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STRUCTURAL AND MECHANICAL PROPERTIES OF CROSSLINKED DRAWN GELATIN FILMS

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Abstract

Differential scanning calorimetry and high angle X-ray diffraction analyses were performed on gelatin films, air dried at different values of constant elongation, crosslinked with glutaraldehyde and examined at constant relative humidity of 75%. Drawing induces a preferential orientation of the chain segments of gelatin parallel to the stretching direction, and a linear increase of the renaturation level, calculated as the ratio between the denaturation enthalpy of gelatin films and that of tendon collagen. The comparison with the results previously obtained on the mechanical properties of the films, puts into evidence the different contributions of orientation and renaturation on the improvement of the mechanical parameters on drawing. The results offer important information on the role of glutaraldehyde (GTA) crosslinking on the stability of collagenous materials.

Keywords: DSC, gelatin films, high angle X-ray

Introduction

Collagen is the most widespread protein in the body occurring in most connective tissues as skin, tendon and bone, where its main role is to provide structural integrity to the tissue. The peculiarity of the amino acid sequence accounts for the characteristic coiled coil structure of the collagen molecule, where three distinct polypeptide chains, each of which is coiled into a left handed helix, are thrown into a right handed superhelix stabilized through interchain hydrogen bonds and covalent crosslinks [1, 2]. Thanks to these interactions and to the highly specific alignment and packing of the molecules in the fibrils, collagen fibrils display high stability and high tensile strength, which determine the mechanical properties of collagenous tissues [3]. Gelatin is obtained by thermal denaturation or physical and chemical degradation of collagen [4, 5]. At temperatures of about 40°C, the gelatin solutions are in the sol state and change into gels when they are cooled at room temperature, provided that their concentration is high enough [6, 7]. The sol-gel transformation is due to a con-

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formational disorder-order transition of the gelatin chains which form thermoreversible networks by associating helices in junction zones stabilized by hydrogen bonds. The mechanism of gelation and the properties of gelatin gels have been extensively investigated [5–9]. Gelatin is used in pharmaceutical industry for the manufacture of soft and hard capsules, and gelatin sponges are widely and successfully utilized in clinical practice [10, 11]. However, further applications of this non-antigenic, biodegradable biopolymer as biomaterial are hindered by its poor mechanical properties. Recently, we have successfully applied a method to induce segmental orientation into gelatin films in order to obtain an improvement of their mechanical properties in the direction of deformation [12]. Gelatin films crosslinked with glutaraldehyde (GTA) after air drying under constant elongation and tested at a constant relative humidity of 75%, display increasing values of Young's modulus and stress at break on increasing deformation. The improvement of the mechanical properties of drawn gelatin films has been related to the renaturation level of the protein, calculated through differential scanning calorimetry (DSC). Thermal analysis was performed on air dried drawn gelatin films and revealed an increase of denaturation enthalpy on increasing stretching [12]. Since water content affects significantly the thermal stability of collagenous material [13, 14], we decided to extend the DSC analysis to drawn gelatin films in the same conditions of hydration, at 75% relative humidity, as those used to measure their mechanical properties. Furthermore, in order to further clarify the contribution of the structural modifications induced by the crosslinking-under-deformation method to the mechanical properties of the gelatin films, we have carried out a high angle X-ray diffraction analysis on the same samples.

Materials and methods

Type A gelatin (Italgelatine S.p.A.) from pig skin was used. Gelatin films were prepared from a 5% aqueous gelatin solution. Films were obtained on the bottom of Petri dishes (diameter=6 cm) after water evaporation at room temperature from 10 ml of gelatin solution. Strip shaped (3×30 mm, thickness around 0.12 mm) air dried films were immersed in a mixture of water and ethanol in the ratio 2:3 for 72 h (constant relative humidity=75%) and stretched in the mixture using an Instrom testing machine, as previously reported [12]. The samples were air dried under constant elongation, with a draw ratio, $\lambda=l/l_0$ where l=final length and l_0=initial length, from 1.2 to 3.0. After air drying, the samples were crosslinked in a 2.5% (w/w) GTA solution in phosphate buffer at pH 7.4 for 24 h at room temperature, while kept at constant elongation. The crosslinked samples were then repeatedly washed with bidistilled water and air dried at room temperature.

Differential scanning calorimetry

Calorimetric measurements were performed using a Perkin Elmer DSC-7 equipped with a model PII intracooler. Temperature and enthalpy calibration was performed by using high purity standards (*n*-decane, benzene and indium). Gelatin (3–4 mg of dried

sample) were immersed in a mixture of water/ethanol in the ratio 2:3 for 72 h (constant relative humidity=75%). The wet samples were wiped with filter paper to remove excess liquid and hermetically sealed in aluminium pans (to prevent any loss of liquid during measurements).

