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# The influence of sodium chloride on the melting temperature of collagen crystalline region in parchments

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**Abstract** In order to observe the influence of sodium chloride on the melting temperature of collagen crystalline region in three new parchments, samples were soaked in water (blanks) and NaCl solutions of different concentrations, then removed, dried in air and measured by means of differential scanning calorimetry (DSC) and dynamic mechanical analysis (DMA). The melting temperature of crystalline region of collagen, $T_m$, was determined as the minimum of the endothermal peak in the range 200–250 °C and as the inflection point of the decrease of storage modulus, respectively. There was observed a decrease in melting temperature of the salt-treated parchments compared to the samples soaked in water, sometimes significant (~20 °C) at certain concentrations of NaCl. Simultaneous TG/DTG/DSC thermal analysis (STA) was also applied for the determination of the amount of sodium chloride in salt-treated parchments, by calculating the mass loss due to the vaporization of NaCl, which occurs above 800 °C. By plotting $T_m$ determined by DSC and DMA versus the NaCl content of the samples, an apparent minimum is observed. Additional information regarding the structural features was also obtained through X-ray diffraction (XRD) and attenuated total reflection fourier transform infrared spectroscopy (ATR-FTIR). XRD data put in evidence the preservation of collagen crystalline region in all salt-treated samples, while FTIR measurements did not showed significant modification of collagen. By removing the sodium chloride from the salt-treated parchments through washing with water, there is a return of the melting temperatures to the values of blank samples, demonstrating the reversibility of this phenomenon.

**Keywords** DMA · DSC · Parchment · Collagen · Melting · Sodium chloride

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## Introduction

Parchment is a complex biomaterial predominantly composed of type I collagen, a hierarchically organized protein. The basic building block of collagen is the triple helix formed of three polypeptide chains coiled together, which further aggregates in structural levels of increasing complexity: microfibrils, fibrils, fibers, and tissue [1].

When heated in dry state, collagen undergoes two main transitions: the loss of residual moisture (at around 100 °C) and the thermal denaturation/melting (above 200 °C). The latter process was reported for the first time by Okamoto and Saeki [2] as a small endotherm (215 °C) in the DTA curve, and more recently using differential scanning calorimetry [3–10], dielectric techniques [10], dynamic mechanical analysis (DMA) [8], and micro-thermal analysis [11]. In this article, we will refer to this transition as "melting of crystalline region of collagen" or simply, "melting".

In our current area of research, the influence of various induced factors (humidity, presence of salts, acids, deterioration) on the melting temperature, $T_m$, and thermal behavior of collagen-based materials (pure collagen, parchment, leather) is studied. There are many articles regarding the influence of various neutral inorganic salts on the denaturation temperature of collagen in solution [12–20]. It was found that the type of ions and their concentration can significantly shift the denaturation temperature, $T_d$. Thus, some salts lower $T_d$, some increase $T_d$, while

others exhibit both effects depending on their concentration. In the particular case of sodium chloride, interesting results were obtained by Komsa-Penkova et al. [13], who have found that NaCl lowers the denaturation temperature of acid-soluble collagen at concentrations up to 0.4 M, due to the screening of charged residues and reduction of collagen stability, then sharply increases $T_d$ above this concentration, due to collagen salting-out and aggregation. However, until now there are no studies concerning the influence of inorganic salts on the thermal behavior of collagen in dry state.

The aim of this preliminary study is to put in evidence that the presence of sodium chloride can influence the melting temperature of collagen crystalline region in parchments, which could bring some insight into the interactions within the hierarchical structure of collagen.

## Materials and methods

Three new parchments (named L1, L2 and C), manufactured from lamb (L1 and L2) and calf (C) skins at the Leather and Footwear Institute (Bucharest, Romania) were used for this study. Sodium chloride was of analytical grade (>99.9 %). Solutions of five-fold increasing concentrations (0.04 M, 0.2 M, 1 M and 5 M) were prepared by dissolving NaCl in distilled water.

