Monitoring of collagen shrinkage by use of second harmonic generation microscopy

Sung-Jan Lin[1,2], Jau-Shiuh Chen[2], Wen Lo[3], Yen Sun[3], Wei-Liang Chen[3], Jung-Yi Chan[1,4], Hsin-Yuan Tan[2,5], Wei-Chou Lin[6], Chih-Jung Hsu[1], Tai-Horng Young[2], Shiou-Hwa Jee[1], Chen-Yuan Dong[3]

1. Department of Dermatology, National Taiwan University Hospital and College of Medicine, Taipei, Taiwan.
2. Institute of Biomedical Engineering, National Taiwan University, Taipei, Taiwan
3. Department of Physics, National Taiwan University, Taipei, Taiwan.
4. Department of Dermatology, Cathay General Hospital, Taipei, Taiwan
5. Department of Ophthalmology, Chang Gung Memorial Hospital, Linko, Taiwan
6. Department of Pathology, National Taiwan University Hospital, Taipei, Taiwan

Abstract

Thermal treatment induced collagen shrinkage has a great number of applications in medical practice. Clinically, the there is lack of reliable non-invasive methods to quantify the shrinkage. Overt treatment by heat application can lead to devastating results. We investigate the serial changes of collagen shrinkage by thermal treatment of rat tail tendons. The change in length is correlated with the finding in second harmonic generation microscopy and histology. Rat tail tendon shortens progressively during initial thermal treatment. After a certain point in time, the length then remains almost constant despite further thermal treatment. The intensity of second harmonic generation signals also progressively decreases initially and then remains merely detectable upon further thermal treatment. It prompts us to develop a mathematic model to quantify the dependence of collagen shrinkage on changes of SHG intensity. Our results show that SHG intensity can be used to predict the degree of collagen shrinkage during thermal treatment for biomedical applications.

Keywords: second harmonic generation, collagen, thermal, shrinkage, denaturation

Optical Interactions with Tissue and Cells XVII, edited by Steven L. Jacques, William P. Roach,
Proc. of SPIE Vol. 6084, 60840S, (2006) · 1605-7422/06/$15 · doi: 10.1117/12.647087

Address correspondence to: JSC cjs0088@yahoo.com.tw and CYD cydong@phys.ntu.edu.tw

1. Introduction

Various clinical procedures aimed at shrinking collagen have been achieved by thermal treatments.[1-3] Advances in the heating modalities, such as the use of various types of laser, intense pulse light, and radiofrequency, have led to their applications in anti-aging therapies.[3-11] Without real-time monitoring of the collagen denaturation process from thermal treatment, identification of appropriate adjustable clinical parameters will continue to be based on trial-and-error practice and years of accumulative experience.[12] Hence, there is a need for a monitoring tool to quantify the effects heating parameters on treatment results.

Collagen I is the most abundant protein in various tissues. Structurally, it is a triple helix, composed of three polypeptide chains, each of approximately 300 nm in length.[12,13] It has been shown that collagen denatures under thermal treatment and the denaturation is accompanied by a gross shrinkage.[14-17]

With the advent of imaging technology such as reflectance confocal microscopy and optical coherence tomography, non-invasive tissue imaging with enhanced resolution in the clinical setting has become possible.[18-20] Recently, the non-linear optical effect of second harmonic generation (SHG) has been extensively used in biomedical imaging.[21-28] Biological structures lacking a central symmetry are effective in generating SHG signals. Unlike fluorescence excitation, no molecular transition is involved in the generation of the second harmonic signal. Therefore, photo-damage is significantly reduced. Collagen is an effective SHG generator, and transitions of structures can be monitored by SHG signals.[21-24,27,29] We have also shown that SHG can be used to monitoring the structural transition of collagen at various temperatures and also the quantification of dermal changes associated with photoaging.[30,31]

In this work, we obtained the serial SHG intensities from rat tail tendon under thermal treatment of 58^{o}C for 0 to 12 mins. The shrinkage of rat tail tendon from the same thermal treatment is also measured.

