Integrated Adiabatic Scanning Calorimetry, Light transmission and Imaging analysis of Collagen Deterioration in Parchment

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Abstract

The collagen denaturation process in several artificially aged modern parchment samples were studied by combining Adiabatic Scanning Calorimetry and Light Transmission Analysis investigations. It was found that the calorimetric evaluations enabled not only to confirm the previously reported specific heat results concerning the collagen denaturation peaks, but also to identify a novel broad feature most likely associated with additional transformations occurring once denaturation takes place. Moreover, the combination of the investigations by the two adopted techniques allowed to validate the light transmission analysis as a novel simple method capable of recording the entire collagen denaturation temperature path of the analyzed samples. Finally, the polarization microscopy imaging, performed simultaneously with the optical transmission characterization, enabled to perform a direct correlation of the morphology changes of the parchment with the different stages of the collagen denaturation.

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Introduction

Parchment is an important collagen-based biomaterial obtained from animal skin and for centuries it has been used as a writing support and for the binding of ancient books. Its peculiar properties are a consequence of the collagen hierarchical arrangement, from the molecules to the fibres. The structural stability of the collagen is fundamental for the preservation of the parchment and, consequently, for the durability of the cultural heritage produced with it. Several factors, generally of biological, chemical and physical nature, are responsible for the changes induced in the different levels of the collagen hierarchical structure and therefore for its deterioration. Such changes can be induced or accelerated by external agents including attacks by bacteria and fungi and non adequate environmental conditions, such as thermo-hygrometric factors and pollutants. These, in turn, may activate deterioration processes like oxidative and hydrolytic reactions and the gelatinization of the collagen [1-4].

The progression stages of the deterioration process of parchment have been generally studied by artificially stimulating the ageing process by keeping the sample in severe thermo-hygrometric conditions for varying time duration. This has been observed to lead to changes in the thermal stability of the collagen, increase in heterogeneity and structural disorder and the loss of the fibrillar structure [5]. The degree of the induced deterioration is generally quantitatively assessed by detecting the changes in some physical-chemical and structural properties of the material [6-8]. The used analytical methods include the Micro Hot Table technique [3, 9, 10], to determine the fibres shrinkage temperature, used also in combination with Differential Scanning Calorimetry (DSC), which detects the collagen denaturation temperature and enthalpy [4, 5, 11, 12]. Moreover, other kind of thermal analysis methods, such as the Thermogravimetric analysis, the Derivative Thermogravimetric analysis (DTG) and the Differential Thermal analysis (DTA) [1, 13, 14] have been used to identify dehydration, decomposition and thermo-oxidative degradation processes. Dynamic Mechanical Thermal Analysis (DMTA) has been employed to measure the shrinkage temperature [15-18], while spectroscopic techniques like Fourier Transform Infrared Spectroscopy (FTIR), UV-Vis-NIR spectroscopy [19, 20] and Photoluminescence [21-23] were used to detect changes occurring at molecular level associated with the collagen deterioration. Finally Nuclear Magnetic Resonance (NMR) [19, 24] and X-Ray Diffraction (XRD) [25] have been used to detect the changes in the molecular structure that occur during the parchment deterioration processes.

Among the thermal analysis methods, DSC has been extensively used to monitor the heat exchange occurring over the collagen denaturation transition, with the goal of determining the involved enthalpy change and consequently the specific heat and the denaturation temperature. To date, however, only the changes of the heat capacity with respect to the initial measuring

temperature have been reported, and no information has been provided concerning the absolute values of the specific heat and its background profile. Moreover, the used temperature scanning rate was quite large, typically in the range of several tenths to a few °C/min. This is two to three order of magnitudes faster than other calorimetric techniques which have been extensively used for the accurate study of the critical behavior of the specific heat over phase transitions such as Adiabatic Scanning Calorimetry (ASC) [26], ac calorimetry, [27, 28] and Photopyroelectric calorimetry [29, 30, 31]. Generally, the large rate of DSC holds the substantial risk that the sample is not in thermal equilibrium during the measurement making, in some cases, the characterization unreliable [32], particularly when the transition has a very sharp temperature profile. In contrast, in ASC measurements the scanning rate over the phase transition can be as low as 10^{-4} - 10^{-3} °C/min and very accurate evaluations of the absolute values of the specific heat and the enthalpy changes can be obtained. Under these conditions it has been possible to resolve the specific heat profile over phase transitions whose width was as small as $\sim 0.01^{\circ}\text{C}$ [33].

