

Chapter 6

Characterization of Goldfish Scales by Vibrational Spectroscopic Analyses



Masayuki Nara, Yusuke Maruyama, and Atsuhiro Hattori

Abstract Scales of bony fishes are calcified tissues that contain osteoblasts, osteoclasts, and bone matrix, all of which are similar to those found in mammalian bone. The scales are composed of hydroxylapatite (HAP) and extracellular matrix which is mainly type I collagen fibers. We investigated the scales from goldfish by Fourier transform infrared (FTIR) and Raman spectroscopic analyses to characterize the components in the scales. The attenuated total reflection (ATR)-FTIR spectrum obtained from the surface coat of the normal goldfish scale was quite different from that of the backside coat of the same scale. The former showed a strong band at 1013 cm^{-1} , which was assignable to HAP, and weak bands at about 1643 , 1415 , and 870 cm^{-1} , whereas the latter showed a typical protein profile: strong bands at about 1631 , 1550 , and 1240 cm^{-1} , which were assignable to amide-I, amide-II, and amide-III of collagen, respectively. We also investigated the local structure of goldfish scales by using micro-Raman spectroscopy. The Raman spectrum of the normal scale showed the amide-I band at about 1640 cm^{-1} from the collagen and the PO_4^{3-} -symmetric stretching band at about 961 cm^{-1} from the HAP. We discuss the implication of Raman and FTIR profiles for normal and regenerating goldfish scales.

Keywords Vibrational spectroscopy · Goldfish scale · Regeneration · Hydroxylapatite · Carbonate apatite

6.1 Introduction

Goldfish scale is a calcified tissue that contains osteoblasts, osteoclasts, and bone matrix, all of which are similar to those found in mammalian bone (Azuma et al. 2007). The scale is composed of hydroxyapatite (HAP) and extracellular matrix which is mainly type I collagen fibers, forming a highly ordered three-dimensional structure. It has been known that scales regenerate after being removed. Therefore,

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the goldfish scale is interesting from the viewpoint of the model system for the regeneration of mammalian bone.

Vibrational spectroscopy is useful for characterizing developmental changes in bone and other mineralized tissues. Infrared spectroscopy is used for examining the mineral properties and collagen maturity of bones (Boskey and Mendelsohn 2005; Paschalis et al. 2011) as well as protein structures such as collagen and osteocalcin (Jackson et al. 1995; Barth 2007; Mizuguchi et al. 2001). Raman spectroscopy is also available for bones and fish scales to provide information about protein and mineral components (Ikoma et al. 2003a, b).

In the present study, FTIR and Raman spectroscopic analyses were applied to scales from goldfish to characterize the components of the normal and regenerating ones. In particular, we focused on the pocket side of them, since the epidermal side of scales is more complicated because of the contribution of chromatophore.

6.2 Materials and Methods

We prepared normal scales and regenerating scales after 1 week and 2 weeks, extracted from goldfishes (*Carassius auratus*). As can be seen in Fig. 6.1, the picture of the regenerating scale after 2 weeks was morphologically different from that of the normal scale. There were about ten circular ridges for regenerated scales after 2 weeks, although a lot of circular ridges were observed for normal scales.

Attenuated total reflection (ATR)-Fourier transform infrared (FTIR) measurements were performed at 25 °C on a PerkinElmer Spectrum-One FTIR spectrometer equipped with a universal ATR unit and a liquid nitrogen-cooled MCT detector at resolution 2 cm⁻¹. A scale sample was placed on a diamond/ZnSe 1-reflection top-plate (PerkinElmer). The sampling depth of the ATR method was approximately 1–2 μm over the range of 2000–1000 cm⁻¹. We obtained the ATR-FTIR spectrum for the spot (about 1.5 mm in diameter) with the accumulations of 200 scans.

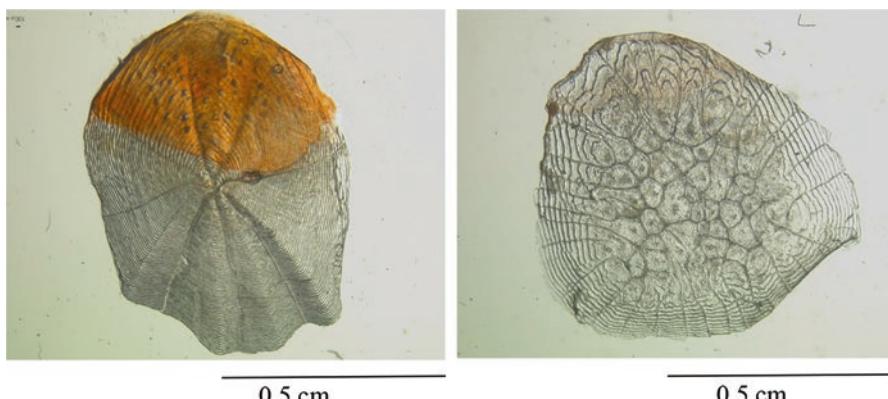


Fig. 6.1 Pictures of a normal scales (left) and a regenerating scale after 2 weeks (right) of goldfish (*Carassius auratus*)

