Cellular and Molecular Bioengineering, Vol. 14, No. 1, February 2021 *(© 2020)* pp. 113–119
https://doi.org/10.1007/s12195-020-00653-w

Original Article

Effect of Heat Level and Expose Time on Denaturation of Collagen Tissues

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(Received 15 July 2020; accepted 16 September 2020; published online 28 September 2020)

Associate Editor Stephanie Michelle Willerth oversaw the review of this article.

Abstract

Introduction—The applied heat level and expose time are main issues in certain operations/applications, such as a laser assisted tissue welding, preparation of collagen-based biomaterials (films, implants). Therefore, the precise investigation of these parameters is crucial. The results can serve as a guideline to assess potential effects while maintaining the functionality of the collagen structures.

Methods—Collagen tissues from rat-tail tendon, calfskin, and bones are soaked in buffer solutions, then examined by microscope at different temperature levels.

Results—Increase in temperature reduced the microscopically observed collagen crimp contrast for calfskin and rat-tail tendons but not for bone tissues. The contrast level for rat tail tendon decreased down to 80% of its initial value at 37, 157, and 266 s for 70, 65, and 60 °C, respectively. The decrease in the crimp contrast was about only 25% and 2% at 55 and 50 °C after 2 h, respectively. 50% drop in contrast level was occurred for the skin samples at 16, 90, 110 and 1900 s for 70, 65, and 60 °C, respectively. The bone samples, did not show any significant differences in contrast levels.

Conclusion—The observed denaturation behaviours are in line with Arrhenius Law. This study could be expanded on to other types of tissues at wider temperature ranges to make a guideline for biological/medical processes that radiate heat in order to assess their side effects on collagen and other proteins.

Keywords—Collagen, Denaturation, Arrhenius, Tissues, Expose time.

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INTRODUCTION

Collagen is the most abundant protein in mammals.[19] It has a high tensile strength that ranges in between 1.4 and 24.6 MPa and can withstand to high forces without plastic deformation.[9] Over 16 types of collagen present in the nature. However, types I, II, and III alone make up 80–90% of total collagen in the human body. Although all the collagen types constitute of polypeptide chains that form triple helix structure, they differ in small sections that can cause them to fold into different three-dimensional forms.[19] These structures are sustained with hydrogen bonds and polypeptide crosslinks.[1] In the denaturation temperature these structures and polypeptide chains break. As a result, collagen turns into a non-homogenous protein called as gelatine.[18] The denaturation temperature varies from tissue to tissue. A great number of studies have focused on defining temperature dependent properties of collagen or collagen-rich tissue (mechanical, electrical, and optical properties). For instance, Chae *et al.*[7] have studied the changes in mechanical properties of cartilage tissue (composed of 3D fibrillary collagen networks) under radiofrequency (or contact heating treatment), which is critical in understanding the permanent effects caused on the tissues. Collagen and gelatine systems were previously examined under different temperatures by using a dilatometer setup to identify phase transitions.[12] Calorimetric investigations were conducted on collagen tissue to reveal collagen denaturation temperature.[2,16] There are also some other studies that focused on age-related changes in denaturation behaviour.[11] High-resolution imaging techniques (Scanning Electron Microscopy,[5,17] Optical Coherence Tomography[8]) as well as spectroscopy (Fourier Transform Infrared,[4]

Circular Dichroism[3] and Fluorescence Spectroscopy[26]) have also been widely utilized to observe the denaturation behaviour of collagen. In these studies, the reported denaturation temperatures vary within a range of 50–70 °C.

The medical literature shows increasing clinical usage of tissue heating to treat a huge number of illnesses and injuries. Laser treatments are progressively utilized in oncology, ophthalmology, dermatology, orthopaedics and other restorative specialties in the treatment of atherosclerotic plaques, prostatic hyperplasia, hyperopia, joint laxity, menorrhagia, Parkinson's disease, malignancies, skin lesions and correction of cornea curvature.[6,10,15] Thermal ablative applications are Radio Frequency Ablation (RFA), Micro Wave Ablation (MWA), High Intensity Focused Ultra Sound (HIFU), LASER Ablation (LA), Cryoablation (CA). The temperatures used in these methods generally range in between 50 and 100 °C.[21] RFA's exposure time limit can be up to 10 min, while it is 1–5 min for MWA, 3 min for LA on average, 1–10 s for HIFU and 7–30 min for CA. Coagulation of soft tissues occurs due to the denaturation of proteins, that takes places within the 60–100 °C temperature range. This causes in a good amount of reduction in bleeding at the margins of ablated tissues during laser ablation application. The coagulation exposure time range in different applications are in between 0.001 and 1000 s depending on the applied temperature.[23]

