Materials Science and Engineering C 43 (2014) 145–152

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Hierarchical structure and cytocompatibility of fish scales from *Carassius auratus*

Zhou Fang [a], Yukun Wang [b], Qingling Feng [a,c,*], Arne Kienzle [d], Werner E.G. Müller [d]

[a] State Key Laboratory of New Ceramics and Fine Processing, School of Materials Science and Engineering, Tsinghua University, Beijing 100084, China
[b] State Key Laboratory of Geological Processes and Mineral Resources, China University of Geosciences, Beijing 100084, China
[c] Key Laboratory of Advanced Materials of Ministry of Education of China, School of Materials Science and Engineering, Tsinghua University, Beijing 100084, China
[d] Institut für Physiologische Chemie, Abteilung Angewandte Molekularbiologie, Johannes Gutenberg-Universität, Duesbergweg 6, Mainz 55099, Germany

ARTICLE INFO

Article history:
Received 22 January 2014
Received in revised form 9 June 2014
Accepted 3 July 2014
Available online 10 July 2014

Keywords:
Hierarchical structure
Fish scale
Cytocompatibility
Carassius auratus

ABSTRACT

To study the structure and the cytocompatibility of fish scales from *Carassius auratus*, scanning electron microscopy (SEM) was used to observe the morphology of fish scales treated with different processing methods. Based on varying morphologies and components, the fish scales can be divided into three regions on the surface and three layers in vertical. The functions of these three individual layers were analyzed. SEM results show that the primary inorganic components are spherical or cubic hydroxyapatite (HA) nanoparticles. The fish scales have an ~60° overlapped plywood structure of lamellas in the fibrillary plate. The plywood structure consists of co-aligned type I collagen fibers, which are parallel to the HA lamellas. X-ray diffraction (XRD), thermogravimetric analysis/differential scanning calorimetry (TGA/DSC) and Fourier transform infrared (FTIR) analysis indicate that the main components are HA and type I collagen fibers. MC3T3-E1 cell culture results show a high cytocompatibility and the ability to guide cell proliferation and migration along the scale ridge channels of the fish scales. This plywood structure provides inspiration for a structure-enhanced composite material.

1. Introduction

Fish scales are mineralized plates from the dermal layer covered in the epidermis. The teleost fish scale is mainly composed of calcium-deficient hydroxyapatite and type I collagen, forming a well-organized micropatterned composite structure. The surface of fish scale is distinguished into three regions: the anterior, lateral and posterior regions [1]. In the vertical direction, the fish scale usually features two different layers: the external osseous layer composed of hydroxyapatite crystals with randomly orientated fibers, and the internal fibrillary plate, an orthogonal or double-twisted plywood structure of co-aligned collagen fibers with lamellas [2–7]. The mineralization of fish scales occurs continuously throughout the whole life span of fish. The collagen fibers are synthesized by the hyposquamal scleroblasts and organized in a plywood pattern [1]. Needle-like or flaky crystals of hydroxyapatite are observed as randomly orientated in the external layer, but preferentially parallel to the collagen fibers with the crystallographic c-axis in the internal fibrillary plate [8,9].

This highly-ordered natural multilayer structure features improved mechanical properties [6,10–14] and is considered as an inspiration in applications such as bio-inspired human body armor [14]. Recent research has focused on the structure of fish scales to analyze their mechanical properties. Meunier suggested that the collagen layers are stiffened by mineralization [15]. Ikoma confirmed that tensile strength is substantially lowered after demineralization of *Pagrus major* fish scale [6]. Garrano performed microscopic Digital Image Correlation (DIC) on the mechanical behavior of scales from *Cyprinus carpio* and found the mechanical properties to vary greatly depending on different scale locations and the moisture content [11]. Unfortunately, no systematical analysis of hierarchical structure of fish scales are reported for *C. carpio*, and thus no model have been created to analyze the relationship between its structure and mechanical properties.

