Structural changes in cartilage and collagen studied by high temperature Raman spectroscopy

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ABSTRACT

Understanding the high temperature behavior of collagen and collagenous tissue is important for surgical procedures and biomaterials processing for the food, pharmaceutical and cosmetics industries. One primary event for proteins is thermal denaturation that involves unfolding the polypeptide chains while maintaining the primary structure intact. Collagen in the extracellular matrix of cartilage and other connective tissue is a hierarchical material containing bundles of triple-helical fibers associated with water and proteoglycan components. Thermal analysis of dehydrated collagen indicates irreversible denaturation at high temperature between 135-200°C, with another reversible event at ~60-80°C for hydrated samples. We report high temperature Raman spectra for freeze-dried cartilage samples that show an increase in laser-excited fluorescence interpreted as conformational changes associated with denaturation above 140°C. Spectra for separated collagen and proteoglycan fractions extracted from cartilage indicate the changes are associated with collagen. The Raman data also show appearance of new features indicating peptide bond hydrolysis at high temperature implying that molecular H_2O is retained within the freeze-dried tissue. This is confirmed by thermogravimetric analysis that show 5-7 wt% H_2O remaining within freeze-dried cartilage that is released progressively upon heating up to 200°C. Spectra obtained after exposure to high temperature and re-hydration following recovery indicate that the capacity of the denatured collagen to re-absorb water is reduced. Our results are important for revealing the presence of bound H_2O within the collagen component of connective tissue even after freeze-drying and its role in denaturation that is accompanied by or perhaps preceded by breakdown of the primary polypeptide chain structure.

1. INTRODUCTION

Understanding the thermal denaturation and breakdown of collagenous tissue is necessary for controlling and developing a wide range of surgical procedures [1,2]. Knowledge of the high temperature properties of collagen-containing materials in relation to their hydration behavior is also needed for processing products for use in the food, pharmaceuticals and cosmetics industries [3,4]. Similarly in some tissues collagen is involved in the hydration, and therefore function, of the extracellular matrix (ECM). In articular cartilage the ECM provides the essential combination of compressive stress resistance and elasticity through the unique interplay of mechanical and chemical properties between the three main tissue components: the fibrillar polypeptide collagen (primarily type II) that is organized into bundles *via* extensive cross-linking [5,6], large proteoglycans (mainly aggrecan) [7], and molecular H_2O. Water molecules are attracted within the tissue by osmotic forces and are primarily coordinated around Na^+, K^+ and other cations that are in turn associated with the sulphate ($-OSO_3^-$) groups bound to glycosaminoglycan (GAG) units of the proteoglycan [8]. The interplay between intercellular water and the macromolecular components of cartilage leads to the essential combination of elastic integrity and compressive stress resistance for dissipation of load at joints during locomotion. The repeating unit of fibrillar collagen consists of three α-helical chains that vary in their amino acid sequence according to the collagen type [5,6]. Cross-links form between the lysine and hydroxylysine residues of these chains [6,9] to strengthen the polymer. The ECM of articular cartilage mainly consists of the fibrillar collagen type II, along with minor amounts of collagens IX, X and XI, whereas skin and tendon mainly contain collagen types I and III [5].

The high temperature properties of collagen have typically been studied by thermal analysis techniques, especially differential scanning calorimetry (DSC) [1, 10-15]. The main process occurring in the lower temperature range before complete oxidation and destruction of the organic species is usually described in terms of a protein denaturation event, where the polypeptide chains become disentangled and unfolded from their functionally active state, but typically without any breakdown of the primary amino acid linkages [16]. The situation for collagen is complicated by the presence of a highly hierarchical structure containing multiply cross-linked units along with incorporated H_2O. Different stages of denaturation have been recorded, depending on the degree of heating and the rate and period of exposure to high temperature conditions [1]. It is suggested that "moderate" heating results in "reversible" denaturation associated with local structural changes, whereas "severe" heating causes irreversible unfolding to yield a random coiled structure. The presence of water also significantly affects the denaturation processes. The stability of the macromolecular assembly of collagen fibrils is thought to be associated with hydrogen bonding

