Second-Harmonic Generation Investigation of Collagen Thermal Denaturation

Wei-Liang Chen[1], Yen Sun[1], Sung-Jan Lin[2,3], Shiou-Hwa Jee[2], Yang-Fang Chen[1], Ling-Chih Lin[1], Peter T. C. So[4], Chen-Yuan Dong[1]

[1]Department of Physics, National Taiwan University, Taipei 106, Taiwan
[2]Department of Dermatology, National Taiwan University Hospital and College of Medicine, Taipei 100, Taiwan
[3]Institute of Biomedical Engineering, College of Medicine and College of Engineering, National Taiwan University, Taipei 100, Taiwan
[4]Department of Mechanical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

ABSTRACT

Using the technique of second-harmonic generation (SHG) microscopy we obtained large area image of type I collagen from rat tail tendon as it is heated from 40°C to 70°C for 0 to 180 minutes. The high resolution images allowed us to investigate the collagen structural change. We observed that heating the tendon below the temperature of 54°C does not produce any change in the averaged SHG intensity. At the heating temperature of 54°C and above, we find that increasing the heating temperature and time leads to decreasing SHG intensity. As the tendon is heated above 54°C, a decrease in the SHG signal occurs uniformly throughout the tendon, but the regions where the SHG signal vanishes form a tiger-tail like pattern. By comparing the relative SHG intensities in small and large areas, we found that the denaturation process responsible for forming the tiger-tail like pattern occurs at a higher rate than the global denaturation process occurring throughout the tendon. Our results show that second-harmonic generation microscopy is effective in monitoring the thermal damage to collagen and has potential applications in biomedicine.

Keywords: second harmonic generation (SHG), collagen, thermal denaturation

1. INTRODUCTION

Collagen is the most abundant protein in mammals. It is responsible for the tensile strength in ligaments and tendons, the elasticity for skin, and the transparency and structural support for the cornea. The most prevalent collagen is Type I collagen, which is found in bones, tendons and scar tissues. The triple helix of Type I collagen molecules are composed of polypeptide chains, each contains the repeated G-X-Y sequence, where G represents glycine, and X and Y corresponds to usually proline or hydroxyproline (1). They combine to form microfibrils few nanometers in diameter, which then combine to form collagen fibrils that are few hundreds nanometers in diameter. The fibrils further bundles to form collagen fibers that are a few to hundreds of micron in diameter.

As the most abundant protein of the extracellular matrix and its relation to the application of several medical procedures, thermally induced conformational changes in collagen have been actively studied (1-4). Examples of medical applications include the use of heating to change cornea curvature, and the use of laser heating to stabilize shoulder joint and to rejuvenate skin.(5-7). The response of collagen to heating has been studied using different methods including differential scanning calorimetry (DSC), x-ray diffraction, NMR, and spectroscopy (1, 3, 4). The denaturation of collagen is complicated amongst other variables by its polymeric nature and cross-linking. Despite numerous efforts, the precise mechanism of collagen denaturation remains unknown (1).

Among the various methods that can be used to study collagen denaturation, second-harmonic generation (SHG) microscopy is unique in that it is a non-linear optical technique that has potential to be applied to collagen studies under *in vivo* conditions (8-14). In short, second-harmonic generation (SHG) is a coherent and nonlinear process where two

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photons at the fundamental frequency are converted into one photon at twice the fundamental frequency. SHG occurs only in structures that lack inversion symmetry. Several biological molecules including collagen have been demonstrated to be efficient in generating second-harmonic signals (8-14).

In this study, we investigate the thermal denaturation of collagen from rat tail tendon using high resolution SHG microscopy. We obtained sub-micron resolution SHG images of collagen fibrils as it undergoes thermally induced structural changes. From the changes in the SHG image, we tried to characterize the mechanism of collagen thermal denaturation.

2. METHOD

2.1 Sample preparation

The rat tail tendons used for this study were removed from frozen rat tails obtained from the National Taiwan University Hospital. The rat tails were frozen at –80 ^{o}C and thawed right before experimentation. The individual tendon fascicles were first removed and placed in phosphate buffer saline (PBS) solutions (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C) inside a micro tube. Then the tube containing the fascicle was immersed for a specific time in a thermal bath set at the desired heating temperature. Finally, the heated tendon fascicle was sealed between a cover glass and a glass slide for imaging, with a PBS wetted tissue paper placed underneath the fascicle to keep it moist.

