Role of Collagen and Inorganic Components in Electrical Polarizability of Bone

Rumi HIRATA1,2), Miho NAKAMURA1)* and Kimihiro YAMASHITA1)

1)Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, 2–3–10 Kanda-Surugadai, Chiyoda, Tokyo 101–0062, Japan
2)School of Veterinary Medicine, Rakuno Gakuen University, 582 Midorimachi, Bunkyoudai, Ebetsu, Hokkaido 069–8501, Japan

(Received 9 May 2013/Accepted 4 October 2013/Published online in J-STAGE 18 October 2013)

MATERIALS AND METHODS
Sample preparation: The bone samples were extracted from the 10-week-old, male, Japanese White rabbits. At least...
one week prior to conducting the experiment, each rabbit was acclimatized to the environment by being housed separately in a temperature controlled facility having 12-hr light/dark cycle. The rabbits were allowed to food and water ad libitum. The animals utilized in this experiment all received humane care. The experimental protocol was approved by the Animal Welfare Regulation Committee of the Tokyo Medical and Dental University. After being preserved at a temperature of −80°C, eight rabbit femurs were cut in half transversely. Some samples were immersed in 2% KOH solution for 4 days (2% koh), immersed in 2% KOH and then baked in electrical furnace at 800°C for 10 min (2% koh+bake). Other samples were decalcified by treatment with EDTA (decalcification). The epiphysis of these samples, as well as the bone with no treatment (untreated), was longitudinally cut and grinded into uniform shapes 4 × 7 × 0.45 mm for measurement with a diamond saw (Buehler, Lake Bluff, IL, U.S.A.) and diamond disk (5 µm). The bone samples were washed with deionized water and then dried at 40°C until use.

Dense ceramic samples of hydroxyapatite (HA) were prepared as a control. HA powder was synthesized from analytical-grade reagents of calcium hydroxide and phosphoric acid by the wet method [16], calcined at 850°C for 1 hr, pressed into a mold at 200 MPa and sintered in a saturated water vapor atmosphere at 1,250°C for 2 hr to suppress dehydration.

**Characterization:** Bone samples were characterized by X-ray diffraction (XRD), Fourier transform-infrared (FT-IR) and scanning electron microscopy (SEM).

XRD measurements for each powdered sample were performed for phase analysis at room temperature with CuKa radiation at 40 kV and 40 mA on a diffractometer (PW-1700, PHILIPS Co., Ltd., Amsterdam, Netherlands) equipped with a graphite monochromator.

FT-IR spectra of each sample were measured in the range of 400-4,000 cm⁻¹ using an infrared spectrophotometer (IR-2000, JEOI Co., Ltd., Tokyo, Japan). The samples were pulverized with a mortar and pestle for the KBr method of which KBr and the samples were mixed in a ratio of 20:1. Then, peak ratios of ν₂CO₃ and ν₃CO₃ to ν₃PO₄ were calculated from the measurements.

After the treatments, bone samples were dried and sputtered with platinum/palladium. Surface morphology of each sample was observed at higher and lower magnifications using a scanning electron microscope (SEM) (Hitachi Instruments Service Co., Ltd., Tokyo, Japan).

**Polarization:** For the polarization measurement, samples were pinched by platinum electrodes, and the outside of each electrode was covered by alumina plates and clamped. A direct current electric field was applied to the samples using stabilized DC power supply units (AE8800, ATTO Co., Ltd., Tokyo, Japan and E3260A, HEWLETT PACKARD Co., Ltd., Palo Alto, CA, U.S.A.) under the chosen polarization condition. Regard, the samples were treated at 400°C with an electric field of 5 kV/cm for 1 hr and at 37°C with an electric field of 5 kV/cm for 1 hr. To avoid polarization relaxation, the electric field was maintained, while the samples were cooled down to the room temperature. The control sample was also treated at 400°C for 1 hr, but without electric field.