The exposed temperature ranges and duration of extracted collagen in this study matches with that used in a variety of thermal ablative modalities (applications) in the literature. In an effort to provide a systematic, rapid, repeatable and low-cost method to characterize protein denaturation, here we've utilized a simple wide-field microscope to monitor the collagen crimp sites. The contrast between crimp stripes, i.e. the "crimp contrast" was reported as a function of time for the applied temperatures on a wide variety of collagen sources (rat-tail tendon, calfskin, and bones of calf, chicken and sheep). The acquired data showed a good fit in the Arrhenius model, which is conventionally used in defining denaturation times and rates at different temperature levels.

METHODS

Preparing Rat Tail Tendon Tissue Sections

Tendons were obtained from Wistar Rat tails ($n = 5$, ~ 10 months old, weight: 380–400 gr), which were received from and dissected at the Central Vivarium of Bogazici University. The dermal layers were first removed from the tail to expose the skeletal frame and the collagen I rich tendons. Then, tendons were pulled from the skeletal structure and stored in 50 mL phosphate buffered saline (PBS). For each experiment, a single tendon was isolated and sandwiched between two glass slides and soaked into a heat-controlled PBS solution. The extracted tendon was folded in a zig-zag shape before it was placed between two glass slides to maximize the amount of tissue that can be visualized under the microscope, as illustrated in Fig. 1.

Preparing Calf Skin Tissues Sections

For the second part of the experiment, the calf-skin tissues were scraped until they were free from their epidermis layer and collagen structure was visible for eye. They were cleaned from epithelial and connective tissues by hand. This step was followed by an acetone bath for 48 h to get rid of the other substances. Next, the skin samples were soaked into the 50 mL PBS solution as same as the rat tail tendons. Then they are sliced and placed into the sample cuvette. To make the collagen fibrils more visible, the samples were stretched from the edges. Each calfskin section mounted between two glass lamellae. In total, 5 calfskin samples were examined. They were heated up to 70, 65, 60, 55 and 50 °C as in the rat tail tendon experiment. The calfskin samples also displayed alternating contrast levels.

Preparing Bone Tissue Sections

Lastly, bone specimens were prepared and tested in the same experimental setup. The soft tissues, epithelial and connective tissues, were cleaned by hand. Chicken, calf and sheep bone samples were cut into small pieces. They were washed 5 times in pure ethanol bath. Each sample were soaked in ethanol solution for 24 h and stirred by magnetic stirrer. After separating from other impurities, bone pieces were reduced to fit the microscope table. A saw or grading tools were not used since such tools could damage the structures. The bone specimens were placed in the sample cuvette in a PBS

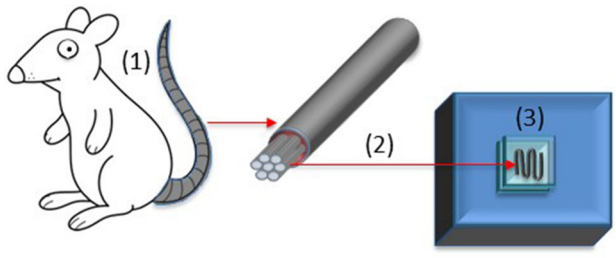

FIGURE 1. Preparation of collagen samples from Wistar rat (1) tail (2) and placement into the sample cuvette (3).

bath. The internal and external parts of the 3 different types of bone samples were examined.

General Setup

An upright microscope illustrated in Fig. 2 was employed to monitor collagen tissues. A thermo-electric-cooler (TEC) was placed under the aluminium sample cuvette and was utilized to control the PBS temperature in which the collagen tissues rested. The temperature was monitored by a thermo-couple unit that is connected to an Arduino platform. Metal fins and a fan were also incorporated into the setup to uniformly distribute the heat, that is radiated from the bottom side of the TEC.

