BM-D-12-00477R1

Journal of Biomechanics (Original Article)

Changes in the viscoelastic properties of cortical bone by selective degradation of matrix protein

Hideki Shirakawa†, Kazuya Furusawa, Akimasa Fukui, Shigeru Tadano‡, and Naoki Sasaki*

Division of Advanced Interdisciplinary Science, Faculty of Advanced Life Science,

Hokkaido University, Sapporo, 060-0810, JAPAN

†Graduate School of Life Science, Hokkaido University, Sapporo, 060-0810, JAPAN

‡Division of Human Mechanical Systems and Design, Faculty of Engineering,

Hokkaido University, Sapporo, 060-8628, JAPAN

* Corresponding author

nasa5131@sci.hokudai.ac.jp

ABSTRACT

We have studied stress relaxation of bovine femoral cortical bone specimens treated with KOH aqueous solution which had been known to degrade selectively protein molecules in bone. With the KOH treatment, we found an increase in specimens' volume. This increase was regarded as swelling of the bone specimen, presumably due to matrix protein network degradation including that of collagen. In an analogy of bone to gel structure, an increasing ratio of specimen volume was used as an indicating parameter for the matrix protein network degradation by the treatment. Although an empirical equation with a linearly combined form of two Kohlrausch-Williams-Watts (KWW) functions has been shown to describe the stress relaxation of bone specimens, a single KWW function was suitable for the bone specimens treated with KOH solution for as little as 3 hours. In KOH treated specimens, both the initial modulus and the relaxation time decreased with the volume-increasing ratio, while the relaxation time distribution did not change. A chemo-rheological consideration attributed the reduction of modulus values to the network degradation in the organic matrix phase. The relaxation time of KOH treated specimens was thought to be related to the longer relaxation time of untreated bones, although there was a discontinuity between the extrapolated relaxation time values for KOH treated specimens and untreated specimens. This discontinuity may have originated from the release of residual stress existing in the bone by the matrix protein degradation. The results of the present study suggest that the state of matrix protein is crucial for integrating the mechanical properties of bone.

Keywords Viscoelasticity of bone/ Stress relaxation/ Relaxation time/ Matrix protein degradation/

Introduction

Bone has been often regarded mechanically as a composite material of hydrated organic matrix mainly composed of collagen and hydroxyapatite (HAp)-like mineral phase. It is thought that the pliant collagen is reinforced by stiff mineral particles, and, as a composite, the brittleness of the mineral is compensated for by the viscoelasticity of the collagen. Recently, the existence of non-collagenous glue proteins that connect mineralized collagen fibres has been revealed (Recker, 1992; Braidotti *et al*., 1997; Fantner *et al*., 2005). Because of the viscoelasticity of collagen fibres and non-fibrous proteins in the bone matrix, bone itself has noticeable viscoelasticity (Currey, 1965; Sasaki, 2000). As for the mechanical role of organic phase in bone, Ji and Gao (2004) predicted on the basis of the tension-shear chain model (Jäger and Frazl, 2000) that the organic phase endows bones with important characteristics such as crack shielding, and energy dissipation, that is, the strength and the toughness. From the result, it can be easily expected that a change in organic phase would affect the mechanical properties of bone, in particular its viscoelasticity. However, there have been not so many studies that treat detailed or thorough experimental results on the relationship between matrix protein degradation and the mechanical properties of bone. Wynnyckyj *et al*. studied the change in toughness of emu femoral bone after the selective degradation of bone collagen by KOH treatment (2009). Following the treatment their stress-strain curves indicated an increase in the elastic energy needed for bone destruction. As the increment was contributed mainly by the stress-strain curve after partial fracture, the toughness increase is considered as a mechanism caused by something like a sacrificial structure. In order to relate such a structural feature to the toughness of bone, direct measurements of viscoelasticity would provide useful information about the relationship. The aim of this study was to obtain the relationship be-

tween the state of matrix protein and the viscoelastic properties of bone. For this, we prepared bone specimens with variously degraded matrix proteins. As KOH is known to affect protein molecules without changing the mineral phase in bone (Abe *et al*., 1992; Wynnyckyj *et al*., 2009), we used KOH solution for the selective degradation of matrix protein in bone. We monitored the degree of degradation of collagen in bone by hydroxyproline assays (Reddy and Enwemeka, 1996) for a measure of matrix protein degradation.

In our previous papers, as a new empirical equation for the description of stress relaxation of cortical bone, we proposed that stress relaxation of cortical bone could generally be described by a linear combination of two Kohlraush-Williams-Watts (KWW) functions (Iyo *et al*., 2004; Iyo *et al*., 2006),

$$E(t) = E_0\{A\exp[-(t/\tau_1)^{\beta}]+(1-A)\exp[-(t/\tau_2)^{\gamma}]\}, \quad [0< A, \beta, \gamma <1] , \qquad (1)$$

where E_0 is the initial modulus value, $E(0)$. τ_1 and τ_2 ($>> \tau_1$) are characteristic times of the relaxation processes, A is the fractional contribution of the fast relaxation to the whole relaxation process, and β and γ are parameters describing the shape of the relaxation modulus. It has been revealed that the first term represents the relaxation in the collagen matrix in bone and the second term is related to the change in a higher-order structure of bone that is responsible for the anisotropic mechanical properties (Iyo *et al*., 2004). It seems to be possible to relate the viscoelastic properties and the hierarchical structure of bone by investigating these mechanical parameters. The expected change in mechanical properties of bone due to the degradation of matrix proteins would be quantified by the parameters in eq. 1.

We performed stress relaxation experiments on bovine femoral cortical bone for its viscoelasticity and analysed the results by determining the parameters in eq. 1. Changes in the relaxation modulus curve, parameters and relaxation spectrum with the degradation of matrix

protein were discussed based on the analogy of mechanical formation of bone to that of gels and network polymers, in particular in terms of the theory of chemo-rheology (Tobolsky, 1960). The changes in the viscoelastic properties of bone brought about by organic phase degradation may suggest an important role of matrix protein in integrating the mechanical properties of bone.

Materials and methods

Materials

The bone samples used in this study were obtained from the anterior area of the mid-diaphysis of 18-month-old bovine femoral cortical bone. Optical microscopic examination showed that all of the samples were generally plexiform but partly transformed into Haversian bone. The samples were cut using a diamond saw. The cut sections were shaped by emery paper under tap water into rectangular plates approximately 0.5 cm wide, 5.0 cm long and 0.1 cm thick. The longer edge of the specimen plate was parallel to the bone axis. The shaped specimens were set in a reaction vessel so that none of the six surfaces of a rectangular specimen would overlie the bottom of the vessel. Specimens were then treated with 0.85M KOH solution for periods of 3, 6, 12, 18 and 24 hours at 37^{o}C under continuous stirring. After the treatment, specimens were washed thoroughly with Ringer's solution. The degree of collagen degradation was determined by monitoring the hydroxyproline concentration in the KOH reactor solution (Wynnyckyj *et al.*, 2009 ; Reddy and Enwemeka, 1996). Details of the specimens are listed in Table 1.