Pieces of these parchments were soaked in water (blank samples) and NaCl solutions for 48 h, then removed and dried in air at room temperature. Each salt-treated piece was cut in two parts, one of which was measured by XRD, FTIR, DSC, STA and DMA, while the other was repeatedly washed with distilled water under stirring for several hours, until no chloride was detected in the eluents by reaction with silver nitrate. The washed samples were removed from water, dried in air at room temperature and also measured by DSC and DMA.

For convenience, the samples were denoted as "parchment name" followed by the concentration of salt solution in which the samples were soaked or by "water" in the case of blank samples; for example: "L2 water", "L2 0.04 M", "L2 0.2 M", etc. No specific name was given for the salt-treated samples which were later water washed.

The TG, DTG, and DSC curves were simultaneously recorded on a STA 409 C (Netzsch, Germany). Samples weighing 10–20 mg were placed in a cylindrical $Al_2O_3$ holder and heated in static air atmosphere, from 20 to 1,000 °C, at a heating rate of 10 K/min.

The DSC curves were recorded using a DSC 204 F1 Phoenix (Netzsch, Germany) in the temperature range 20–260 °C at 10 K $min^{-1}$ in open aluminum pans and nitrogen flow (20 mL $min^{-1}$; nitrogen purity of 99.999 %). The samples for DSC typically weighed 3–10 mg.

The DMA measurements were performed using a DMA Q800 (TA Instruments, USA) in tensile mode between room temperature and 260 °C at 3 K $min^{-1}$, under a controlled strain of 0.2 % and 0.3 N static force, at 1 Hz frequency, in static air atmosphere.

The X-ray powder diffraction patterns were collected on a Bruker D8 Advance diffractometer using an X-ray tube with Cu anode (with wavelength of the Cu K$\alpha$ radiation at 1.5406 Å). For all samples, the $2\theta$ angle was scanned between 1.5° and 60°, with 0.040° step size and 1 s/step. The samples had typical dimensions of about 10 × 10 mm and were measured on the flesh side.

Attenuated total reflection Fourier transform infrared spectra were collected on a Bruker Tensor 27 FTIR spectrometer equipped with PIKE MIRacle single reflexion ATR unit, in the wavenumber range 4,000–600 $cm^{-1}$ (resolution < 1 $cm^{-1}$), on the flesh side of the parchment samples. Each spectrum resulted from 32 consecutive scans.

The instruments were calibrated using the manufacturer-specified calibration procedures.

## Results

### X-ray diffraction (XRD)

Figure 1 shows the XRD patterns obtained for L1 samples which have been soaked in water and NaCl solutions. Similar patterns were observed for the other two series of parchments. All samples exhibit characteristic diffraction peaks of collagen at $2\theta$ values of around 14°, 17° and 26°, which were described previously [8]. No clear correlation was observed between the absolute intensities of these peaks, obtained for the three series of parchments, and the concentration of NaCl solutions. Different intensities of collagen peaks are most probably due to the fact that after soaking in solutions and drying, the surface of parchment samples was not flat, which affected the quality of XRD data. It was noticed that the position of the main collagen peak for the three series of samples (16.96 ± 0.08° for L1, 16.93 ± 0.04° for L2 and 17.00 ± 0.11° for C) does not change significantly. Although very qualitative, the XRD data confirm the existence of a crystalline region of collagen in these samples, which is not altered by the salting procedure. In their study using small and wide-angle X-ray diffraction, Maxwell et al. [21] found that in a dry salted bovine hide the distance between amino acid residues, the intermolecular lateral packing distance between the collagen molecules, the *d* spacing and the collagen crystallinity remain unaffected as compared to the dry untreated bovine hide.

