Unilateral NMR and Micro DSC Study of Artificially Aged Parchments

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New and artificially aged parchments were studied by unilateral NMR and micro DSC. Samples were obtained by exposing new parchments to 80 °C and alternate 40 and 80% relative humidity for increasing times up to 32 days. The impact of accelerated ageing was assessed by measuring both the proton transverse relaxation times (T_{2eff}) and thermodynamic parameters associated with thermal denaturation of fibrillar collagen. Depending on the ageing time, significant structural changes correlated with thermal stability and collagen heterogeneity were observed, in good agreement with changes in phase composition and relaxation times. The results suggest two ageing regimes depending on the balance between the two main processes which drive the behavior of parchments during thermal ageing, i.e. thermally induced cross linking and thermal destabilization and denaturation.

Keywords: parchment, collagen, unilateral NMR, micro DSC, thermal ageing

The structural stability of collagen in parchments is crucial to their preservation and thus for carrying out the functional and artistic rôle for which they were intended, i.e. as texts reading, bookbinding, decorative objects, etc., the longest possible. For this conservators require accurate results and the sample amounts made available by curators are limited. Techniques which cope with both these limitations are micro differential scanning calorimetry (micro DSC) and unilateral Nuclear Magnetic Resonance (NMR). Their combined use for damage detection and its quantitative evaluation represents a powerful analytical application in the field of heritage science as it can help to resolve the important question of the environmental stability of collagen-based artefacts (e.g. parchment, leather, mummified skin, bone). While DSC is the most direct and sensitive approach to study the protein stability [1-3], unilateral NMR has been recently used in a wide range of applications due to its high efficiency, non-destructive sample characterization and accuracy [4-8]. As far as collagen-based materials are concerned, DSC has been succesfully applied to assess and quantify damage in parchments [9-12] and vegetable tanned leather [13-14], while pilot studies used portable unilateral NMR to assess the deterioration of collagen in historical parchments [15-16]. Recently, we reported unilateral NMR studies of vegetable tanned leather exposed to dehydrothermal treatment [17] and gamma irradiation [18], and showed that relaxation times are discriminative for collagen in various materials as parchment, leather and gelatin [19].

The aim of this study is to investigate the ability of unilateral NMR to asses collagen deterioration in parchment during ageing. For this purpose we have correlated the results obtained by unilateral NMR technique with those obtained by micro DSC, a consolidated technique for the characterization of collagen denaturation in parchment during ageing [6-8].

Experimental part

Materials and methods

Parchments

The parchments used for the artificial ageing treatments were prepared by traditional methods by the Dutch parchment craftsman Z.H. de Groot. The artificially aged samples were obtained by exposing the new calf parchments to accelerated ageing by heating at fixed temperature, i.e T = 80 °C, in a controlled RH atmosphere (i.e. 40% and 80% RH) that alternates every two days for 2, 4, 8, 16 and 32 days, using Servathin humidity and temperature test chambers.

Unilateral NMR measurements

NMR measurements were performed at room temperature using a bar magnet NMR-MOUSE sensor with a double-D radio-frequency coil working at 20.05 MHz ^{1}H resonance frequency and controlled by a Bruker Minispec spectrometer. The NMR signal stems from a depth of about (0.5 – 1) mm from the surface of the sensor. Effective ^{1}H spin-spin relaxation T_{2eff} measurements have been measured using the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with an echo-time (TE) of about 25 μs. A short echo-time ensures that the decays are not affected by diffusion effects. The waiting time between two consecutive scans was 3 s which is needed to avoid heating effects during the measurement.

The experimental CPMG curves (fig. 1) could be best analyzed by a combination of double exponential functions according to the equation:

$$A(t) = A_{short} \exp\left(-\frac{t}{T_{2eff\_short}}\right) + A_{long} \exp\left(-\frac{t}{T_{2eff\_long}}\right)$$

where t is the time and A is the amplitude of the recorded signal. $A_{short} + A_{long} = 100\%$.