**TSDC measurement:** Samples were once again pinched by platinum electrodes, and the outside of the electrodes was covered by alumina plates and clamped. TSDC measurement of the samples was accomplished using a min electric current measuring unit (4140B pA METER, HEWLETT PACKARD Co., Ltd., Palo Alto, CA, U.S.A.). The polarization relaxation current was measured, while the sample temperature was increased at a constant 5°C/min from room temperature to 650°C. The amount of stored charge in the samples was calculated by surface integration of the TSDC spectrum.

**Change of mass:** In order to obtain the remaining organic ratios, each prepared bone sample was baked at 1,000°C for 30 min with weight measured before and after baking.

**Proportion measurement of collagen:** The amount of hydroxyproline, a main component of collagen of each sample, was quantitatively determined by the following procedure. Steps for this measurement are described below. The sample was combined with 6 M HCl and heated at 110°C for 24 hr to enable amino acid analysis. The reaction mixture was then dried in a rotary evaporator and dissolved in 0.02 M HCl. Amino acid analysis was then performed on an L-8800 amino acid analyzer (Hitachi Instruments Service Co., Tokyo, Japan) using citrate buffers and a sodium chloride gradient. Amino acids in the eluate were monitored by post-column reaction with ninhydrin. After measurement, the weight percentage of collagen in each sample was calculated.

**RESULTS**

**Characterization:** Infrared absorption peaks corresponding to ν₂CO₃ and ν₃CO₃ modes were detected to confirm the existence of carbonate acid group in the untreated and 2% koh samples (Fig. 1). The strength of ν₂CO₃ and ν₃CO₃ in comparison with ν₃PO₄ signal was 13 and 58.8% for the untreated samples, 8.8 and 48.8% for the 2% koh samples and 0 and 13.3% for the 2% koh+bake sample, respectively (Table 1). Thus, the remaining organic substance was highest in the 2% koh+bake sample.

X-ray diffraction peaks detected for every sample were attributed to HA, as the patterns were able to be matched to publish data ICDD no.9-432, and demonstrated that the surfaces of the specimens consisted of a single phase of hexagonal HA (indicated by open circles) (Fig. 2). The 2% koh sample showed broad pattern indicating amorphous compositions which were similar to that of the untreated sample, while crystallization was observed in the 2% koh+bake sample. Figure 3 shows the resulting SEM images. Surface of the untreated sample was rough, and that of the 2% koh sample was almost uniform. Collagen fibers were exposed in the decalcification sample. Many small voids in the 2% koh+bake sample seemed to be the results of collagen removal.

**TSDC measurement and the amount of stored charge:** The amount of the stored charges of all samples (Fig. 5) was calculated from their TSDC spectra (Fig. 4). The stored
The charge of the unpolarized 2% koh sample was 0.04 \( \mu \text{C/cm}^2 \), but those of the untreated, 2% koh, 2% koh+bake and HA (or decalcified) samples treated at 400 vs. 37°C for 1 hr with an electric field of 5 kV/cm were 1.72 vs. 0.96, 138 vs. 1.63, 11.31 vs. 11.69 and 18 vs. 5.8 \( \mu \text{C/cm}^2 \), respectively. Besides, those of the 2% koh samples treated at 37°C for 1 and 10 min with an electric field of 5 kV/cm were 0.96 and 1.57 \( \mu \text{C/cm}^2 \). It was apparently that polarization in a harsher 400°C condition was not possible as the sample was burned and destroyed (Fig. 5). Peak of the TSDC spectrum of the untreated, 2% koh and 2% koh+bake samples was found in the vicinity of 100, 450–500 and near 580°C, respectively (Fig. 4). The second peak could not be observed, since the samples were destroyed at higher temperature.

Change of mass: The untreated, 2% koh and 2% koh+bake samples were burned at 1,000°C for 30 min, and the bone mass ratio between before and after burning was calculated for each sample. Those ratios were 62% for untreated, 65% for 2% koh and 97% for 2% koh+bake samples (Table 1).

