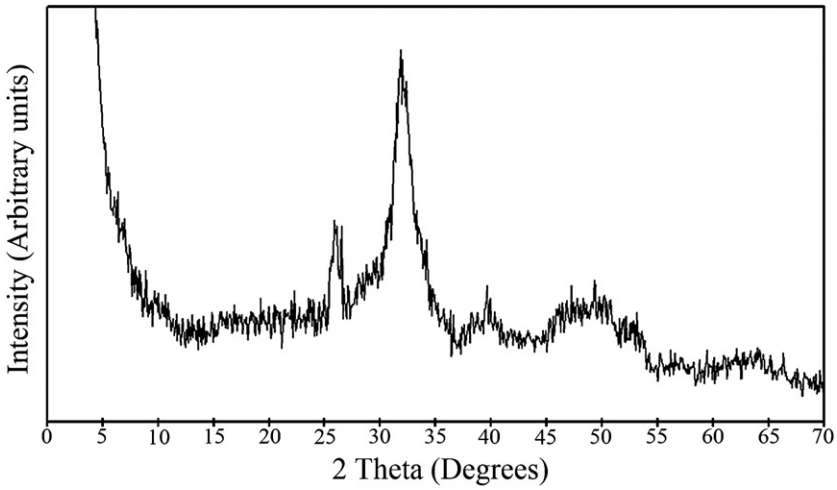

Fig. 1. XRD spectra of *A. Gigas* scale.

ultra-microtome with diamond knife was used to cut ultra-thin sections of the scales.

3. Results and discussion

3.1. X-ray diffraction

The X-ray diffractogram of *A. Gigas* scales is shown in Fig. 1. The broad peaks corresponding to the apatite structure reveled that scales show relatively low crystallinity levels, due to the fact that hydroxyapatite (HA) crystals could possibly be as small as the HA crystals present in other mineralized collagen structures which are 1.5–4 nm in thickness [3,4].

The peaks are found in $2\theta = 25.8°$, 31.8°, 39.6°, 47.2°, 49.3° and 53.1° with corresponding d spacings of 0.345, 0.281, 0.227, 0.192, 0.184 and 0.172 nm. Previous studies that were carried out on collagen from calf skin associate the diffraction pattern of collagen with a characteristic peak at $2\theta = 23$ °[7,8]. However, the obtained results are comparable with previous data for fish scales [1] and other biological apatite containing structures [7–10].

3.2. FTIR-spectra

Ikoma et al. [1] suggest that is clear from IR that fish scales have both organic and inorganic components. The IR spectrum (Fig. 2) shows characteristic peaks corresponding to the organic components of the fish scales (1662, 1560 and 1242 cm^{-1}), namely amide I, II and III bands of collagen [1,7–10]. The absorption peak at 1662 cm^{-1} is associated with the C=O stretching vibrations of the amide I protein, the amide II absorption (1560 cm^{-1}) is due to the N–H bending vibration and the C–N stretching vibrations; the amide III peak (1242 cm^{-1}) have components of C–N stretching and N–H in plane bending, as well as absorptions arising from CH_2 groups from the glycine back bone and proline side-chains [11,12].

The inorganic content of scales is mainly accounted to the hydroxyapatite. Its absorption bands shown in Fig. 2 correspond to phosphate groups (560–603 and 1032 cm^{-1}) and carbonate anions (873, 1430 and 1449 cm^{-1}).

3.3. Water sorption and desorption

The absorption of water by a polymeric material is usually carried out according to a transient diffusion mechanism. When a flat material is exposed to an environment rich in diffusants, penetration occurs towards the midplane of the flat material. The concentration of diffusants varies with time, thickness and the diffusion coefficient (D) of the material [13].

Diffusion is a thermally activated process; hence D depends on the process temperature [14]. The case studied here is that of sorption and desorption by a flat plate or membrane. If the region $-b<\times<b$ is initially at a uniform concentration C_o, and the surfaces are kept at a constant concentration C_1 the solution becomes as shown in Eq. (1):

$$\frac{C - C_o}{C_1 - C_0} = 1 - \frac{4}{\pi}\sum_0^{\infty} \frac{(-1)^n}{2n+1} \exp\left\{-D(2n+1)^2\pi^2 t/4b^2\right\} \times \cos\frac{(2n+1)\pi x}{2b} \quad (1)$$