2.2 Imaging setup

Figure 1 shows the second-harmonic generation (SHG) microscopy system used in this study. It is consisted of a custom-built laser scanning microscopic imaging system based on an upright microscope (E800; Nikon, Tokyo, Japan). A diode-pumped solid-state (DPSS)-pumped (Millennia X; Spectra Physics, Mountain View, CA) titanium-sapphire (Ti-Sa) laser (Tsunami; Spectra Physics) producing 80-MHz, femtosecond laser pulses was used as the excitation source. For this study, the laser was tuned to 760 nm for SHG imaging. The beam power and polarization properties were controlled by the combination of a half-wave plate, a linear polarizer, and a quarter-wave plate. The scanning system mirrors and galvanometers (Model 6220; Cambridge Technology, Cambridge, MA) were controlled by the computer, in conjunction with the signal acquisition and image formation. The scanned laser beam is expanded and then reflected by a dichroic mirror (700DCSPXRUV-3p; Chroma Technology, Rockingham, VT) into the back aperture of a oil-immersion objective (S Fluor 40x, NA 1.3; Nikon). The backward SHG signals are collected by the same objective and registered by a single-photon-counting photomultiplier tube (R7400P; Hamamatsu, Hamamatsu City, Japan). Before reaching the photodetector, the SHG signals are selected by a secondary dichroic mirror (435DCXR; Chroma Technology) and a narrow band pass filter (HQ380/20; Chroma Technology). For large-area scanning, a computer controlled motorized sample stage (H101, Prior Scientific Instruments, Cambridge, UK) is used in conjunction with the scanning software.

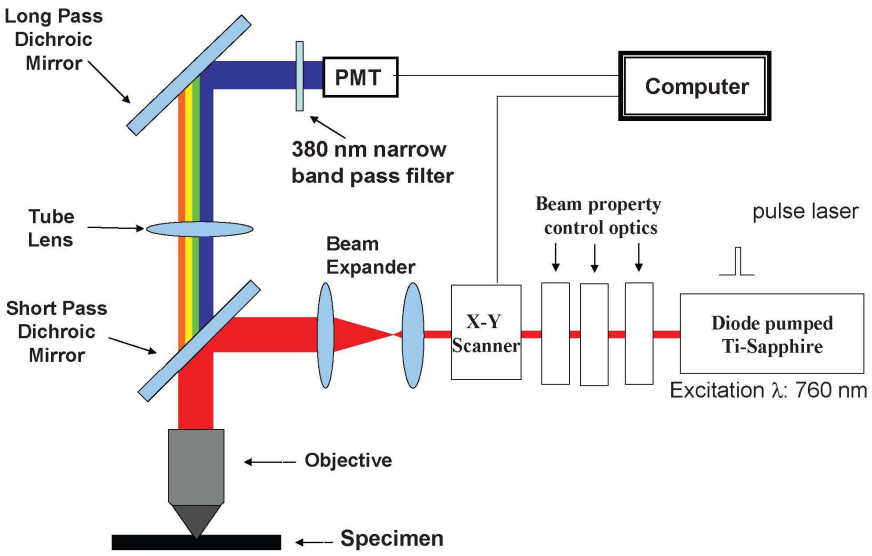

Fig.1 Schematic diagram of the experimental setup.

3. RESULTS AND DISCUSSION

Figure 2 shows the 660 μm × 660 μm SHG images of rat tail tendon fibril heated from 50°C to 57.5°C for different heating times. Each image is a montage of thirty-six non-overlapping 110μm × 110μm image. The fibrils in the images are oriented along the vertical direction. A magnified view of a section of the T=55°C sample is shown in Figure 3, where the direction of the fibrils are clearly visible. For heating below T=54°C, increasing the amount of heating time up to 140 minutes did not produce observable structural alterations as shown by the SHG images. At temperatures of 54°C and higher, the SHG images show the fibrils start to disintegrate in a "tiger-tail" like band pattern perpendicular to the collagen fibers. The areas with little or no SHG signal tends to extend horizontally in a direction perpendicular to that of the fiber. A magnified view of part of one of these dark bands is visible in Fig.3. The pattern suggests that there are positions along the fibers that are more prone toward thermal denaturation.

