Analysis of tissue condition based on interaction between inorganic and organic matter in cuttlefish bone

Maya Mizuno · Kaori Fukunaga

Received: 16 May 2012 / Accepted: 20 September 2012 / Published online: 20 October 2012
© Springer Science+Business Media Dordrecht 2012

Abstract The absorption properties of an inner layer of cuttlefish bone were measured using a transmission terahertz time-domain spectrometer in a band from approximately 0.1 to 4 THz. For oriented samples, an absorption peak related to the behavior of calcium carbonate appeared at approximately 2 THz. The peak magnitude and frequency depended on the direction of the incident terahertz electric field, indicating that calcium carbonate crystals constituting the inner layer were oriented in a certain direction. The absorbance of a sample heated to 350 °C for 0 to 2 h to remove organic matter tended to decrease with heating time in the oriented direction, while the peak frequency shifted to higher frequencies. Furthermore, we showed that the peak frequency depended on the interaction area within the unheated sample and we thus obtained a two-dimensional image reflecting crystal regularity inside the cuttlefish bone from the spectral data at each position.

Keywords Cuttlefish bone · Aragonite · Absorbance · Terahertz spectroscopy

1 Introduction

Hard tissues such as bone and shells are called biominerals and are found in various species ranging from microorganisms to animals. Both the growth kinetics of formation and the morphology of biominerals have been speculated to relate to the interaction between the organic and inorganic matter present within biominerals [1]. Hence, it is necessary to study this interaction and the interaction between different kinds of inorganic matter to identify the crystal states of biominerals. Biominerals with complicated structures such as cuttlefish

M. Mizuno (✉) · K. Fukunaga
Applied Electromagnetic Research Institute,
National Institute of Information and Communications Technology,
4-2-1, Nukui-kitamachi, Koganei, Tokyo, 184-8795, Japan
e-mail: mmizuno@nict.go.jp

bone [2] have recently been studied owing to their potential application as scaffold materials in bone regenerative engineering [3, 4]. These studies on crystals are important because scaffold material affects the properties of regenerated tissue, enabling safe recycling of biominerals in regenerative medicine. The crystal structure and molecular behavior are presently determined employing special equipment for analysis such as X-ray diffractometers and Fourier transform infrared (FT-IR) spectrometers [5]. X-ray diffraction can be used to examine and verify the crystal structure of materials and FT-IR spectroscopy in the mid-infrared region can be used to identify lattice and molecular vibrations through a comparison of spectral patterns. However, it is difficult to sensitively observe weak interactions between molecules such as those due to hydrogen bonds and van der Waals forces inside the scaffold material. Conversely, terahertz waves can propagate through various non-metallic specimens and terahertz absorption reflects lattice vibrations and skeletal vibrations such as stretching, bending, and torsion of macromolecules. Furthermore, both the deterioration of materials and the interaction between organic and inorganic matter affect the spectral patterns [6, 7]. Therefore, we employed a terahertz transmission spectrometer and verified the possibility of non-destructive evaluation of the condition inside cuttlefish bone from changes in the absorbance spectra on the basis of the interactions between inorganic crystal and organic matter.

2 Specimens and method

Cuttlefish bone consists of a dorsal shield and an internal lamellar matrix, which are separated by numerous pillars. The material of the internal lamellar matrix was found by X-ray diffraction measurements to be aragonite, which is a polymorph of calcium carbonate (CaCO_3) that has an orthorhombic crystal structure. The pillars and thin septum of the internal lamellar matrix are coated with organic matter. We measured the absorbance of an inner layer that had a thickness (interval between the septa) of approximately 0.6 mm, as shown in Fig. 1 (left), a septum and a ground powder sample of cuttlefish bone. The ground powder was sieved to have a grain size less than $20\text{ }\mu\text{m}$. Subsequently, 4 mg of this material was homogenized with polyethylene powder and formed into a pellet with thicknesses of approximately 1.6 mm. The ground-powder-to-polyethylene ratio, pressure and pressing time were 0.02, 47 kN/cm^2 and 10 min, respectively.

Fig. 1 Microscope image of the cross-section of an inner layer of cuttlefish bone and schematic diagram of the aragonite structure

Absorbance spectra were measured using a terahertz time-domain spectrometer (Rayfact SpecTera RS-01020, Tochigi Nikon, Otawara, Japan) in a range of approximately 0.1 to 4 THz. The system, which used a femtosecond laser and a low-temperature-grown GaAs photoconductive dipole antenna, could acquire a time waveform for a linear polarized terahertz electric field [8]. By comparing the Fourier spectra of the time waveform with and without the sample, the absorbance spectra were obtained. The signal-to-noise ratio in the spectral domain was greater than that of the FT-IR spectroscopy below 3 THz [9]. A measurement image was obtained from time-resolved waveform data for each pixel using a similar system synchronized with a scanning stage (T-Ray 4000, Picometrix LLC). The terahertz wave was irradiated perpendicular to the samples in all measurements.