For all three parchment series, the samples which were soaked in 5 and 1 M NaCl solutions also exhibit the characteristic peaks of sodium chloride at ~32° and 46° $2\theta$

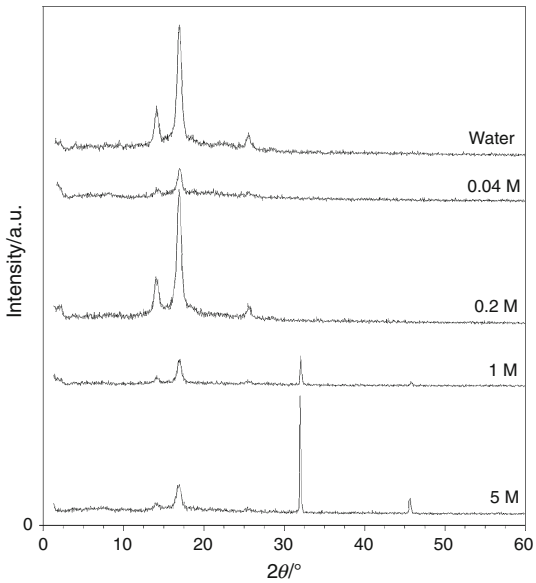


**Fig. 1** XRD patterns of L1 samples previously soaked in water and salt solutions

(JCPDS No. 05-0628), while those soaked in 0.04 M and 0.2 M NaCl solutions do not, due to their low content of sodium chloride.

## Fourier transform infrared spectroscopy

The ATR-FTIR spectra of parchment L2 samples which have been soaked in water and NaCl solutions are represented in Fig. 2. Similar results were obtained for the other two parchments. The fingerprint region in the spectral range 1,700–900 $cm^{-1}$ was plotted, which includes the characteristic bands of collagen (amide I at $\sim$1,630 $cm^{-1}$ and amide II at $\sim$1,540 $cm^{-1}$). The spectra were normalized to the amide I band. It is known that the separation between amide I and II bands and their ratio are sensitive markers of collagen alteration. Thus, the increase in the amide I–amide II separation indicates denaturation of collagen to gelatin [22], while the increase of the amide I/amide II ratio is associated with hydrolysis [23]. As it is seen from the figure, there are no significant changes of the positions and the intensities of the amide I and II bands for these samples. The corresponding values of the separation and of the ratio between amide I and II bands for the three series of salt-treated parchments are as follows: 89.6 ± 0.9 $cm^{-1}$, 1.05 ± 0.03 for L1; 94.2 ± 0.5 $cm^{-1}$, 1.00 ± 0.01 for L2 and 89.8 ± 0.4 $cm^{-1}$, 1.09 ± 0.03 for C. These values are in good agreement with those obtained for some reference (new) parchments [5, 23]. The positions of bands from the fingerprint region are also unaltered. These facts suggest that the salting procedure does not induce any important structural and chemical modification of the parchments' collagen.

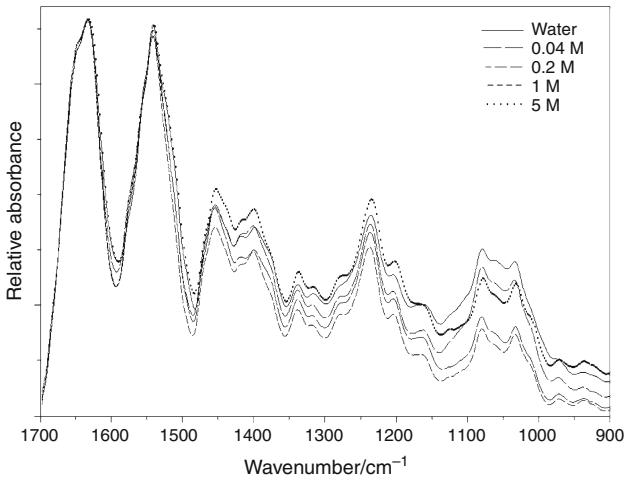


**Fig. 2** ATR-FTIR spectra of parchment L2 samples previously soaked in water and NaCl solutions

## Simultaneous TG/DTG/DSC thermal analysis (STA)

An example of TG/DTG/DSC curves for a salt-treated parchment (sample "L1 5 M") is represented in Fig. 3. Similar curves were observed for the other samples. The first three mass losses are typical for parchments [5] and correspond to dehydration, thermo-oxidation and pyrolytic decomposition of parchment's collagen in air. The plateau in the TG curve observed between about 600 and 750 °C suggests that in this temperature region all the organic part of the parchment is lost. The final (forth) mass loss, which occurs above 800 °C, is preceded by a sharp endothermic peak in the DSC curve at 801.7 °C and is accompanied by a DTG peak at 885.0 °C and a broad DSC endotherm at 868.9 °C. These facts suggest that the final mass loss is due to the vaporization of sodium chloride, which occurs immediately after its melting (801.6 °C), as it has been previously observed through thermal analysis of NaCl [24].