within specific domains as well as water bridges formed between hydroxyproline residues in the triple helical structures [13,17]. The DSC measurements typically show an endothermic feature appearing in the range 135-200°C for samples prepared with low water content, that is correlated with the process of "irreversible" denaturation [13-15]. In experiments on dried rat tail collagen (11.5 w%), Miles *et al* found the endothermic peak to occur at 120 $^{\circ}$C [13]. However, significantly lowered denaturation temperatures have been observed as the hydration level was increased. Trebacz and Wojtowicz found an endothermic DSC peak at 137 $^{\circ}$C for a "dry" (5 wt% H_2O content) sample of bovine Achilles tendon, but recorded a value of 82 $^{\circ}$C for wet (58%) tissue [15]. The process of freeze-drying is typically used to remove molecular H_2O from cartilage and collagen materials. However, it has been suggested that some water molecules might remain strongly bound within the fibrillar collagen sublattice, and would only be released at high temperature [18]. The interpretation of these results in terms of collagen denaturation processes has been supported by results of circular dichroism (CD) experiments carried out for hydrated samples [17,19], but they have not yet been extended to dehydrated specimens. In our study we applied microbeam laser-excited Raman and fluorescence spectroscopy [20-24] both *in situ* at high temperature and following quenching and recovery to ambient conditions to study changes in the molecular structure associated with denaturation as well as chemical reaction processs in freeze-dried cartilage and collagen materials.

2. EXPERIMENTAL

We first examined freeze-dried samples of human articular cartilage obtained from biopsies of macroscopically normal tissue from femoral condyles following donor consent from the Stanmore Musculoskeletal BioBank, (Institute of Orthopaedics and Musculoskeletal Research, Royal National Orthopaedic Hospital, Stanmore, UK) and institutional approval (Research and Welfare Ethics Committee, Royal Veterinary College, URN 2010 0004H). Full-depth biopsies of cartilage from a 49 year donor were prepared using a 6 mm biopsy punch and placed in microfuge tubes sealed with perforated Parafilm®. Samples were freeze-dried at -70 °C and a pressure of 1.3 x 10^{-7} MPa (Virtis Genesis 35XL freeze-dryer, New York, USA) for 24 hours and then stored in a desiccator under vacuum. Separate collagen and proteoglycan fractions were extracted from a further human femoral condyle sample from a 58 year donor. The tissue was frozen in liquid nitrogen and pulverized using a ball mill dismembrator (Mikro-Dismembrator, Sartorius, U.S.A.) at 2500 oscillations/min for 2 mins. The powdered cartilage was transferred into a Falcon tube (20 mL) and extraction buffer added (4 M guanidine hydrochloride (GdHCl), 50 mM sodium acetate, 0.05 M ethylene diamine tetra-acetic acid, pH 5.8). The ratio of tissue to buffer solution was 1:15 (1.45 g to 21.75 ml). The

mixture was rotated for 48 h at 4 °C to extract soluble proteins and the soluble *vs* insoluble fractions separated by centrifugation (15,000 g for 20 mins). The insoluble fraction contains mostly the collagenous component of cartilage. This was washed with excess 95% ethanol during repeated washing/centrifugation steps (21 washes) to fully remove GdHCl as confirmed by Raman spectroscopy. This process resulted in a collagen[f] fraction that predominantly consists of collagen type II that was investigated in our spectroscopic study. The soluble component was then made up with 90% ethanol and chilled for 18 h at -80 °C to precipitate proteins that were recovered by centrifugation. The precipitate was washed with excess 95% ethanol to remove residual GdHCl and this provided the aggrecan rich proteoglycan fraction (aggrecan[f]) examined in the study. We also investigated a commercial sample of collagen type III of human placental origin (Sigma Aldrich, Dorset, UK) to compare its high temperature behavior with that of type II collagen, obtained as our collagen[f] sample. This triple-helical variety has close homology with the type II material [25].

Raman spectra were obtained using an InVia microbeam spectrometer system (Renishaw, Gloucestershire, UK) system with a 1200 grooves/mm diffraction grating and a CCD detector. The microscope was equipped with a 0.5 N.A/50x long working distance objective (Leica, Germany). A diode laser with wavelength 785 nm was used for excitation. Exposure times were set to 30s/scan and 4 accumulations were averaged for each spectral acquisition, for a total acquisition time of 16 minutes in all of these runs. For analysis of the less densely packed commercial collagen type III sample, Raman spectra were averaged over 2 spectral acquisitions (i.e., 8 accumulations, 32 minutes).