Heating was carried out at 5°C min^{-1} in the temperature range –5–120°C. Denaturation temperature (T_D) was determined as the peak value of the corresponding endothermic phenomena. The value of denaturation enthalpy was calculated with respect to the mass of air dried (containing 14% mass water) gelatin.

X-ray diffraction analysis

High angle X-ray diffraction patterns were recorded on a flat camera with a sample to film distance of 60 mm using Ni filtered CuK_α radiation. The mean degree of orientation of some reflections was evaluated from the X-ray patterns obtained using the fiber specimens attachment FS-3 mounted on the Rigaku wide angle goniometer D/max-C. Measurements were performed at $2\theta=7.6°$, corresponding to the 1.16 nm spacing of collagen, with a sample rotation from $\beta=0°$ to $\beta=360°$. The data were processed using the Rigaku D/max System application software texture analysis program.

Determination of extent of crosslinking

The extent of crosslinking in drawn gelatin films was determined by a UV assay of uncrosslinked ∈-amino groups before and after crosslinking [15]. Following reaction with 0.5% TNBS, gelatin was hydrolysed with 6 M HCl, and extracted with ethyl ether. The absorbance of the diluted solution was measured at 346 nm in a Perkin Elmer model 552A double beam spectrophotometer *vs.* a blank. The relationship between absorbance and moles of ∈-amino groups per gram of gelatin is

$$\frac{\text{moles} \in\text{-amino groups}}{\text{g gelatin}} = \frac{2(\text{absorbance})(0.020\ \text{L})}{1.46{\cdot}10^4\ \text{L mol}^{-1}\ \text{cm}^{-1}(b)(x)}$$

where $1.46{\cdot}10^4$ L mol^{-1} cm^{-1} is the molar absorptivity of TNP-lys, b is the cell path length in cm, and x is the sample mass in grams.

Results

X-ray diffraction patterns from crosslinked undrawn gelatin films diplay the presence of two rings corresponding to the periodicities of about 0.29 and 1.16 nm, characteristic of collagen molecular structure. The spacing, 1.16 nm, of the equatorial reflection is shorter than that characteristic of wet collagen, about 1.4 nm, due to GTA treatment. The high angle X-ray diffraction patterns of the drawn films show a preferential orientation of the 0.29 and 1.16 nm reflections along the directions respectively parallel and orthogonal to the stretching one, in agreement with a preferential orientation of the chain segments along the stretching direction. Figure 1 shows the high an-

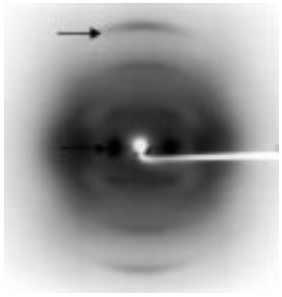

Fig. 1 High angle X-ray diffraction patterns of a gelatin film crosslinked under a draw ratio of 2.0. The arrows indicate the meridional reflection at 0.29 nm and the equatorial reflection at 1.1 nm. The vertical direction of the pattern coincides with the direction of elongation

gle X-ray diffraction pattern from a gelatin film crosslinked under a draw ratio, λ, of 2. A quantitative evaluation of the degree of orientation of the chain segments has been performed through the analysis of the relative intensity distribution along the Debye ring of the equatorial reflection at 1.16 nm. Figure 2 shows the relative intensity distributions of the reflection obtained from the films at a draw ratio of 1.2 (a) and 2 (b). The degree of preferred orientation (A) has been calculated as

$$A(\%)=\frac{180-\alpha}{180}100$$

where α is the width at half maximum intensity. The results, plotted in Fig. 3 as a function of λ, indicate that a very low draw ratio is sufficient to induce a preferential

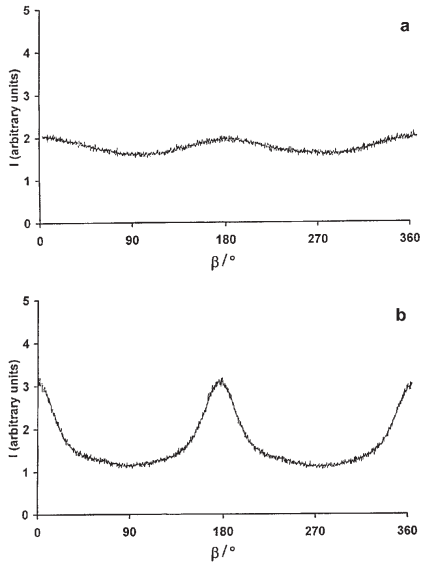

Fig. 2 Relative intensity distributions along the Debye ring of the collagen equatorial reflection at 1.16 nm recorded from drawn films. (a) λ=1.2, (b) λ=2.0

alignment of the chain segments parallel to the direction of deformation. The degree of orientation increases up to a value of λ of about 2, after which it remains almost constant.

