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Effect of Water Content on Enthalpic Relaxations in Porcine Septal Cartilage

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Abstract

Cartilage thermoforming is an emerging surgical technology which uses heat to accelerate stress relaxation in mechanically deformed tissue specimens. Heat induced shape change in cartilage is associated with complex thermo mechanical behavior of which the mechanisms are still a subject of debate. Differential scanning calorimetry (DSC) was used to characterize the threshold temperatures and enthalpies in cartilage as a function of water content. The DSC identified two enthalpic events in porcine nasal septal cartilage, which depend on the water content. The change in the water content of cartilage impacts the interactions between matrix macromolecules and water molecules, which may be associated with a bound-free water transformation (reversible process) and a denaturation of cartilage (irreversible process).

Keywords

cartilage; cartilage reshaping; enthalpic relaxation; nasal septum; thermal transition; threshold temperature; TMDSC

Introduction

A novel method to reshape cartilage using laser irradiation was introduced by Helidonis *et al.* in 1993 [1]. Laser heating of mechanically deformed cartilage specimens accelerates stress relaxation and leads to permanent shape change and occurs below the ablation threshold. It has been hypothesized that cartilage reshaping occurs due to a heat induced transition that leads to rearrangement of molecular bonds in the cartilage matrix macromolecules [2–5]. The two different forms of water in cartilage, free and bound (e.g., exchangeable and non-exchangeable) are the subjects of intense discussion in the literature. It is known that hydrogen bonds result in the organization of individual water molecules in a pseudo crystalline configuration and the formation of ‘bound water’ [6–8].

Recently, nuclear magnetic resonance (NMR) spectroscopy of simple collagen-water systems has shown that water molecules exist either as bulk water, which does not interact with collagen, or as interior hydration water molecules [9–11]. Collagen fibrillar water (CFW) is thought to consist of water molecules involved in both inter- and intrahelical hydrogen bond formation within collagen fibrils. Bagratashvili *et al.* [2] found that the proportion of bound water in cartilage is around 4% using differential microcalorimetry and FTIR spectroscopy based on a single endothermic DSC peak in cartilage. Water liberation and adsorption was controlled by diffusion through the tissue and by a presumed bound-to-free water transformation. The water movement in cartilage is related to the alternating breakage and reformation of weak bonds between water molecules and proteoglycans.

While hydrogen bonding may be one contributing factor to the stabilization of the cartilage matrix macromolecular structure, the reduction in water content accompanying either heating or dehydration may also lead to a change in the thermal behavior of cartilage. However, there is limited data available on the thermal behavior of cartilage and there is an incomplete understanding of processes such as the disruption of hydrogen bonds, which may be responsible for heat-induced stress relaxation. In addition, few studies have been performed regarding the relationship between water liberation and heat-induced stress relaxation.

Thermoanalytical techniques measure the change in physical or chemical properties as a function of temperature. Differential scanning calorimetry (DSC) has widely been established in the study of thermal decomposition of biological macromolecules and provides insight into the changes in the bonds between water and matrix macromolecules. Previous calorimetry studies of cartilage established calorimetric standards of the healthy cartilage [12–13] and demonstrated differences between various stages of osteoarthritis [14–15]. Ignat'eva *et al.* observed a single weak endothermic peak of porcine septal cartilage (collagen type II) and cornea (collagen type I) and investigated curves of laser treated and enzymatically digested cartilage specimens using DSC. The endothermic process was assumed to be the sum of three endothermic peaks at 60, 65 and 75°C [16, 17]. Sillinger *et al.* observed three separable thermal denaturation events in one wider endotherm of knee-joint cartilage and demonstrated that the transition temperature of the infected cartilage produced by septic arthritis shifted toward higher temperatures. However, the authors did not report thermal transitions associated with the presence of two types of hydrogen bonds, which may relate to weakly and strongly bound water compartments [14].

In this study, we focus on characterizing the thermal properties of cartilage in order to better understand the heat-induced stress relaxation that contributes to the shape change process during clinical cartilage thermoforming. While thermoanalysis has been used to examine normal and degenerative cartilage, the impact of water content has not been examined. The objectives of this study are to examine the importance of water content in contributing to the threshold temperatures and enthalpies of cartilage thermal transitions as a function of water content and to determine the impact of water content on the strength of the bonds between water molecules and matrix macromolecules.