Methods

The relaxation Young's modulus of the rectangular sample plate was measured by a three-point bending method. A Kyowa Electric Works (KEW) LTS-1K strain gauge transducer was used as the force sensor. The LTS-1K gauge was set on a Sigma Kouki Auto-microstage CTS-50X, and the auto-microstage was operated by a Sigma Kouki Co. Stage Controller Mark-12. By moving the microstage downward, the indenter set at the force sensor probe pushed the specimen plate at the center to cause bending deformation. The force sensor detected the recovering force of the specimen. According to the stress-strain curves of bovine femora in the literature, yield strains are around 0.6% (Reilly and Burstein, 1975). As for the yield strain for KOH treated bones, it was almost similar to those of untreated specimens (Wynnyckyj *et al*., 2009). In our experiments, a maximum strain less than 0.23% was applied within 0.03 sec. The relaxation modulus measurement was performed to a maximum time of 5×10^4 sec. All of the measurements were made in Ringer's solution at 37 ± 0.5^{o}C. The obtained relaxation modulus was analysed by fitting the empirical eq. 1 to the data. The mechanical effect by KOH treatment was discussed by observing the dependence of mechanical parameters in the equation on the degree of degradation of collagen. Parameter fitting was carried out using Gnuplot, a scientific graphing software program (Ver. 4.2, patchlevel 5, ©Thomas Williams, Colin Kelly and many others).

Results

Hydroxyproline assay

Figure 1 shows the weight ratio of collagen dissolved in the reactor solution against collagen in bone, [Dcol], as a function of reaction time. [Dcol] was estimated from the hydroxylproline concentration, [Hyp], in the solution (Wynnyckyj *et al*., 2009 ; Reddy and Enwemeka, 1996).

[Hyp] was determined by the hydroxylproline assay indicated above. The [Dcol] values for the initial two points, the reactions for 3 and 6 hours, were not different from those of controls and seemed to be below the detection limit of the assay, i.e., concealed by the background level. The increasing tendency of [Dcol] with reaction time thereafter is understandable. We also confirmed that dissolution of Ca^{2+} from bone specimen was not detected during the treatment with KOH solution.

After the KOH treatment, we found an increase in specimens' thickness, width, and then cross-sectional area. The width and thickness were measured after the soaking of bone specimens into Ringer's solution for 12 hours. These increased values of width and thickness were used for modulus calculation from the detected force values. Figure 2 shows the normalized cross-sectional area of specimen plotted against treatment time. The result implies that degradation of matrix proteins in bone brings about this increase. It is worth noting that the increase in the cross sectional area of treated specimens was almost completely reversible with soaking-drying- soaking cycles of specimens.

Stress relaxation

Figure 3 shows the relaxation Young's modulus curves of specimens for the indicated times of KOH treatment. The curve for bone specimens not treated with KOH (control) is also shown. This figure shows that the Young's modulus values decreased with KOH treatment. Moreover, significant changes were observed in the relaxation curves compared with that of the controls. This indicates that KOH treatment affects not only the modulus value but also the relaxation process itself. In order to evaluate the change more quantitatively, we fitted eq. 1 to the obtained relaxation data. Untreated specimens were described by eq. 1 with the pa-

rameter values listed in Table II, which are consistent with those in the literature (Iyo *et al*., 2004; Iyo *et al*., 2006). However, even for specimens treated for as little as 3 hours, a very small value for A in the order of 10^{-12} was needed for the successful fitting. This result indicates that for KOH treated bone specimens, an equation

$$E(t) = E_0 \exp\left[-\left(\frac{t}{\tau}\right)^{\delta}\right] \quad , \quad (0 < \delta < 1) \qquad (2)$$

can adequately describe the relaxation modulus data. We used eq. 2 for fitting instead. The parameters determined are listed in Table III. Considering equations 1 and 2, the former seems more universal than the latter, having more parameters. A satisfying fitting by eq. 1 with insufficient one by eq. 2 might be generally expected. In this case, eq. 1 did fit the data. However, obtained formula with infinitesimal parameter value as above should be actually pointing eq. 2.

Discussion

Matrix protein degradation

As shown in Figure 1, the deviation of [Dcol] values of specimens treated for 3 and 6 hours from that of untreated specimens was too small to be detected by the hydroxyproline assay. However, even though the increment in [Dcol] value is negligibly small, the mechanical properties of bone specimens after only 3 hours of treatment with KOH drastically changed. This fact indicates that for specimens treated for 3 hours and 6 hours changes that cannot be reflected on [Dcol] would be resulted. There are at least two types of matrix proteins; collagen fibers and non-collagenous proteins gluing mineralized collagen fibers. The former is armoured by mineral particles while the latter is connecting the formers. The non-collagenous

glue proteins are easily accessible to degrading agents. Then, specimens treated for 3 and 6 hours, non-collagenous glue proteins could mainly degraded and collagen molecules would be degraded after 12 hours of treatments as [Dcol] significantly increased. Ji proposed a mechanical importance of interface between mineral and organic phase (Ji, 2008). There might be also a possibility that during early stage of KOH treatment the interface would mainly degraded. But, geometrical accessibility discussion above could eliminate the possibility.

Jäger and Fratzl proposed a structural mechanical model at the nanoscale for bone, taking account of the geometrical arrangement of collagen matrix and mineral particles in mineralized collagen fibers (Jäger and Fratzl, 2000). Forces are transferred in a zigzag manner by both collagen matrix and mineral particles. Gao discussed thoroughly such a staggered (a tension-shear-chain) model as representing the optimum structure of biological composite materials (Ji and Gao, 2004; Gao, 2006) and similar staggered model at the different level of bone's hierarchical structure has been applied to elucidate the mechanical characteristics (Gupta, 2006). Based on the knowledge that the organic phase in bone is continuous and that force is carried by both organic phase and mineral (Lees, 1979; Gao *et al*., 2003), a gel-like structural mechanical model of bone can be analogically considered: paths of load in bone collagen and non-collagenous protein correspond to inter cross-link chains in gel and mineral particles to cross-link points. On the basis of this model, the increase in bone cross-sectional area observed can be considered similar to the increase in degree of swelling of a gel; the matrix protein degradation in bone corresponds to the network disconnection in gels, where the matrix protein degradation brings about a matrix protein network swelling, *i. e*. bone swelling. As the increase in specimen cross-sectional area can be clearly and reproducibly observed for specimens treated with KOH even for only 3 hours, the value

$$q \equiv \left(\frac{A}{A_0} \right)^{3/2}, \qquad (3)$$

is used as the measure of matrix protein degradation, where A_0 is the cross-sectional area for the specimen before KOH treatment and A is that after the treatment. q defined in eq. 3 represents the swelling ratio of bone specimen. In the case of a typical gel system, according to Flory and Rehner (1943), such an increase in the swelling ratio underlies a decrease in the cross-link density of the gel. There is only a small space for water in native bone other than pre-existing pores; the absorption isotherm of native bone was revealed as the Langmuir type and water fraction at the plateau region of the isotherm was 0.1 (g/g dry weight) (Sasaki and Enyo, 1995). However, by the collagen and the non-collagenous glue protein degradation, water could penetrate into bone and build pools of water. This process may be observed as the swelling of bone after the treatment.