If M_t denotes the total amount of diffusing substance that has entered the sheet at time t, and M_{eq} is the mass uptake at equilibrium (considering an infinite diffusion time), then:

$$\frac{M_t}{M_{eq}} = 1 - \sum_0^{\infty} \frac{8}{(2n+1)^2\pi^2} \exp\left\{-D(2n+1)^2\pi^2 t/4t^2\right\} \quad (2)$$

It is also possible to deduce an average diffusion coefficient from the initial gradient of the sorption curve when plotted

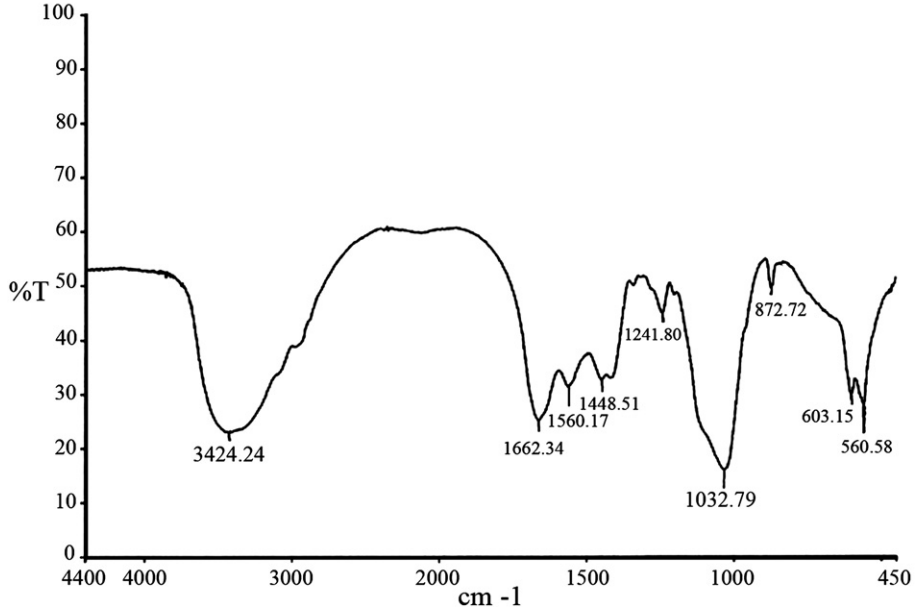

Fig. 2. AFTIR spectra of *A. Gigas* scale.

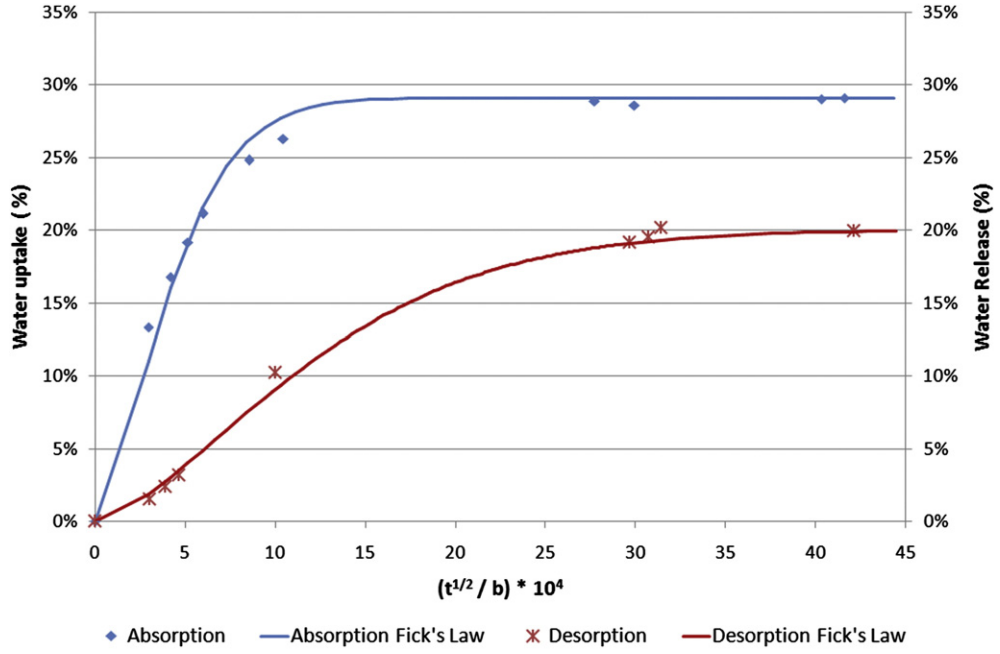

Fig. 3. Absorption and desorption curves of *A. Gigas* scales. Solid lines represent the theoretical Fick's Law calculation.

against the square root of time. Thus, in the early stages, for a constant diffusion coefficient D and a sheet of thickness l, we have:

$$\frac{M_t}{M_{eq}} = \frac{4}{\pi^{1/2}}\left(\frac{Dt}{b^2}\right)^{1/2} \quad (3)$$

If the sorption curve when plotted against $\left(\frac{t}{b^2}\right)^{1/2}$ is approximately linear as far as $\frac{M_t}{M_{eq}} = \frac{1}{2}$, then D can be calculated from Eq. (3)) [15].