To quantify our observation, we computed and plotted the relative intensities with respect to the unheated 57.5°C data, measured over the 660 μm × 660 μm images. In cases such as the 40 minute heating of the 54°C data, where the edge of the fiber is shown, the intensity is averaged only over the fiber areas of the images. Figure 4 shows a logarithmic plot at the heating temperature of 40°C to 70°C, for the heating time intervals of 0 to 180 minutes. At the heating temperature of 54°C and higher, there is a steady decrease in the SHG signal as the heating time lengthens.

As shown in Fig. 2, the decrease of SHG intensity with increasing heating time for heating temperature at and above 54°C are caused by the increase in regions lacking SHG signal. The location and pattern of these dark areas suggest that a mechanism of the thermal denaturation of tendon fibril occurred along the tiger-tail patterns running perpendicular to the direction of the tendon. Previous studies have found that when viewed under crossed polarizers, rat tail tendon fascicles show banding patterns perpendicular to the fiber direction with the bands spaced by approximately one hundred microns(15). Since the banding pattern is likely formed by the macromolecular ordering of collagen assembly, it is possible that the location most susceptible to bond breaking also occurs in a banding pattern perpendicular to the fiber.

To further investigate the thermal denaturation process, we calculated the SHG intensities in smaller regions where the signal has not completely disappeared. Fig. 5 shows a selection example where the average SHG intensity was calculated for the circled region, while several nearby regions have little or no SHG signal. Under each heating condition, the relative intensities were calculated by averaging over five smaller selected regions each being 914 μm^2 in area. The average intensities of these smaller, selected areas for various heating conditions are shown in Figure 6. The results of our calculations show that regardless of the selected regions, the average SHG intensities begins to drop off at long heating times for heating temperature at and higher than 54°C.

This observation is qualitatively similarly to that observed when the average SHG intensity is determined from the large area calculations including the dark tiger-tail patterned regions where the collagen has denatured. By comparing Fig.4 and Fig.6, we see that the global change of the SHG signal has a higher rate of intensity drop. These results suggest that although Figure 2 shows the denaturation process appears to first occur only along certain selected regions, a global melting of the collagen fibril also occurs concurrently. Our results suggest that type I collagen in rat tail tendon undergo thermal denaturation by two mechanisms. First, thermal effects can induce breakages at weak points along the tendon fiber and additional heating would result in collagen denaturation spreading from the broken positions. In addition, collagen can undergo a global denaturation independent of the presence of the break points.

To perform further analysis, we plot in Fig. 7 on semi-logarithmic scale the ratio of the small to large area average intensity (as shown in Figure 4 and 6) for the different heating conditions. For heating temperatures above 54 °C, the calculated ratio rises with increasing heating time. With increasing temperatures, the ratio increases more quickly with the heating times. The rise in the ratio with heating time suggests that the denaturation along the tiger-tail pattern occurs at a higher rate than the global melting of the collagen fibril.

In conclusion, by use of SHG microscopy, we have shown that both the global and local mechanisms contribute to the thermal denaturation for type I collagen in rat tail tendon. Our results show the potential applications of SHG microscopy in studying the thermal effects of collagen for biomedical applications.