On the other hand, the main components of fish scale, hydroxyapatite and type I collagen, are similar to the composition of human bone and dentin. Some previous works studied the biological properties of hydroxyapatite [16], others focused on fish scale collagens [17–21]. Typically, both show good biological properties, although some organic extracts (mainly type I collagen) from different species of fishes may cause fish allergies [22]. However, fish scales still have a number of potential uses, such as providing HA and biological polymers as a bio-resource. Collagens from the fish scales of *Rohu* and *Catla* were isolated and fabricated into collagen scaffolds by Falguni et al., in which the fish scale collagen was observed to have considerable biocompatibility and minimal inflammatory response [23]. Furthermore, their natural composite structure might be used as an inspiration for new materials showing good mechanical properties.

* Corresponding author at: School of Materials Science and Engineering, Tsinghua University, Beijing 100084, China.
E-mail address: biomater@mail.tsinghua.edu.cn (Q. Feng).

http://dx.doi.org/10.1016/j.msec.2014.07.015
0928-4931/

Carassius auratus is a popular edible freshwater fish in China, but has not been the subject of as many studies as other edible fishes such as salmon, cod, flounder, and sea bream [24,25], and the scales are commonly discarded as waste. In this study, the hierarchical structure of fish scales from *C. auratus* is analyzed using scanning electron microscopy (SEM). The components are analyzed using X-ray diffraction (XRD), thermogravimetric analysis/differential scanning calorimetry (TGA/DSC), Fourier transform infrared (FTIR) and energy dispersive spectroscopy (EDS). Furthermore, the cytocompatibility of fish scales is tested by MC3T3-E1 cell culture using CCK-8 assay and DAPI staining to analyze if they can be used in tissue engineering. Scales of *C. auratus* differ from the common orthogonal or double-twisted plywood structure. This new plywood structure provides a new model for a structure-enhanced composite material. A corresponding model can be built by analyzing the hierarchical structure of scales. As far as we know, it is the first time to observe three individual layers in a fish scale. This might provide information to analyze and understand the mechanism of scale formation. Moreover, a proper comprehension of the structure will reveal its corresponding mechanical properties.

2. Materials and methods

2.1. Preparation of fish scale samples

The fish scales were extracted from *C. auratus* purchased in Beijing (body length ~ 20 cm) and processed in four distinct ways: fresh fish scales ("f-fish"), alkali fish scales ("a-fish"), sintered fish scales ("s-fish"), and decalcified fish scales ("de-fish"). The alkali fish scales were immersed in a 10 wt.% NaOH solution at room temperature for 48 h, then washed with deionized water and kept dry at room temperature. Also, fish scales were heated to 700 °C, soaked for 1 h in a furnace (SX-G02163, Tianjin Central Furnace Co., LTD) with a heating rate of 5 °C/min and then cooled down to prepare the s-fish scales. The cellular components of some fish scales were removed based on a previously in literature described method [3]. The fish scales were then immersed in 5vt% nitric acid at room temperature for 10 h. To decalcify the scales, they were immersed in a 10 wt.% EDTA and 2vt% nitric acid solution for 3 days, renewing the solution daily at a temperature of 4 °C. After decalcification, the fish scales were washed with 70% ethanol and deionized water. All the fish scales, except for the s-fish scales, were then kept at −20 °C for 24 h and transferred into a freeze-dryer at −60 °C and 60 bar for 48 h to obtain the final specimens.

2.2. Fish scale characterization

The morphologies of the differently treated fish scales were observed by field-emission scanning electron microscopy (FESEM; LEO-1530, 20.0 kV, SEI) after gold or carbon sputter-coating. The surface elements of the gold-coated fish scales were analyzed with an energy dispersive spectrometer (EDS; resolution: 133 eV, Be4–U92). The f-fish scales were dried under pressure to keep them flat at room temperature while the s-fish scales were grinded into powder in an agate mortar. Both scales were then characterized by X-ray diffraction (D/max 2500 Rigaku X-RAY DIFFRACTOMETER, CuKα, 5°/min, 0.02 per step). The inorganic and organic contents of the f-fish scales and de-fish scales were determined by thermogravimetric analysis (TGA Q5000 IR/DSC Q2000) using a heating rate of 10 °C/min in static air. The chemical components of the f-fish scales and de-fish scales were studied with diffuse-reflectance FTIR spectroscopy (VERTEX70V).