3 Results and discussion

Figure 2 shows the absorbance spectra for the inner layer, septum and pellet samples. In the figure, multiple elements of reflection between sample surfaces that introduce delays in the time domain are removed. Increases in absorption as the frequency approached 3 THz were observed for all samples. This indicates that aragonite undergoes absorption corresponding to CO_3 and Ca vibrations around 3 THz as reported in a previous paper [10]. Only for the inner-layer spectrum, above approximately 2.5 THz, the strength of the signal transmitted from the inner layer sample was insufficient owing to strong absorption and scattering. We also verified that cuttlefish bone has a large absorption peak at approximately 2 THz only when the crystal is highly oriented, while the Bragg reflection peak for the (002) plane is large in X-ray diffraction measurement. The magnitude of the absorption peak depended on the polarization angle of the incident terahertz wave; θ is the angle between the polarization direction and the transverse direction of cuttlefish bone as shown in Fig. 3. The absorption at 2 THz was greatest when θ was 0° , indicating that the aragonite crystal comprising the lamellar matrix was oriented in this direction. Additionally, a small absorption peak was observed from 40° to 90° at frequencies lower than 2 THz. We speculate from the absorption direction and the aragonite structure described in a previous paper [11] that ion polarization easily occurs in the C–O direction. We simulated molecular vibration modes in an aragonite crystal lattice using semi-empirical quantum chemistry software, based

Fig. 2 Absorbance spectra of samples of the inner layer of cuttlefish bone, septum and pellet of mixed ground powder

Fig. 3 Spectra of the inner layer of cuttlefish bone at θ of 0° , 20° , 40° , 60° , 80° and 90°

on NDDO approximation, called the Molecular Orbital Package (MOPAC) [12] with a parameterized model 6 (PM6) [13] Hamiltonian and visualized the vibration direction and infrared spectrum using Facio software [14]; the results are shown in Fig. 4. The figure shows that it is possible to observe absorption peaks related to displacement of the CO_3 group in a C–O direction at 1.4 and 2.9 THz. However, the frequencies do not match the absorption peak for the oriented inner layer.

We heated the inner layer to 350°C to change the amount of organic matter such as β -chitin coated on the aragonite crystals and verified spectral variations caused by the organic matter. Chitin is the main organic substance ($<4.5\text{ wt}\%$) in cuttlefish bone and does not have any large absorption peaks at frequencies below 2.7 THz [15]. The absorbance of a sample heated for 0 to 2 h tended to decrease with heating time when θ was 0° , while the peak frequencies shifted to higher frequencies (Fig. 5a). The microscope images in Fig. 5a indicate that the amount of organic matter coated on the septum and pillars decreased because of heat treatment. Furthermore, we verified that the difference in the reflection angle 2θ from the (002) plane between the samples before and after heating was approximately 0.04° in X-ray diffraction measurements even though the measurement was

Fig. 4 **a** Schematic representation of molecular motion at 2.9 THz and **b** the infrared spectrum of aragonite

Fig. 5 Microscope images and spectra of the inner-layer sample heated to 350 °C for (a) 0 to 2 h and (b) absorption peak distribution with heating time at θ of 0°

near the sample surface. From these results, we speculate that the relationship between organic matter and the crystal lattice in the inner layer affects the spectral pattern relating to the behavior of the CO_3 group. The spectral pattern of a biological sample has large individual variation. Therefore, we also measured the distribution of the absorption peak at 0° with heating time (Fig. 5b). The dashed line indicates the absorbance spectrum at 90° taken as a baseline. We verified that the magnitude and frequency of the absorption peak tended to depend on heating time under all tissue conditions, even though it was difficult to infer the condition for two samples. Thus, we have the possibility of determining the tissue condition using the absorption properties that arise when organic matter is connected to inorganic matter.

We carried out spectral and imaging measurements for unheated cuttlefish bone using a terahertz time-domain spectroscopy and imaging system. Microscope images of the sample are shown in Fig. 6. The S-shaped pillars in region B, defined as circular areas with center at O and diameters of approximately 5 mm, were smaller and more dispersed than those in region A as seen in microscope images of the sample surface (Fig. 6 (left)). In spectral measurement, we observed that the absorption peak frequency around 2 THz in region B was lower than that in region A (Fig. 7) and the total area of interactions such as those

Fig. 6 Photographs of the inner-layer sample used for spectroscopy and imaging measurement

