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Comparative NEXAFS study of the selected icefish hard tissues and hydroxyapatite

O V Petrova¹, S V Nekipelov^{1,2}, D V Sivkov¹, A E Mingaleva¹, A Nikolaev³, O V Frank-Kamenetskaya³, V V Bazhenov^{4,5}, D V Vyalikh^{6,7}, S L Molodtsov^{4,5}, V N Sivkov¹, H Ehrlich⁴

¹Institute of Physics and Mathematics, Komi Science Centre Ural Division, RAS, 167982, Syktyvkar, Russia

²Syktyvkar State University, Syktyvkar, Russia

³Institute of Earth Sciences, Department of Crystallography, St. Petersburg State University, St. Petersburg, Russia

⁴Institute of Experimental Physics, TU Bergakademie Freiberg, Freiberg, Germany

⁵European XFEL GmbH, Albert-Einstein-Ring 19, 22761 Hamburg, Germany

⁶IKERBASQUE, Basque Foundation for Science, 48011 Bilbao, Spain

⁷Departamento de Fisica de Materiales and CFM-MPC UPV/EHU, Donostia International Physics Center (DIPC), 20080 SanSebastian, Spain

Abstract. The structure of native *Champsocephalus gunnari* icefish otoliths, scales, teeth, bones and pristine hydroxyapatite (HA) were examined using Near Edge X-ray Absorption Fine Structure (NEXAFS) spectroscopy. NEXAFS C1s-absorption spectra of the selected icefish hard tissues indicate that otoliths contain anion $[\text{CO}_3]^{2-}$. NEXAFS P2p-spectra clearly indicate the absence of phosphorus atoms only within otoliths and scales samples. However, the icefish teeth and bones P2p-spectra demonstrate identical spectral feature typical for the HA. NEXAFS Ca2p-spectra of the icefish hard tissues studied also shows features, which are in good correspondence with HA spectra. Interestingly, there is a red shift ≈ 0.1 eV of the $2p_{1/2,3/2} \rightarrow 3d$ transition energies in NEXAFS Ca2p-spectra of teethes and bones of the *C. gunnari* in comparison to HA.

1. Introduction

Near Edge X-ray Absorption Fine Structure (NEXAFS) spectroscopy become to be an important and highly informative method in modern studies related to chemistry and structure of diverse biomacromolecules including biominerals and biocomposites. Recent records as discoveries of novel hydroxylated collagen in deep-sea glass sponges [1], or the oldest exceptionally preserved chitin in Cambrian fossilized sponges [2] have been made also using NEXAFS. Our preliminary studies also provide strong evidence that NEXAFS spectroscopy at the C1s, P2p and Ca2p-edge represents the most suitable method to examine structural changes and differences within organic and inorganic functional groups in both natural and biomimetically developed Ca-containing biomaterials [3-5]. Currently attractive studies on the biomineral-based composites are related to synthesis under ambient laboratory conditions. Surprisingly, extreme biomineralization, which seems to be one of the most ancient naturally occurring phenomenon, and which is to be found up today in diverse natural



extremes (hot springs, Arctic and Antarctic seas, etc.) is less considered. Psychrophilic biomineralization is one direction of extreme biomineralization that includes chemical, biogeochemical and biochemical processes at very low temperatures. Typical examples of calcium-based biominerals within psychrophilic icefishes are their teeth, scales, otoliths and bones (see Figure 1.). However, information on characterization of their structure and chemical composition is still limited. Antarctic ice fishes habituate under extreme condition in deep, oxygen-reach, cold waters and due to specific adaptation can withstand temperature about $-1,9^{\circ}\text{C}$ that freeze the blood of all other types of fishes. To our best knowledge, there is still no any direct study with regard to investigation of chemical and mineral composition and nanostructure of ice- fish hard tissues. Our preliminary X-ray diffraction and NEXAFS studies showed that mineral component of the icefish bone is presented by the poorly crystallized hydroxyapatite (HA) [5].