In Fig. 4 the TG curves of parchment C samples which were soaked in water and NaCl solutions are represented. First, it was noticed that the moisture content of these samples, measured as the first mass loss and corrected taking into account the salt content, did not show any clear variation within the series. Also, as expected, the final mass loss, corresponding to the NaCl content, diminishes with decreasing the concentration of salt solutions in which the samples were soaked. It was found that even the blank sample ("C water") exhibit a small mass loss in this region (0.45 %), but at lower temperature (DTG peak at 792.5 °C). This mass loss was also described in [5] and was attributed to the decomposition of a small quantity of calcium carbonate usually present in parchments. For salt-treated parchment samples these two processes are overlapped and one cannot separate the mass losses corresponding to vaporization of NaCl and to decomposition

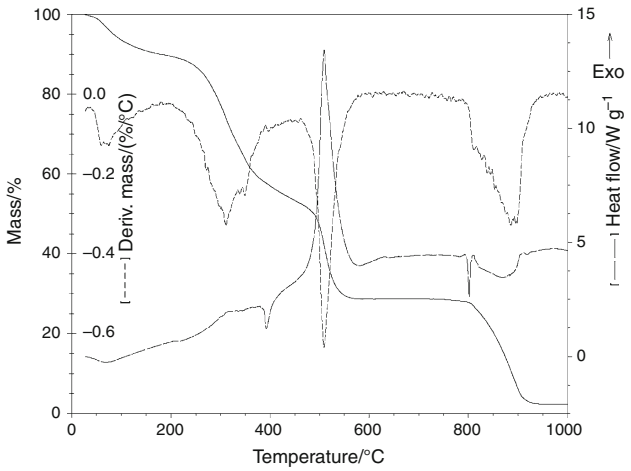


**Fig. 3** The TG/DTG/DSC curves of parchment sample "L1 5 M"

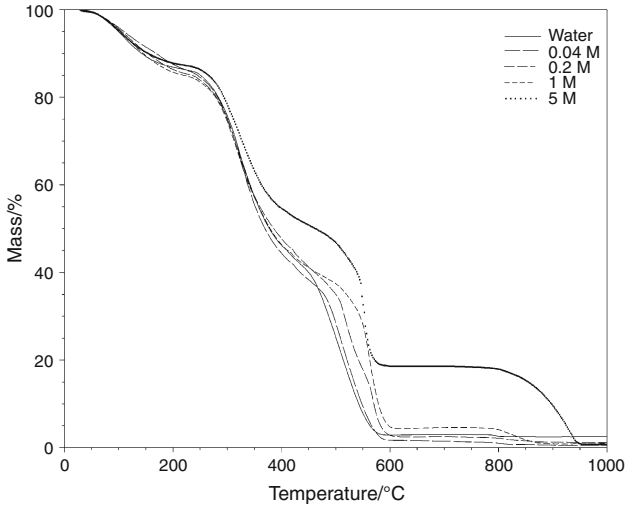


**Fig. 4** TG curves of the parchment C samples previously soaked in water and NaCl solutions

of $CaCO_3$. It was assumed by us that the quantity of $CaCO_3$ in salt-treated parchments is the same as that in the blank sample and thus the amount of NaCl could be calculated by subtracting the final mass losses of blank sample (due to $CaCO_3$) from the final mass losses of salt-treated samples (due to NaCl and $CaCO_3$).