2. Materials and methods

The second harmonic imaging system used in this study is a modified version of

a home-built laser scanning microscopic imaging system based on an upright microscope (E800, Nikon, Japan) described previously.[31] The 760 nm output of the ti-sa laser is scanned in the focal plane by a galvanometer-driver x-y mirror scanning system (Model 6220, Cambridge Technology, Cambridge, MA). An oil immersion objective (S Fluor 40x, NA 1.3, Nikon) is selected for SHG microscopy. To ensure even excitation of collagen fibers at different orientations, a $\lambda/4$ wave plate is used to convert the linearly polarized ti-sa laser beam into one with circularly polarization. The generated SHG signal is then collected in the back-scattering geometry where the dichroic mirror, a short-pass filter (E680SP, Chroma Technology), and a 380 nm bandpass filter (HQ380/20, Chroma Technology) are used to isolate the SHG signal. The signal photons are processed by a single-photon counting PMT (R7400P, Hamamatsu, Japan) and a home-built discriminator.

Rat tail tendon is chosen because the organization of rat-tail tendon is relatively simple, consisting of bundles of parallel collagen fascicles.[26,30] Tail tendons of a Winstar rat are acquired and cut into strips 3-cm in length and placed in PBS buffer prior to being subjected to 58^{o}C thermal baths. The temperature is selected because collagen denaturation at this temperature is observed in our previous work.[31] The periods of thermal treatment range from 0 to 12mins with a 90-second increment. At the end of thermal treatment, the tendon specimens are removed and the length of each specimen is measured. Then, the specimens are mounted on a glass slide and covered with a No. 1.5 cover glass for viewing. We acquired second harmonic signals and images of the tendons with different periods of thermal treatment. Five randomly chosen large area scans composed of 6 by 6 array of neighboring SHG images are acquired and assembled in each specimen. The average SHG signal per pixel is computed and plotted. After the imaging process is completed, the specimen is fixed in buffered formalin solution (10% in PBS) and further processed for histological examination (hematoxylin and eosin stains).

3. Result

Shown in **Figure 1** are the serial changes of length of rat tail tendon along its longitudinal axis after thermal treatment for 0, 1.5, 3, 4.5, 6, 7.5, 9, 10.5, and 12 mins. Rat tail tendon progressively shortens from 0 to 9 mins. However, beyond 9 mins of thermal treatment, the length of rat tail tendons remains almost constant. This suggests that there is a limit to which thermal treatment can be used to shrink collagen

fibers.

The corresponding changes of SHG intensity during thermal treatment are shown in **Figure 2.** Interestingly, SHG intensities also show a linear trend of decrease from 0 to 9 mins of thermal treatment. The SHG intensities are almost undetectable at 9 mins and remains at an undetectable baseline value upon further thermal treatment. Hence, the collagen structure in rat tail tendons responsible for SHG is completely disrupted after thermal treatment at 58^{o}C for 9 mins.

By comparing **Figure 1** and **Figure 2**, several interesting results can be obtained. Both shrinkage of collagen fibers and decrease in overall SHG intensities show a trend of linear dependence on duration of initial thermal treatment up to 7.5 mins. After heating the samples for 9 mins and beyond, SHG diminishes to nearly undetectable levels. At the same time, collagen fibers also shrink to a terminal length. Further thermal treatment beyond 9 mins fails to shorten collagen fibers to the extent caused by shorter thermal treatment periods. This result suggests that collagen shrinkage by thermal treatment can not be efficiently achieved after the collagen structure responsible for SHG is completely disrupted.