The background subtraction procedure of the Origin® program software was first used to remove any instrumental background in the spectral region of interest by first obtaining a blank spectrum for each sample set-up without any sample in place. We then established a background signature due to sample autofluorescence by manually fitting points throughout the fingerprint region to result in a flat baseline to the Raman data, for several experimental datasets. This constructed fluorescence signature remained constant for all points for which it was established, and this was stored as a background profile that was adjusted in intensity and removed from each obtained dataset to result in the background subtracted spectra shown in Figure 3.

High temperature studies were carried out using a Linkam TS1500 (Epsom, UK) microscope heating stage. Typically, spectra were obtained in 10 °C increments, by heating at 5°C/min followed by 2 min equilibration. Spectra were then acquired for a total of 16 min exposure of the sample to the laser beam at each temperature. The laser power was maintained at 1-2 mW at the sample surface to avoid beam damage. This was checked in several runs with different incident laser power and exposure time and no unexpected changes in the Raman signals were observed.

For programmed heating-recovery studies separate biopsy samples of freeze-dried cartilage from the 49 years donor were used. These were heated directly at a rate of 10 °C/min to the target temperatures of 100, 150 and 200 °C; also one sample was maintained at 200 °C for 30 mins. The samples were then quenched in water and left at 4 °C for 12 hrs before acquiring their spectra. To maintain a constant level of hydration the samples were kept submerged in water throughout the spectral acquisitions. Data collection proceeded as before but the counting period was extended to 4 spectral acquisitions (16 accumulations, 64 minutes) to compensate for the lowered signal intensity. In this case, each heating experiment used a different aliquot of the freeze-dried cartilage sample. In all of the studies, several sets of replicate Raman experiments were carried out with different samples, or on different spots within the same sample, to confirm reproducibility of the results.

TGA studies were carried out using a Netzsch STA449C thermal analyzer (Leipzig, Germany), attached to a quadrupole mass spectrometer Netzsch QMS 403C for analysis of evolved gases. A biopsy sample of the freeze-dried cartilage sample (49 years donor) weighed 5.4 mg before analysis and was heated at a rate of 10°C/min within the range 20-400 °C.

3. RESULTS AND DISCUSSION

3.1 Fluorescence and Raman spectra collected *in situ* at high temperature

Initially we attempted to collect Raman spectra using visible (514.5 nm, 633 nm) laser excitation, but the background fluorescence was found to be too intense to observe the weak Raman features (Fig. 1). Collagen and cartilage contain several well known fluorophores including amino acid residues containing aromatic side chains (tryptophan Trp, tyrosine Tyr and phenylalanine Phe) as well as pyrrole- and pyridinoline-containing cross-linking groups that give rise to autofluorescence when excited by UV to visible light [26-28]. However only the Trp species are expected to give rise to significant fluorescence when excited at wavelengths >295 nm, at least before denaturation occurs [26].

We collected our spectra for freeze-dried cartilage samples using 785 nm laser irradiation, where only weak fluorescence remains at ambient temperature, causing a gently sloping background extending throughout the 100-3000 cm^{-1} region (Fig. 1). As the temperature was raised unpon heating to 100 °C, the fluorescence background initially increased slightly, but this then diminished rapidly upon exposure to the exciting laser (with a constant 16 min exposure time on the same sample spot). We believe that this is due to photobleaching effects (Fig. 2a). However, upon heating to above 140 °C, the fluorescence intensity increased more rapidly, and photobleaching no longer occurred (Fig. 2b). The signal eventually became so strong that the weak Raman features could no longer be distinguished above 180 °C, and this persisted after recovery of the samples to

ambient temperature. Samples treated at the higher end of the temperature range became yellowish to dark brown in color, indicating that thermal degradation and oxidation reactions had also occurred. In the temperature range between 100-180 °C we could assign the onset of the strong fluorescence excited by 785 nm laser irradiation and extending throughout the long wavelength range to conformational reorientation effects within the collagen macromolecules associated with irreversible denaturation. However, the UV-excited fluorescence of Tyr both unbound and contained within (hydrated) calf skin (type I) collagen has been shown to decrease in intensity with temperature in the 9-60 °C range [27]. Second harmonic generation (SHG) studies that investigate the presence of non-linear optically active centers without inversion symmetry have been carried out for hydrated type I collagen did show an increase in SHG intensity at the proposed denaturation temperature (54 °C), although the results were complicated by the kinetics of fibril reorientation effects that may occur on a different timescale to localized denaturation of the protein structures [29]. Our observations on freeze-dried human articular cartilage samples suggest that changes in the fluorescence properties excited by deep red to near-IR illumination may result from irreversible thermal denaturation of collagen in the 140-180 °C temperature range, as suggested from the DSC results [1, 13-15].