In this work ASC is applied to the study of the collagen denaturation effects in several artificially aged modern parchment samples. This kind of analysis is combined with that obtained by the Light Transmission Analysis (LTA) [34, 35], a recently introduced method which enables, simultaneously, to measure the denaturation temperature of the collagen and to visualize the morphological changes occurring in the parchment fibres. It was found that the calorimetric evaluations enabled not only to confirm the previously reported specific heat results concerning the collagen denaturation peaks, but also to identify a novel broad feature most likely associated with additional transformations occurring once the collagen denaturation takes place. Moreover, the combination of the investigations by the two adopted techniques allowed to validate LTA as a novel simple optical method capable of recording the entire collagen denaturation temperature path of the analyzed samples. Finally, the polarization microscopy imaging performed during the LTA characterization enabled to perform a direct correlation of the morphology changes of the parchment with the different stages of the collagen denaturation.

Experimental

The specific heat and enthalpy measurements of the samples were carried out by ASC, a proven reliable technique for the high-resolution measurements of such parameters in solids and liquids [26, 36-38]. An implemented version of the standard configuration, the so called Peltier element ASC (pASC) [38] was used for the present work. The parchment samples, in full excess water condition, were sealed inside Mettler-Toledo 120 μl stainless steel medium-pressure crucibles, which were kept in good thermal contact with a platform containing a heater and a thermometer. In

the pASC configuration, this assembly is surrounded by an adiabatic shield, temperature-controlled by a Peltier element acting as a differential thermometer between shield and sample platform. A constant heating power is supplied and the resulting temperature rate recorded [38]. In this way, unlike most temperature scanning techniques where constant heating rates are imposed, the sample is free to respond according to its properties, without the constant heating rate constraint, resulting in better equilibrium conditions. The constant power supply is achieved by passing a constant electrical current through the sample heater. By keeping the adiabatic shield at the same temperature as the sample, all heat leaks from the sample platform are eliminated, so that the heating power P is supplied to the sample platform only and it can be determined with great accuracy. The temperature evolution $T(t)$ is continuously monitored, so the heat capacity C and the enthalpy H can be calculated through the expressions:

$$C(T) = \frac{P}{\dot{T}} \quad \text{and} \quad H(T) = H(T_0) + Pt(T)$$

where $\dot{T}$ is the temperature time derivative. The specific heat values c and of the enthalpy H are obtained by calibrating against the background values of the empty calorimeter and sample cell and the sample mass. In the vicinity of phase transitions, where the heat capacity increases, $\dot{T}$ decreases, with a consequent increase of the temperature resolution of the measurements. Details of the pASC technique and measurements can be found also in Refs. 39-42. For this work, the constant power supplied to the sample platform was 1 mW, inducing a temperature rate change around 0.08 - 0.09 K/min sufficient to resolve specific heat features a few degrees in width typical of the collagen denaturation reactions.

The Light Transmission Analysis, like the calorimetric investigations, enables to determine the denaturation temperature of the collagen fibres [34, 35].

However, at the same time, the adopted set-up also allows a polarization microscopy inspection of the sample so as to monitor the morphology changes occurring in the parchment fibres during the denaturation process. The measurement is performed on samples consisting of collagen fibres in aqueous suspension, prepared by mechanically pulping a 1mm² parchment sample by a blade in conditions of saturated hydration. The sample was placed in a sealed quartz cell of known thickness

Fig. 1 Sketch of the LTA set-up

(typically 100µm) and maintained in condition of full water excess. A semi-transparent metal (titanium) layer, a few hundreds nm thick, was deposited at the bottom of the cell to ensure both

Fig.2 Specific heat Vs temperature for samples with different ageing (in days). Broken line represents the common background

halogen lamp, coming from the opposite side of the cell, which was necessary for the microscopy imaging by a CCD camera. The two optical beams, propagating along opposite directions, superimpose on the same sample area. The temperature rate change for the LTA measurements was around 0.7 - 0.9 K/min.