Collagen Degradation and Modulus Values

Figure 4 shows the q dependence of initial modulus value, E_0. Young's modulus $E(t)$ was estimated by

$$E(t) = \frac{FL^3}{4ab^3d}, \qquad (4)$$

where F is the observed force, L the inter fulcrum distance of the three point bending tester, a the width, b the thickness of the specimen, and d the deflection of the specimen at the center (Ferry, 1980). As indicated in the results section, specimens increased in volume with treatment time. The relaxation modulus values in Figure 3 and E_0 values in Figure 4 were calculated by using the increased width, a, and thickness, b, values. The observed reduction in

modulus values in these Figures would be caused by the increases in a and b with the KOH treatment. In Figure 4, filled circles represent values

$$[ab^3/a_0b_0^3]\,E_0=r(ab^3)E_0 \qquad (5)$$

where a_0 and b_0 are respectively width and thickness values before treatment, then $r(ab^3)$ is the increasing ratio of the moment of inertia of the cross sectional area. Eq. 5 compensates for the change in the moment of inertia of the cross-sectional area of KOH treated specimens. However, $r(ab^3)E(t)$ values are still reduced with q after the compensation as

$$r(ab^3)E_0=185\exp(-2.619\,q), \qquad (R^2=0.9936). \qquad (6)$$

It is known that the mechanical properties of bone are affected by Ca^{2+} elimination (Gustafson *et al*., 1996; Sasaki *et al*., 2008). We measured the Ca^{2+} concentration of the reactor solution of KOH treatment and did not detect even a trace of Ca^{2+}. Thus, the reduction in $r(ab^3)E_0$ is concluded not to be due to an elimination of mineral during KOH treatment. The relaxation modulus value was found to decrease as an exponential function of q. In gels and network polymers, the characteristic feature of the stress relaxation is a relaxation originating from segment motion among cross-link points followed by a gel plateau (Anseth *et al*., 1996). Stress relaxations observed for bone and KOH treated bones are considered to correspond to the gel plateau region in gels and network polymers because the process changed with the matrix protein degradation. Based on the analogy between bone structure and that of gels, this relation, eq. 6, is reminiscent of the stress relaxation of natural rubber at high temperatures. Natural rubber has a network structure that causes rubber elasticity. When incubated at more than 100^{o}C, part of the network structure is degraded. According to Tobolsky, the relaxation modulus value of incubated rubber reduces with the exponential function of the network degradation proceeding linearly with time (Tobolsky, 1960). In the case of bone, applying the

chemo-rheological consideration, a load-carrying structure will be partly destroyed by the KOH treatment in the organic phase. This could lead to the reduction in $r(ab^3)E_0$ value with q as eq. 6.

Collagen Degradation and the Relaxation Process

In bone stress relaxation, the most remarkable event observed for treated specimens could be described by the change in the relaxation modulus function from eq.1 to eq.2. Untreated bone specimens had two distinct relaxation processes. After 3 hours of treatment, the relaxation modulus became to contain only one process. Figures 5 and 6 show the q dependencies of relaxation time, τ, and relaxation time distribution, δ, respectively. τ decreased with q rapidly at first but levelled off later. At the same time, the distribution of relaxation time became broad: from $\delta \sim 0.5$ for untreated bone to 0.3 for KOH treated specimens. Reduction of the relaxation time has been reported for gels and network polymers with the decrease in cross-link density (Scholtens and Booij, 1980; Lee and McKenna, 1988; Thirion and Casset, 1970; Levelut *et al*., 1996; Heinrich and Vilgis, 1992; Liu *et al*., 2009; Curro and Pincus, 1998). The decrease in τ can be explained as follows: with the decrease in crosslink density, constraints on the molecular motion of network chains in rubber and polymers could decrease, and then the average relaxation time of the chains would decrease. The decrease in τ with the decrease in crosslink density have been reported to be described by an exponential function of q (Thirion and Chasset, 1970; Levelut, *et al*. 1996) or a power law of function of q (Heinrich and Vilgis, 1992; Liu *et al*., 2009; Curro and Pincus, 1998) depending on the link structure in gels and network polymers.

In the case of bone, as the relaxation time decreased with progressive degradation of

matrix protein, the relaxation time after the KOH treatment should be related to τ_2 in untreated specimens. The reduction in τ along with the matrix protein degradation is thought to be caused by the decreasing constraints on the molecular motion of matrix proteins in bone. The remarkable reduction in τ is observed for specimen treated for 3 and 6 hours. This indicates that the decrease in τ is related to the degradation of non-collagenous protein gluing mineralized collagen fibers as discussed above.

In our result for KOH treated bone specimens, τ could be well-described by both an exponential function and the power law relation of q,

$$\tau = 5.12\times10^{5} q^{-33.3} + 3.98\times10^{4} \qquad (R^2=0.999) \qquad (7)$$

$$\tau = 5.09\times10^{5} \exp(-32.5q) + 4.02\times10^{4} \qquad (R^2=0.999). \qquad (8)$$

In both cases, however, for a successful fitting, we needed an equilibrium relaxation time τ_e of the order 10^4 sec for both the exponential function and the power law relation. The requirement for τ_e indicates the existence of a certain limit of relaxation time reduction. As the constraint decrease in the matrix with the protein degradation is thought to cause the reduction in τ, this limitation may be a breakdown of the integration of bone structure by organic components. The obtained values for τ_e would indicate the characteristic time for an irreversible rearrangement of mineralized collagen fibers without gluing proteins.

In contrast, at $q\rightarrow1$, τ had a value of 5×10^5 sec in both cases. This value $\tau=5\times10^5$ sec is much smaller than $\tau_2=1.42\times10^7$ sec, the longer relaxation time of the untreated bone specimens. The inconsistency of relaxation time suggests that there is a discontinuity in the states of a bone between untreated and protein-degraded, presumably the degradation of non-collagenous glue proteins. After experiencing the discontinuity, the state of bone would not change as was reflected on the constancy in δ, that is, the discontinuous change is irre-

294 versible. The existence of residual stress in bone was recently demonstrated (Yamada *et al*.,
295 2011). This means that bone is integrated in a pre-stressed state. Following a loss of continu-
296 ity in bone structure, this pre-stressed state can be released. Selective, systematic elimination
297 of minerals from bone resulted in an applicability of the time-mineral content, ϕ_m, reduction
298 to stress relaxation curves (Sasaki and Yoshikawa, 1993). The shift factor there, however, was
299 a continual function of ϕ_m from untreated bone to slightly demineralized bone. These experi-
300 mental results of destruction of bone structure by matrix protein degradation in the present
301 work and mineral elimination by Sasaki and Yoshikawa (1993) show the importance of or-
302 ganic matrix in integrating the bones' mechanical construction.
303

304 **Conflict of interest statement**

305 The authors do not have any conflict of interest about the material within the manuscript
306 submitted.
307

308 **Acknowledgements**

309 A part of this work was supported by the Grant-in-Aid for Scientific Research (A) (No.
310 19200035) and (C) (No. 21500401) both from JSPS.
311

References

Abe, K., Hashizume, H., Ushiki, T. (1992) An EDTA-KOH method to expose bone cells for scanning electron microscopy. *Journal of Electron Microscopy* **41,** 113-115.