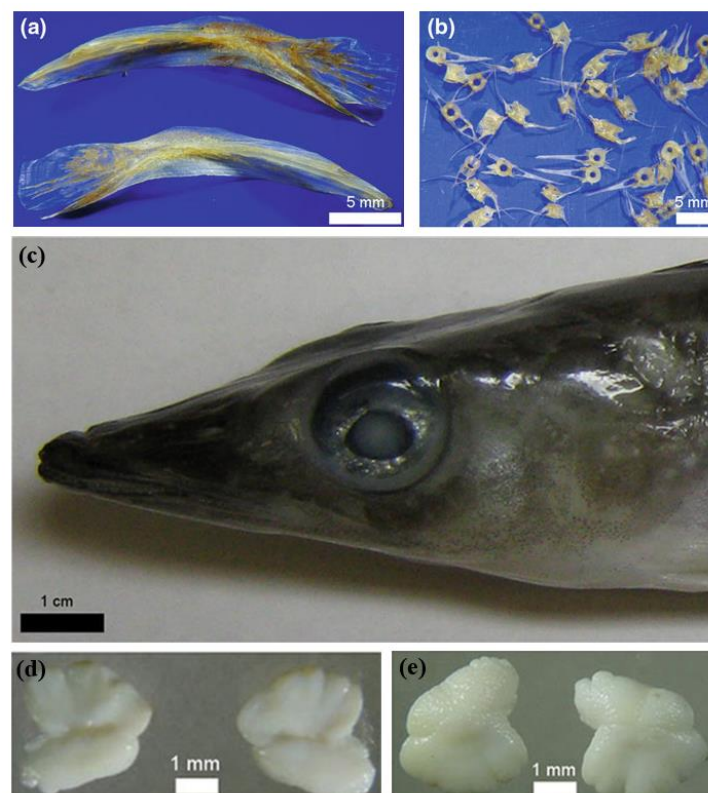


Figure 1(a, b, c, d, e). (c) Native icefish (*Champsocephalus gunnari*); icefish hard tissues: (a, b) bones and (d, e) otoliths [5] © Springer International Publishing AG 2017, with permission of Springer.

The theoretical and experimental investigations have shown that in bone tissue a close relationship between its hierarchical organization and local electronic structure of its mineralized phase exists [6]. According to the 3D-super-lattice (3DSL) model, the size-dependent ratio d/L of the hydrated layer thickness d to nanocrystallite average size in bone L is the important tool for functionalizing of the mineralized bone electronic structure. The 3DSL model predicts the size-dependent spectral changes in electronic structure of mineralized phase relative to the band structure of HA. In particular it predicts the red shift δE_{3d} of the band in the assembly of nanocrystallites and roughly estimate it as $\delta E_{3d} = E_{3d} \cdot d/L$, where E_{3d} is the energy above the bottom of the conduction band HA. According to the band structure calculations of HA [7, 8], the energy $E_{3d} \approx 2$ eV above the bottom of the conduction band. In addition to the red shift, the narrowing of the band in the assembly is also expected [6]. These effects can be investigated by studying the NEXAFS Ca2p-spectra of the pure HA deposited layer and

bone nanocrystallites. NEXAFS allows probing the atomic composition and angular-momentum-character specific densities of unoccupied electronic states and may give information about chemical bonds in investigated samples [9]. This method is using to measure the photoabsorption cross-section for the excitation of primarily bound core electrons that makes NEXAFS spectra element specific since each atom has characteristic binding energies of core levels. Thus, we have a great opportunity to apply this method to obtain information about individual units and substitutional groups, and to explore their fingerprinting character using the building block concept. The purpose of this comparative work is to study the NEXAFS Ca2p-spectra of pure HA and selected *C. gunnari* icefish hard tissues and to determine the average sizes of nanocrystallites in them.

2. Experimental details

The samples for absorption measurements were prepared ex situ in air by pressing of powders into the clean copper plate surface. The *C. gunnari* icefish bones were put into test tubes with H₂O₂ solution for 24 hours and then mechanically cleaned from muscles. For finalize cleaning of attached tissues the separated bones were put into the test tubes with 50% ethanol solution or NaOH solution, exposed in ultrasonic bath for 2-4 hours, washed in distilled water and, finally, dried at 50°C. For investigation of Ca2p-, P2p- and N1s-spectra the selected powdered icefish structures were mixed with pure fullerene C₆₀ for better conductivity and NEXAFS P2p-spectra calibration (C1s in second order diffraction). For investigation of C1s-spectra the pure powdered icefish samples were used. Icefish otoliths and scales have been extracted mechanically, separated, washed in 50% ethanol solution and dried. Powdered otoliths samples were mixed with C₆₀ too, while scales were not powdered, but covered by the gold layer except thin central band, for better charge draining. The NEXAFS studies on the powdered samples were carried out using monochromatic synchrotron radiation (SR) at the RBL at the BESSY-II electron storage ring (the Helmholtz-Zentrum Berlin, German) [9]. All measurements of the absorption spectra were performed under vacuum conditions $\sim 10^{-9}$ torr in the experimental chamber. The samples were placed at the angle of $\sim 45^\circ$ with respect to the incident beam of SR and the size of the focal spot on the sample was around 100×100 mkm². The NEXAFS-spectra at the Ca2p-, P2p- and C1s-absorption edge of the bone samples were measured by the total electron yield (TEY), which is proportional to the absorption cross section [9, 10]. All the X-ray absorption spectra were recorded with an energy resolution about 0.01 eV. The spectra were normalized to the incident photon flux, which was defined as a ratio between TEY-signal from a clean Au-plate and Au-atom cross section. The suppression and estimation of the nonmonochromatic background in TEY-signal was carried out by absorptionTi-filter method [3]. The energy over the range of the Ca2p-spectrum was calibrated by the first narrow peak in the F1s spectra of solid K₂TiF₆ (683.9 eV [13]) in the second order diffraction, C1s- and P2p- spectra by the peak in the C1s-absorption spectra of fullerite C₆₀ (284.5 eV [14]) in the first and second order diffraction, respectively. All spectra were collected by TEY-signal monitoring by the drain current detection with Keithley 617 microammeter.