5 rat-tail, 5 calfskins, 1 calf bone, 1 chicken bone and 1 sheep bone were examined where 3 different sites per each sample was monitored at 50, 55, 60, 65, and 70 °C in maximum duration of ~ 2 h (for 50 °C). Under the microscope, collagen fibers appear crimped, with alternating dark and bright regions that were nearly orthogonal to the fiber direction.[13] It was shown that the crimps, observed *via* Scanning Electron Microscopy (SEM), were sites where collagen fibrils suddenly change orientations.[22,25] Changes in the crimp patterns and structures had been monitored to investigate the effects on strain,[14] and rupture [20] of collagen fibrils. In this study, the crimp contrast change was used as an indicator of denaturation of collagen. The contrast of the crimp patterns improves upon using a polarized microscope, yet the crimps are also observable under a regular wide-field microscope, as shown in Fig. 2. Here, the crimp contrast (γ) is defined as:

$$\gamma = \frac{I_{\max} - I_{\min}}{I_{\max} + I_{\min}} \tag{1}$$

where $I_{\max}$ is the average intensity of the selected bright stripe and $I_{\min}$ is the average intensity of the adjacent dark stripe of the collagen crimp. In the experiments, the crimp contrast was time-lapse monitored for all rat-tail, calfskin and bone structures for the listed temperatures, and three defined locations per specimen. Figure 2 illustrates a tendon, and its intensity for the dashed cross-section. It is noted that increase in temperature, results in a diminished crimp contrast.

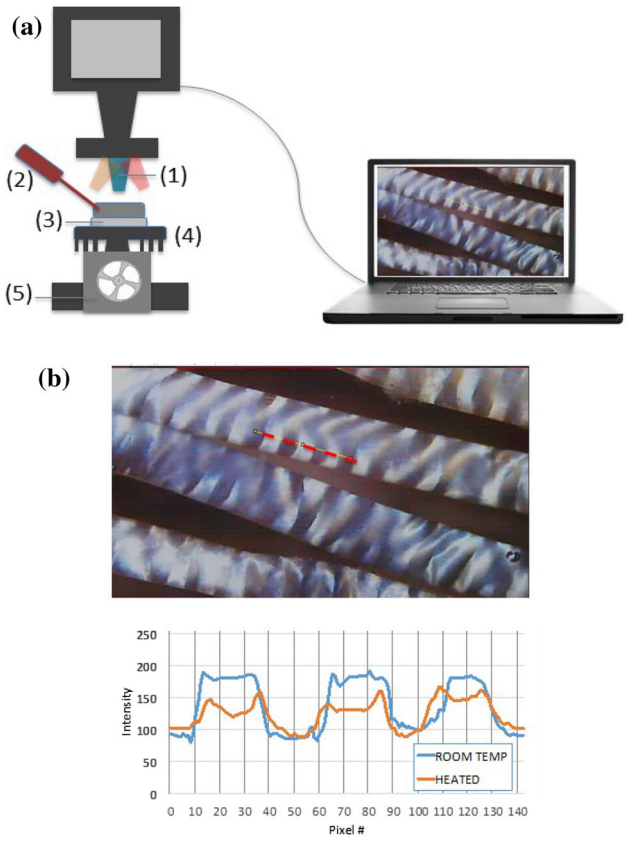

FIGURE 2. (a) Microscopy setup involved in the experiments, (1) comprising of an objective lens, (2) temperature sensor, (3) TEC, (4) aluminum fins, (5) fan. (b) An exemplary screen-shot from the microscope and plot of the highlighted cross- section for nominal temperature and heated tissue shows the decrease in crimp contrast.

Statistical Analysis

All data were taken in triplicate and reported as mean ± standard deviation in the text while error bars in graphs show standard error. Different treatment groups were compared using a one-way analysis of variance (ANOVA). Pairwise comparisons were made between groups using Fisher's Least Significant Difference (LSD) post hoc test. p-values less than 0.05 were considered significant.