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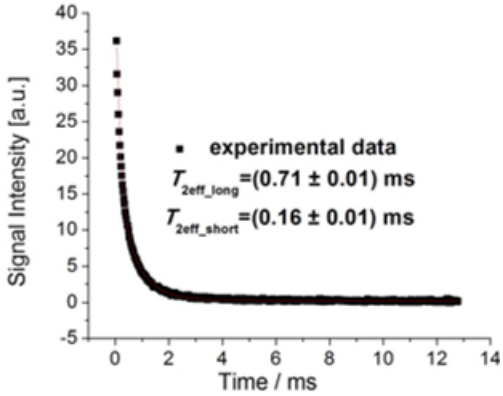

Fig. 1. Typical ^{1}H CPMG decay curve and the fit analysis used to determine the T_{2eff} spin-spin relaxation times for a newly manufactured calf parchment

The relaxation times T_{2eff} are related to the mobilities of each detected phase of collagen. The relative fractions of the relaxation components $A_i/(A_{short} + A_{long})$ represent the relative numbers of hydrogen atoms (molar fractions) of the detected phases with different molecular mobilities [20].

Micro DSC measurements

Micro DSC measurements were performed in the temperature range (20 – 100)°C with a high-sensitivity SETARAM Micro DSC III microcalorimeter by using 850 µL stainless steel (Hastelloy C) cells. Parchment samples of about 2 mg were suspended in 0.5 M acetate buffer with *p*H = 5.0 directly in the measure cell and left for 2 h to secure fully hydrated conditions and thus reproducible calorimetric values for both temperature and enthalpy of denaturation as previously reported [9-10]. Measurements were made with a 0.5 K x min^{-1} scanning rate which provides a very good resolution of different denaturation peaks. Certified reference materials such as naphthalene, benzoic acid and gallium were used to check the supplier calibration constants in the working temperature region. Several melting runs performed under same experimental conditions as for the parchment measurements showed a very good agreement with the IUPAC recommended values [20] for temperature and enthalpy.

Experimental DSC data acquired with the SETARAM Set-Soft2000 software were analysed using PeakFit 4.1 (Jandel Scientific) to obtain the experimental heat capacity values of the sample C_p^{ex}(T) (J x K^{-1}x g^{-1}) in the scanned temperature interval and derive the DSC peak parameters featuring collagen in parchment hydrothermal denaturation as reported earlier [7,9]: maximum peak temperature T_d (°C), denaturation enthalpy, ΔH (J x g^{-1}), calculated by integrating $C_p^{ex}(T)$ curve across the denaturation temperature range, peak half-width, $\Delta T_{1/2}$ (°C) and maximum height, max (J x K^{-1} x g^{-1}).

The typical denaturation endotherm for the new calf parchment illustrated in figure 2a displays a narrow and asymmetric peak in the range (50-55) °C with a pronounced shoulder at about 60°C. During hydrothermal ageing, the collagen within parchment changes due to various processes such as thermal stabilization, thermal destabilization, denaturation, and gelatin formation resulting in formation of multiple collagen populations with distinct thermal stability [9-11]. To estimate the percent fraction of collagen populations and thus provide the pattern and dynamics of the deterioration of fibrillar collagen during artificial ageing, mathematical