## Differential scanning calorimetry (DSC)

The DSC curves of the studied samples generally resemble the typical ones of parchments [3]. Thus, two main endothermal peaks are observed: a large one below 100 °C corresponding to the loss of moisture, and a smaller one at above 200 °C, which is due to the melting of the collagen crystalline region. Figure 5a depicts the region 190–260 °C of the DSC curves of parchment L1 samples which have been soaked in water and NaCl solutions. As it can be observed, the melting peak shifts from 230.9 °C (the value for the blank sample) towards lower temperatures on increasing the concentration of salt solutions in which the samples were soaked. The minimum temperature is reached for the sample "L1 1 M", which exhibit a melting temperature as low as 211.1 °C, meaning an almost 20 °C difference. Quite unexpectedly, the melting peak for sample "L1 5 M" is at higher temperature (217.4 °C) than the previous and is very broad. It was noted that the melting enthalpies of the samples of this series are comparable, being situated in the range −4.7 to −5.5 J/g, without a clear variation with the salt content, which demonstrates that the crystalline region is roughly preserved.

A similar order of variation of $T_m$ with the concentration of NaCl is observed for parchment C (for sample "C 5 M" the melting peak is split into two, probably due to the heterogeneity of the sample), while for L2 series a continuous decrease of $T_m$ is put in evidence. The melting enthalpies for the samples of these 2 parchments did not also show a variation with the salt content; however, the scattering of the values is larger (between −4.0 and −6.7 J/g for L2 and between −3.0 and −8.3 J/g for C samples).

After washing with water and drying, the melting peaks of L1 samples which were previously soaked in NaCl solutions revert to a very narrow range (<1.5 °C) in which the one of the blank sample appears (Fig. 5b), and the enthalpies are also comparable. Similar results were obtained for L2 and C samples. This demonstrates that the collagen structure in parchments is fully restored.

## Dynamical mechanical analysis (DMA)

The DMA curves of the samples soaked in water and in NaCl solutions resemble in general the typical curve for parchments [8]. Figure 6a shows the storage modulus $E'$ curves of L2 samples in the 180–240 °C region, which are characterized by the decrease of $E'$ due to the melting of the crystalline region of collagen [8]. The inflection point of this decrease was taken as melting temperature, $T_m$ (DMA), as in [8]. It can be seen that DMA data confirm the lowering of the melting temperature of the salt-treated samples, with respect to the blank sample. Thus, the value of T for "L2 0.04 M" is *cca* 5 °C lower, and that of "L2 0.2 M" is 13.5 °C lower than that of "L2 water". At higher concentrations of NaCl, a slight increase of $T_m$ is observed. The absolute values of modulus were not compared, since they depend on the sample dimensions, which where difficult to determine with high precision due to the fact that after soaking and drying, the surface of parchment samples was uneven.

After washing of salt-treated samples with distilled water and drying, the DMA curves become very similar.