RESULTS

Rat Tail Tendon Results

Figure 3 illustrates the change in the normalized crimp contrast (γ) on five rat-tails, three locations per tail, observed at five different temperatures. As expected, the decay in contrast is more rapid at high temperatures, and significantly slows down at lower temperatures. Finally, we observe that there are not significant signs of contrast change at 50 °C for a total duration of 7200 s (> 2 h).

The error bars in the Fig. 3 reflect the standard deviation of the contrast, for 15 different data from 5 different tissues.

Calf Skin Results

Crimp contrast values of calfskin, taken from five animals, were normalized as in Fig. 3. The Contrast decreases sharper as the temperature increases. There were not any significant changes at 50 and 55 °C before 1000 s. After 60 °C, the contrast values decrease drastically. It indicates that the denaturation of collagen is faster at 60 °C. The results, shown in Fig. 4, are in-line with those observed for collagen extracted from rat tail tendon.

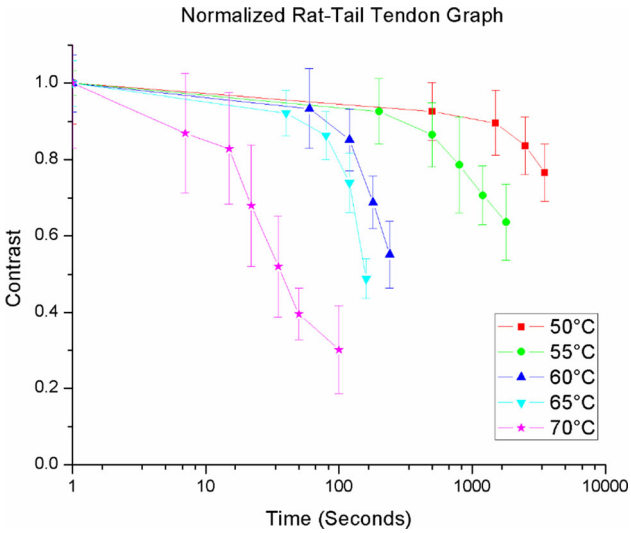

FIGURE 3. Decay in tendon crimp contrast (normalized to initial value) as a function of time, at different buffers solution temperatures.

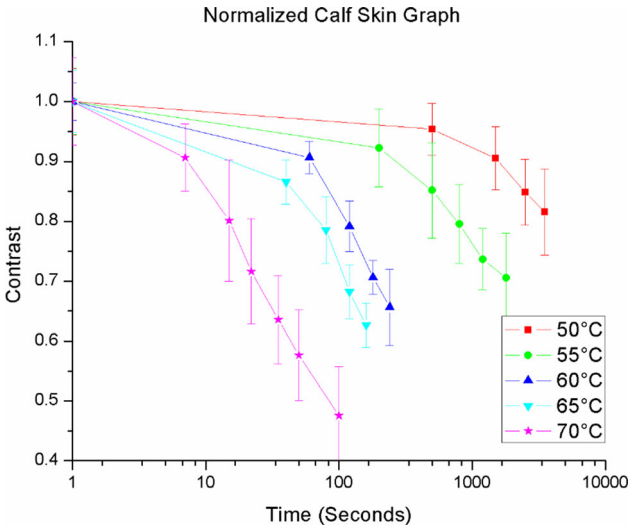

FIGURE 4. Decay in calfskin crimp contrast (normalized to initial value) as a function of time, at different buffers solution temperatures.

Bone Tissue Results

3 different bone tissues from calf, chicken and sheep and three different locations for each bone sample were monitored. Both external and internal surfaces of the bones were examined. There was not any significant change in contrast at defined temperatures, as shown in Fig. 5.

Statistical Analysis Results

In Figs. 3, 4 and 5, the error bars reflect the standard deviation of the contrast, acquired from three different tissues (calf skin, rat tail, and bone tissues) at three different locations per tissue, totaling 9 data points. P fit accuracy value of 2.44×10^{-7} for tendon, 1.37×10^{-6} for calfskin and 4.4×10^{-4} for bone. P value calculated as 3.99×10^{-9} for all the groups.