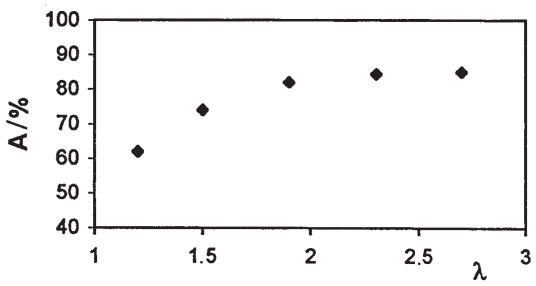

Fig. 3 Degree of orientation, *A*, of the chain segments of the crosslinked gelatin films as a function of the draw ratio, λ.

Figure 4a displays a typical DSC curve recorded from a wet undrawn gelatin sample, which exhibits an endothermic peak at about 67.8°C associated to the helix-coil transition of gelatin with a denaturation enthalpy ΔH_D=5.0 J g^{-1}. The renaturation level, *X*, of the different samples was calculated as

$$X\,(\%)=\frac{\Delta H_D}{\Delta H_T}100$$

where ΔH_T is the denaturation enthalpy of tendon collagen (Fig. 4b) which was examined in the same conditions as gelatin samples, that is hydrated in water/ethanol after crosslinking with GTA [12, 16]. The values of denaturation temperature, T_D, denaturation enthalpy, ΔH_D, and renaturation level, *X*, obtained for the drawn gelatin samples are reported in Table 1 together with the thermal parameters obtained for tendon collagen. Undrawn crosslinked gelatin films display a very low renaturation level, in

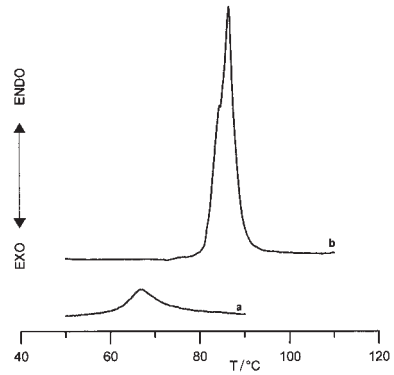

Fig. 4 DSC curves of (a) undrawn gelatin film and (b) tendon collagen, maintained at 75% constant relative humidity

agreement with the low value of its denaturation enthalpy compared to that of tendon collagen. However, the renaturation level increases with the draw ratio up to about 38%, whereas the values of the denaturation temperature show a slight, but appreciable reduction at high draw ratios.

Table 1 Denaturation temperature, T_D, denaturation enthalpy, ΔH_D, and renaturation level, X, of crosslinked gelatin films as a function of the draw ratio, λ. Each value is the mean of ten determinations and is reported with its standard deviation

λ	T_D/C	ΔH_D/J g^{-1}	X/%
1.0	67.8±0.5	5.0±0.1	11.9
1.2	69.2±1.0	5.7±1.0	13.6
1.5	67.4±0.3	8.1±0.5	19.3
1.8	64.5±0.1	11.0±0.5	26.2
2.0	63.8±0.4	13.7±0.2	32.6
2.5	61.3±0.1	14.5±0.2	34.5
3.0	61.8±0.1	15.8±0.2	37.6
Tendon collagen	85.0±0.5	42.0±0.5	

In order to better clarify the influence of GTA crosslinking on the thermal behaviour of the samples, further DSC analyses have been carried out on wet uncrosslinked undrawn gelatin films, as well as on tendon collagen in the same conditions. In absence of GTA treatment, the endothermic peak of wet gelatin is at about 43°C and has an associated denaturation enthalpy of 29 J g^{-1}. The values of denaturation temperature and enthalpy obtained for wet uncrosslinked tendon collagen are 77°C and 44 J g^{-1}, respectively.

The extent of crosslinking has been calculated from the moles of free $\in$-amino groups per g of gelatin. A loss of 91±2% amino groups has been determined after crosslinking of undrawn gelatin films. The extent of crosslinking has been found to decrease on increasing the draw ratio of the films down to the value of 70±3% obtained for λ=3.0, as reported in Table 2.

Table 2 Extent of crosslinking, expressed as percent of free $\in$-amino groups lost after crosslinking as a function of the draw ratio

λ	Extent of crosslinking/%
1.0	91±2
1.5	88±3
1.8	80±3
2.5	72±3
3.0	70±3

Discussion

The results of this paper put into evidence the different contributions of renaturation level and degree of orientation on the mechanical properties of crosslinked drawn gelatin films.