Experimental

Materials and methods

Preparations of tissue specimens—Nasal septal cartilage were extracted from pig crania obtained from a local abattoir (Clougherty Packing Company, Vernon, CA) as previously described [18]. All protocols and experimental design settings were reviewed and approved by the University of California, Irvine Institutional Animal Care and Use Committee. Disc shaped cartilage specimens (3–4 mm in diameter, 3–10 mg for temperature modulated differential scanning calorimetry (TMDSC) were fashioned using a dermatologic biopsy punch. In the TMDSC experiments, thin specimens were used to reduce axial temperature gradients that might occur during heating. The specimens were maintained in ambient temperature saline solution until just immediately before TMDSC measurements. Before TMDSC studies, the surface water was initially removed by a laboratory paper and the remaining mass reduction of cartilage occurred naturally via water evaporation. All samples were separately placed in the saline solution and stored at a temperature of 4°C for no longer than 24 h before use in experiments.

Temperature modulated differential scanning calorimetry (TMDSC)

Studying the impact of water content can be performed by using hermetically sealed pans that do not allow the escape of water. DSC curves were recorded between 30 and 95°C using a temperature-modulated differential scanning calorimeter (mDSC 2920, TA Instruments, New Castle, Delaware) with a sinusoidal modulated heating rate of 2°C min⁻¹ (amplitude 0.3–0.45°C and periods 60–100 s). The sample specimens were sealed in a hermetically sealed DSC cell and kept at ambient temperature for 30 min to reach thermal equilibrium before placement into the vestibule of the calorimeter. DSC cells were weighed immediately before and after DSC measurements.

Samples weighing 3 to 10 mg were hermetically sealed in aluminum pans (TA Instruments) and an empty pan was used as a reference. The samples were then subject to a dry N₂ flux. The recording, deconvolution and analysis of the heat flow signals were performed using software (Thermal Solutions, TA Instrument). The onset temperature was defined as the point of intersection between the extrapolated baseline and the tangent to the point of the greatest slope on the leading edge of the peak. The baseline corresponds to the portions of the DSC curve for which T is approximately zero.

$W_{TOT}(t)$ is the total water mass in the specimen at time t . Since we assume that the total water content in all porcine septal cartilage tissues is essentially identical, measuring the fractional water content of this tissue will allow us to estimate the total water content (W_{TOT}) in a specimen of known mass. In an analogous manner, the fractional water loss (w_{LOSS}) in a specimen can be expressed as:

$$w_{LOSS} = (W_{TOT}(t=0) - W_{TOT}(t)) / W_{TOT}(t=0) \quad (1)$$

For example, when w_{LOSS} is 0 there is no reduction in the mass of the cartilage specimen. Likewise, when w_{LOSS} is one, only trace water remains in the specimen.

Statistical analysis

The results were statistically evaluated by Microsoft Excel and expressed as mean values $\pm$ SEM. Data were analyzed with 1-way analysis of variance (ANOVA). The level of significance was set at $P < 0.05$.

Results and discussion

DSC curves and complex heat capacity of porcine nasal septal cartilage

Temperature modulated DSC (TMDSC) was used to estimate the enthalpies and corresponding peak temperatures for cartilage specimens in the absence of any dehydration (e.g., fully hydrated cartilage tissue) (Fig. 1). Two enthalpic events were identified and these are labeled in Fig. 1. The enthalpies were calculated as the total time integrated power (per unit mass) beneath each peak (in J g^{-1}) (Table 1). The local maxima for these two distinct enthalpic events occurred in the temperature ranges 59–60 and 69–71°C for the first and second peaks, respectively. Integrating the area below TMDSC curve relative to the baseline trend, we obtain enthalpies of $0.105 \pm 0.03 \cdot 10^3 \text{ J kg}^{-1}$ and $0.140 \pm 0.05 \text{ J} \cdot 10^3 \text{ kg}^{-1}$ for the first and second events, respectively. The onset temperature (calculated by extrapolating the peak temperature back to the baseline) of the first enthalpic event in cartilage was 50–52°C. These peaks may correspond to enthalpic events associated with tissue shape change during thermoforming procedures.