Raman spectra were collected at room temperature ($25\text{ }^{\circ}\text{C}$) with a Raman microscope (Kaiser HoloLab 5000 of Kaiser Optical System Inc.) using 532 nm Nd-YAG laser (2–5 mW at the sample surface), holographic transmission grating, and charge-coupled device (CCD). The spectral resolution in the present system is approximately 4.8 cm^{-1} . We obtained the Raman spectrum for a spot (about $2\text{ }\mu\text{m}$ in diameter) with two accumulations of 30 s each. The sampling depth of the Raman measurement was about $100\text{ }\mu\text{m}$. A scale was set on a slide glass covered with aluminum foil, and the pocket sides of the surface were measured by Raman spectroscopy.

6.3 Results and Discussion

6.3.1 Characterization of a Normal Scale from Goldfish by FTIR Spectra

Figure 6.2 shows ATR-FTIR spectra of the surface and backside coats of a normal goldfish scale in the pocket side. Figure 6.2a showed a strong band at 1013 cm^{-1} , which was assignable to HAP, and weak bands at 1647 , 1546 , 1415 , and 871 cm^{-1} . The bands at 1647 and 1546 cm^{-1} were probably assignable to proteins. The bands

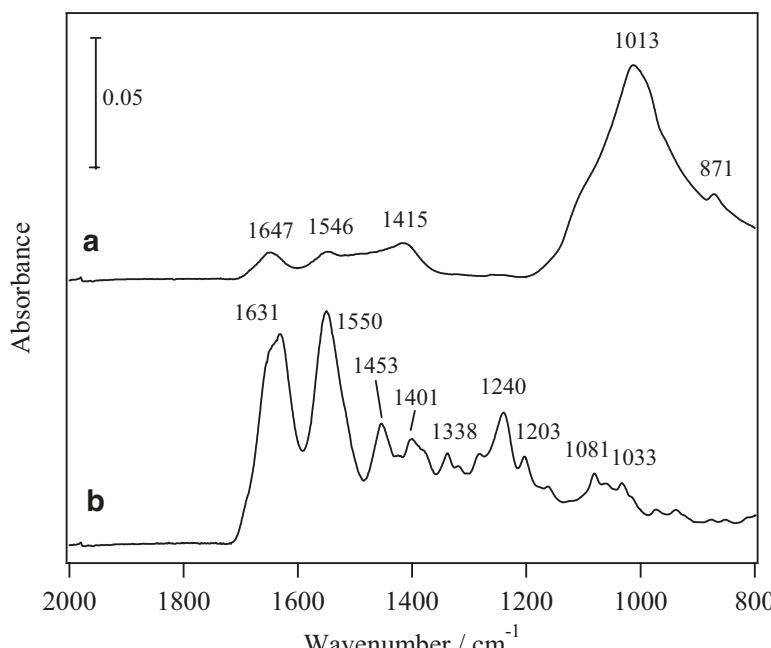


Fig. 6.2 ATR-FTIR spectra from (a) the surface coat and (b) the backside coat of the normal goldfish scale