Fig. 7 Spectra for regions A and B in an inner-layer sample

between the pillar and organic matter coated on the pillars was thus large in region B. We therefore assumed that we could observe an absorption change due to the interaction, regardless of the change in crystal lattice due to heating. In addition, we speculate that the deviation mentioned above was mainly due to interaction differences. On the basis of these results, we verified the possibility of carrying out imaging measurements to observe differences in crystal regularity through a change in absorption, over a large area. The sample was placed approximately 0.5 mm from an aluminum plate (Fig. 8). We detected only the terahertz wave reflected from the aluminum plate and we imaged the sample according to the integral intensity of reflection in the range from 1 to 2 THz; this frequency range was limited by the equipment and a more sensitive observation can be made using apparatus at a higher frequency. Figure 9 shows terahertz images of the cuttlefish bone acquired at a resolution of 0.2 mm/pixel. The terahertz polarization angle here was set to $\theta = 0^\circ$ and 45° . The dark regions in these images indicate strong absorption. Although there was a significant absorption change at the edge of the sample due to the addition of the waves reflected from the surface of the aluminum plate, the absorption did not depend on the angle dependence. We verified from these results that the absorption in region B was stronger than that in region A in the range from 1 to 2 THz. In particular, the contrast at 0° was greater than that at 45° owing to the absorption peak around 2 THz being observed at 0° . This reflects the fact that the frequency of the absorption peak in region B was lower than that in region A, owing to the broad area of interactions between the crystal and organic matter. We were able to distinguish the distribution of pillars from this absorption property as well as the magnitude of interactions between organic matter and crystal, even at the absorption-edge frequency.

Fig. 8 **a** Schematic of the experimental setup and **b** example of the time waveform used for terahertz imaging

Fig. 9 Terahertz images of the inner-layer sample at θ of 0° and 45°

4 Conclusions

We acquired the absorption spectra of inner-layer samples of cuttlefish bone using a transmission terahertz time-domain spectrometer. By comparing the spectra measured in different polarization directions and after different heating treatment times, we verified that the magnitude and frequency of the absorption peak at approximately 2 THz were affected by crystal orientation, the crystal lattice, and interaction with organic matter. On the basis of these dependencies, we imaged an inner-layer sample and demonstrated that it was possible to sensitively image the crystal regularity through crystal interaction with organic matter. Hereafter, we intend to study the relation between crystal regularity and properties such as the conduction and induction of bone as a scaffold material.

References

1. Kim, Y.Y., et al.: An artificial biomineral formed by incorporation of copolymer micelles in calcite crystals. *Nat. Mater.* **10**, 890–896 (2011)
2. Birchall, J.D., Thomas, N.L.: On the architecture and function of cuttlefish bone. *J. Mater. Sci.* **18**, 2081–2086 (1983)
3. Okumuş, Z., Yildirim, Ö.S.: The cuttlefish backbone: a new bone xenograft material? *Turk. J. Vet. Anim. Sci.* **29**, 1177–1184 (2005)
4. Ivankovic, H., et al.: Preparation of highly porous hydroxyapatite from cuttlefish bone. *J. Mater. Sci. Mater. Med.* **20**, 1039–1046 (2009)
5. Florek, M., et al.: Complementary microstructural and chemical analyses of *Sepia officinalis* endoskeleton. *Mater. Sci. Eng. C* **29**, 1220–1226 (2009)
6. Ohki, Y., et al.: Terahertz time-domain spectroscopic analysis of molecular behavior in polyamide nanocomposites. *Appl. Phys. Express* **1**, 122401 (2008)
7. Nomura, K., et al.: Effect of annealing on optical absorption of LaAlO_3 at terahertz frequencies. *Jpn. J. Appl. Phys.* **50**, 021502 (2011)
8. Nishizawa, S., et al.: Terahertz Time-Domain Spectroscopy. *Terahertz Optoelectronics, Topics in Applied Physics*, vol. 97, pp. 203–270. Springer, Berlin (2005)
9. Han, P.Y., et al.: A direct comparison between terahertz time-domain spectroscopy and far-infrared fourier transform spectroscopy. *J. Appl. Phys.* **89**, 2357–2359 (2001)
10. Yamamoto, A., et al.: Optically active vibrations and elastic constants of calcite and aragonite. *B. Chem. Soc. Jpn.* **47**, 265–273 (1974)
11. Negro, A.D., Ungaretti, L.: Refinement of the crystal structure of aragonite. *Am. Mineral.* **56**, 768–772 (1971)
12. Stewart, J.J.P.: MOPAC: a general molecular orbital package (version 6.0). *QCPE Bull.* **10**, 86–87 (1990)

13. Stewart, J.J.P.: Optimization of parameters for semiempirical methods V: modification of NDDO approximations and application to 70 elements. *J. Mol. Model.* **13**, 1173–1213 (2007)
14. Suenaga, M.: Facio: new computational chemistry environment for PC GAMESS. *J. Comput. Chem. Jpn.* **4**, 25–32 (2005)
15. Fischer, B., et al.: Chemical recognition in terahertz time-domain spectroscopy and imaging. *Semicond. Sci. Tech.* **20**, S246–S253 (2005)