In Fig. 1, the change in the heat flow baseline is observed resulting from the change in the heat capacity of cartilage, which may be accompanied by changes in mechanical properties [19]. Complex heat capacity (C^*) is determined by dividing the modulated heat flow amplitude by the modulated heating rate amplitude and can be split into an in-phase, real component C' (usually considered the thermodynamic heat capacity) and an out-of-phase, imaginary component C'' . The complex heat capacity (C^*) of fully hydrated porcine septal cartilage (i.e., water content 85% of total mass) was $2.9 \pm 0.3 \cdot 10^3 \text{ J kg}^{-1} \text{ K}^{-1}$ at 27°C and $3.0 \pm 0.5 \cdot 10^3 \text{ J kg}^{-1} \text{ K}^{-1}$ at 70°C, the value is smaller than that of water $4.17 \cdot 10^3 \text{ J kg}^{-1} \text{ K}^{-1}$ (27°C), $4.19 \cdot 10^3 \text{ J kg}^{-1} \text{ K}^{-1}$ (70°C). The changes in heat capacity were observed at about 52–54°C and 68–70°C (Fig. 1), however the changes were insignificant.

Water content dependent DSC curves

The total heat flow curves as a function of temperature (Fig. 2) take on a distinctly different appearance when the initial water content of the specimen is altered. This underscores the importance of water in this process. Water content was manipulated by allowing the specimen to dehydrate at ambient temperature prior to calorimetry. Both the peak temperatures for two endothermic events and their corresponding enthalpies were dependent on the degree of hydration. As the water content decreases in cartilage, an enthalpic peak at low temperature shifted to a higher temperature and the corresponding enthalpy increased, while the enthalpic event at the high temperature did not demonstrate water loss dependence. The onset temperatures for each endotherm along with the corresponding enthalpies were plotted as a function of fractional water loss, w_{LOSS} as represented in Figs 3a and b, respectively. Notably, when water loss exceeded about 35–40%, only one endothermic event could be distinguished.

In Fig. 3a, the first peak temperature was independent of water content below 20% water loss and proportional to water loss between 20 and 35%, whereas the peak temperature of the second enthalpic event is independent of the change in water content below 35% water loss. Similarly, in Fig. 3b the corresponding enthalpy of the first enthalpic event is independent of water content below 20% water loss and proportional to water loss between 20 and 35%, whereas the enthalpy of the second enthalpic event is independent of water content below water loss 35%. In Figs 3a and b, above 35–40% water loss, one enthalpic event was observed and the corresponding peak temperature and enthalpy increased with increasing water loss, reaching $75 \pm 2^\circ\text{C}$ and $3 \pm 0.5 \cdot 10^3 \text{ J kg}^{-1}$, respectively. The water loss in excess of 50% results in the one onset temperature at about 72°C . Likewise, the enthalpies were approximately $0.05\text{--}0.2 \cdot 10^3 \text{ J kg}^{-1}$ for 1st endothermic event and $0.12\text{--}0.25 \cdot 10^3 \text{ J kg}^{-1}$ for 2nd endothermic event when the water loss was less than 20–25%. The value of enthalpy increased to $1.25\text{--}1.6 \cdot 10^3 \text{ J kg}^{-1}$ when water content fell from 35% to 50% and reached $3 \pm 0.5 \cdot 10^3 \text{ J kg}^{-1}$ at a loss of 50% (Fig. 3b).

Laser heating of cartilage creates a localized region of dehydration within cartilage tissue in the area of light distribution. These local variations in water content in cartilage lead to marked changes in both the temperature and enthalpy, which accompany the mechanical changes, observed during surgical reshaping procedures. Hence, water content may be as important as temperature elevations in the thermoforming of cartilage. This focal loss of water may alter the weak non-covalent interactions (e.g., hydrogen bonds) between the collagens and proteoglycans within the matrix.

Two distinct enthalpic events in nasal cartilage

Bagratashvili *et al.* [2] used differential microcalorimetry and FTIR spectroscopy to estimate the amount of bound water in cartilage which was about 4% by mass. They found that most water molecules in cartilage exhibit the characteristics of free water and have little interaction with matrix macromolecules. This is in agreement with our assumption that the decrease in mass during heating may result from the loss of free water by evaporation.