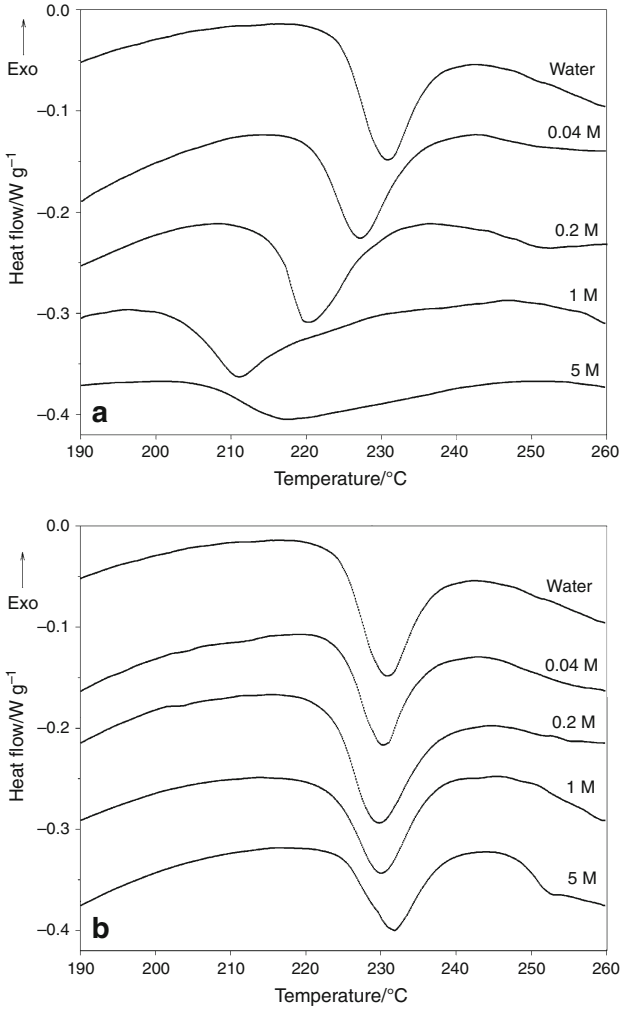


**Fig. 5** DSC curves of parchment L1 samples previously soaked in water and NaCl solutions (**a**) and of salt-treated L1 samples after washing and drying (the curve for sample "L1 water" is also given for comparison) (**b**)

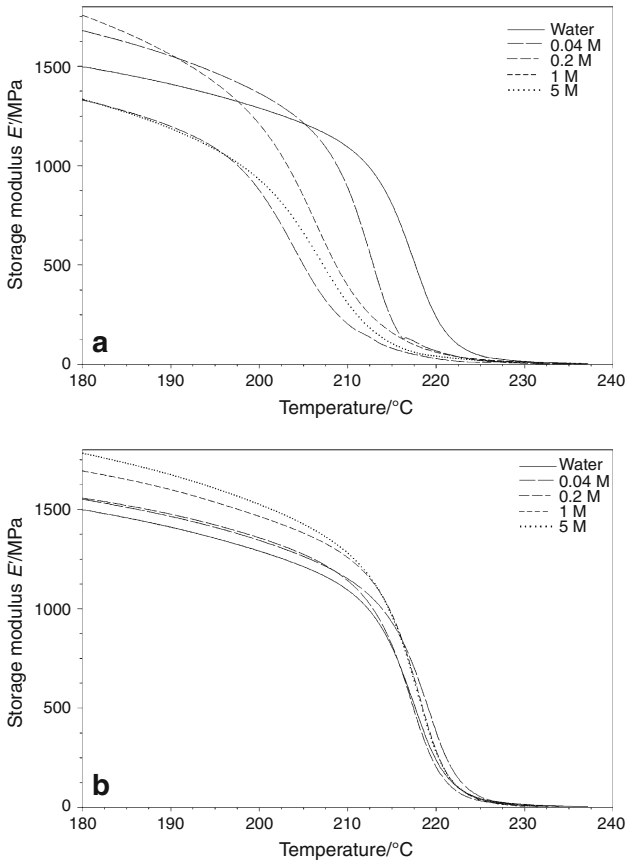


**Fig. 6** Storage modulus $E'$ curves of L2 samples previously soaked in water and NaCl solutions (**a**) and of salt-treated L2 samples after washing and drying (**b**)

The decreases of modulus occur in a narrow region of temperature (Fig. 6b): the $T_m$ values of the washed samples are within 2 °C of that of blank sample. Similar results were obtained for L1 and C samples.

By plotting the variation of the melting temperature, measured by DSC and DMA with the NaCl content, determined from TG measurements for the three series of parchment samples, the following features are observed: at low salt content there is a sharp decrease of $T_m$, followed by a minimum and a slight increase at high NaCl content (Fig. 7).

It can be noted from Fig. 7 that the values of $T_m$ measured by DMA are lower by about 10–15 °C than those measured by DSC. This is explained by different heating rates used in the two techniques, as well as by different masses of samples.

## Discussion

As it was mentioned before, the influence of various neutral inorganic salts (including NaCl) on the denaturation temperature $T_d$ of collagen in solution has been intensively studied. However, the mechanisms proposed in these works can not be applied to the process of melting of dry/non-hydrated collagen. At temperatures above 200 °C the parchment is already dehydrated, as indicated by TG measurements, and obviously no interaction between NaCl and collagen exist in liquid phase.