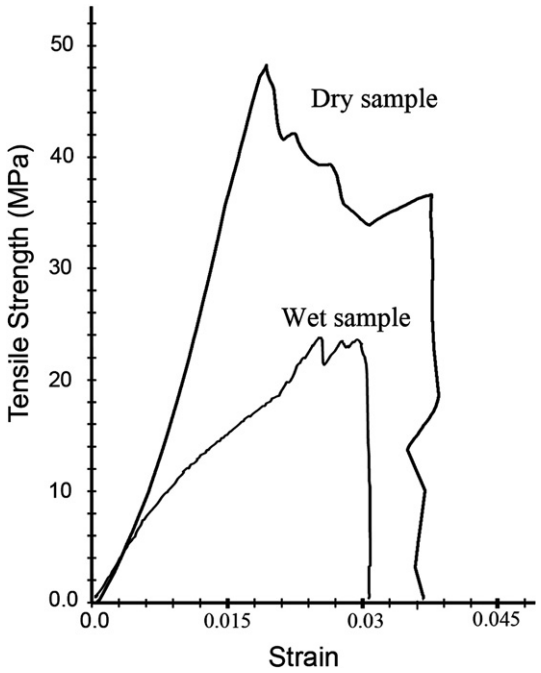

Fig. 4. Stress–strain curves of *A. Gigas* tensile test in the dry and wet state.

The water sorption and desorption behaviour of the scales can be observed in Fig. 3. Both, the water uptake percentage and the water release percentage are potted against $\left(\frac{t}{b^2}\right)^{1/2}$. The initial linear regions confirm that absorption and desorption of water are Fickian transport processes [16,17].

It is observed that absorption is faster than desorption for fish scales. Furthermore, after 4 days of being removed from the water, the samples did not release as much water as they had absorbed originally. This could be due to some type of interaction between water molecules and collagen fibrils, presumably in the form of hydrogen bonds [18].

The diffusion coefficient of water in the scales was 2.95×10^{-7} cm^2/s. This value is in agreement with reported data for cortical bone (2.87×10^{-7}–3.35×10^{-7} cm^2/s) [18] which have the same building blocks (mineralized collagen) as fish scales.

3.4. Tensile tests

Tensile tests confirmed that these scales behave as a laminated composite material. In Fig. 4 two representative stress–strain curves are shown for dry and wet scales. Both curves present several peaks related with the breakage of successive lamellae. The sliding of collagen layers and the pulling out of their fibers are confirmed by SEM micrograph.

Table 1
Maximum tensile strength, maximum strain and Young's modulus from tensile tests of *A. Gigas* scales

	Maximum tensile strength (MPa)	Maximum strain	Young's modulus (GPa)
Dry scale	53.86±8.36	0.0315±0.006	1.38±0.21
Wet scale	22.26±3.94	0.0258±0.005	0.83±0.12

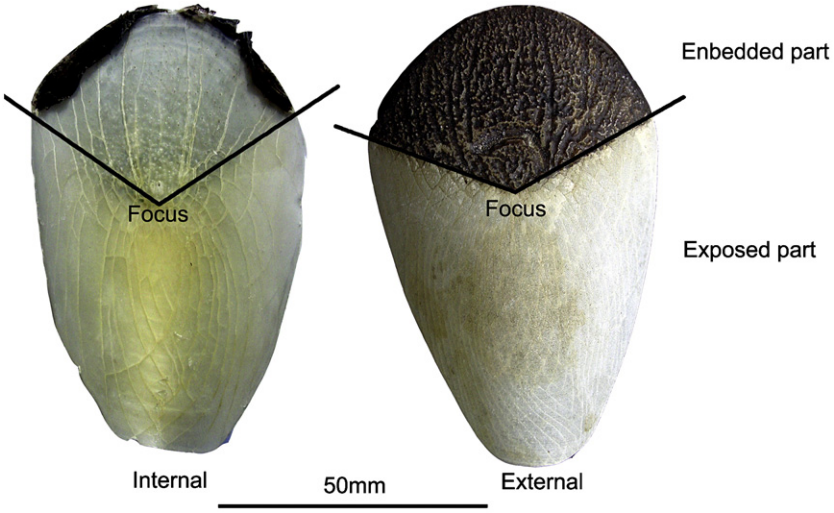

Fig. 5. Photograph of the whole mount of an *A. Gigas* scale showing the internal and external layers.