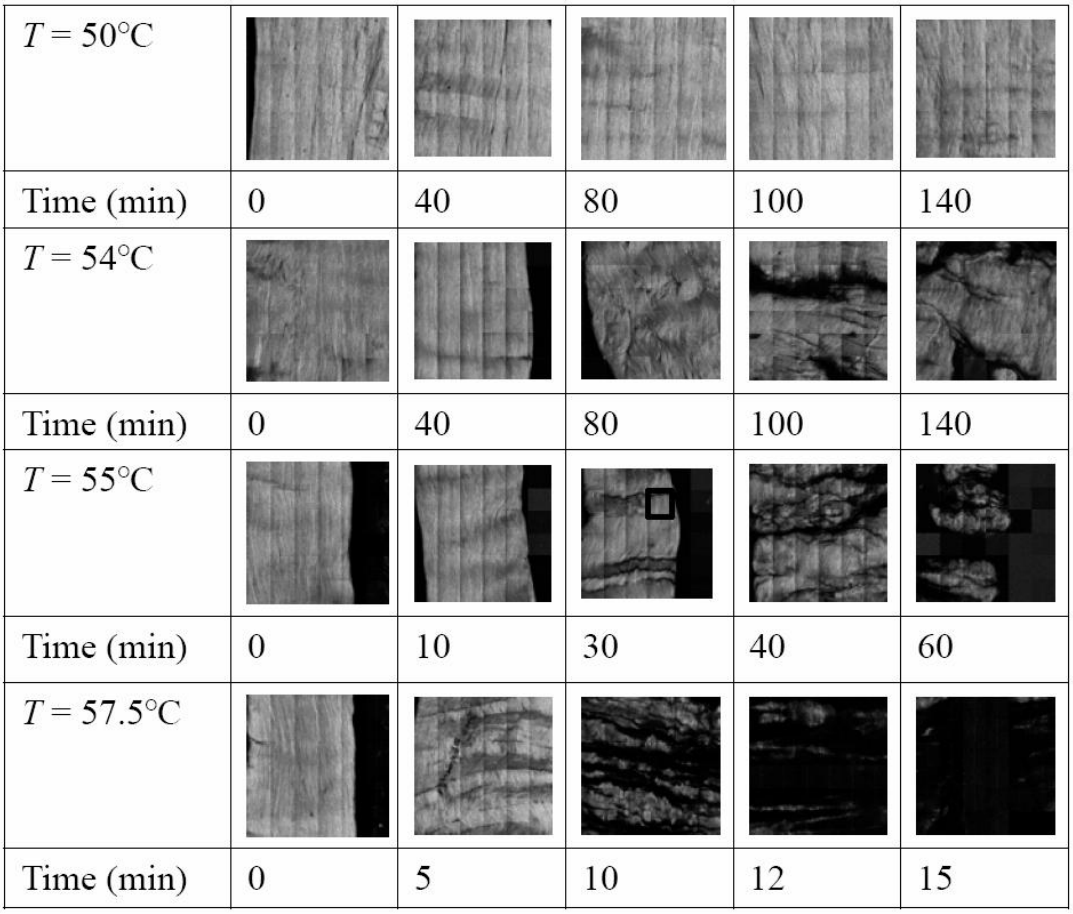

Fig. 2. Second-harmonic generation images of rat tail tendon treated at different heating temperatures and times. Structural change at the fibril level of the rat tail tendon is visible in the SHG signal as the fibril is heated at above 54°C. Very little structural change is observed when the heating temperature is below 54°C. The images are 660 μm × 660 μm in size, and each one is a montage of 36 individually scanned images.

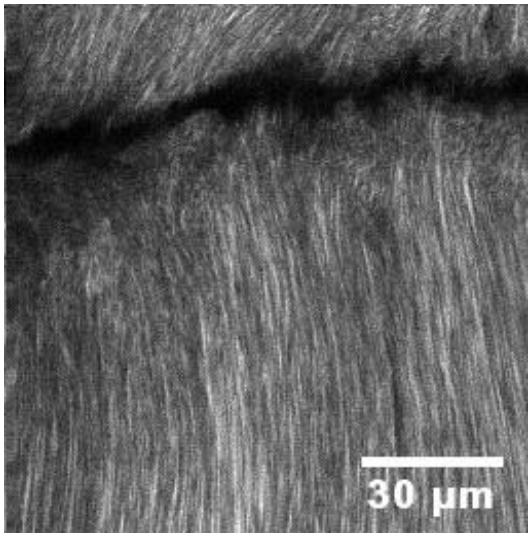

Fig. 3. A magnified view of the section indicated by the square region in the T=55°C and 30 minute heating image of Fig. 2.