Proportion measurement of collagen: The amount of hydroxyproline, a main component of collagen, was quantitatively determined for each sample, and the weight proportion of collagen was calculated. These results were 13.84% for untreated, 13.86% for 2% koh, 0% for 2% koh+bake and decalcification samples (Fig. 6).

DISCUSSION

In this experiment, untreated, 2% koh and 2% koh+bake bone samples were prepared from rabbit femur, and all could be polarized after treatment at 400°C with an applied electric field of 5 kV/cm for 1 hr. These are conditions under which HA can be polarized. However, in addition, all the samples could be polarized at a lower temperature of 37°C, at which HA cannot be polarized [23].

| CO\(_3\) ratio of each sample to v\(_3\)PO\(_4\) and amounts of the remaining organic substances in the untreated, 2% koh and 2% koh+bake samples |
|-----------------|-----------------|-----------------|-----------------|
| Untreated (\%)  | 2% koh (\%)     | 2% koh+bake (\%)|
| v\(_2\)CO\(_3\) | 13.0            | 8.8             | 0.0             |
| v\(_3\)CO\(_3\) | 58.8            | 48.8            | 13.3            |
| Remaining organic substance | 62.0 | 65.0 | 97.0 |

Fig. 1. FT-IR measurement results for the untreated, 2% koh and 2% koh+bake samples. Infrared absorption peaks in v\(_2\)CO\(_3\) and v\(_3\)CO\(_3\) modes confirmed the existence of carbonic acid group in the untreated and 2% koh samples.

Fig. 2. XRD patterns of untreated, 2% koh and 2% koh+bake samples. The bone samples consisted of a single phase of hexagonal HA (indicated by open circles).
The 2% koh sample exhibited stored charge of 138 µC/cm² after being subjected to the strongest treatment conditions, a very high degree of charge storage compared to the other samples. This sample could also be polarized at lower...
temperature conditions with shorter treatment time. At 37°C, this sample was treated for 1 hr with the electric fields of 5 kV/cm for 10 min with 5 kV/cm and for 1 min with 1 kV/cm (Fig. 5). These results mean that the polarization of bones is possible even at body temperature. It was also found that the 2% koh sample had almost the same quantity of CO3 as the untreated sample (Fig. 1, Table 1) and structurally was in a broad amorphous state with no crystallization observed by XRD. Those findings indicated that the 2% koh sample was not crystallized and could retain similar characteristics to that in the untreated sample (Fig. 2). In addition, the 3% higher in weight ratio of the 2% koh sample either before or after burning than the untreated sample, and a similar weight proportion of collagen in both samples (Fig. 6) would indicate a similar condition of collagen components in bone structure of both samples. However, this experiment still cannot explain clearly why the amount of the stored charge of the untreated sample was much lower than that of the 2% koh sample.

The 2% koh+bake sample showed similar results in IR and XRD measurements to those of HA. This sample could be polarized at the usual high temperature conditions, giving stored charge of 11.31 μC/cm². This value was similar to that of HA, which gave 18 μC/cm². On the other hand, the 2% koh+bake sample could be polarized under the lower temperature conditions, giving stored charge of 11.96 μC/cm², similar to the high temperature treatment, while HA could not be polarized at temperatures below 200°C. The 2% koh+bake sample had a mass ratio of 97%. This sample also showed 0% collagen content. These findings suggest that this sample was rendered a completely inorganic material by the sample treatment. Thus, the polarization capability of this sample is due to inorganic components of the bone. However, XRD measurement showed that the sample was crystallized in the same way as HA, while IR measurement revealed an extremely low CO3 content. This meant that the sample had not retained the properties which were similar to those of bones.