We then investigated structural changes occurring in the low temperature range using Raman spectroscopy. Representative background-subtracted spectra obtained in the temperature ranges 20-100 and 120-180 °C are illustrated in Figure 3. These are shown between 700-1850 cm^{-1} to highlight the main characteristic vibrations of collagen as well as aggrecan components contained within cartilage [20-24]. Raman spectra obtained above 120 °C exhibited a new peak appearing at 1788 cm^{-1}, that we can identify as due to the ν_{CO} stretching vibration of non-ionized carboxylic acid (-COOH) units [20]. These species can only appear following partial hydrolysis of peptide links within the samples. A similar peak was found to be present for an amino acid mixture representing the primary sequence of collagen molecular fragments in acidic solution [30]. We could not confirm the expected complementary formation of NH_2 groups because their vibrations are masked by the strong amide bands in the Raman spectrum.

Because our high-temperature Raman spectroscopic investigation had revealed the likely occurrence of peptide bond hydrolysis we investigated the possible presence of H_2O within the nominally anhydrous freeze-dried sample. The most obvious way to do this would be by examining Raman active vibrations in the O-H stretching region, at ~3000-3700 cm^{-1} from the exciting line [31]. However, our experiments required us to use 785 nm laser excitation to avoid intense autofluorescence effects, and our CCD detector could no longer operate efficiently at these near-IR wavelengths (3500 cm^{-1} Stokes-shifted from 785 nm excitation lies at 807 nm), that in fact lies outside the manufacturers specified range. In addition, strong N-H stretching vibrations also occur

throughout this spectral range. We did attempt some FTIR experiments on quenched samples, but those required using cells constructed from quartz glass rather than KBr to ensure proper hydration levels of the cartilage and collagen samples, and the SiO_2 material contained its own trace O-H signature that had to be subtracted, along with the strong N-H stretching features. We decided that vibrational spectroscopy in the O-H stretching range was not the best option to study the possible presence of remaining H_2O component bound within the collagen matrix.

We applied thermogravimetric analysis (TGA) coupled with mass spectrometry (MS) to identify the evolved gas species as a function of temperature (Fig. 4). The results clearly demonstrated that 5-7% by weight of the molecular H_2O component was retained within the freeze-dried material, and this was released progressively from the onset of heating and continuing up to ~220 °C. That result is consistent with the previous suggestion that freeze drying does not completely remove all water associated with the fibrillar collagen matrix [17]. Above 220 °C CO_2 begins to be detected among the gaseous products indicating the onset of tissue combustion that also contributes to further and final loss of H_2O component.

We then examined changes in the Raman spectra occurring at lower temperature in more detail, focussing on peaks in the 700-1850 cm^{-1} region. Above 60 °C we observed a slight decrease in intensity of the 1635 cm^{-1} shoulder on the amide I band. This can be attributed to a reduced contribution from the bending vibration of molecular H_2O [31] (Fig. 2). We also observed a decrease in intensity of the 934 cm^{-1} peak attributed to C-C vibrations adjacent to the carbonyl group on proline [32], as well as broadening accompanied by a shift to lower wavenumber in the peaks near 1240 cm^{-1} that are assigned as part of the amide III doublet [21]. Similar spectral changes have been observed to occur in Raman spectra from collagen as a function of decreasing water content at ambient temperature [32].