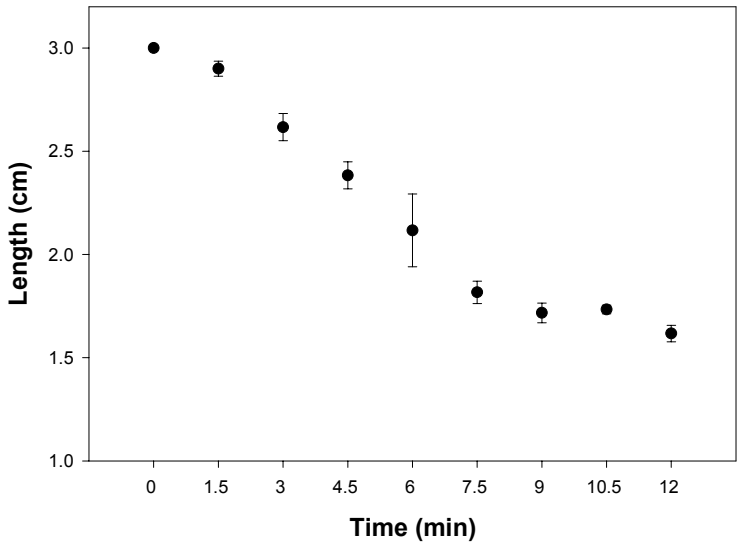

Figure 1. Dependence of lengths of rat tail tendon on duration of thermal treatment at 58^{o}C.

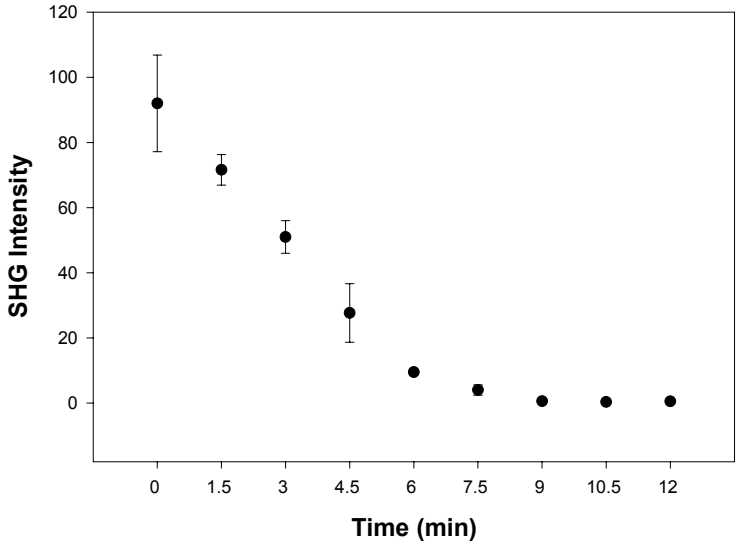

Figure 2. Dependence of of SHG intensities of rat tail tendon on duration of thermal treatment at 58^{o}C.

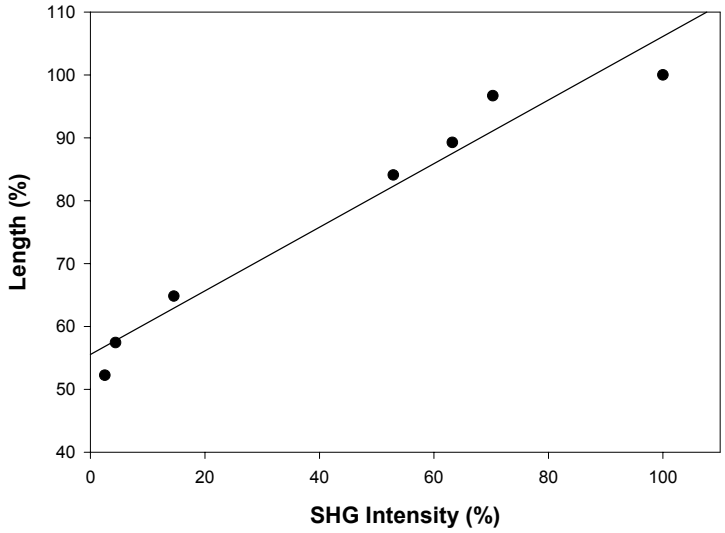

Figure 3. Linear regression of mean collagen shrinkage and mean SHG intensity from thermal treatment of 58^{o}C for 0 to 9mins. Mean rat tail tendon length (shown as percentage of initial length) is plotted as a function of mean SHG intensity (shown as percentage of initial intensity). The solid line is linear least-square fits.