The high weight proportion of collagen in the decalcification sample (74.2%) would indicate a successful removal of inorganic components from the bone; thus, leaving mainly organic components in situ. It is a matter of fact that such decalcified bone containing almost purely collagen could not tolerate a high temperature treatment. Thus, induction of polarization could merely perform only in a lower temperature. This statement indeed supports the concept that polarization of the bone relies largely on its organic components [17].

Double peaks of the untreated and 2% koh samples were found in the vicinity of 100 and 500°C. In contrast, the 2% koh+bake sample had only single peak in the region of 580°C (Fig. 4). At high temperature, it is certainly that collagen in the rabbit’s bone could not be preserved; therefore, there was only one TSDC peak at the vicinity of 500°C. For the decalcified bone, a TSDC peak was found near the 100°C region. Similar polarization pattern of collagen portion of decalcified bone of various animal species had also been shown by Mascarenhas et al. [17]. The study in the decalcified bone also confirmed the polarizability of its collagen content. From these findings, it is conceivable that collagen is responsible for the polarization at around 100°C while the inorganic components are responsible at around 500°C.

Along with the already described phenomena regarding piezoelectric activity and streaming potential, this experiment could found evidences of polarization which enable electrical storage of the bone. Polarization of the bone could be induced even at 37°C, but not that of the HA. The study in decalcified bone could confirm that not only inorganic components but also collagen played a major role in this particular. However, the explanation for the polarization of bone at low temperature, but not of HA is very limited. The presence of residual CO3 or the structural orientation of the bone would be a fundamental of this induction. Electrical storages by polarization from the electrophysiological standpoint would be a result of mechanical stimulation important to remodeling of the bones which lead to the known phenomena. In terms of clinical applications, polarization induction of autografting bone would possibly enhance new bone formation around the implanted area of bone defects upon the effects of the osteogenic cells in bone remodeling [16].

In conclusion, our experiment could exhibit the phenomenon of polarization with certain capability of electrical storages in rabbit femurs. The bones could be polarized even under low temperature conditions, but this phenomenon did not occur with HA. In addition, both collagen and inorganic components could play significant roles in polarization in different temperature conditions.

ACKNOWLEDGMENTS. This study was partly supported by the Shiseido Female Researcher Science Grant, the Inamori Foundation, the Asahi Glass Foundation, a Grants-in-Aid from the Japan Society for the Promotion of Science (No. 21700485) and the Kazuchika Okura Memorial Foundation.
REFERENCES

1. Ahn, A. C. and Grodzinsky, A. J. 2009. Relevance of collagen piezoelectricity to “Wolff's Law”: a critical review. Med. Eng. Phys. 31: 733–741. [Medline] [CrossRef]

2. Bassett, C. A. L., Pawluk, R. J. and Becker, R. O. 1964. Effects of electric currents on bone in vivo. Nature 204: 652–654. [Medline] [CrossRef]

3. Bassett, C. A. L., Pawluk, R. J. and Pilla, A. A. 1974. Augmentation of bone repair by inductively coupled electromagnetic fields. Science 184: 575–577. [Medline] [CrossRef]

4. Bowen, C. R., Gittings, J. P., Turner, I. G., Baxter, F. and Chaudhuri, J. B. 2006. Dielectric and piezoelectric properties of hydroxyapatite-BaTiO3 composites. Appl. Phys. Lett. 89: 132906. [CrossRef]

5. Brighton, C. T., Friedenberg, Z. B., Mitchell, E. I. and Booth, R. E. 1977. Treatment of nonunion with constant direct current. Clin. Orthop. Relat. Res. 124: 106–123. [Medline]

6. Cerquiglini, S., Cignitti, M. and Marchetti, M. 1967. On the origin of electrical effects produced by stress in the hard tissues of living organisms. Life Sci. 6: 2651–2660. [Medline] [CrossRef]

7. Connolly, J. F. 1981. Selection, evaluation and indications for electrical stimulation of ununited fractures. Clin. Orthop. Relat. Res. 161: 39–53. [Medline]