Table 1 shows the results of the tensile tests of dry and wet scales. The maximum tensile strength and Young's modulus of the *A. Gigas* scales are 53.86 MPa and 1.38 GPa, respectively. These values are lower than those reported by Ikoma et al. for *P. Major* scales (93 MPa and 2.26 GPa respectively) [1].

The maximum tensile strength and the Young's modulus decreased after soaking the scales for 4 days in distilled water. This confirms the results of the absorption tests, where some interaction between water and collagen was argued. The fact that soaked samples have a water content of around 30% of its dry weight, reduces the volume fraction of hydroxyapatite crystals which is accompanied with a reduction in mechanical properties.

Although the ultimate tensile strength of wet scales decreases in around 60% with regard to their dry value, the maximum strain value of scales in the wet state is only 18% lower than that in the dry state. Thus, the same level of strain can be obtained with a much lower stress allowing the scales to adapt to the shape of the body during swimming.

3.5. Morphology

Due to their large size, some morphological characteristics of the scales can be observed at very low magnifications (Fig. 5). The outer part, known as external layer, is usually in contact with water and has a rough texture, while the inner part (i.e. basal plate) is smooth. The dark region in Fig. 5 serves as an attachment to the epidermis of the fish and is referred in the literature as limiting layer [19]. The focus indicates that the scale grows towards the periphery forming the annulus marks (see arrows in Fig. 5).

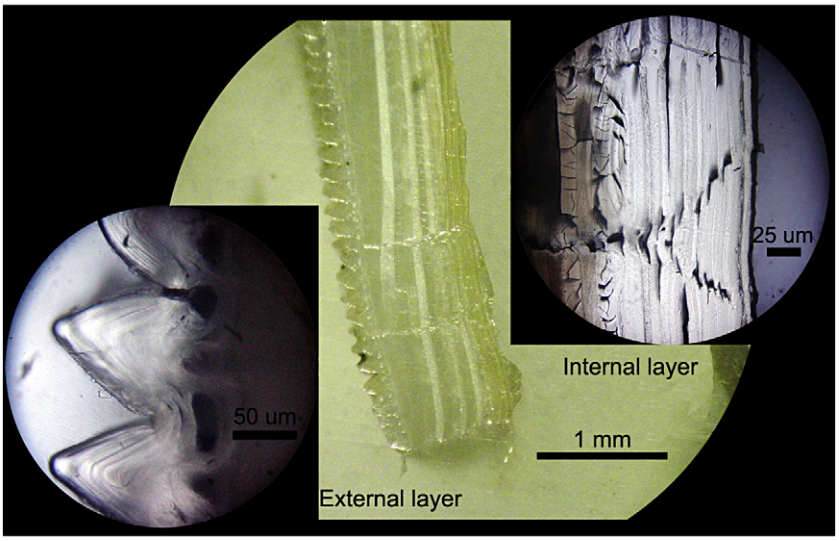

Fig. 6. Cross-sections of an *A. Gigas* scale showing the internal and external layers.

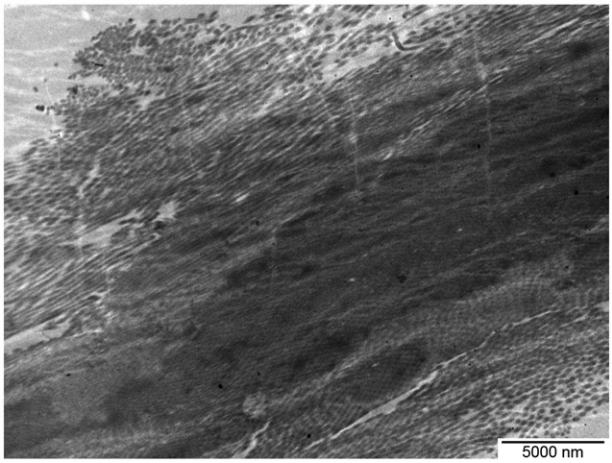

Fig. 7. TEM micrograph of a cross-section of an *A. Gigas* scale.