In order to determine which component of articular cartilage was involved in the peptide bond hydrolysis reaction, collagen[f], mainly type II, and the proteoglycan (mainly aggrecan[f]) fractions were extracted from one of our cartilage samples, freeze-dried and examined by high temperature Raman spectroscopy (Fig. 5). We observed the appearance of the same 1788 cm^{-1} Raman peak for the collagen[f] sample when it was heated above 120 °C, but not for aggrecan[f]. We also observed reduction in the 934 cm^{-1} peak intensity for collagen[f] during heating (Fig. 5). We observed similar changes in the spectra obtained for a purified and nominally anhydrous commercial sample of collagen type III that has a triple-helical fibrillar structure analogous to collagen II, and found that heating resulted in similar appearance of the 1788 cm^{-1} peak and reduction in the 934 cm^{-1} feature above 100-120 °C (Fig. 6).

Our results indicate that peptide bond hydrolysis occurs among different collagen-containing samples, even when these have been freeze-dried to nominally remove all H_2O content.

Our TGA and Raman data in fact indicate that some (5-7 wt%) H_2O component is retained within the macromolecular matrix associated with the collagen component, and is gradually released during heating to within the 60-220 °C range. That then results in hydrolysis of some of the peptide linkages, causing disruption of the polypeptide backbone that then facilitates the irreversible denaturation recorded at higher temperature. We note that the permanent fluorescence background increased significantly for all samples studied: cartilage, collagen[f], aggrecan[f] and collagen III when heated above 100 °C.

3.2 Water uptake and Raman spectroscopy of samples following heat treatment

A further series of initially freeze-dried cartilage samples were heated to specified temperatures, then recovered to ambient temperature and placed in water. Raman spectra were then obtained for the samples submerged in water to maintain a constant level of hydration (Fig. 7). Samples heated to 20, 80 and 150 °C exhibited no obvious changes in their Raman spectra. However, following exposure to 200 °C we could clearly observe the appearance of the 1788 cm^{-1} peak, along with spectral changes in the 934 cm^{-1} and 1410 cm^{-1} regions as before (Fig. 7). The peak became more prominent after maintaining the sample at 200 °C for 30 mins. We note that the temperature at which the peptide bond hydrolysis occurs is significantly higher than that observed in the *in situ* heating experiments. This could be related to the different timescales implied by the heating schedules. In the first series of experiments, the temperature was raised at 10 ^{o}C/min then maintained at the target value during each equilibration and acquisition period (approximately 16-20 mins) before raising to the next temperature. That resulted in a slow overall heating rate. In the the present scans, the temperature was raised directly to the target value at 10 ^{o}C/min, then quenched immediately in water before acquisition. Each experiment was carried out with a fresh sample of freeze-dried cartilage. That resulted in a heating rate that better approximated the timescale of the DSC experiments (1-10 ^{o}C/min) used previously to study high temperature behavior and denaturation phenomena [1, 10-15]. In particular, Miles *et al* noted an irreversible change in structure of freeze-dried rat tail collagen at 150-200°C, that they attributed to unfolding of the triple helical polypeptide chains [13]. Our results now suggest that this process was at least partly assisted by partial hydrolysis of the peptide links and breakdown of the primary chain structure.

In our study, samples exposed to different temperatures showed different responses to rehydration at ambient conditions. Following exposure to 200 °C, the peak at 934 cm^{-1} decreased markedly in intensity (Fig. 7), whereas the 1420 peak has shifted to 1410 cm^{-1} and the 1635 cm^{-1} feature has decreased (Fig. 8). These spectral changes were enhanced following heating at 200 °C for 30 mins. Similar changes have previously been shown to be dependent on the degree of

hydration of collagen samples [32]. The changes occur concurrently with the appearance of the 1788 cm^{-1} peak, indicating that the irreversible structural changes occurring within this temperature range limits the ability of cartilage samples to reabsorb water. Other inter-relationships between the collagen triple-helical fibril architecture and proteolysis reactions have been discussed in the literature [33].