As mentioned above, both the degree of collagen shrinkage and the decrease in SHG intensity show a linear dependence on duration of thermal treatment from 0 to

9min, suggesting that there may be a linear correlation between collagen shrinkage and SHG intensities. **Figure 3** shows association of collagen shrinkage and SHG intensity during thermal treatment at 58^{o}C for up to 9 mins and the linear regression is calculated using linear least-square fits. As shown in **Figure 3** when mean rat tail tendon lengths during thermal treatment is plotted as a function of mean SHG intensities, very good linear fits can be obtained (r^2= 0.96). Further, our data suggests that the denatured collagen has an ultimate length approximately 50% of the initial length (the average of tendon lengths at 9, 10.5 and 12 mins). This result is also consistent with previous report showing bovine joint capsule collagen has an ultimate length of about 50% of its initial length from thermal treatment.[32] Assuming the linear dependence of collagen shrinkage on SHG intensity and an ultimate length of 50% from thermal treatment, we can better quantify the correlation of collagen shrinkage with the changes in SHG intensity by using the following equation:

$$\mathbf{L_d = (0.5I_d/I_0 + 0.5)^* L_0} \qquad (1)$$

(L_d: rat tail tendon length after thermal treatment; L_0: initial rat tail tendon length before thermal treatment; I_d: SHG intensity after thermal treatment; I_0: initial SHG intensity before thermal treatment)

By use of Equation (1), L_d equals L_0 when no thermal treatment is applied. For thermal treatment before collagen is fully denatured, there is a linear dependence of collagen shrinkage on the decrease of SHG intensity. When collagen is fully denatured as revealed by the absence of SHG signals, there is an ultimate length of collagen fibers of 50% of its initial length. Hence, by comparing the initial SHG intensity and final SHG intensity, we can easily predict the degree of collagen shrinkage during thermal treatment.

4. Discussion

In the traditional approach of biochemical assays, methodologies were developed to investigate the kinetic denaturation of collagen. Changes in viscosity can reflect the relative concentration of native and denatured states of collagen in the liquid states.[33,34] In this work, we have succeeded in monitoring the collagen denaturation process by use of SHG microscopy. Quantitative analysis can be obtained and the correlation with the gross tissue shrinkage can be made.

Heat-induced collagen denaturation depends both on the temperature used and the duration of thermal treatment.[15] In terms of thermal treatment in clinical application, it is quite possible that the response of collagen to heating can be affected by the age, anatomical distribution and other associated diseases in the patients. This may accounts for the different responses of patients to the same heating protocol.[11] Therefore, the development of a real-time modality to monitor the response to thermal treatment will contribute greatly to individualized treatment. Our results suggest that the quantitative determination of the denatured collagen in the tissue from SHG imaging can be used to estimate the extent of collagen shrinkage and the degree of tissue damage.

The progressive loss of SHG in rat tails during thermal treatment indicates that the collagen structures responsible for SHG signals become gradually disrupted. Since there is a linear correlation between collagen shrinkage and the changes in overall SHG intensities, the approach proposed in this manuscript enables us to predict collagen shrinkage by determining the changes in the sample's SHG intensities.

Our study also shows that after SHG signals completely fade, further heating is inefficient in shrinking the collagen. Further thermal treatment can at most shrink the collagen tissue by a small amount. This is consistent with previous report that there is a ultimate length of collagen shrinkage even if the tissue is heated at different temperatures.[15]

In conclusion, shrinkage of thermally treated rat tail tendon at 58^{o}C shows a linear correlation with the decrease of SHG intensity. Our results show that SHG imaging is an effective method in predicting the shrinkage of thermally treated collagen and in monitoring the mechanisms of collagen denaturation. Our results suggest that quantitative SHG microscopy can be developed into a non-invasive method to monitor the thermal treatment of collagen in medical practices.

Acknowledgement

This work is supported by the National Research Program of Genomic Medicine of the National Science Council, Taiwan (NSC 94-3112-B-002-015-Y and NSC 93-3112-B-002-034).

Address correspondence to Dr. Jau-Shiuh Chen at cjs0088@yahoo.com.tw and Dr. Chen-Yuan Dong at cydong@phys.ntu.edu.tw.