Anseth, K. S., Bowmann, C. N., Brannon-Peppas, L. (1996) Mechanical Properties of hydrogels and their experimental determination. *Biomaterials* **17**, 1647-1657.

Braidotti, P., Branca, F. P., Stagnit, L., (1997) Scanning electron microscopy of human cortical bone failure surfaces. *Journal of Biomechanics* **30**, 155-162.

Currey, JD. (1965) Anelasticity in bone and echinoderm skeletons. *Journal of Experimental Biology* **43**, 279-292.

Curro, J. G., Pincus, P. (1998) A theoretical basis for viscoelastic relaxation of elastomers in the long-time limit. *Macromoleculs* **16**, 559-562.

Ferry, J. D. (1980) Viscoelastic properties of polymers. Third Edition, John Wiley & Sons, New York, Chapter 7.

Flory, P. J., Rehner, J., Jr. (1943) Statistical mechanics of cross-linked polymer networks II. Swelling. *Journal of Chemical Physics* **11**, 521-526.

Fanter, G., Hassenkam, T., Kindt, J., Weaver, J. C., Birkedal, H., Pechenik, L., Cutroni, J. A., Cidade, G. A. G., Stucky, G. D., Morse, D. E., Hansma, P. K. (2005) Sacrificial bonds and hidden length dissipate energy as mineralized fibrils separate during bone fracture. *Nature Materials* **4**, 612-615.

Gao, H. (2006) Application of fracture mechanics concepts to hierarchical biomechanics of bone and bone-like materials. *International Journal of Fracture* **138**, 101-137.

Gao, H., Ji, B., Jager, I. L., Arzt, E., Fratzl, P. (2003) Materials become insensitive to flaws at nanoscale: Lessons from nature. *Proceedings of National Academy of Science* U. S. A. **100**,

5597-5600.

Gupta, H. S., Seto, J., Wagermaier, W., Zaslansky, P., Boesecke, P., Fratzl, P. (2006) Cooperative deformation of mineral and collagen in bone at the nanoscale. *Proceedings of National Academy of Science* U. S. A. **103**, 17741-17746.

Gustafson, M. B., Martin, R. B., Gibson, V., Stroms, D. H., Stover, S. M., Gibeling, J., Griffin, L. (1996) Calcium bufferingis required to maintain bone stiffness in saline solution. *Journal of Biomechanics* **29**, 1191-1194.

Heinrich, G., Vilgis, T. A. (1992) Long-time relaxation of polymer networks. *Macromolecules* **25**, 404-407.

Iyo, T., Sasaki, N., Maki, Y., Nakata, M. (2006) Mathematical description of stress relaxation of bovine femoral cortical bone. *Biorheology* **43**, 117-132.

Iyo, T., Maki, Y., Sasaki, N., Nakata, M. (2004) Anisotropic viscoelastic properties of cortical bone. *Journal of Biomechanics* **37**, 1433-1437.

Jäger, I., Fratzl, P. (2000) Mineralized collagen fibrils : A mechanical model with a staggered arrangement of mineral particles. *Biophysical Journal*. **79**, 1737-1746.

Ji, B. (2008) A study of the interface strength between protein and mineral in biological materials. *Journal of Biomechanics* **41**, 259-266.

Ji, B., Gao, H. (2004) Mechanical properties of nanostructure of biological materials. *Journal of Mechanics and Physics of Solids* **52**, 1963-1990.

Lee, A., McKenna, G. B. (1988) Effect of crosslink density on physical ageing of epoxy networks. *Polymer* **29**, 1812-1817.

Lees, S. (1979) A model for the distribution of HAP crystallites in bone—An hypothesis. *Calcified Tissue International* **27**, 53-56.

Levelut, C., Scheyer, Y., Boissier, M., Pelous, J., Durand, D. (1996) A Brillouin scattering investigation of relaxation versus crosslink density in glass- and gel-forming polymers. Journal of Physics : *Condensed Matter* **8**, 941-957.

Liu, J., Cao, D., Zhang, L. (2009) Static and dynamic properties of model elastomer with various cross-link densities ; A molecular dynamic study. *Journal of Chemical Physics* **131**, 034903 1-10.

Recker, R. R., (1992) Embryology, anatomy, and microstructure of bone. In *Disorders of bone and mineral metabolism*. (Edited by Coe, F. L. And Favus, M. J.) 219-240, Raven Press, New York.

Reddy, G. K., Enwemeka, C. S. (1996) A simplified method for the analysis of hydroxyproline in biological tissues. *Clinical Biochemistry* **29,** 225-229.

Reilly, D. T., Burstein, A. H. (1975) The elastic and ultimate properties of compact bone tissue. *Journal of Biomechanics* **8**, 393-405.

Sasaki, N., Nozoe, T., Nishihara, R., Fukui, A. (2008) Effect of mineral dissolution from bone specimens on the viscoelastic properties of cortical bone. *Journal of Biomechanics* **41**, 3511-3514.

Sasaki, N. (2000) Viscoelastic properties of bone and testing method. In *Mechanical testing of bone and the bone-implant interface*. (Edited by An, Y. H. and Draughn, R. A.) Boca Raton FL: CRC Press; p. 329-348.

Sasaki, N., Enyo, A. (1995) Viscoelastic properties of bone as a function of water content. *Journal of Biomechanics* **28**, 809-815.

Sasaki, N., Yoshikawa, M. (1993) Stress relaxation in native and EDTA-treated bone as a function of mineral content. *Journal of Biomechanics* **26**, 77-83.