Cross-section micrographs of the scales are presented in Fig. 6. The basal plate is about 900 μm wide and is clearly formed by partially mineralized tissue organized in several layers of collagen fibrils in a plywood pattern. By contrast, the external well-mineralized layer is about 100 μm wide and does not show any organization at this magnification. The roughness of this layer can be explained by the presence of protrusions.

A thin cross-section of a TEM micrograph is depicted in Fig. 7. TEM micrographs confirm that the plywood pattern of collagen layers is formed by collagen fibers co-aligned within each individual layer. These layers alternately rotate at angles of around 90° between each layer as it was also found for *P reticulata* and *P major* [1].

Fig. 8 shows a SEM micrograph of the internal layer of a scale. Voids are depicted all over the surface. The annulus of growth is shown in the middle where the fibrous nature of the scale is appreciated. The building block of this fibrous matrix is collagen. A single mineralized collagen bundle is shown in Fig. 9.

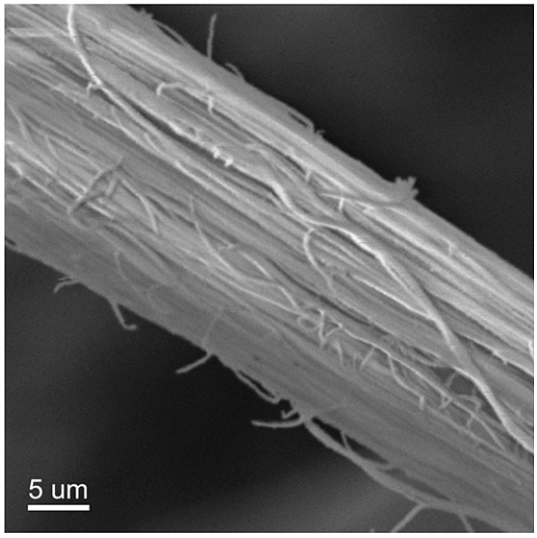

Fig. 9. SEM micrograph of a single collagen bundle of *A. Gigas* scales.

The fracture surface depicted in Fig. 10 confirms the organization of these structures. The layers forming the laminated composites can be observed as well as some collagen fibers torn apart during the tensile test.

In order to assess the mineral phase content of scales, all organic components were extracted by burning them in an oven at 600 °C for 35 min. Ashes were collected, weighted and observed under the microscope. Weight ratios of water, organic and inorganic components were 16%, 45% and 39%, respectively, which is in agreement with thermogravimetric analysis from a previous study [1]. As the organic and inorganic phase are mainly composed by collagen and hydroxyapatite, receptively, the density of collagen (1.33×10^3 kg m^3) and hydroxyapatite (3.17×10^3 kg m^3) were used to estimate the volume fraction of the components [20]. The average hydroxyapatite volume fraction is 21.5% which is much lower than those found in lamellar bones (45%–50%) [21].

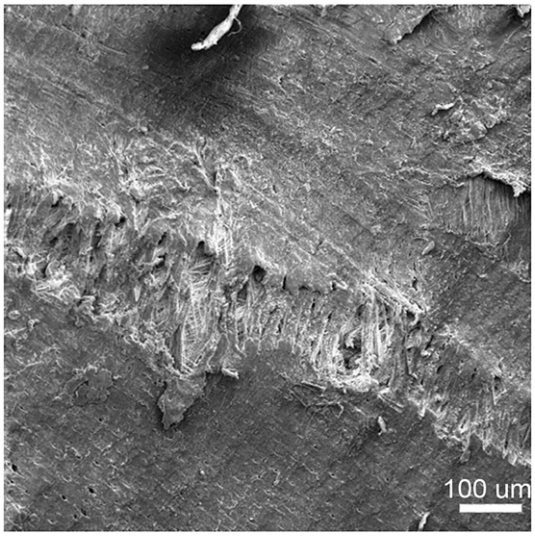

Fig. 8. SEM micrograph of the internal layer of an *A. Gigas* scale.