In the present investigation, calorimetry identified two distinct enthalpic events in normal cartilage (Fig. 1), which suggest that cartilage may have two distinct moieties of water associated with matrix macromolecules (collagen and proteoglycan), i.e., a weakly bound water compartment and strongly bound water compartment with quantitatively different characteristics in cartilage matrix. A third compartment of free or bulk water exists in the tissue as well, but does not interact with matrix proteins.

In DSC curves, the first peak observed around $55\text{--}65^\circ\text{C}$ may correspond to a step in the denaturation process where weakly bound water molecules are cleaved from matrix macromolecules (i.e., PG and collagen). The second peak observed around $72\text{--}75^\circ\text{C}$ may correspond to the cleavage of more strongly bound water leading to the complete denaturation of cartilage. In addition, the existence of the ‘shoulder’ in the first enthalpic event suggests that the hydrogen bond strength associated with weakly bound water might not be either uniform or stable. The changes in complex heat capacity (Fig. 1) are very small due to weak enthalpic events potentially associated with the disruptions of weakly and strongly bound water molecules to macromolecules in cartilage.

The enthalpic events observed during the heating of cartilage have a strong relationship with the previously measured changes in cartilage mechanical behavior [20–24] in terms of temperature thresholds. We hypothesize that the disruption of weakly bound water with macromolecules in cartilage may be associated with the process of a bound to free water transformation, which leads to heat-induced shaping of cartilage and is a step along the cascade of events leading to complete denaturation.

Cartilage hydration and DSC curves

The impact of water content on thermal behavior is difficult to estimate, specifically because little is known about thermal behavior of cartilage tissue. However, collagen is the most abundant component of cartilage tissue (apart from water) and the thermal behavior of cartilage might be expected to parallel some of the trends observed in the calorimetric study of isolated collagen [25]. Experiments focusing on cartilage (Figs 3a and b) show that the peak temperature and corresponding enthalpy as a function of water content is similar to heat flow trends observed in simple water-collagen molecule mixture studies [10, 25–29]. The change in the peak temperature of the first enthalpic event with water content is in close agreement with what has previously been reported in simple collagen systems. In contrast, the peak temperature of the second enthalpic event in cartilage shows very low water content dependence in cartilage.

In this study, the first enthalpic peak shifts to a higher temperature and the corresponding enthalpies increase with water loss. Figures 3a and b may be indicative of the increase in the hydrogen bond strength and/or the number of hydrogen bonds between water and macromolecules (collagen and PG). Up to a water loss of 20%, cartilage remains sufficiently hydrated and can contribute free water to stabilize the matrix macromolecules. In contrast, hydrogen bond strength and number may be affected by the large decrease in free water during dehydration. This leads to a change in intermolecular charge–charge repulsive force in cartilage produced by neighboring and adjacent hydrogen bonds in PG and in cartilage [30]. The change in these surrounding conditions, that is, intermolecular charge–charge repulsive force, may alter the process of adsorption and desorption of water to macromolecules.

The second enthalpic event may be merged with the first enthalpic event because the peak temperature of the first enthalpic event gradually increased when water loss exceeded 20%, while the peak temperature of the second enthalpic event appears to be independent of water content. The behavior of the enthalpic event observed between 35 and 45% water loss is assumed to be associated with weakly bound water. Between a water loss of 20 to 45%, negatively charged proteoglycans produced by the release of bound water due to dehydration may be locally neutralized by free Na^+ and Ca^{2+} ions. The remaining negatively charged proteoglycans may have an impact and may produce an increase in the bond strength between water molecules and PG neighboring or adjacent hydrogen bonds. In addition, the decrease in free water may induce the unstable hydrogen bonds associated with PG into a stable state, which may result in an overall increase in the number of stable hydrogen bonds.