381 Sasaki, N., Nakayama, Y., Yoshikawa, M., Enyo, A. (1993) Stress relaxation function of bone
382 and bone collagen. *Journal of Biomechanics* **26**, 1369-1376.
383 Scholtens, B. J. R., Booij, H. C. (1982) Approximative method for the evaluation of
384 equilibrium moduli of relaxing rubber networks. In *Elastomers and rubber elasticity*.
385 (Edited by Mark, J. E. and Lai, J.) Amer. Chem. Soc., Wasington D. C., pp. 517-532.
386 Scholtens, B. J. R., Booij, H. C. (1980) Time-temperature superposition and linear
387 viscoelastic behavior of EPDM networks with various degree of crosslinking. *Polymer*
388 *Bulletin* **3**, 465-471.
389 Thirion, P., Chasset, R. (1970) Recent observations on the mechanical properties of polymer
390 networks. *Pure and Applied Chemistry* **23**, 183-199.
391 Tobolsky, A. V. (1960) Properties and structure of polymers, John Wiley & Sons, New York,
392 Chap. 5.
393 Wynnyckyj C, Omelon, S, Savage, K, Chachra, D, Grympas, MD. (2009) A new tool to
394 assess the mechanical properties of bone due to collagen degradation. *Bone*. **44,** 840-848.
395 Yamada, S., Tadano, S., Fujisaki, K. (2011) Residual stress distribution in rabbit limb bones.
396 *Journal of Biomechanics* **44**, 1285-1290.
397

Captions to Figures

Figure 1. The weight-by-weight ratio of dissolved collagen molecules by the KOH treatment against the amount of collagen in untreated bone specimen plotted against the treated time. The amount of dissolved collagen was estimated from hydroxyproline in the reacter solution.

Figure 2. The ratio of cross sectional area of specimens after KOH treatment swollen with Ringer's solution against that before the treatment plotted against the treated time.

Figure 3. Relaxation Young's modulus of bone specimens treated for 3 hours (○), 6 hours (●), 12 hours (△), 18 hours (▲), and 24 hours (□). Vertical lines on the curves represent the standard errors. Relaxation Young's modulus for untreated specimen was also plotted as a control (×).

Figure 4. Initial Young's modulus values, E_0, determined by fitting plotted against the swelling ratio. Measured value (○) and compensated values by the inertia of moment of the cross section of each specimen (●). E_0 value for untreated specimen was also plotted (△).

Figure 5. Relaxation time, τ, in eq. 2 plotted against the swelling ratio. Vertical lines represent the standard errors. The decay in τ with q can be described by a

power law relation, eq. 7, (-------) or an exponential decay, eq. 8, (———).

Figure 6. Stretched exponent value, δ, in eq. 2 plotted against the swelling ratio. The horizontal line indicates the stretched exponent value of the slow process, γ, in eq. 1 for untreated specimens.

Figure 1

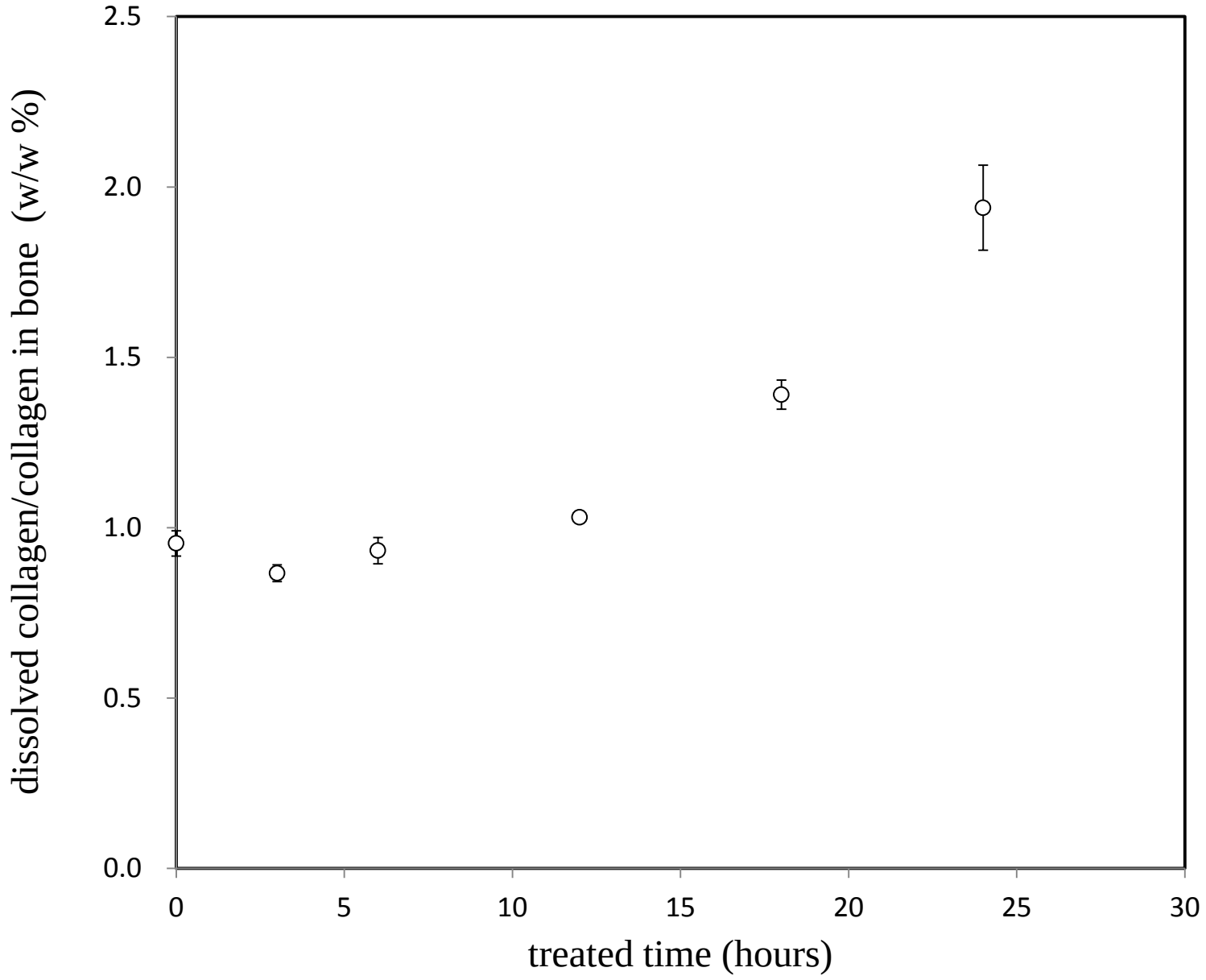
dissolved collagen/collagen in bone (w/w %)
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
treated time (hours)