The artificial ageing of the samples was obtained by keeping them in an environment at 80°C and 80% relative humidity for periods ranging from 2 to 32 days. This was carried out in a specifically designed home-made oven in Rome.

Results and discussion

I - ASC Results

Fig. 2 displays the specific heat results obtained for the parchment samples obtained from sheep [43], which had undergone artificial ageing treatments of different duration. All the curves displayed in Fig. 2 show a main peak followed by a secondary peak at larger temperature. Moreover, the possibility to measure the absolute specific heat values in the entire investigated range enables the identification also

the transmission of the incident laser beam, necessary for the LTA procedure, and the reflection of the illumination beam for the image recording, as sketched in Fig. 1. For the proposed experimental setup, a 5 mW He-Ne laser beam, whose amplitude was modulated by an acousto-optic device at a frequency typically of a few tens of Hertz, was employed for the LTA. The semi-transparent metallic layer at the cell bottom allowed a transmission of 20-30% of the

incident laser beam so that it could traverse the sample. As shown in Fig. 1, the metallic film also allowed the reflection of the light from a

Fig. 3 Specific heat Vs temperature for samples with different ageing (in days) after background subtraction

of the background profile (dashed curve). It turns out that the profile is very similar for all the curves both on the lower and on the larger temperature ranges with respect to the peaked features. In particular, a broad profile in their background is shown with a maximum around 75 °C. The profile of the background underlying the peaked features was obtained by interpolating the data below and above the peaks, using a double-spline function. The obtained profiles were very similar for all the curves and a typical result is displayed by the broken line in Fig. 2. By subtracting that particular background curve from all the data sets, the specific heat variation Δc associated with the collagen denaturation only, was obtained for all the samples as reported in Fig. 3. The zero values obtained for all the curves, below and above the peaked features range, confirms that the single employed background profile is representative for all the data sets.

I-A Collagen denaturation peaks

The profile of Δc displays a main peak which is followed, at larger temperature, by a shoulder feature that is most likely associated with the presence of an additional peak. The contribution of the latter superimposes with that of the first one to form the obtained overall profile. The shape of the two peaks was deconvolved by a mathematical regression routine, where general asymmetric peak profiles were assumed. The results are reported in Fig. 4a and 4b for the non-aged sample and for that aged for 32 days, the maximum duration. The first peak corresponds to the so called

Fig. 4 Specific heat Vs temperature for samples: a) non aged; b) aged 32 days. Broken lines represent the deconvolved peaks

“native” collagen, the collagen located in the parchment fibres nucleus and in between the fibres, generally the less stable population whose denaturation occurs at lower temperature [44,45]. The second peak has been referred to in the literature as corresponding to a “stabilized” collagen population, since it denaturates at larger temperature. It has been reported to correspond to the collagen of the external sheath of the fibres [45]. Our measurements show that, with increasing

ageing, the denaturation temperature, T_d , at the lower temperature peak decreases and so does that at the higher temperature peak, T_2 . Moreover, there is an evident depression of the maximum Δc values and a broadening of the combined overall peak profile. The observations are in agreement with earlier reports [44, 45]. In Fig. 5a and 5b, the evolution of the two above mentioned temperatures with increasing ageing duration is shown for all the samples investigated by ASC as well as by LTA, as described later on. T_d shows a continually decreasing trend, which is pretty steep up to the 8 days ageing, and then considerably slower for the longer ageing durations. T_2 also shows an overall decreasing trend. In order to explain the observed results, it should be kept in mind that