Drawing induces a preferential orientation of the collagen molecular portions parallel to the stretching direction. The chain segments appear preferentially aligned along the stretching direction even at very small values of draw ratio, the degree of orientation increases up to about λ=2.0, and then it remains almost constant. Drawn gelatin films exhibit mechanical properties which change as a function of the draw ratio [12]. In particular, the Young's modulus, *E*, and the stress at break, σ_b, were found to increase linearly with λ up to about five times the values characteristic of undrawn samples. The non-linear variation of the degree of orientation of the chain segments with λ (Fig. 3), suggests that the variation of this parameter is not sufficient to justify the further improvement of the mechanical parameters of gelatin films at draw ratios higher than 2.0.

The results of the DSC analysis carried out on wet samples indicate that the renaturation level of drawn gelatin films increases as a function of the draw ratio (Table 1). The endothermic process present in the DSC curve of collagenous material and associated to the helix-coil transition, has been suggested to be mainly due to H-bonds rupture [16, 17]. On the other hand, the increased thermal stability and decreased endothermic heat change exhibited by collagen after air-drying, have been ascribed to the presence of bonds, such as crosslinks, which break exothermically [18]. Furthermore, the increased thermal stability of collagen with ageing has been related to covalent crosslinks, which become increasing thermostable [19]. In agreement, the thermal parameters of collagen were found to change as a function of chemical crosslinking [20, 21].

Our data indicate that GTA crosslinking has a great stabilizing effect on wet gelatin films, which exhibit reduced enthalpy variation and increased denaturation temperature with respect to uncrosslinked films. The reduction in denaturation enthalpy due to GTA treatment is dramatic in comparison to that observed for tendon collagen, which shows also a much smaller increase in the value of denaturation temperature. The different modifications of thermal properties induced by GTA on gelatin and collagen, which account for the apparent low level of renaturation of gelatin films, can be easily explained on the basis of the structural differences between the two materials. In fact, gelatin is much less crystalline than collagen and has much more free amino groups, which can react with GTA. Therefore, the number of covalent crosslinks which GTA can form with the chain segments in gelatin is undoubtely higher than that it can estabilish with collagen molecules in fibrils.

The DSC results previously obtained on air dried films [12] suggest a similar stabilizing effect of crosslinking on the thermal behaviour of dried gelatin. However, the GTA induced reduction in denaturation enthalpy of dried gelatin was lower and, as a consequence, the calculated renaturation level greater than that obtained for wet films. The greater influence of GTA on wet films can be ascribed to the fact that

crosslinking reduces the amount of water associated with the protein in wet conditions, with a consequent decrease in denaturation enthalpy and increase in denaturation temperature [21]. The reduction of water content is confirmed by the spacing of the high angle equatorial reflection of wet crosslinked gelatin films, which is smaller than that characteristic of wet uncrosslinked collagen [14].

The denaturation enthalpy of wet drawn gelatin films increases with increasing λ, so that the renaturation level increases from about 11 in undrawn films to about 38% in films stretched up to about 200%. Furthermore, the samples with a draw ratio higher than 1.5 exhibit a slight reduction in the value of denaturation temperature, which could be due to the observed reduction in extent of crosslinking on increasing deformation: stretching induces a partial renaturation of gelatin in a collagen-like structure and, as a consequence, a reduction in the number of sites available for GTA crosslinking.

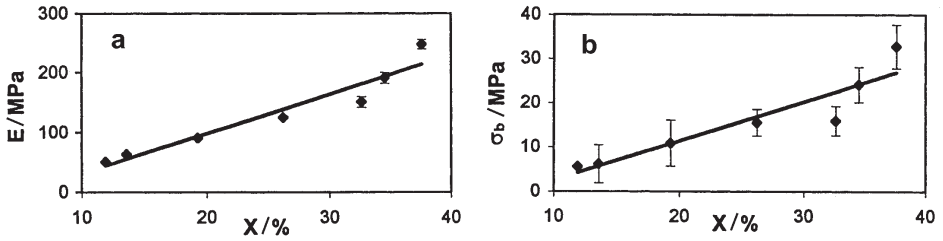

Fig. 5 Variations of Young's modulus, *E*, (a) and stress at break, σ_b, (b) as a function of the renaturation level, *X* of the drawn gelatin films. The lines have been calculated by means of linear regression analysis. The values of the mechanical parameters are from reference [12]

Figure 5 reports the variation of the Young's modulus, E, and the stress at break, σ_b, previously obtained for drawn gelatin films [12] as a function of the renaturation level. It is evident the close relationship between the mechanical parameters and the renaturation level of drawn gelatin. It can be concluded that uniaxial stretching induces a preferential orientation of the chain segments parallel to the direction of elongation, which is the first essential step towards the process of gelatin renaturation. Although the degree of orientation does not increase any longer at draw ratios higher than 2, further stretching likely promotes the lateral approach between the chain segments, as suggested by the reduction of the thickness of the gelatin layers on increasing λ [12], with a consequent further increase in the renaturation level, which seems to be the main parameter in determining the mechanical properties of the gelatin films.

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