Figure 2

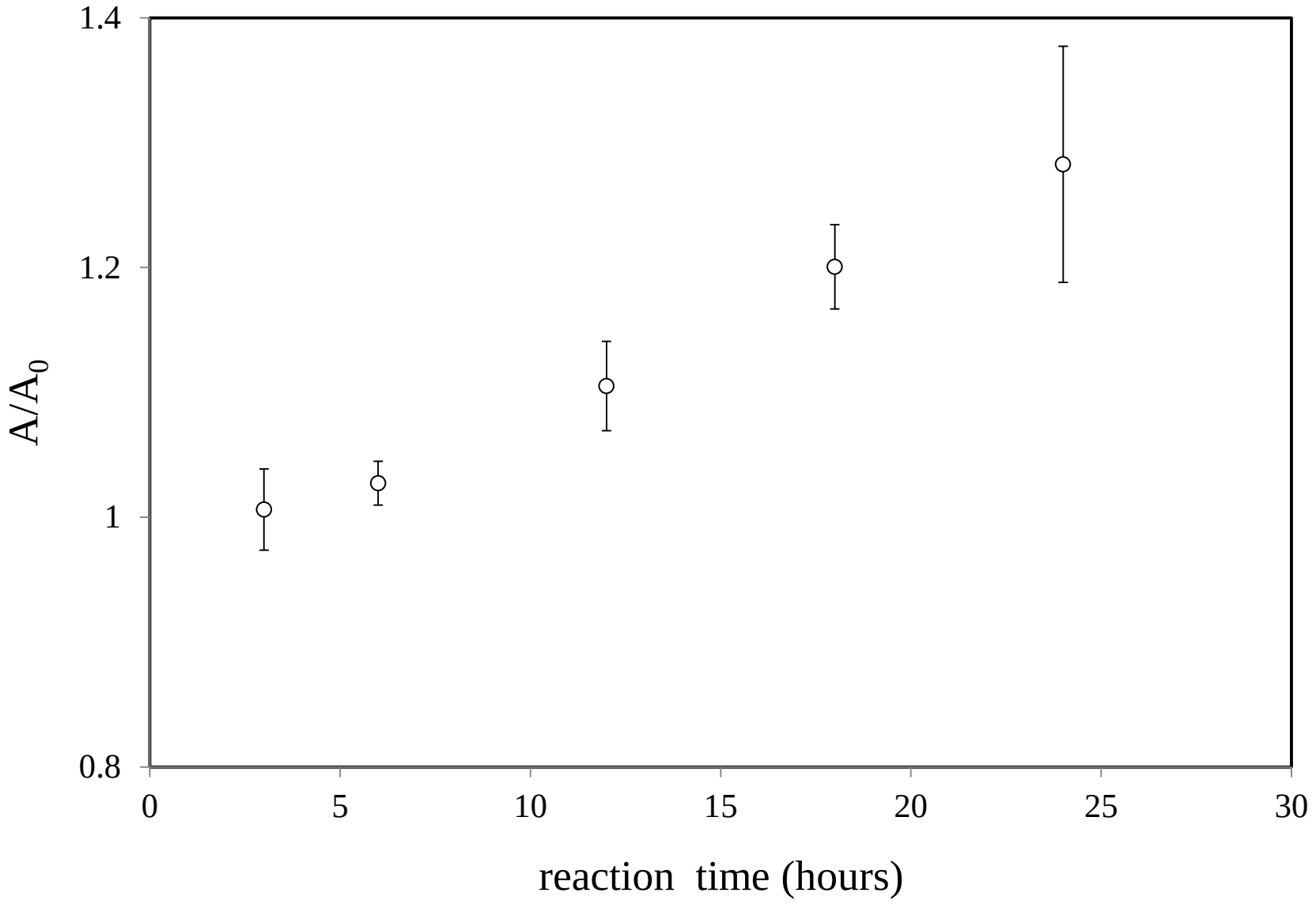

Figure 3

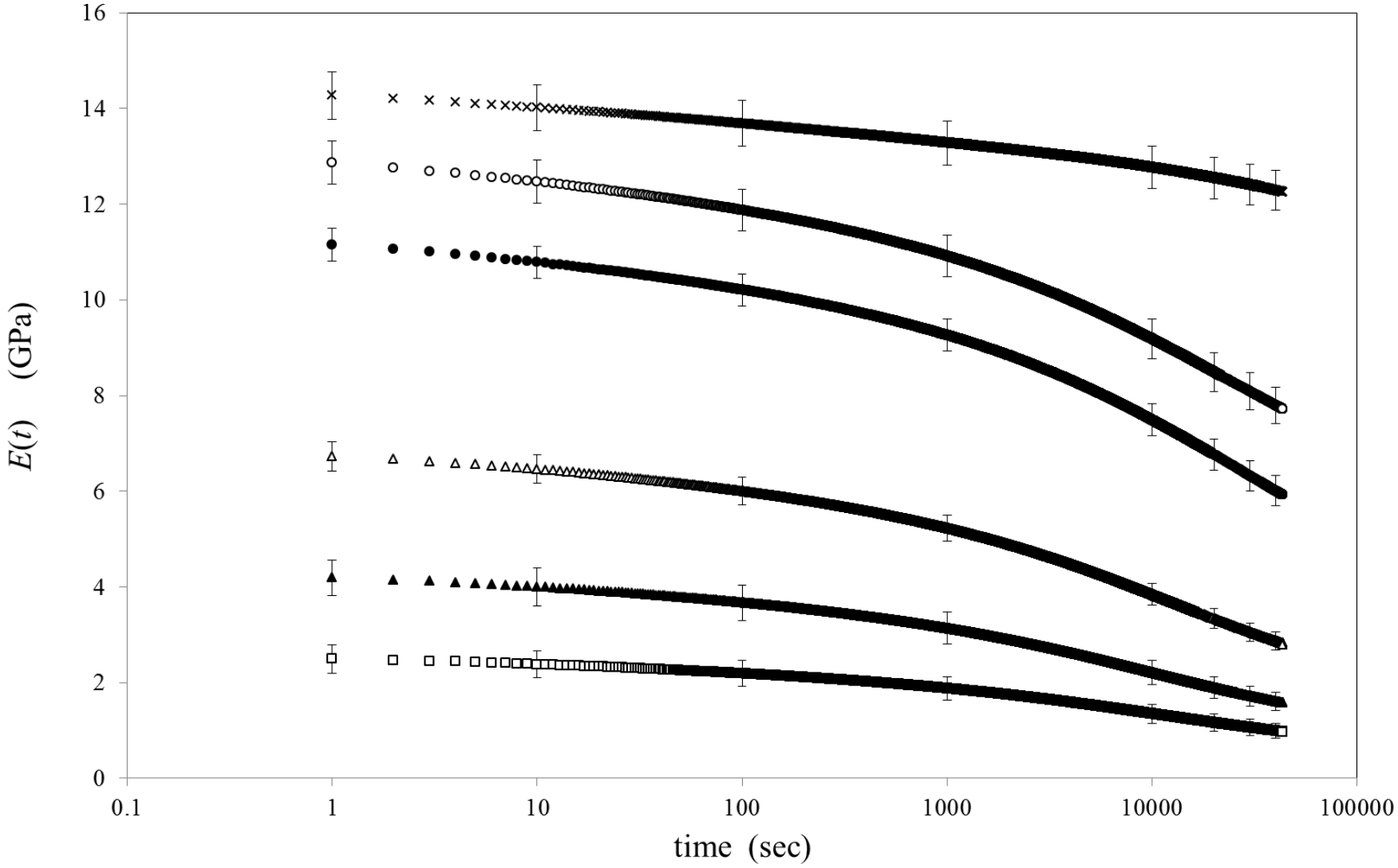

Figure 4

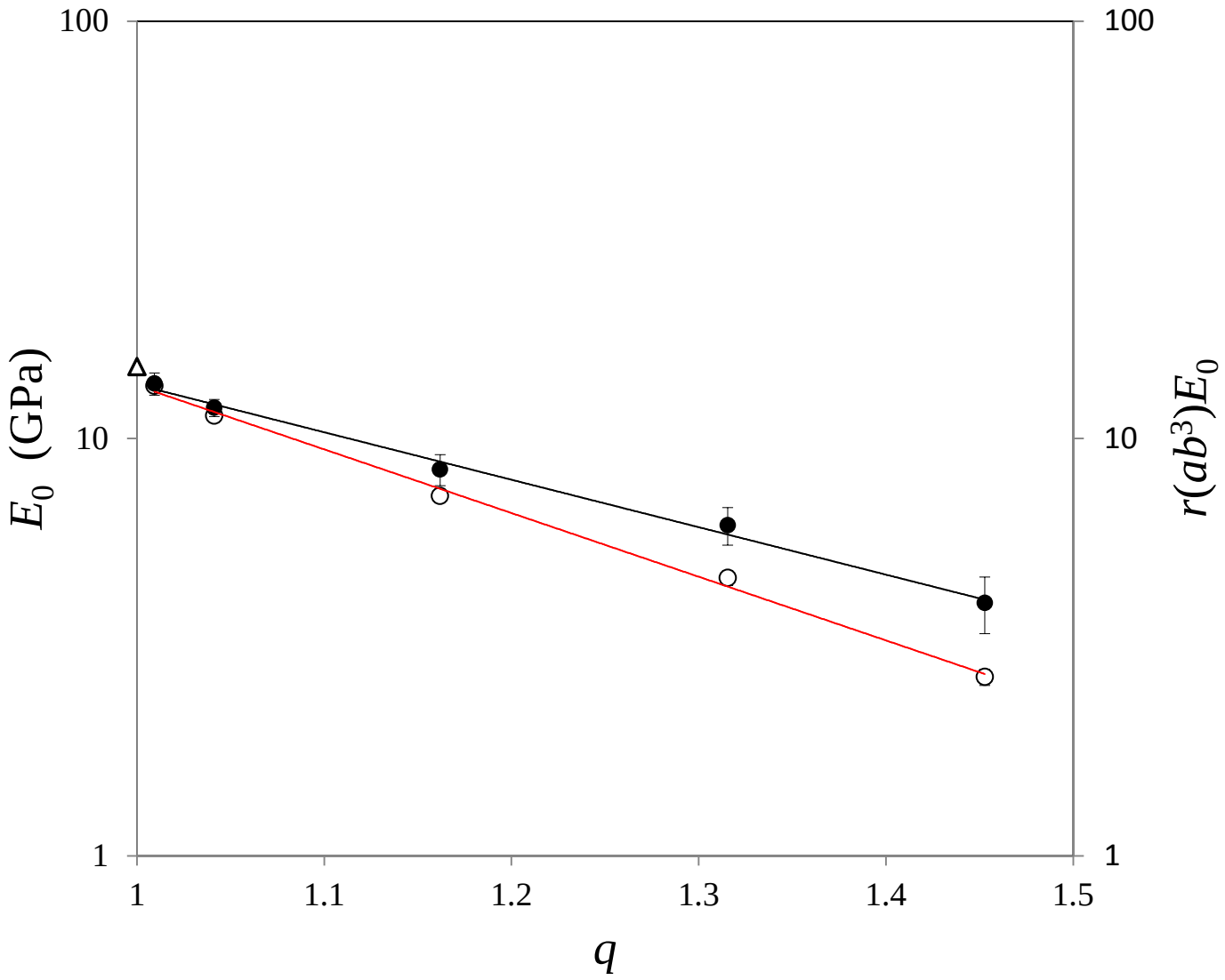