Reference

1. T. R. Lyons, P. L. Griffith, F. H. Savoie, and L. D. Field, "Laser-assisted capsulorrhaphy for multidirectional instability of the shoulder," *Arthroscopy* 17, 25-30 (2001)
2. M. B. McDonald, P. S. Hersh, E. E. Manche, R. K. Maloney, J. Davidorf, M. Sabry, and the Conductive Keratoplasty United States Investigators Group, "Conductive keratoplasty for the correction of low to moderate hyperopia: U.S. clinical trial 1-year results on 355 eyes," *Ophthalmology*, 109, 1978-1989 (2002).
3. R.E. Fitzpatrick, M.P. Goldman, N.M. Satur, and W.D. Tope, "Pulsed carbon dioxide laser resurfacing of photoaged facial skin," *Arch. Dermatol.* 132, 395-402, (1996).
4. T.S. Alster and S. Garg, "Treatment of facial rhytides with a high-energy pulsed carbon dioxide laser," *Plast. Reconstr. Surg.* 98, 791-794, (1996).
5. K.A. Khatri, E.V. Ross, J.M. Grevelink, C.M. Magro, and R.R. Anderson, "Comparison of erbium: YAG and carbon dioxide lasers in resurfacing of facial rhytides," *Arch. Dermatol.* 135, 391-397, (1999).
6. D.J. Goldberg, "Full-face nonablative dermal remodeling with a 1320nm Nd:YAG laser," *Dermatol. Surg*. 26, 915-918, (2000).
7. E.L. Tanzi, C.M. Williams, and T.S. Alster, "Treatment of facial rhytides with a nonablative 1450nm diode laser: a controlled clinical and histologic study," *Dermatol. Surg.* 29, 124-128, (2003).
8. J.R. Lupton, C.M. Williams, and T.S. Alster, "Nonablative laser skin resurfacing using a 1540nm erbium:glass laser: a clinical and histologic analysis," *Dermatol. Surg.* 28, 833-835, (2002).
9. D.J. Goldberg and K. B. Cutler, "Nonablative treatment of rhytides with intense pulsed light," *Lasers. Surg. Med.* 26, 196-200, (2000).
10. T.S. Alster and E. Tanzi, "Improvement of neck and cheek laxity with a nonablative radiofrequency device: A lifting experience," *Dermatol. Surg.* 30, 503-507, (2004).
11. R. Fitzpatrick, R. Geronemus, D. Goldberg, M. Kaminer, S. Kilmer, and J. Ruiz-Esparza, "Multicenter study of noninvasive radiofrequency for periorbital tissue tightening," *Lasers. Surg. Med.* 33, 232-242, (2003).
12. N.T. Wright and J.D. Humphrey, "Denaturation of collagen via heating: An irreversible rate process," *Annu. Rev. Biomed. Eng.* 4, 109-128, (2002).
13. J. Uitto, L. Pulkkinen and M.L. Chu, "Collagen," in *Fitzpatrick's dermatology in general medicine*, 6th ed., I.M. Freedberg, A.Z. Eisen, K. Wolff, K.F. Austen, L.A.