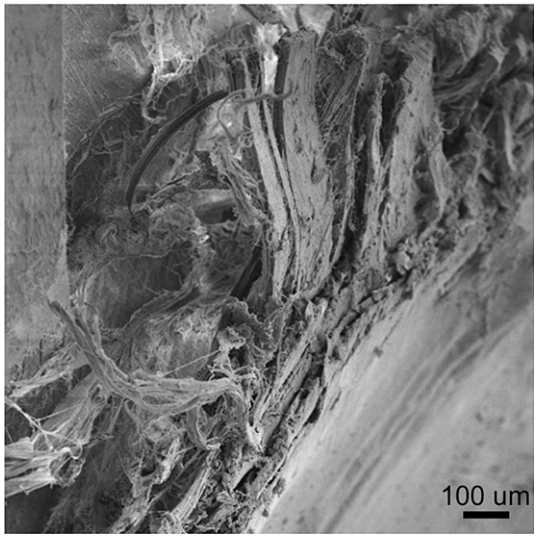

Fig. 10. SEM micrograph of a fracture surface of *A. Gigas* scales.

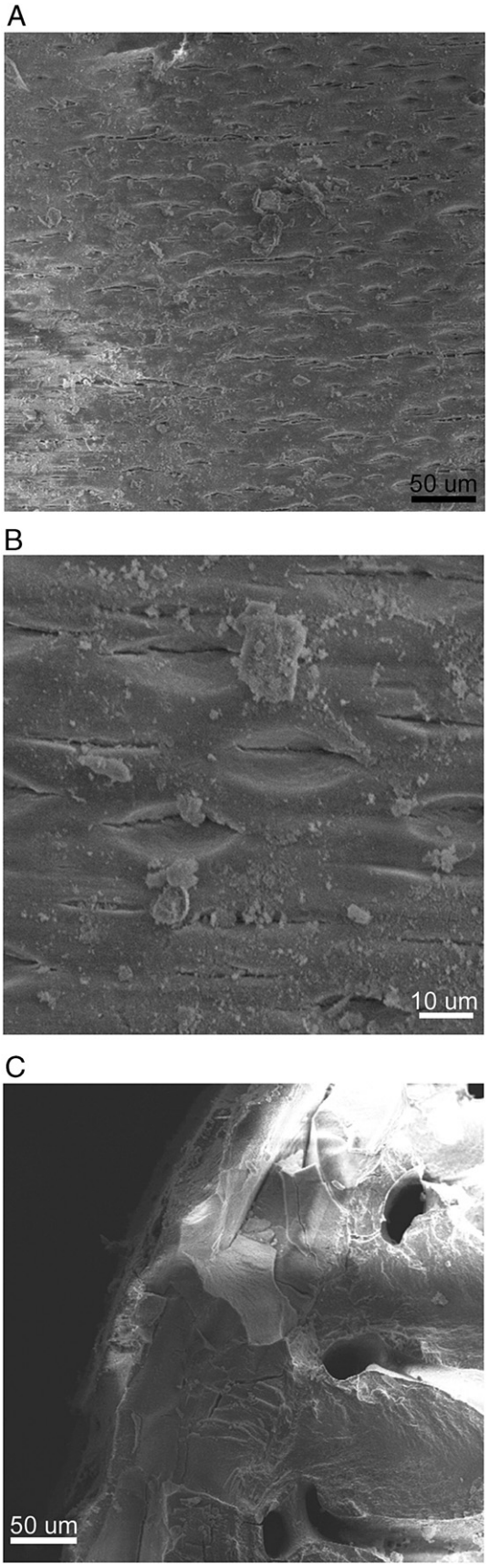

Fig. 11. A. SEM micrographs of the mineral structure of *A. Gigas* scales. B. SEM micrographs of the mineral structure of *A. Gigas* scales. C. SEM micrographs of the mineral structure of *A. Gigas* scales.

Fig. 11A, B and C shows SEM micrographs of the scale mineral phase. The voids present in Fig. 11A and B have a regular form and are aligned in a pattern with similar distances between each void. Fig. 11C shows voids and grooves that seem to have been left after the thermal degradation of the organic material.

4. Conclusions

Fish scales can be thought as laminated composites structures formed by mineralized collagen fibers in a plywood pattern of layers. These fibers are co-aligned within each individual layer that alternately rotates at angles of around 90°. The scales are reinforced with nano-crystals of hydroxyapatite. X-ray diffraction and Infrared spectroscopy of the *A. Gigas* scales confirmed the presence of collagen and hydroxyapatite. Absorption and desorption of water proved to be Fickian diffusion phenomena, although the diffusion coefficients calculated were different in both cases.

Tensile tests and morphological analysis showed a typical laminated composite behavior with successive rupture of the layers. The maximal tensile strength and Young's modulus reached relatively low values with regard to previous investigations. As expected, wet scales had lower mechanical properties (i.e. maximal strength and Young's modulus) than dry scales. However, the maximum strain values reached by wet and dry scales were close, showing that almost the same level of strain can be obtained with lower stress.

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