Figure 5

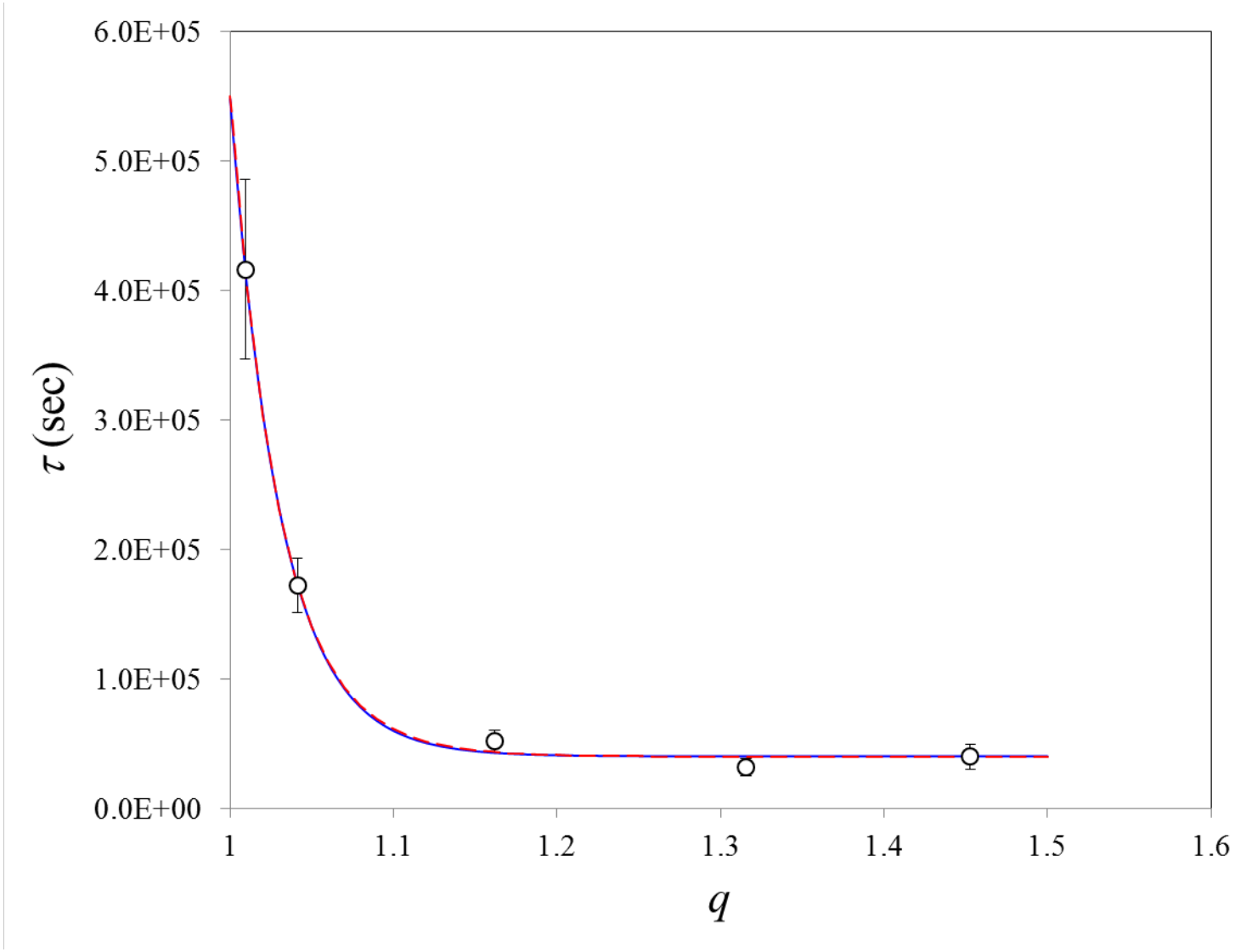
6.0E+05
5.0E+05
4.0E+05
3.0E+05
2.0E+05
1.0E+05
0.0E+00
τ (sec)
1
1.1
1.2
1.3
1.4
1.5
1.6
q