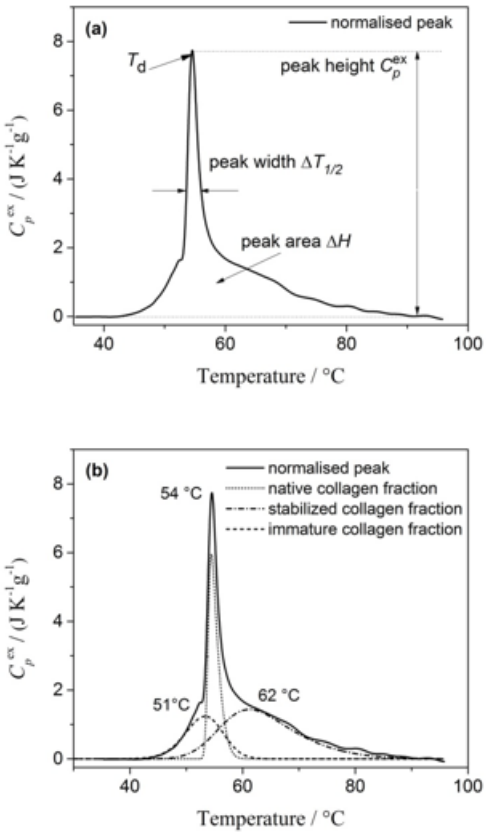

Fig. 2. Normalized DSC peak of thermal denaturation of fully hydrated collagen from a new calf parchment showing multiple character. (a) Denaturation parameters derived for the overall DSC peak: T_d = 54.4 °C, ΔH = 49.3 J·g^{-1}, $\Delta T_{1/2}$ = 2.3 °C, $_{max}$ = 7.7 J·K^{-1}·g^{-1}. (b) Multiple denaturation transitions revealed by deconvolution corresponding to the following native collagen fractions: labile or immature (T_{max} = 51 °C), stable (T_{max} = 54 °C) and stabilized (T_{max} = 62 °C)

deconvolution (fig. 2b) and analysis of DSC peaks was performed as previously reported [9-11].

Results and discussions

Parchment is a complex biomaterial consisting of a matrix of collagen fibres, the structural protein of the dermis layer in skin [11]. The structure and hierarchical organisation of collagen in various tissues is well documented [21]. The building blocks of a collagen tissue are collagen molecules formed by the right-handed twisting of three collagen polypeptide chains each comprising over 1050 amino acids of a specific sequence, i.e. (G-X-Y)$_n$, where G is glycine and X and Y are often proline and hydroxiproline. The resulted collagen molecule is a rigid rope like triple helix motif. The alternate direction of the twist and the steric local arrangement of amino acids in the chain cause the helix to lock into a specific structure where water has a significant relationship with the collagen molecule. Collagen molecules then aggregate in a very specific and highly directed way to form an insoluble collagen fibril whose stability is provide by ionic interactions, hydrophobic regions and covalent intra and intermolecular cross links. In skin, collagen fibrils are slender structures of about 120 nm wide and at least several microns long. Each fibril comprises several thousand collagen molecules in cross-section and extends over several collagen molecules in length. Fibrils are organised less specifically into bundles to form a collagen fibre, and finally collagen fibres form a collagen tissue. The relationship of collagen with water at specific levels of the hierarchical organisation within a

tissue confers great stability. Water molecules associate with collagen in a tissue in four different phases. Structural water (phase I) is tightly bound within the collagen triple helix, either through a double or triple hydrogen bond. Bound water (phase II) is represented by water bridges between neighbouring polypeptide chains which act as receptors for CH-O hydrogen bonds [22]. Free water (phase III) molecules are fixed by one hydrogen bond between polypeptide chains or fixed in the hole zones at the end of the polypeptide chain. Transition water or unperturbed bulk water (phase IV) are water molecules bound between the fibrils [23-24]. The total amount of structural and bound water (phase I and II) contained in collagen is approximately 0.5 g/g [25]. The remaining amount of free and bulk water (phase III and IV) held in skin is dependent upon various factors (i.e. age, sex, hydration, health, etc.), while in parchment it mainly depends on deterioration and environmental RH [26-27]. In the unfolded collagen structure, more polar groups are exposed to water, and can therefore bind to significantly more water molecules than the original collagen structure. Moreover, gelatin holds less water than collagen at the same RH and T [23]. These imply that in aged parchments, which are heterogeneous materials constituted by collagen and gelatin [10-11], the differential behaviour of native collagen and deteriorated collagen/gelatin, and consequently the effect on the interfaces between them, is a key factor to be considered for damage assessment and stability against specific environmental scenarios. In fact, a morphology similar with that of semi-crystalline polymers was earlier proposed to explain the deterioration of parchment and leather by C. Popescu *et al.* [28]. This consists of rigid collagen filaments that are embedded in an amorphous matrix with an interface zone between the rigid and amorphous regions for leather [28]. Contrary to this, only the rigid and the amorphous regions could be detected for parchment from measurements performed at room temperature. Against this background, we consider the relation between the changes shown by the transverse relaxation times and hydrothermal stability to draw conclusions with respect to the hydrothermal ageing effect on collagen within parchment.