3. Results and discussion

C1s-absorption spectra of the selected *C. gunnari* icefish biomineralized structures and calcite CaCO₃ as reference are shown in Figure. 2(a). Almost all icefish samples spectra, with exception of the scales, demonstrate feature at 290.2 eV similar to flat group [CO₃]²⁻-anion that indicates the presence of carbonates in otoliths, bones and teeth samples and carbonates absence in scales. Concerning C1s-spectra of CaCO₃, the structure presented before strong resonance at 290.2 eV is connected with sample contamination. In the case of the icefish samples the same structure is determined by the presence of specific chemical compounds in them. P2p-absorption spectra of the icefish bones, teeth and hydroxyapatite (HA, Ca₁₀(PO₄)₆OH₂) spectrum [15] contain identical structure (see Figure. 2(b)). This structure include 3 groups of features: **a** (136.5 eV) and **a'** (137.5 eV), **b** (138.2 eV) and **b'** (139.1 eV), **c** (141.5 eV) and **d** (146.8 eV), which correspond with P2p_{1/2,3/2} electron transition to molecular unoccupied states of [PO₄]³⁻-anion with predominant contribution of P3s, P3p and P3d component, respectively.

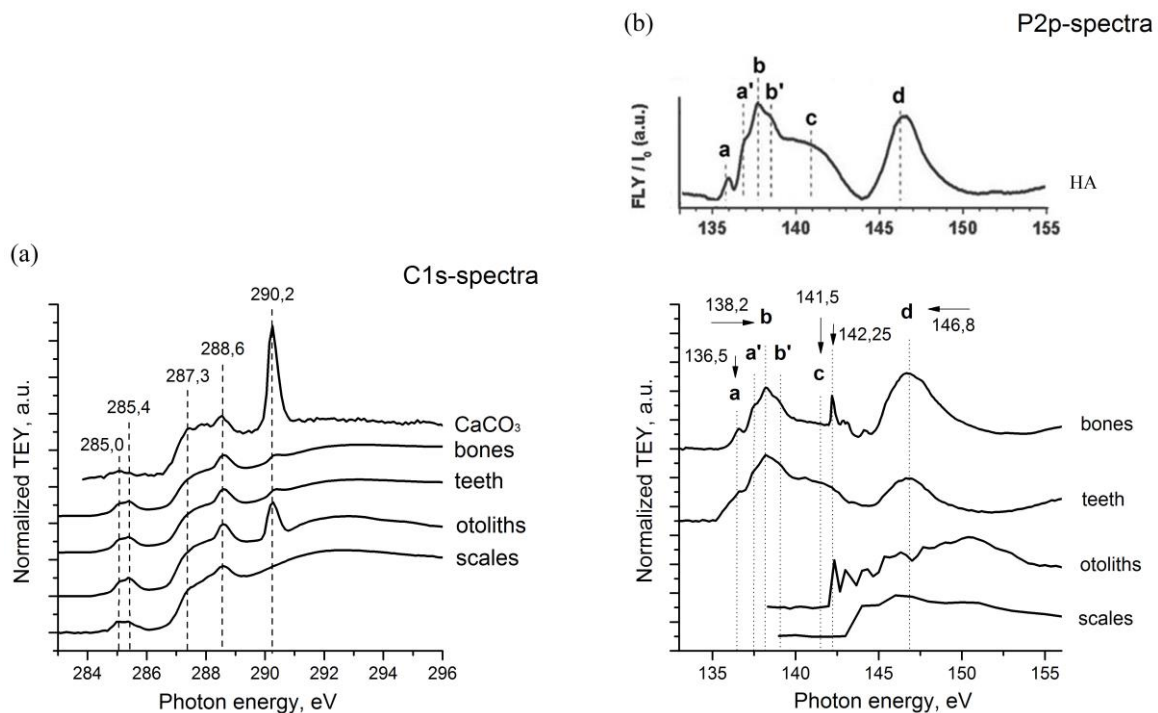


Figure 2(a, b). (a) NEXAFS C1s-absorption spectra of the *C. gunnari* icefish biomineralized structures and CaCO_3 ; (b) NEXAFS P2p-absorption spectra of the icefish biomineralized structures and the standard hydroxyapatite (HA) spectrum [15]. Reprinted from [15] Copyright © 2010, with permission from Elsevier.