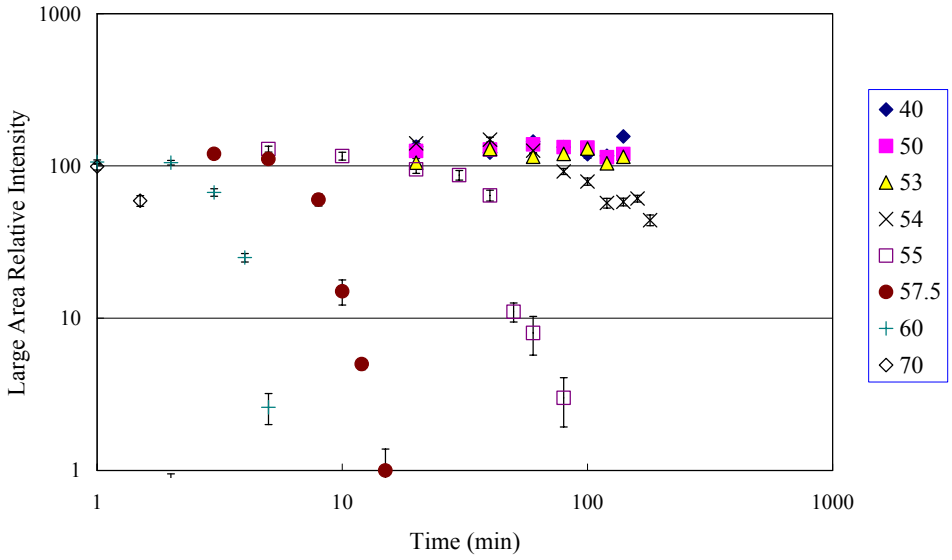

Fig. 4. SHG intensity of rat tail tendon treated for different heating temperatures and times, plotted on a logarithmic scale. For temperature above 54 °C, the SHG intensity falls off rapidly with increasing heating time. Below 54 °C, the intensities remain fairly constant with heating time. The relative intensities are normalized with respect to the SHG intensity measured for the T=57.5 °C and time = 0 min sample.

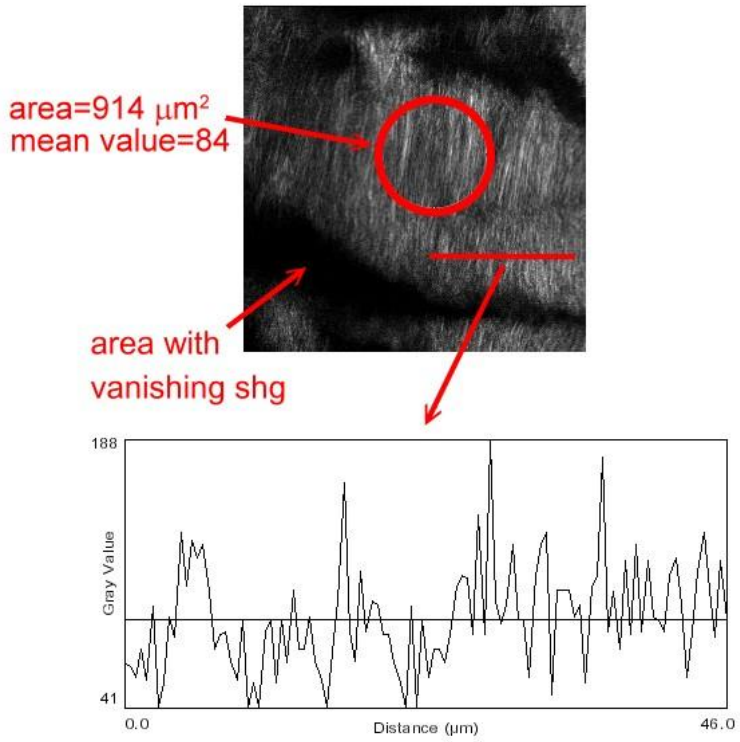

Fig. 5. A 110 μm × 110 μm SHG image from a thermally treated rat tail tendon specimen where parts of image have little or no second-harmonic signal. The circled area shows an example of selected small area used to calculate the SHG intensity. The profile plot corresponding to the intensity profile of the selected line segment is shown in the SHG image. The horizontal line in the profile plot is the average intensity along the selected line. It is used as a reference for counting the number of fibrils.