Figure 6

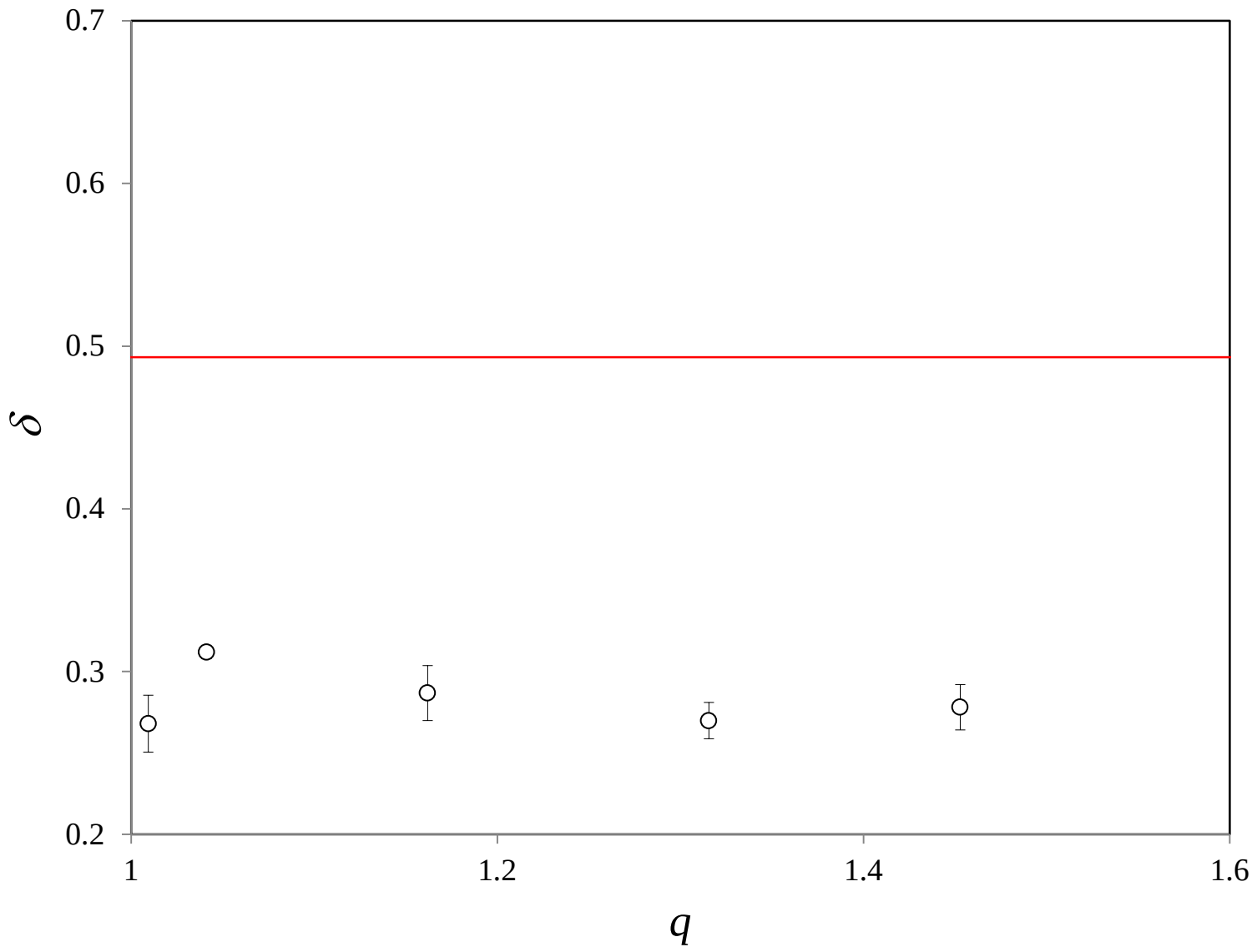

Table I. Detaile of bovine cortical bone specimens.

treatment time	sample size	specimen size before the treatment		A/A_0 *
		width (mm)	thickness (mm)	
control	8	4.80 ±0.27	1.27 ±0.04	1
3 hours	5	4.87 ±0.08	1.26 ±0.01	1.01 ±0.01
6 hours	5	4.90 ±0.05	1.27 ±0.01	1.03 ±0.01
12 hpurs	5	4.83 ±0.03	1.26 ±0.02	1.11 ±0.04
18 hours	5	4.85 ±0.04	1.26 ±0.02	1.20 ±0.04
24 hours	5	4.87 ±0.03	1.26 ±0.02	1.28 ±0.10

∗ A_0 and A are cross-sectional area values of speciemns before and after the treatment, respectively. The ratio was calculated for each specimen and then averaged.

Table II. Mechanical parameters in eq. 1 for untreated specimens.

E_0(GPa)	A	τ_1(sec)	β	τ_2(sec)	γ
14.8 ±0.5	0.12 ±0.01	117 ±24	0.27 ±0.05	$(1.42\pm0.5)\times10^{7}$	0.49 ±0.03

Table III. Mechanical parameters in eq. 2 for KOH treated specimens.

specimen treatment time	E_0(GPa)	τ(sec)	δ
3 hours	13.4 ±0.4	$(4.2\pm0.7)\times10^5$	0.27 ±0.02
6 hours	11.3 ±0.3	$(1.7\pm0.2)\times10^5$	0.312 ±0.004
12 hours	7.3 ±0.4	$(5.2\pm0.9)\times10^4$	0.29 ±0.02
18 hours	4.6 ±0.4	$(3.2\pm0.7)\times10^4$	0.27 ±0.01
24 hours	2.7 ±0.3	$(4.0\pm1.0)\times10^4$	0.28 ±0.01