In order to propose a mechanism for the results described in this article, we will refer again to the aforementioned XRD study [21]. There it has been found that in a wet salted bovine hide the intermolecular lateral packing between collagen molecules is larger (1.49 nm) than in a wet untreated sample (1.40 nm). This indicates that during soaking in NaCl solutions the fibrils are expanding more than in water, allowing not only the water molecules but also salt (in the form of hydrated ions) to penetrate between the collagen molecules within the fibrils. On air-drying of both untreated and salted hide the intermolecular lateral

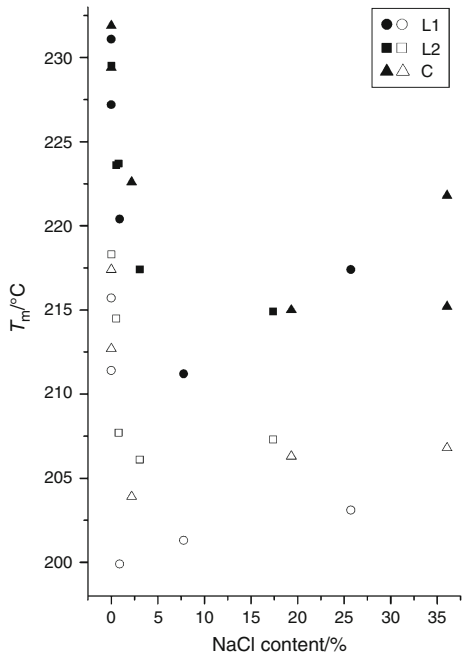


**Fig. 7** The plot of melting temperatures of the studied parchments measured by DSC, $T_m$(DSC), (*filled points*) and DMA, $T_m$(DMA), (*open points*) versus the NaCl content (0 % for blank samples)

packing is reduced to a same distance of 1.20 nm. This suggests that the excess water between the molecules is stripped away and collagen molecules are brought into closer contact, surrounded only by the hydration shell. For the salted sample, this result also suggests that salt does not remain between the collagen molecules, but crystallizes between fibrils and fibers. On further dehydration by heating, the collagen molecules lose their hydration shell, approach each other to maximum extent and an "ordered" collapsed structure results. According to the "polymer-in-a-box" mechanism, proposed by Miles and Ghelashvili [25], the collagen molecule in a fully dehydrated fiber is highly constrained by adjacent molecules as if it was confined within a "box". The free-volume available for denaturing is very reduced and thus the Gibbs free energy of activation is increased and $T_m$ is high. In salt-treated parchments, the salt crystallites between the fibrils act as solid spacers between them, as it was proposed for the drying of salted skins [26]. In the case of samples soaked in low-concentration NaCl solutions, a small amount of salt crystallites surround the collagen fibril but sufficiently to prevent the fibrils from approaching completely one to another, resulting in a less ordered structure (with gaps). It can be supposed that collagen molecules in a less constrained fibril will denature easier and thus $T_m$ will decrease. Unfortunately, in [21] no data were given for the distance between the fibrils for the dry untreated and salted sample, which could confirm the above supposition. Hence, other destabilization mechanisms of the collagen structure by low NaCl amounts are also possible. At high salt content, the salt crystals may "cement" the fibrils and act on their turn as "walls" of the "box", thus reducing again the available free-volume and increasing $T_m$. On washing, the salt is removed, the native hierarchical structure of collagen is fully restored and thus the melting temperatures revert to the values of the blank samples.

## Conclusions

The influence of sodium chloride concentration on the melting temperature of parchments, measured by DSC and DMA, is discussed. A decrease in melting temperature of these parchments compared to the samples soaked in water was observed. By plotting the melting temperatures versus the actual salt concentration in the samples, an apparent minimum results, corresponding to the largest decrease of melting temperature. At higher content of sodium chloride $T_m$ has a tendency of increasing. The reversibility of the phenomenon of decreased melting temperature was put in evidence, by washing the salt-treated samples with water, drying and then measuring by DSC and DMA. These studies can provide information about the processes taking place within the hierarchical structure of collagen in parchment.

Further research using other neutral inorganic salts on a larger number of collagen-based materials is in progress, in order to verify the above-discussed mechanism and to find new correlations.

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