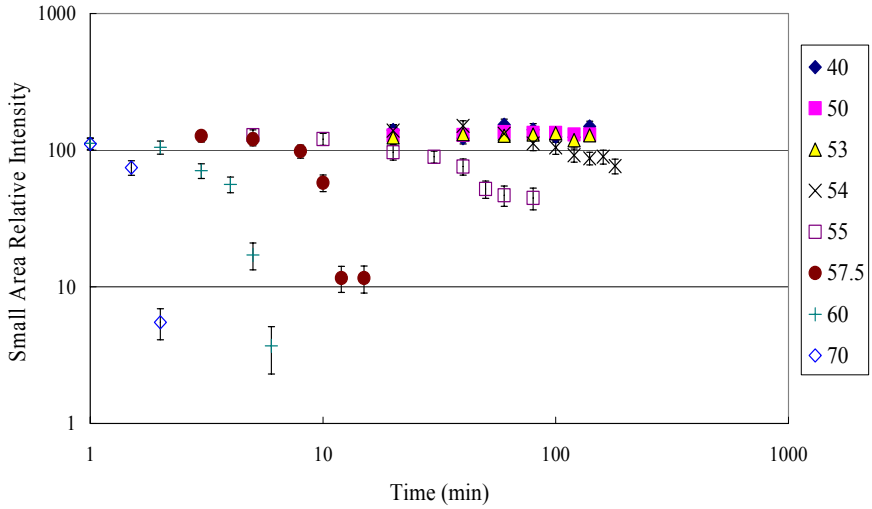

Fig. 6. The SHG intensity averaged over five selected small area as shown in Fig. 5, for different heating temperature and time. The plot is on a log-log scale. The SHG intensity falls off more slowly with increasing heating time than the case shown in Figure 4.

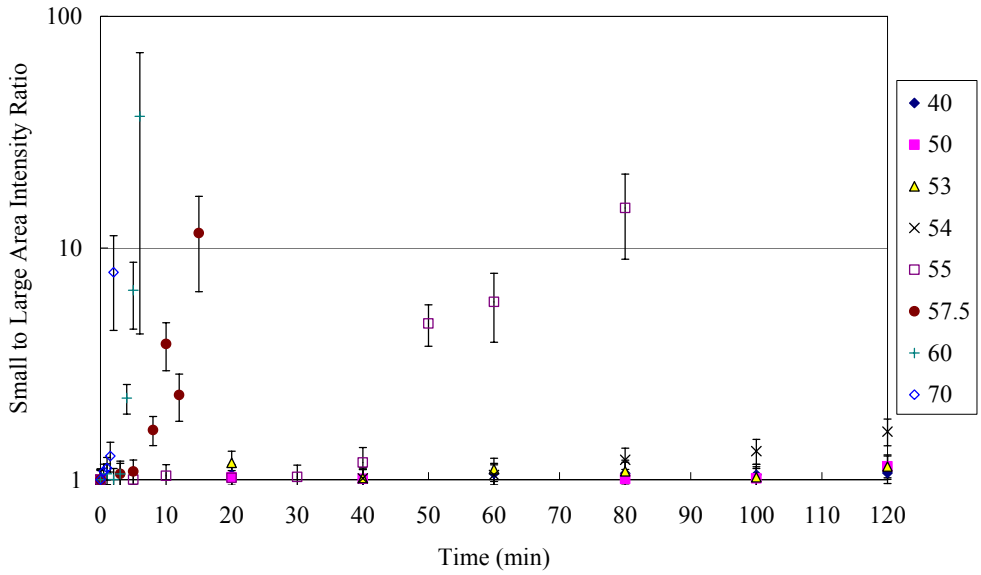

Fig. 7 Ratio of small to large area SHG intensity for different heating conditions plotted on a semi-logarithmic scale. For heating temperature above 54 °C, the ratio rise with increasing heating time. The rise in the ratio with heating time suggests that the denaturation along the tiger-tail pattern occurs at a higher rate than the global melting of the collagen fibril.

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