Fig. 5 Denaturation temperature Vs ageing for: a) native collagen population ; b) stabilized collagen population. Striped band in 5a) denotes the collagen denaturation temperature range for historical parchments

the artificial ageing treatments on the samples are known to produce deterioration in the collagen structure in the form of cleavage of its molecules, which causes their fragmentation, and of gelatinization of the fibres [2, 3], such changes being similar to the ones occurring naturally in ancient parchments [3]. When the parchment samples are then heated in excess water in order to determine the specific heat temperature dependence, the denaturation of the collagen progresses further turning it into a gelatin form. This process converts the fibrillar arrangement of the collagen molecules, which are originally structured in an ordered triple helix form, to a disordered random structure. This is due to the synergistic action of water tending to break the existing hydrogen bonds of the molecules and of the temperature increase making such a reaction more likely. Moreover, the progression of the gelatinization process is more effective in partially deteriorated collagen molecules [2] as the energy required to denaturize a shortened tripled helix is smaller than that required for intact molecules. Therefore, the greater the ageing degree of the parchment samples, the smaller is the additional thermal energy necessary to induce the collagen denaturation, so that the temperatures of the observed peaks are found at lower temperature as depicted in Fig. 5a and 5b.

Hence the usefulness of the determination of the denaturation temperature to characterize the deterioration state of parchments, whether induced artificially or occurring naturally in ancient artefacts. In this respect, in Fig. 5a the striped band delimits the range of values of the denaturation temperature reported in the literature [46] for ancient western parchments aged naturally throughout the centuries because of the sustained environmental conditions. Such values fall within the range obtained for the artificially ageing parchment samples obtained in this work, in particular in the temperature region corresponding to a pronounced deterioration stage. Finally, it is also worthwhile remarking that the maximum shift in the denaturation temperature for the stabilized collagen ($\sim 3^\circ\text{C}$), shown in Fig. 5b, is smaller than that observed for the native collagen ($\sim 7^\circ\text{C}$), in Fig. 5a. This is to be associated with the greater resilience of the stabilized collagen to the deterioration effects induced by the severe thermo-hygrometric conditions.

The denaturation enthalpy, ΔH_d , can be determined in two ways from the results obtained by ASC,

Fig. 7 Enthalpy profile over the denaturation range for non aged parchment. The arrow indicates the enthalpy change at T_d .

Fig. 6 Native collagen denaturation enthalpy Vs ageing

namely, by the temperature integral of Δc or by the direct evaluation of the heat exchanged with the sample during the calorimetric measurements. Fig. 6 displays the results obtained for all the analysed samples by the Δc temperature integral. The value obtained for the non aged sample (42 J/g) is very close to that (44 J/g) reported for parchment obtained from sheep in ref. 9. A progressive decrease of ΔH_d , with ageing is shown, also in

agreement with earlier reports [45]. This should be ascribed to the fact that the collagen in aged samples is partially denaturated so that the

enthalpy required to complete the denaturation is smaller than in undeteriorated samples. In Fig. 7, as an example, the enthalpy exchanged with the non aged sample once the contribution of the specific heat background (see Fig. 2) had been subtracted, is also reported. The denaturation enthalpy can be estimated as the difference in the enthalpy levels, determined at T_d , of the profiles extrapolated from the high and low temperature sides of the data (the flex point of the data curve) [39]. The value $\Delta H_d = 40$ J/g was obtained, very close to the 42 J/g obtained with the previous method, thus confirming the soundness of the results.

I-B Specific heat background

As regards the specific heat background shown in Fig. 2, it has never been reported in the literature on calorimetric studies performed on parchment, as only the changes induced in the specific heat by the denaturation reaction can be found to date. Its profile shows a broad maximum and it may suggest a possible association with some additional material transformations occurring because of the severe thermo-hygrometric conditions endured by the samples during the calorimetric measurements. The circumstance that, as discussed earlier on, its shape and position is basically the same for all the investigated samples points to the fact that the possible undergone transformation would be independent from the sample initial ageing and, therefore, deterioration conditions. Moreover, the maximum, though very broad, occurs around 75 °C, above the maximum denaturation temperatures for the native and stabilized collagen populations. This suggests that the additional transformations take place when the denaturation/gelatinization of the collagen is well underway or has terminated. In the literature [2] it is indicated that the occurrence of the collagen denaturation can lead to further changes including the formation of extra crosslinks between the deteriorated molecules and changes of conformation of the residual amino acids. While the former can occur as a result of dry heating in vacuum, UV irradiation or by chemical cross-linking agents, the latter occurs as a result of gelatinization. In fact, as collagen turns into gelatin, molecular mobility is gained and the amino acids can thus change their conformation switching from the left-handed one to the right-handed one [2, 47]. We have also estimated the enthalpy change associated with the occurring transformation by evaluating the temperature integral of the background profile of the specific heat after a linear baseline had been subtracted. The obtained value, $\Delta H = 50$ J/g, is not distant from the one corresponding to the collagen denaturation for the non aged sample. This may imply that the additional transformations involve molecular changes of nature similar to those occurring during the denaturation involving bonds rearrangements between molecular chains. The described processes must be considered as only some of those possibly taking place and more