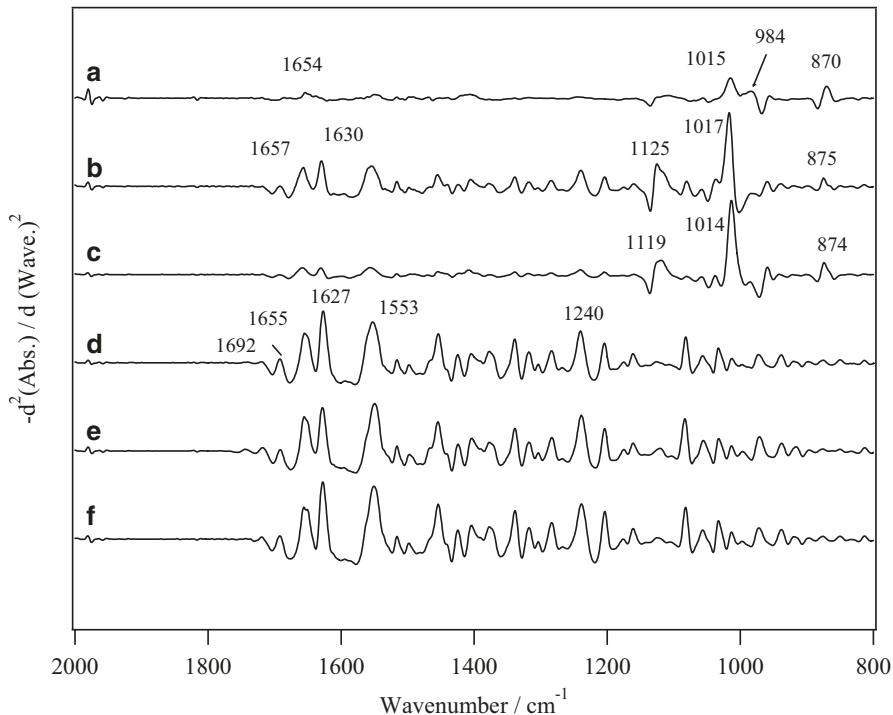


Fig. 6.3 ATR-FTIR second-derivative spectra of (a–c) the surface coat and (d–f) the back coat in the pocket side of three type of scales from goldfish: (a, d) a normal scale, (b, e) a regenerating scale after 1 week, and (c, f) a regenerating scale after 2 weeks. The second derivatives were multiplied by -1

at 1415 and 871 cm^{-1} were assigned to carbonate (CO_3^{2-}). Figure 6.2b showed a protein profile of collagen: strong bands at 1631 , 1550 , and 1240 cm^{-1} , which are undoubtedly assignable to amide-I, amide-II, and amide-III, respectively.

Figure 6.3a, d shows the ATR-FTIR second-derivative spectra of the corresponding spectra in Fig. 6.2. The second-derivative spectrum of surface coat showed the bands at 1654 , 1015 , 981 , and 781 cm^{-1} in Fig. 6.3a, whereas that of the backside coat showed three bands at 1696 , 1652 , and 1625 cm^{-1} in the region of amide-I, one band at 1550 cm^{-1} in the region of amide-II, and one band at 1240 cm^{-1} . Obviously, the spectral profile for the surface coat reflected the HAP as the main component of the calcified layer, while the spectral profile for the back coat reflected the collagen as the main component of the fibrillary layer. The amide-I band observed from the surface coat may be due to proteins other than collagen, since the profile was different from that of collagen. The bands at 1415 and 871 cm^{-1} are probably originated from CO_3^{2-} (Ikoma et al. 2003b).

6.3.2 Characterization of Regenerating Scales from Goldfish by FTIR Spectra

Figure 6.3b, e shows the second-derivative spectra of the surface and back coats of the regenerating scale after 1 week in the pocket side, respectively. The band at 1017 cm^{-1} caused by HAP in Fig. 6.3b was quite sharper than the corresponding band of normal scale in Fig. 6.3a. The spectral profile of the amide-I region was similar to that of the back coat, which suggested that the ATR spectra reflected the fibrillary layer as well as the calcified layer, since the thickness of the calcified one was less than $2\text{ }\mu\text{m}$. The spectral profile of the back coat of regenerating scales after 1 week (Fig. 6.3e) was almost the same as that of the back coat of a normal scale (Fig. 6.3d).

Figure 6.3c, f shows the second-derivative spectra of the surface and back coats of the regenerating scale after 2 weeks in the pocket side, respectively. The absorbance of amide-I band was weaker than that of Fig. 6.3b, which suggested that the calcified layer became thicker. The spectral profile of the back coat was almost the same as that of normal scale (Fig. 6.3d).

It was difficult to obtain an infrared spectrum of fish scale by using transmission mode, because the thickness of the normal scale is $>100\text{ }\mu\text{m}$ and the absorbance of most interesting bands was saturated. ATR measurements have an advantage over transmission measurement, because ATR technique makes it possible to obtain not only the spectral profiles of fish scales but also the information about the surface and backside coats of the scales separately. Therefore, ATR-FTIR spectroscopy is promising for monitoring HAP and collagen components during the regeneration process of goldfish scale.