Changes in mobility and phase composition due to thermal ageing

The effective transverse relaxation times of the rigid (T_{2eff_short}) and amorphous phases (T_{2eff_long}) of the parchment samples aged at 80 °C and alternate 40% and 80% RH are presented in figure 3 as a function of ageing time. The relaxation time of the amorphous phase is the most labile upon ageing and shows the most significant changes. It decreases at 2 days ageing and then starts to smoothly increase suggesting two ageing regimes: from 2 to 8 ageing days the amorphous phase is characterized by lower T_{2eff} values compared to the not treated parchment, while higher T_{2eff} values are obtained at 16 and 32 days ageing. On the other hand, a very smooth increasing trend is displayed by the transverse relaxation times T_{2eff} of the rigid phase. The reduction of chain mobility in the long relaxation component of the parchment samples after 2 ageing days can be interpreted in terms of a tighter packing of collagen fibrils due to thermally induced cross-linking. As the exposure time increases, T_{2eff} of the amorphous phase turns to increase. This change in trend could be assigned to a gradual increase of the primary peptide bonds cleavage within the collagen triple helix which leads to a decrease in the fraction of the rigid phase (fig. 4) and also due to chain scission in the amorphous phase. Thus, the lower

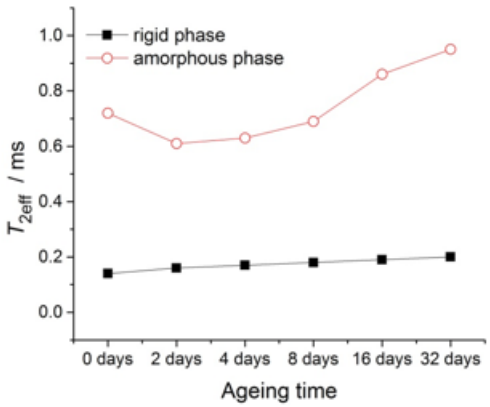

Fig. 3. Changes in relaxation time T_{2eff} with ageing time for parchments aged at 80 °C and alternate 40% and 80% RH

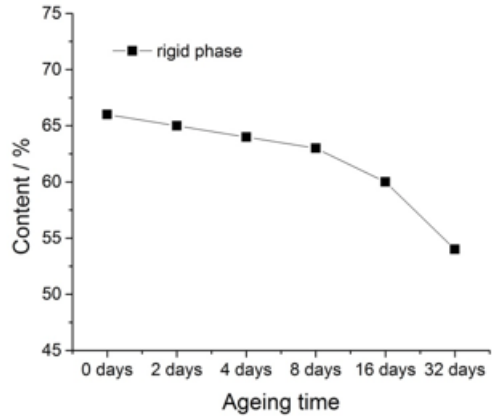

Fig. 4. Changes in the rigid phase contribution with ageing time for parchments aged at 80 °C and alternate 40% and 80% RH

fraction of rigid phase and the chain scissions are together responsible for the increased mobility of the amorphous chains. The accumulation of peptide bonds scissions was found to firstly lead to the formation of thermally destabilized intermediate states and, once sufficient damage has been inflicted, beyond the hypothetical critical level of holding the triple helical structure, to gelatin and random coil structures [11, 13, 29]. The polypeptide chain cleavage (promoted by the high T and high RH treatment) competes with cross-links formation process (promoted by the high T and low RH treatment) resulting in a progressive enhancement of the chain mobility as confirmed by the increase of T_{2eff} of the amorphous phase for the parchment samples aged for longer times (fig. 3). On the other side, the gradual increase in the short relaxation time suggests the appearance of structural disorder in the rigid phase.

A quantitative characterization of the phase composition is important for understanding how the parchment morphology is affected by thermal ageing. A very smooth and then faster decrease in the rigid phase contribution is observed upon ageing (fig. 4). This seems to correlate with the reduction in the amount of native collagen due to the thermal destabilization (fig. 5).