detailed studies are certainly necessary to shed further light on the true origin of the observed background structure.

Thus, the calorimetric evaluations by ASC enabled, on the one hand, to confirm the previously reported specific heat results concerning the collagen denaturation peaks in artificially aged parchments, but also to identify a novel broad feature in the background most likely associated with additional transformations taking place after the parchment gelatinization has occurred.

II - LTA Results

All the parchment samples analyzed by ASC have also been subject to LTA characterizations which, as stated earlier on, enabled both the optical transmission and optical imaging evaluations.

II-A Optical Transmission

We first present the optical transmission results in order to show that they are very effective to determine the collagen denaturation temperature path, to date determined mainly by thermal analysis techniques. Because of the order reduction of the collagen during its denaturation, its ability to scatter light is reduced so as to induce an increase in the amount of light traversing the parchment fibres sample. The signal resulting from the detection of the light of the probe laser beam transmitted through the sample was processed as follows. The background linear baseline was first subtracted and the data were then differentiated with respect to the sample temperature. In this way curves representing the temperature rate dependence of the light transmission signal, $\frac{dL}{dT}$ were

obtained. After further subtraction of the derivative background component, so as to obtain changes associated only with the collagen denaturation, similarly to what previously performed in the case of the specific heat data, the results shown in Fig. 8 are finally obtained. The position of the peaks represent the temperatures where the transmission temperature rate change is maximum, similarly to the specific heat peaks representing the temperature where the enthalpy temperature rate change is maximum. From the peaks position the denaturation temperatures can thus be identified. From a general point of view, the

Fig. 8 Rate of change with temperature of the optical transmission Vs temperature for parchment samples with different ageing (in days)

LTA results are similar to those obtained by *ASC*, in the sense that they show double peaked features like those obtained in the specific heat. The main difference is that the *LTA* curves are considerably broader than the corresponding ones obtained by *ASC*. This should be ascribed basically to the fact that the two techniques characterize the denaturation process by probing different kind of physical parameters which can respond differently. The very different temperature change rate adopted during the two kind of characterization has been tested not to cause substantial difference in the widths as the adoption of a rate very similar to that used for the calorimetric investigations (0.1 °C/min) did not produce remarkably different results. However, in spite of such a difference, when comparing, for example, the results obtained for the non aged parchment sample and for the one

Fig. 9 Optical transmission rate change Vs temperature for samples : a) non aged; b) aged 32 days. Broken lines represent the deconvolved peaks

aged for 32 days (Fig. 9a and 9b) with the corresponding calorimetric results (Fig. 4a and 4b), the double peak envelope shapes are evident in all cases though with somehow different profiles. Moreover, after the deconvolution of the profiles of the two peaks is performed for all the analyzed samples to determine the denaturation temperatures at the two peaks, it turns out that the ageing dependence for such temperatures determined by *LTA* is very similar to the ones obtained by the calorimetric investigations as shown in Fig. 5a and 5b. The only difference is the larger values of the temperatures determined for the stabilized parchment population by *LTA* as compared with those determined by *ASC*, with the overall trend remaining nonetheless similar. Finally, a broadening of the combined overall peak profile with aging occurs like the specific heat results.