8. Frost, H. M. 1983. A determinant of bone architecture: the minimum effective strain. Clin. Orthop. Relat. Res. 175: 286–292. [Medline]

9. Frost, H. M. 1987. Bone “mass” and the “mechanostat”: a proposal. Anat. Rec. 219: 1–9. [Medline] [CrossRef]

10. Frost, H. M. 1990. Skeletal structural adaptations to mechanical usage (SATMU): 1. Redefining Wolff's law: the bone modeling problem. Anat. Rec. 226: 403–413. [Medline] [CrossRef]

11. Frost, H. M. 1990. Skeletal structural adaptations to mechanical usage (SATMU): 2. Redefining Wolff’s law: the remodeling problem. Anat. Rec. 226: 414–422. [Medline] [CrossRef]

12. Fukada, E. and Yasuda, I. 1957. On the piezoelectric effect on bone. J. Phys. Soc. Jpn. 12: 1158–1162. [CrossRef]

13. Fukada, E. and Yasuda, I. 1964. Piezoelectric effects in collagen. Jpn. J. Appl. Phys. Soc. 3: 117–121. [CrossRef]

14. Isaacson, B. M. and Bloebaum, R. D. 2010. Bone bioelectricity: what have we learned in the past 160 years? J. Biomed. Mater. Res. A 95: 1270–1279. [Medline]

15. Itoh, S., Nakamura, S., Kobayashi, T., Shinomiya, K., Yamashita, K. and Itoh, S. 2006. Effect of electrical polarization of hydroxyapatite ceramics on new bone formation. Calcif. Tissue Int. 78: 133–142. [Medline] [CrossRef]

16. Kobayashi, T., Itoh, S., Nakamura, S., Nakamura, M., Shinomiya, K. and Yamashita, K. 2007. Enhanced bone bonding of hydroxyapatite-coated titanium implants by electrical polarization. J. Biomed. Mater. Res. A 82: 145–151. [Medline]

17. Mascarenhas, S. 1974. The electret effect in bone and biopolymers and the bound-water problem. Ann. N. Y. Acad. Sci. 238: 36–52. [CrossRef]

18. Nakamura, S., Takeda, H. and Yamashita, K. 2001. Proton Transport Polarization and depolarization of hydroxyapatite ceramics. J. Appl. Phys. 89: 5386–5392. [CrossRef]

19. Paterson, D. 1982. Clinical use of the osteostem, an implanted bone growth stimulation, for impaired bone healing. Instr. Course Lect. 31: 103–113. [Medline]

20. Ross, F. P. and Christiano, A. M. 2006. Nothing but skin and bone. J. Clin. Invest. 116: 1140–1149. [Medline] [CrossRef]

21. Sela, J., Gross, U. M., Kohavi, D., Shani, J., Dean, D. D., Boyan, B. D. and Schwartz, Z. 2000. Primary mineralization at the surface of implants. Crit. Rev. Oral Biol. Med. 11: 423–436. [Medline] [CrossRef]

22. Tofail, S. A. M., Haverty, D., Cox, F., Erhart, J., Hana, P. and Ryzhenko, V. 2009. Direct and ultrasonic measurements of macroscopic piezoelectricity in sintered hydroxyapatite. J. Appl. Phys. 105: 064103. [CrossRef]

23. Ueshima, M., Nakamura, S. and Yamashita, K. 2002. Huge, Milli-coulomb Charge storage in ceramic hydroxyapatite by bimodal electric polarization. Adv. Mater. 14: 591–595. [CrossRef]

24. Williams, W. S. and Breger, L. 1975. Piezoelectricity in tendon and bone. J. Biomech. 8: 407–413. [Medline] [CrossRef]

25. Yamashita, K., Okawa, N. and Umegaki, T. 1996. Acceleration and deceleration of bone-like crystal growth on ceramic hydroxyapatite by electric poling. Chem. Mater. 8: 2697–2700. [CrossRef]