Changes in thermal stability and structural heterogeneity

In order to better correlate the NMR results and collagen changes during ageing, micro DSC was used to directly measure thermal stability and structural heterogeneity of parchments. Recently, based on micro DSC data, Badea et al. [11] showed that ageing-induced crosslinking and polypeptide chain scission mechanisms can run either individually or in parallel in parchment, depending on the ageing conditions and time. Complex variations with the

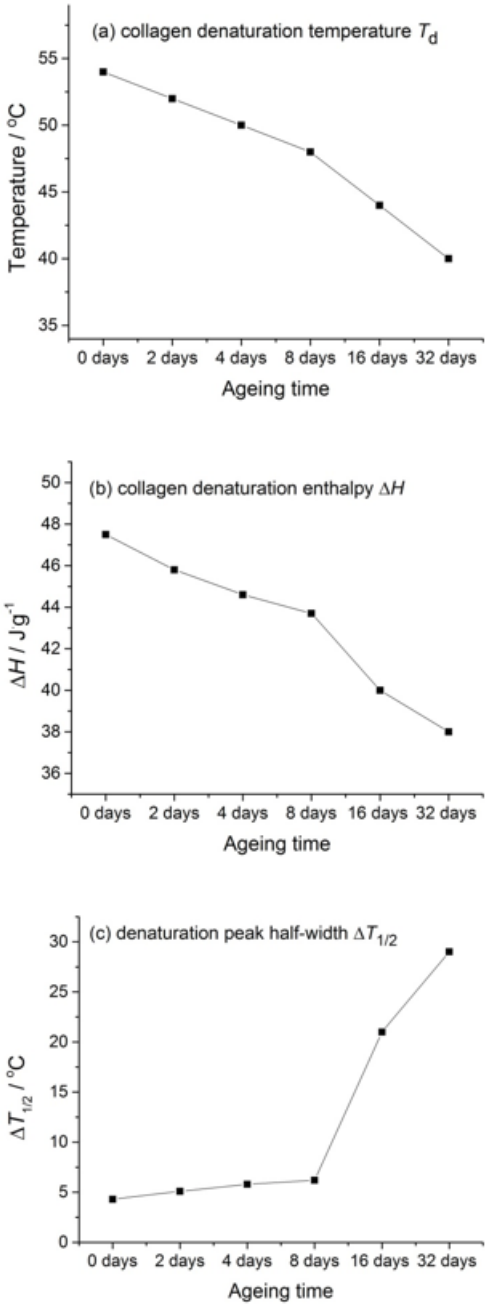

Fig. 5. Variations of DSC parameters featuring overall denaturation of collagen in parchments exposed to accelerated ageing at 80 °C and alternate 40% and 80% RH for 2 to 32 days: (a) denaturation temperature T_d; (b) denaturation enthalpy ΔH; (c) denaturation peak half-width $\Delta T_{1/2}$

ageing time was also observed by some of us in vegetable leathers being specifically related to the process dominating the structural reorganization at different exposure times [13,17,18]. In particular, cross linking leads to an increased thermal stability of collagen-based materials measured by a simultaneous increase of the denaturation temperature T_d and decrease of peak half-width $\Delta T_{1/2}$ without affecting the enthalpy of denaturation ΔH, while peptide chain scission leads to a decrease of T_d and ΔH accompanied by $\Delta T_{1/2}$ increase [9-13, 15, 30-33].

The variation of micro DSC parameters of the investigated parchments as a function of ageing temperature and time is illustrated in figures 5a-c. The plots indicate two regimes characterized by smooth changes for samples aged up to 8 days and strong variations for samples aged for 16 and 32 days. Such behaviour supports the competition between thermally induced cross linking and peptide bonds scission. In the first 8 days the DSC peak maintains its narrow shape while the variations of T_d and ΔH are not significant (i.e. less than 10%). The sudden increase of $\Delta T_{1/2}$ and lowering of both T_d and ΔH (up to 25%) are due to the progressive conversion of native collagen into less and less unstable and disorganized collagen until its partial full denaturation. The cross-linking that prevails in the first case causes dehydration of the fibres by drawing the collagen molecules closer together and thus provides thermal stabilization [34] that allows parchment to withstand disorganization and extensive swelling, and hence thermal ageing up to 8 days. On further ageing, thermal destabilization becomes prevailing. This is supported by the changes in the distribution of collagen populations with distinct thermal stability reported in figure 6. The collagen population percent distribution was obtained by the deconvolution of the DSC peaks which allows the calculation of denaturation temperature and enthalpy for each of their components [10]. Based on the T_d value, the collagen fractions in aged parchment were previously grouped in four classes: stable/native (N) for collagen with 48 °C < T_d ≤ 56 °C; stabilized (S) for collagen with T_d > 56 °C; unstable (U) for collagen with 30 °C < T_d ≤ 48 °C and gelatin-like (G) for T_d ≤ 30 °C [9-10].