6.3.3 Raman Spectra from Normal and Regenerating Scales of Goldfish

Figure 6.4a, b shows Raman spectrum from the area between adjacent circular ridges in a normal scale. The bands at about 1669 cm^{-1} and 1242 cm^{-1} were assignable to collagen amide-I and amide-III, respectively, and the intense band at about 961 cm^{-1} was originated from the PO_4^{3-} symmetric stretching band for HAP (Penel et al. 1998). The intensity at 961 cm^{-1} was quite weak in the most outer part of the circular ridges, suggesting that HAP layer is not formed or thin on the collagen layers. The band at 1070 cm^{-1} was observed at the center of the scale, which was caused by type-B carbonate apatite (Awonusi et al. 2007). This interpretation was also confirmed by the FTIR bands at 1415 and 871 cm^{-1} (Fig. 6.2a). Furthermore, we found that the HAP layer became slightly thicker along the ridge line than the area between adjacent circular ridges (data not shown).

Fig. 6.4 Raman spectra from (a, b) the normal goldfish scale and from (c–e) the regenerating scale after 2 weeks. The spot position for (a, c) were on the outer part of the scale in the pocket side, and the spot positions for (b, d, e) were on the inner part of the scale in the pocket side. The spot position on (d) was in the middle of (c, e)

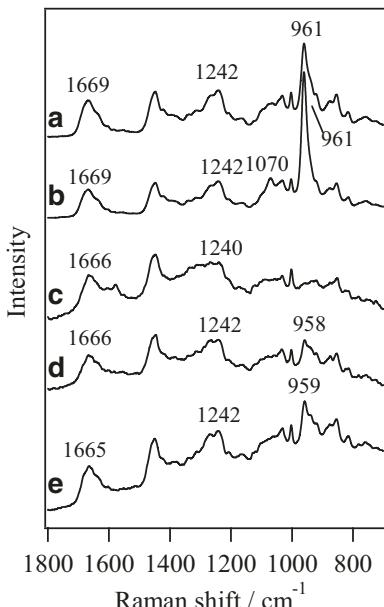


Figure 6.4c–e shows the Raman spectra from the area between adjacent circular ridges in regenerating scales after 2 weeks. The spectral profiles of the regenerating scales were similar to those of the normal ones except for the band at 961 cm^{-1} , which were weaker than that of normal scale (Fig. 6.4a, b). The intensities of the band at 961 cm^{-1} were the order (c) < (d) < (e). This result demonstrated that the HAP layers were formed from the center part to the outer part during the regeneration process of the scale.

Micro-Raman spectroscopy is a useful method for understanding the local components of goldfish scale because Raman spectroscopic method has higher resolution in the area compared with FTIR spectroscopic method. ATR-FTIR provides information regarding the surface component, because the sampling depth of Raman measurements was $100\text{ }\mu\text{m}$. Therefore, Raman spectroscopy in combination with ATR-FTIR spectroscopy is powerful for analyzing HAP and carbonate apatite of the regenerating scales.

References

- Awonusi A, Morris MD, Tecklenburg MMJ (2007) Carbonate assignment and calibration in the Raman spectrum of apatite. *Calcif Tissue Int* 81:46–52
- Azuma K, Kobayashi M, Nakamura M, Suzuki N, Yashima S, Iwamuro S, Ikegami M, Yamamoto T, Hattori A (2007) Two osteoclastic markers expressed in multinucleate osteoclasts of goldfish scales. *Biochem Biophys Res Commun* 362:594–600
- Barth A (2007) Infrared spectroscopy of proteins. *Biochim Biophys Acta* 1767:1073–1101

- Boskey AL, Mendelsohn R (2005) Infrared spectroscopic characterization of mineralized tissues. *Vib Spectrosc* 38:107–114
- Ikoma T, Kobayashi H, Tanaka J, Walsh D, Mann S (2003a) Microstructure, mechanical, and biomimetic properties of fish scales from *Pagrus major*. *J Struct Biol* 142:327–333
- Ikoma T, Kobayashi H, Tanaka J, Walsh D, Mann S (2003b) Physical properties of type I collagen extracted from fish scales of *Pagrus major* and *Oreochromis niloticus*. *Int J Biol Macromol* 32:199–204
- Jackson M, Choo LP, Watson PH, Halliday WC, Matsch HH (1995) Beware of connective tissue proteins: assignment and implications of collagen absorptions in infrared spectra of human tissues. *Biochim Biophys Acta* 1270:1–6
- Mizuguchi M, Fujisawa R, Nara M, Kawano K, Nitta K (2001) Fourier-transform infrared spectroscopic study of Ca^{2+} -binding to osteocalcin. *Calcif Tissue Int* 69:337–342
- Paschalis EP, Mendelsohn R, Boskey AL (2011) Infrared assessment of bone quality: a review. *Clin Orthop Relat Res* 469:2170–2178
- Penel G, Leroy G, Rey C, Bres E (1998) Micro Raman spectral study of the PO_4 and CO_3 vibrational modes in synthetic and biological apatites. *Calcif Tissue Int* 63:475–481

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