2.3. Cell culture

The de-fish scales were placed in a 24-well plate (Nunc, Denmark) and exposed to ^{60}Co irradiation at 3.5 kGy before cell culturing. MC3T3-E1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Invitrogen, USA), 100 μg/mL streptomycin, and 100 U/mL penicillin (growth medium, GM) in an incubator supplied with 5% CO_2 and a water-saturated atmosphere at 37 °C. After seeding the MC3T3-E1 cells (10^4 cells/well) they were cultivated for 24, 72 and 120 h. The wells without fish scales were considered to be the blank test.

A cell count kit-8 (CCK-8, Dojindo, Japan) was used to quantitatively evaluate the cell proliferation. At certain times after seeding, CCK-8 with a 10% vol. of the medium was added into the wells and incubated for 4 h at 37 °C. CCK-8 was transformed to orange-color formazan by the dehydrogenase in cells. The amount of the formazan, which was measured by the absorbance (OD) of the solution by a microplate reader at 450 nm, was directly proportional to the number of living cells.

DAPI (4′,6-diamidino-2-phenylindole) was applied to stain the karyon after different times of cultivation. 300 μL 4% paraformaldehyde was added to each well and fixed for 30 min. After being washed with PBS, DAPI was added and incubated in darkness for 30 min. The DAPI-stained cells were then observed with a fluorescence microscope.

3. Results and discussion

3.1. Intact characterization of fish scale

Generally, the surface of a fish scale can be divided into three regions: anterior, posterior and lateral [4], as shown in Fig. 1(a). The relative orientation of fish scales and the fish body are shown in Fig. 1(b). The anterior region is covered under the posterior region of the front scale. The whole scale is divided into several interradial areas by radial grooves. In contrast, radial grooves only appear in the anterior region of scales of *Channa argus* [5] and *C. carpio communis* [4]. The scale ridges, considered as growth rings, grow on the surfaces of both the anterior and lateral regions, forming concentric arcs with the scale focus in the center. The scale ridges are relatively smooth on top, whereas in some species there are many obvious individual denticles on the scale ridges [3,5]. In addition, the posterior region features convex particles and many tiny holes.

TGA results give the weight ratios of water, organic components and inorganic components (Fig. 2(a)) to be 12.3, 52.1, and 35.6% in the f-fish scale and 8.3, 88.2, and 3.5% in the de-fish scale, respectively. The absorbed water desorbs before 250 °C and the thermal decomposition of organic components occurs until 660 °C in both scales. Two exothermic peaks were observed in the DSC results for the f-fish scale, as shown in Fig. 2(b). The exothermic peak at 380 °C is due to labile organic components such as small molecular proteins or amino acids, and the other exothermic peak at 480 °C is due to crystalline collagen fibers. This also suggests that collagen is the main organic component of the f-fish scale.

XRD patterns show broad peaks for the f-fish scale and relatively sharp peaks for the s-fish scale, as shown in Fig. 3. The XRD result of the f-fish scale shows broad diffraction peaks corresponding to its organic components. According to the TGA result, only inorganic components remained after sintering at 700 °C. This is the reason for the XRD results of the s-fish scale to show relatively sharp peaks, like standard hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$, JCPDS No. 54-0022). Dehydroxylation of hydroxyapatite in air takes place in a temperature range of 850–900 °C [26], whereas no thermal decomposition occurred when HA was sintered at 700 °C. Furthermore, because calcium-deficient apatite can change into tricalcium phosphate (TCP) at high temperatures and none of this TCP phase was observed, the hydroxyapatite is not calcium-deficient apatite. XRD results indicate that the inorganic component is hydroxyapatite, different from the calcium-deficient carbonate-doped apatite in *P. major* [6] or calcium-deficient hydroxyapatite in *Chub* [27].

FTIR results show strong absorption bands at 1632, 1238 and 1547 cm^{-1}, corresponding to amides I, II and III of type I collagen, respectively (Fig. 4). These peaks are similar to the characteristic bands of collagen content in the fish scales of *Lates calcarifer* [2] and *P. major* [6]. Furthermore, the peak at ~1000 exhibits phosphate groups of the