At 2 days ageing a conversion of N collagen to S collagen is observed confirming the cross-links formation. At further ageing the S population remains practically constant, while N population starts to convert into U collagen at 8 days ageing. After 16 days ageing N population was fully converted into U collagen which, in turn, progressively converts to fully denatured collagen which is no longer detected by DSC technique. The collagen loss by denaturation is quantified by the loss of ΔH compared to the non aged parchment.

The ageing patterns revealed by both unilateral NMR and micro DSC indicate two regimes in parchment thermal ageing ruled by the competition between two processes: thermally induced cross-linking, which prevails up to 8 days ageing, and thermal destabilization and denaturation, which dominates at longer ageing times. In fact, at short ageing times the reduction of the axial separation of molecules by cross-linking increases collagen-collagen interactions and stabilizes collagen fibrils by restricting the access of water molecules to the specific sites of the collagen intermediate states [34]. This prevents swelling and peptide bonds breaking. At longer ageing times, more and more polar groups are exposed to water due to increasing scissions of peptide bonds resulting in extensive swelling and hence increased sensitivity to thermal denaturation.

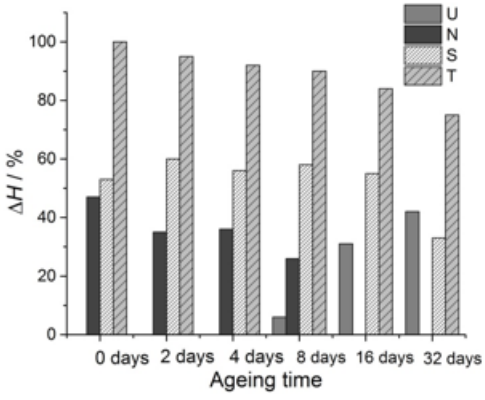

Fig. 6. Percent variation of collagen fractions N, S and U with distinct thermal stability in parchments exposed to heating at 80 °C and alternate 40% and 80% RH for 2 to 32 days. T represents the percent of total collagen. Values are reported to those of a not treated parchment. N (stable/native collagen) 48 °C < T_d ≤ 56 °C; S (stabilised collagen) T_d > 56 °C; U (unstable collagen) with 30 °C < T_d ≤ 48 °C.

Conclusions

The effects of temperature and ageing time on the chain dynamics and phase composition of parchment were studied by unilateral NMR. The changes in transverse relaxation time and phase composition well correlate with the changes in collagen thermal stability and structural order derived from the analysis of the DSC denaturation peaks. The relaxation behavior of parchment samples initially shows restrictions in the macromolecular chains mobility of the amorphous phase attributed to the thermally induced cross-linking. This is supported by the collagen thermal stabilization revealed by the percent distribution of collagen populations with distinct thermal stability, i.e. the stabilized S fraction percent increases at the expenses of native N fraction. On longer ageing times, the macromolecular chains mobility increases and simultaneously the contribution of the rigid phases decreases suggesting that thermal degradation becomes the dominant process. This is supported by the collagen thermal destabilization (the percent of both N and S fractions diminish while new unstable U fraction occurs and gradually increases) and fall of the enthalpy of denaturation which indicates an increase of structural disorder.

The results obtained show that the unilateral NMR technique is suitable for characterizing the parchment thermal ageing and are expected to be of use for assessing the storage/display conditions and validating the conservation and restoration treatments of historical parchments as well as for setting up a non-invasive protocol for *in situ* testing of collagen-based historical/ archaeological objects and artefacts.

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