4. CONCLUSIONS

The background fluorescence excited by a 785 nm laser increased rapidly and irreversibly when cartilage and collagen samples were heated above approximately 140 ^{o}C. This can be attributed to conformational changes in the fibrillar triple-helical polypeptide structures associated with denaturation previously observed by calorimetry studies. In a similar temperature range, the Raman spectra show appearance of a peak at 1788 cm^{-1} due to the C=O stretching vibration of non-ionized carboxylic acid groups, caused by hydrolysis of peptide bonds. Separating the cartilage sample into collagen^{f} and aggregan^{f} fractions shows that these spectral changes are associated with the collagen (predominantly type II) component. Our comparison with a commercial sample of collagen type III demonstrates that the peptide bond dissociation reaction is common among samples of different colagen types and origin. The occurrence of the peptide bond hydrolysis reaction requires the presence of molecular H_2O maintained within the nominally anhydrous freeze-dried tissue. That is confirmed by TGA studies that show the presence of 5-7 wt% H_2O that is released gradually between 60-200 ^{o}C. Programmed heating-recovery experiments designed to better approximate the timescales used in DSC measurements show the appearance of the 1788 cm^{-1} peak and corresponding hydrolysis reaction at higher temperature, close to the range recorded for the denaturation event in DSC studies. That result suggests that the phenomenon of irreversible denaturation of collagen and cartilage is accompanied by and perhaps assisted by breakdown of the primary polypeptide chain. The increasingly denatured samples exposed for longer time at higher temperature show a reduced capacity to re-absorb water as indicated by changes in the Raman spectra. The results give new insight into the role of H_2O component that is attracted strongly into the collagen and cartilage tissue and is maintained even after freeze-drying, that is then released at high temperature to participate in peptide bond hydrolysis reactions that participate in the denaturation process. The resulting materials show a decreased ability to re-hydrate following recovery after heating, with implications for design of cartilage- and collagen-based biomaterials.

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FIGURE CAPTIONS

Figure 1. Laser excited fluorescence for a freeze-dried sample of human articular cartilage. Spectra obtained using 514.5 and 633 nm laser excitation show high fluorescence background intensity that precluded Raman measurements; only for 785 nm laser excitation were Raman peaks apparent.

Figure 2. Raman spectra for a freeze-dried sample of human articular cartilage obtained during heating between 20-180 °C. (a) Comparison of Raman spectra obtained at ambient (20 °C) and high temperature (100 °C) showing a decrease in fluorescence background due to photobleaching effects. (b) Spectra obtained above 140 °C showed a rapid increase in background fluorescence that did not decrease during the collection period. Above 180 °C Raman peaks could no longer be distinguished from the intense fluorescence background.