Arrhenius Modelling

The decrease of contrast to 80% of its initial value is accepted as average denaturation time ($\tau_{0.8}$) and its inverse is accepted as the denaturation rate ($\tau_{0.8}^{-1}$) in Arrhenius Modelling. The experimental results reveal that average denaturation times ($\tau_{0.8}$) for tendon are 17, 101, 117,1890 s and average denaturation times for calfskin are 16, 90, 110,1900 s for 70, 65, 60, and 55 °C, respectively. The average denaturation times were not reached within the experimental time frame for lower temperatures. The decay of the contrast to other values rather than 80% of the initial value could also be chosen, especially for specific biomedical applications that requires shorter duration times.

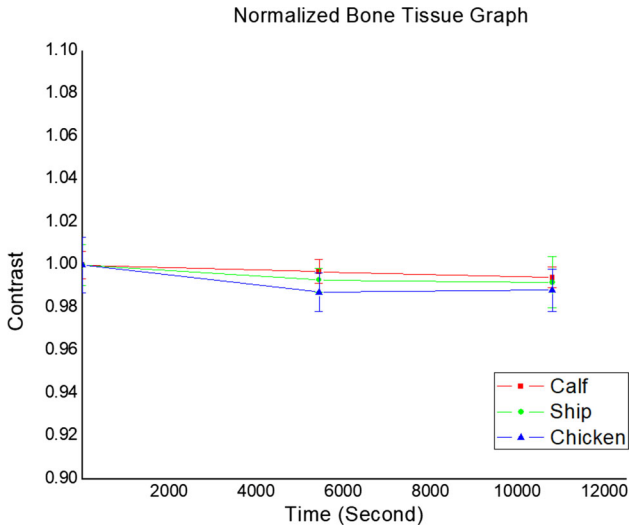

FIGURE 5. Decay in bone contrast (normalized to initial value) as a function of time, at different buffers solution temperatures.

Arrhenius plot is a simple model and has been widely used in describing thermal denaturation of various proteins and cells.[24] The acquired crimp contrast rates were modelled based on Arrhenius equation that links reaction rate to inverse temperature, such that:

$$k = Ae^{(B/T)} \tag{2}$$

where k is the rate constant, A is the pre-exponential factor, B is the exponential factor (activation energy) that defines the rate, and T is temperature in Kelvins. The denaturation rate vs. Temperature was plotted in Fig. 6, where R^2 fit accuracy value of 0.735 for tendon (Fig. 6a) and 0.7416 for calfskin (Fig. 6b) were obtained.

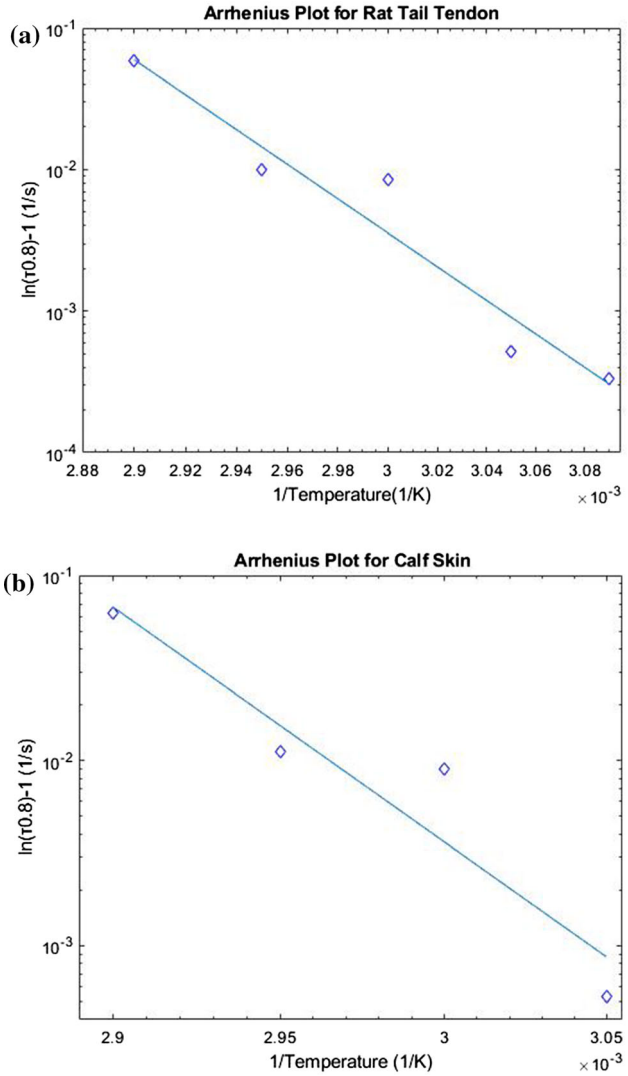

FIGURE 6. Arrhenius plots of duration rates for (a) rat-tail tendon and (b) calfskin.