As suggested by the behavior of the first peak in Figs 3a and b, the hydrogen bond strength and the number of water molecules associated with strongly bound water likely did not change with water content up to 35% water loss. In contrast, the second enthalpic event changes little with water loss. Cartilage matrix macromolecules may be expected to remain maximally swollen until W_{LOSS} reaches 35% (Figs 3a and b). Between 45 and 55%, the two enthalpic events merge to produce one peak and then the peak temperature became independent of water content. Consequently, the reversible and simultaneously occurring adsorption and desorption of water to proteoglycans stabilizes, which may be due to a fixed number of weakly and strongly bound water molecules.

We observed an increase in the first peak temperature with water loss in cartilage indicating a substantial increase in thermal stability. This is in agreement with what is observed in water-collagen mixtures [10, 25–29]. In contrast, the observed increase in the enthalpies with water loss is exactly opposite of what occurs in water-collagen mixtures [27]. It has been reported that in the water-collagen molecules mixtures, changes in enthalpy as a function of water content may be a consequence of either hydrogen bonding or by both hydrogen bonding and hydrophobic interactions [27]. In a complex material like cartilage, the potential contribution of hydrophobic bond interactions may be a contributing factor. We hypothesize that the changes in the peak temperatures and enthalpies are a consequence of alterations in the binding forces between water molecules and macromolecules (collagen and proteoglycan), which is dependent on water content.

In the present study, when the mass fraction of water in cartilage is 0.55, one dominant endothermic peak was observed at 65°C with an enthalpy of $1.5 \cdot 10^3 \text{ J kg}^{-1} \text{ K}^{-1}$, which is close to Ignat'eva's reported values [16]. It is unclear what the tissue water content was in their studies, which may be one reason for why they differ from the present result. A secondary but perhaps important difference may be the use of TMDSC in this study. TMDSC is more sensitive to subtle enthalpic changes in materials. Finally, their heating rates ($10^\circ\text{C min}^{-1}$) were much higher than what was used to obtain our data.

DSC curves obtained in hydrated cartilage specimens identified two distinct thermodynamic events. The first enthalpic event may be a reversible adsorption and desorption process of water to macromolecules ('bound-free water transformation') because it is recovered by the rehydration process. The second enthalpic event may correspond to thermal denaturation (irreversible process). Since water content is an important factor influencing cartilage transition behavior, hydrogen bonds and their disruption or formation may be critical elements responsible for shape change during cartilage thermoforming. The disruption of the intra- and intermolecular hydrogen bonds, particularly within the helical structure of cartilage collagen type II fibers, may be responsible for the two enthalpic transitions identified using calorimetry.

DSC curves obtained in cartilage specimens with different initial water content identified marked changes in both the temperature and energy of each enthalpic relaxation. The first enthalpic event, likely resulting from the disruption of weakly bound water with the macromolecules of the cartilage matrix, was dependent on water content while the second enthalpic event was not. Compared with strongly bound water to collagen, the disruption of

hydrogen bond between water and PG occurs at relatively lower temperature due to weak interaction between water and PG, which may be responsible for bound-to-free water transformation. This change in the interaction between water molecules and proteoglycans leads to a change in the heat-induced stress relaxation behavior of cartilage. While the heating rates used in calorimetry are much slower than those used in clinical laser cartilage reshaping procedures, the information gained during calorimetry provides a starting point at which to begin to understand the processes involved in shape change. Further investigations should be focused on elucidating these molecular mechanisms.

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Fig. 1. DSC curve and complex heat capacity of porcine septal cartilage (fully hydrated specimen) as a function of temperature

Fig. 2. TMDSC curves of porcine septal cartilage with different percentage of water loss ($W_{LOSS}\%$); a – 0–20% b – 20–55%

Fig. 3. a – peak temperatures and b – enthalpies of porcine septal cartilage as a function of the water loss percentage (Diamond, square and triangle marks correspond respectively with the first and the second enthalpic events and one merged enthalpic events)

Table 1
Thermal properties of porcine septal cartilage: transition temperatures and enthalpies

Type	1 st peak $T/^{\circ}\text{C}$	1 st peak $H/\text{kJ kg}^{-1}$	2 nd peak $T/^{\circ}\text{C}$	2 nd peak $H/\text{kJ kg}^{-1}$
Temperature modulated DSC	58.5 ± 0.2	0.105 ± 0.03	69.5 ± 0.5	0.14 ± 0.05