Figure 3. Background subtracted Raman spectra of freeze-dried articular cartilage in the 700-1850 cm^{-1} region of interest demonstrating structural changes as a function of temperature between 20-180 ^{o}C. The temperature ranges have been separated into datasets obtained between 20-100 ^{o}C and 120-180 ^{o}C for clarity. All spectra were normalized to the Phe peak at 1003 cm^{-1}. Gray shaded areas highlight regions of interest discussed in the text.
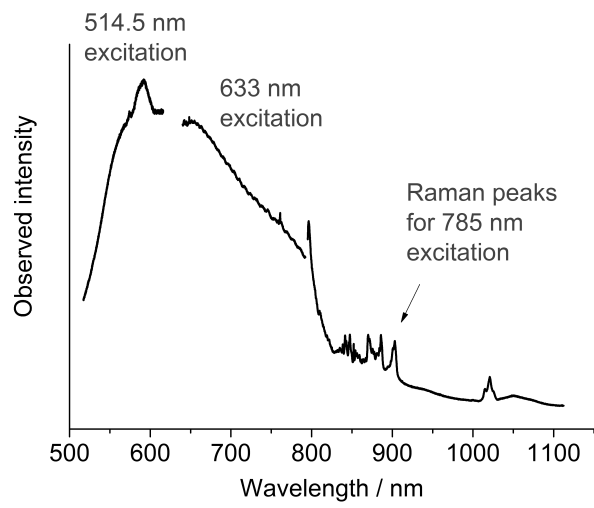
Figure 4. Thermogravimetric analysis of freeze-dried human articular cartilage combined with mass spectrometric analysis of the evolved gases. Mass loss is shown as a percentage of the initial value. Evolved H_2O and CO_2 were detected as singly charged ions as ion currents measured *via* mass spectrometry.
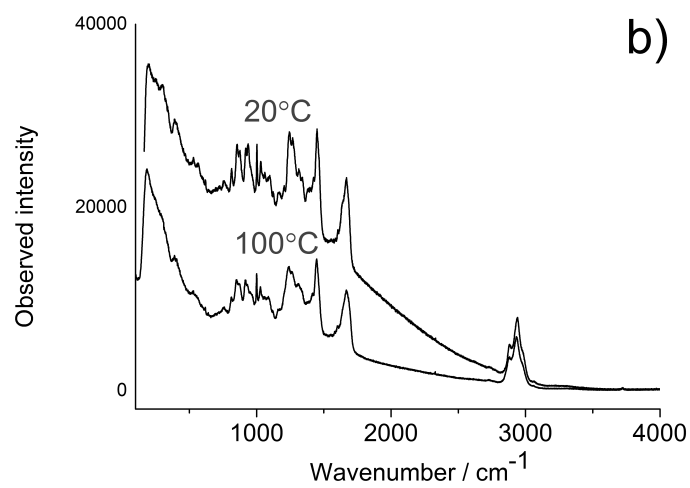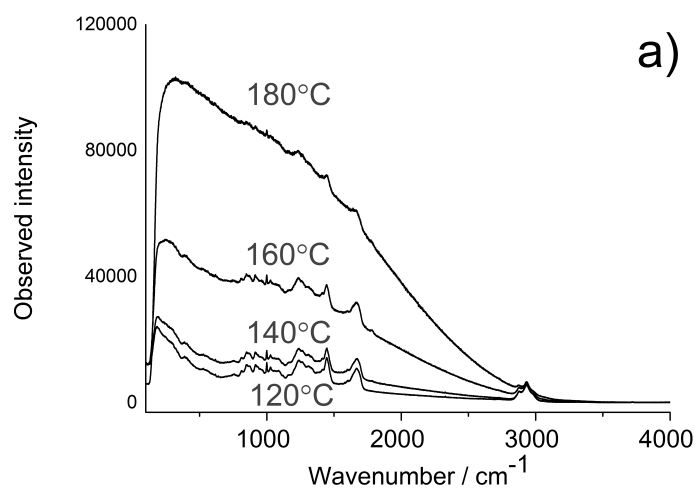
Figure 5. Background subtracted Raman spectra of freeze-dried collagen[f] and aggrecan[f] in the 700-1850 cm^{-1} region of interest at selected temperatures (20, 100, 120 and 140°C). All spectra were normalized to the Phe peak at 1003 cm^{-1} and the gray shaded areas highlight regions in which spectral changes occur as a function of temperature.

Figure 6. Background subtracted Raman spectra of collagen type III in the 700-1850 cm-1 region as a function of temperature (20, 100, 120 and 140 °C). All spectra were normalized to the phenylalanine (Phe) peak at 1003 cm^{-1}.

Figure 7. Raman spectra of freeze-dried articular cartilage samples heated to 100, 150, 200°C, and 200°C* (denotes sample heated at 200°C for 30 mins). All samples were exposed to H_2O after recovery to ambient temperature and analyzed while immersed in water. Gray shading highlights regions where the main spectral changes were observed.

Figure 8. Detailed comparison of Raman spectra in a) 1350-1500 cm^{-1} and b) 1500-1900 cm^{-1} regions for cartilage samples heated to 100, 150 and 200 ^{o}C. One sample was maintained at 200°C for 30 mins. All samples were returned to ambient temperature and rehydrated before obtaining the spectra.

514.5 nm
excitation

633 nm
excitation

Raman peaks
for 785 nm
excitation

Observed intensity

Wavelength / nm

Cartilage Biopsy Sample 1

1003

1788 (ν C=O)

Temp.

180°C
160°C
140°C
120°C

Raman intensity

Wavenumber / cm^{-1}

Cartilage Biopsy Sample 1

934
1003
1240

Temp.

100°C
80°C
60°C
40°C
20°C

Raman intensity

Wavenumber / cm^{-1}

Figure 4

Collagen^f

934

1788

Temp.

140°C

120°C

100°C

20°C

Aggrecan^f

140°C

120°C

100°C

20°C

Raman intensity

Wavenumber / cm^{-1}

800   1000   1200   1400   1600   1800

Collagen type III

934

1788

Temp.

140°C

120°C

100°C

20°C

Raman intensity

Wavenumber / cm⁻¹

Cartilage post-heat treatment

934 · 1410 · 1635 · 1788 (ν C=O)

Temp.

200°C
(30 mins)

200°C

150°C

100°C

20°C

Raman intensity

Wavenumber / cm^{-1}