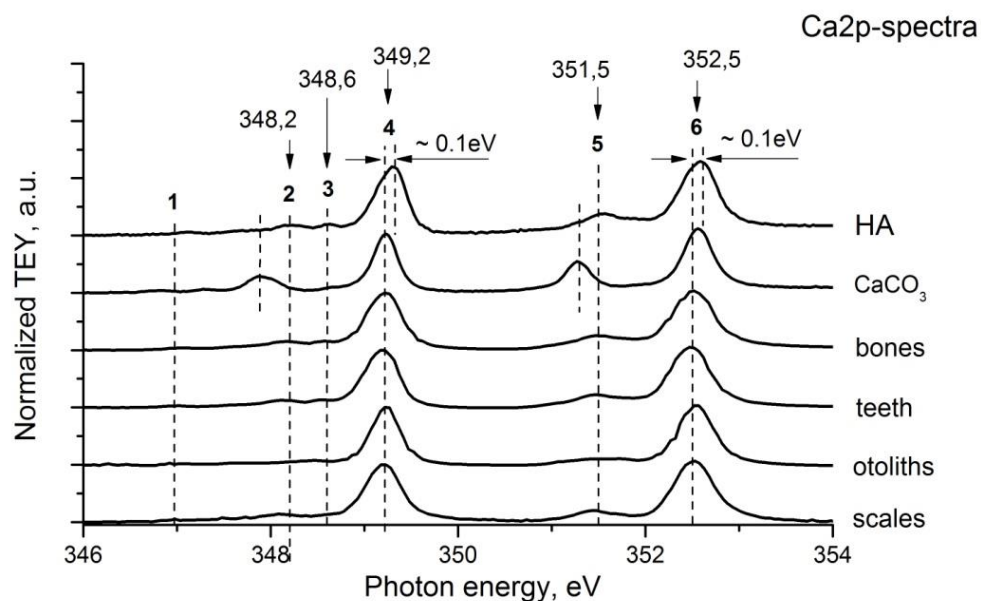


Figure 3. NEXAFS Ca2p-absorption spectra of the icefish biomineralized structures and HA.

Figure 3. shows the NEXAFS Ca2p spectra of the different icefish specimens (bones, teeth, otoliths and scales) as well as the HA reference. In all absorption spectra dominate two main spin-orbit-related peaks. The icefish otoliths and scales absorption spectra indicate the lack of phosphorus in them and HA presence in icefish bones and teeth. The absorption spectra of HA was taken from [15] and measured by Fluorescent Yield detection mode. The structure presented in the energy range of 140-160 eV is connected with the second order contribution of fullerene C1s-edge. peaks 4 and 6, along with a number of smaller peaks preceding the $2p_{3/2}$ and $2p_{1/2}$ main peaks. The origin of these multi-peak patterns is known to be the crystal field arising from the symmetry of the Ca^{2+} ion surrounded by oxygen atoms with close to octahedral configuration in the first coordination sphere [16]. Small shift ≈ 0.1 eV detected for the $\text{Ca } 2p_{1/2,3/2} \rightarrow 3d$ transition energies in the icefish biomineralized structures relative to HA is a very interesting phenomena. It is clearly seen that the magnitude of the red shift differs for diverse icefish hard tissues: for otoliths, the shift is minimal and increases when passing from the scales and bones to the teeth. The red shift also were detected in Ca2p-spectra of the bones of white mongrel male rats [6] and has been interpreted by the influence of the super periodicity on the band structure of HA and are assigned with the hierarchy-induced effect on the ground level in the bone tissue. Taking into account the fact that $E_{3d} = 2$ eV, our experimental results allow us to determine the value of $L/d \approx 20$.

4. Conclusion

Comparative NEXAFS C1s-absorption spectra study of the selected icefish hard tissues, hydroxyapatite and calcite show the presence of carbonates in otoliths, bones and teeth samples and absence in scales. The selected icefish hard tissues NEXAFS P2p-absorption spectra indicate the lack of phosphorus in otoliths and scales as well as HA presence in icefish bones and teeth. NEXAFS Ca2p-absorption spectra comparative analysis of the icefish bones and teeth and hydroxyapatite indicate the red shift for the Ca2p-spectra main features in icefishes bones and teeth relative to HA. This red shift is estimated ≈ 0.1 eV. According to 3DSL-model the presence of 0.1 eV red shift allow to define the nanocrystallite size as $L=20d$ where d is hydrated layer thickness between neighboring HA nanocrystallites. The obtained experimental results demonstrate the prospects of using of NEXAFS-spectroscopy for studying the hierarchical organization structure of HA containing icefishes hard tissues and, thus, stimulate further research in this area.

Acknowledgments

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