Goldsmith and S. I. Katz, Eds., pp. 165-179, McGraw-Hill, New York (2003).
14. K. Pankhurst, "Incipient shrinkage of collagen and gelatin," *Nature* 159, 538, (1947).
15. C.E. Weir, "Rate of shrinkage of tendon collagen- heat, entropy, and free energy of activation of the shrinkage of untreated tendon. Effect of acid, salt, pickle, and tannage on the activation of tendon collagen," *J. Am. Leather. Chem. Assoc.* 44, 108-140, (1949).
16. H. Hormann and H. Schlebusch, "Reversible and irreversible denaturation of collagen fibers," *Biochemistry* 10, 932–937, (1971).
17. K. Hayashi, G. Thabit III, J.J. Bogdanske, L.N. Mascio, and M.D. Markel, "The effect of nonablative laser energy on the ultrastructure of joint capsular collagen," *Arthroscopy* 12, 474-481, (1996a).
18. M. Rajadhyaksha, M. Grossman, D. Esterowitz, R.H. Webb, and R.R. Anderson, "In vivo confocal scanning laser microscopy of human skin: Melanin provides strong contrast," *J. Invest. Dermatol.*104, 946–952, (1995).
19. S. Hollingsworth, J.G., Bonshek, R.E., and Efron, N, "Correlation of the appearance of the keratoconic cornea in vivo by confocal microscopy and in vitro by light microscopy," *Cornea* 24, 397-405 (2005).
20. A. W. Drexler, U. Morgner, R.K. Ghanta, F.X. Kartner, J.S. Schuman, and J.G. Fujimoto, "Ultrahigh-resolution ophthalmic optical coherence tomography," *Nat. Med.* 7**,** 502-507 (2001)
21. A. Zoumi, A. Yeh, and B.J. Tromberg,"Imaging cells and extracellular matrix in vivo by using second-harmonic generation and two-photon excited fluorescence," *P. Natl. Aca. Sci. U.S.A.* 99, 11014-11019, (2002).
22. E. Brown, T. McKee, E. diTomaso, A. Pluen, B. Seed, Y. Boucher, and R.K. Jain, "Dynamic imaging of collagen and its modulation in tumors in vivo using second-harmonic generation," *Nat. Med.* 9, 796-800, (2003).
23. P.J. Campagnola and L.M. Loew, "Second-harmonic imaging microscopy for visualizing biomolecular arrays in cells, tissues and organisms," *Nat. Biotechnol.* 21, 1356-1360, (2003).
24. W.R. Zipfel, R.M. Williams, R. Christie, A.Y. Nikitin, B.T. Hyman, and W.W. Webb, "Live tissue intrinsic emission microscopy using multiphoton-excited native fluorescence and second harmonic generation," *P. Natl. Aca. Sci. U.S.A.* 100, 7075-7080, (2003).
25. K. Konig and I. Riemann, "High-resolution multiphoton tomography of human

skin with subcellular spatial resolution and picosecond time resolution," *J. Biomed. Opt.* 8, 432-439, (2003).

26. P. Stoller, B.M. Kim, A.M. Rubenchik, K.M. Reiser, and L.B. Da Silva, "Polarization-dependent optical second-harmonic imaging of a rat-tail tendon," *J. Biomed. Opt.* 7, 205–214, (2002).
27. A.T. Yeh, B. Choi, J.S. Nelson, and B.J. Tromberg, "Reversible dissociation of collagen in tissues," *J. Invest. Dermatol.* 121, 1332 -1335, (2003).
28. T. Yasui, Y. Tohno, and T. Araki, "Characterization of collagen orientation in human dermis by two-dimensional second-harmonic-generation polarimetry," *J. Biomed. Opt.* 9, 259-264, (2004).
29. B.M. Kim, J. Eichler, K.M. Reiser, A.M. Rubenchik, and L.B. Da Silva, "Collagen structure and nonlinear susceptibility: Effects of heat, glycation, and enzymatic cleavage on second harmonic signal intensity," *Lasers. Surg. Med.* 27, 329-335, (2000).
30. S.J. Lin, R.J. Wu, H.Y. Tan, W. Lo, W.C. Lin, T.H. Young, C.J Hsu, J.S. Chen, S.H. Jee, and C.Y. Dong, "Evaluating cutaneous photoaging by use of multiphoton fluorescence and second harmonic generation microscopy," *Opt. Lett.* 30, 2275-2277, (2005).
31. S.J. Lin, C.Y. Hsiao, Y. Sun, W. Lo, W.C. Lin, G.J. Jan, S.H. Jee, and C.Y. Dong, "Monitoring the thermally induced structural transitions of collagen using second harmonic generation microscopy," *Opt. Lett.* 30, 622-624, (2005).
32. G.S. Naseef III, T.E. Foster, K. Trauner, S. Solhpour, R.R. Anderson, and B. Zarins, "The thermal properties of bovine joint capsule: The basic science of laser- and radiofrequency-induced capsular shrinkage," *Am. J. Sports. Med.* 25, 670-674, (1997).
33. H. Boedtker and P. Doty, "The native and denatured state of soluble collagen," *J. Am. Chem. Soc.* 78, 4267–4280, (1956).
34. R.E. Burge and R.D. Hynes, "The thermal denaturation of collagen in solution and its structural implications," *J. Mol. Biol.* 1, 155–164, (1959).