The similarity of the results obtained by the two techniques confirms that also the *LTA* technique is reliable for the parchment denaturation studies. This is important considering that it is the only optical technique capable, like the ones based on thermal analyses, of recording the *entire* denaturation temperature path of the analyzed samples. With respect to the thermal analysis

techniques, however, LTA requires a much simpler and less expensive setup. Moreover, unlike the thermal analysis techniques, *LTA* is capable to perform simultaneously also the imaging analysis of the samples useful to monitor the parchment morphological changes induced by the deterioration process, as described in the following.

II-B Polarization microscopy imaging

The results of the polarization microscopy imaging at the different temperatures are reported in Fig. 10, with the indication of the temperature position of the various collected images within the collagen denaturation path monitored by the optical transmission results. At low temperatures (around 35 °C), several birefringent areas, typical of the crystalline structure of the non-deterioreated parchment, can be identified. With increasing hydrothermal denaturation, the sample progressively loses the birefringent capability (picture at 56,5 °C). This evolution progresses throughout the entire denaturation path, as displayed in the pictures obtained in correspondence of the maximum rate of denaturation (58,6 °C) and later on during the stage of decreasing rate of denaturation of the native collagen (60,7 °C). Over the large temperature peak (65,4 °C), typical of the denaturation of the stabilized collagen, only moderate additional changes are observed in the birefringent properties of the parchment. Very little further changes are observed near 75 °C where

Fig. 10 Polarization microscopy imaging for non-aged parchment fibres during the collagen denaturation stages

the denaturation has been completed also by the stabilized collagen population. The sample has transformed into a state where, beside the almost total loss of birefringence, it has also undergone a decrease of its size (shrinkage), both typical of the gelatine state. It is thus confirmed that the gelatinization process is basically concluded once the collagen denaturation is completed. The possibility granted by the LTA analysis to perform simultaneously the imaging and optical transmission evaluations has made it possible to directly correlate the morphological changes in the collagen to the different stages of its denaturation.

Conclusions

The collagen denaturation effects in several artificially aged modern parchment samples were studied by combining Adiabatic Scanning Calorimetry and Light Transmission Analysis investigations. The former allows the detailed evaluation of the absolute specific heat and of the exchanged enthalpy while the latter enables to detect, simultaneously, the variation of the light scattering in the sample and to record the images of the morphology changes associated with the denaturation. It was found that the calorimetric evaluations enabled to confirm the previously reported specific heat results concerning the collagen denaturation peaks, as well as the enthalpy exchange. Moreover, the possibility to measure also the specific heat background profile, not reported in the earlier studies, has enabled to identify a novel feature with a broad maximum at a temperature well above those characteristic of the denaturation. Such features were independent of the extent of the ageing of the samples and they were most likely associated with additional transformations occurring once the denaturation had been completed. The combination of the calorimetric and transmission investigations allowed to show that the denaturation temperature profile obtained by the two techniques were consistent with each other so as to be able to validate the optical transmission technique as a reliable novel simple tool for the recording of the entire collagen denaturation temperature path. Finally, the polarization microscopy imaging performed during the transmission characterization enabled to perform a direct correlation of the morphology changes of the parchment with the different stages of the collagen denaturation.

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Figure captions

Fig. 1 Sketch of the LTA set-up

Fig. 2 Specific heat Vs temperature for samples with different ageing (in days). Broken line represents the common background

Fig. 3 Specific heat Vs temperature for samples with different ageing (in days) after background subtraction

Fig. 4 Specific heat Vs temperature for samples: a) non aged; b) aged 32 days. Broken lines represent the deconvolved peaks

Fig. 5 Denaturation temperature Vs ageing for: a) native collagen population ; b) stabilized collagen population. Striped band in 5a) denotes the collagen denaturation temperature range for historical parchments

Fig. 6 Native collagen denaturation enthalpy Vs ageing

Fig. 7 Enthalpy profile over denaturation range for non aged parchment. The arrow indicates the enthalpy change at T_d .

Fig. 8 Rate of change with temperature of the optical transmission Vs temperature for parchment samples with different ageing (in days)

Fig. 9 Optical transmission rate change Vs temperature for samples : a) non aged; b) aged 32 days. Broken lines represent the deconvolved peaks

Fig.10 Polarization microscopy imaging for non-aged parchment fibres during the collagen denaturation stages