DISCUSSION

The decay in crimp contrast of rat-tail tendons, calfskins and different bone tissues at different temperatures as a function of time is analysed. 80% decrease of its initial value in crimp contrast was observed for rat tail tendons within 17, 101, 117,1890 s. It was observed for calf skin in 16, 90, 110,1900 s at 70, 65, 60 and 55 °C respectively. Indeed, the creep contrast values are very similar for both tissues. The decay in crimp contrast was less than 25% at 50 °C for both samples. There was not any significant change in bone tissues (calf, sheep and chicken bones). It was also difficult to observe and follow the collagen fibrils under microscope, since it has special arrangement and closely packed in mineralized bundles, comparing with relatively loose structure in tendon and skin. The experimental results showed a good fit in the Arrhenius model, which has been mainly utilized for cell and protein denaturation studies.

Thermal denaturation of collagen is studied by many techniques including NMR, spectroscopy, differential scanning calorimetry. In addition to these studies, Yen Sun *et al.*, used second-harmonic generation (SHG) imaging to observe the denaturation of rat tail tendon temperatures between 40 and 70 °C for 180 min.[27] When we compare with our suggested method, Yen Sun and co-workers were able to successfully characterize the change in collagen structures in scales of sub microns to hundred microns while heating. At the end, they have not observed any notable change in SGH intensity below 54 °C. In our work, we used an LCD microscope (Bresser) with 10× objective lens and we obtained comparable results especially for the rat tail tendon by using a significantly lower-cost setup. Overall, assessing crimp contrast by the proposed setup reveals similar results at a good sensitivity that are comparable by observing the tissue denaturation under a SHG microscope (coupled with a pulsed laser, scanning mechanism along with a number of alignment mirrors and lenses) at much lower expenses.[27,28]

The proposed method can be applied to different Collagen types (apart from Collagen I) to obtain a wider representation of denaturation rates for the most abundant protein found in the body. Conducting specialized studies for a wider variety of collagen-rich tissue can potentially establish a detailed guideline to accurately design targeted collagen therapies for effective heat treatment with minimized tissue damage. At this point, a challenge arises in the implementation of this system for diagnostic or therapeutic purposes. To make the proposed system fully functional in medical environments, adjustments might be necessary. Knowledge of the heat responses of many types

of collagen, would greatly improve the accuracy in collagen enriched tissues and allows better hybrid systems for effective heat treatment with minimized tissue damage.

CONCLUSION

The assessment of denaturation rates verse to the denaturation temperature provides guidelines for a number of biomedical applications, such as adjustment of temperature and expose time during a laser-assisted tissue welding, or the heat treatment steps while manufacturing collagen-based tissue regeneration products (films, implants, cross-linkers).

This method, that utilizes a simple upright microscope to monitor the crimp contrast, has certain advantages over high-tech technologies such as SEM, OCT as well as other spectroscopic techniques, due to its simplicity, denaturation evaluation capability in real time (30 fps) and repeatability.

This study could be expanded on to other types of tissues at wider temperature ranges to make a guideline for biological/medical processes that radiate heat in order to assess their side effects on collagen and other proteins.

CONFLICT OF INTEREST İrem Deniz Derman, Esat Can Şenel, Onur Ferhanoğlu, İnci Çilesiz, and Murat Kazanci declare that they have no conflicts of interest.

ETHICAL STANDARDS No human studies were carried out by the authors for this article and no animal studies were carried out by the authors for this article.

ACKNOWLEDGMENTS

We thank Enes Atas (scientist in Biomedical Engineering Department at Istanbul Medeniyet University) for helping us to prepare bone samples and Ekin Opar (research assistant in Biomedical Engineering Program at Istanbul Technical University) for analysis and discussing the results.

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