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DIFFERENTIAL SCANNING CALORIMETRY AND OPTICAL PROPERTIES OF COLLAGEN-DICHROIC AZO PONCEAU SS COMPLEXES

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ABSTRACT

The purpose of this research was to find correlations between thermocalorimetric properties, linear dichroism and birefringence of collagen-Ponceaus SS complex as compared to pure collagen fibers. In this work, we show that the binding of the dichroic sulfo-azo dye Ponceau SS to highly oriented collagen fibers results in increased crystallinity, as measured by differential scanning calorimetry and optical anisotropy. Collagen fibers formed a complex with Ponceau SS that showed higher crystallinity and molecular order than the fibers alone. Differential scanning calorimetry revealed that the denaturation (peak) temperatures (Tm) of the complex were higher than for the pure collagen fibers. In addition, the dye-collagen complex showed high linear dichroism and stark interference colors in birefringence dispersion. These photo-optical properties are caused by periodic differences in the refractive indices along the molecules in the ordered collagen-dye complex, and indicate that this complex is a non-linear optical supramolecular chiral object.

Key words: Collagen, differential scanning calorimetry, linear dichroism, molecular order, crystallinity, Ponceau SS-collagen complex

INTRODUCTION

Dichroic azo dyes have been extensively studied because of their optical properties. The molecular ordering of dye molecules has been observed in liquid crystals (LC) containing azo dyes, as shown by the birefringence, preferential absorption, and dichroic indices of these crystals [1,7,8]. In addition, the properties and functionality of multilayered films assembled with dichroic dyes and LC have been investigated [1,2,5,22], although the self-assembly of functional dye Ponceau SS in a multilayered structure with electro-optical properties was unrelated to the dyes linear dichroism (LD) and birefringence dispersion properties [22]. The complexation of dichroic azo dyes with highly oriented and optically anisotropic biopolymers could increase the overall optical anisotropy and even produce photoelectric properties by combining the characteristics and properties of the dyes and biopolymer. Type I collagen is an example of a biopolymer that can interact with an azo dye to form a complex endowed with crystallinity and high optical anisotropy.

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Type I collagen self-assembles to form superstructures, such as tendons, that can be considered to be multilayered helical films [14,18-21]. The ordered assembly of acidic sulfonic azo dyes with collagen fibers can provide information on the relationship between supramolecular order and functionality. In addition, the analysis of multi-layered superstructures, such as Ponceau SS-collagen fiber complexes can contribute to our understanding of the optical LD of dye-liquid crystal interactions.

In this work, we used differential scanning calorimetry, linear dichroism and birefringence to examine the changes in the crystallinity, denaturation (peak) also temperature (Tm) and enthalpy of collagen fibers complexed with Ponceau SS. We examined changes inthe linear dichroism (LD) and birefringence of the Ponceau SS molecules complexed with collagen fibers.

MATERIAL AND METHODS

Collagen bundles were isolated from the tails of 40-day-old male rats and cleaned of all extraneous tissue. Distilled water was used during all procedures. Differential scanning calorimetry (DSC) measurements of 5 mg samples were done using a DSC 2910 from TA Instruments, with an argon flow of 50 mL/min and a heating rate of 10°C/min. The parameters determined include the phase transition temperature (T_m), which corresponded to the maximum

temperature at which there was complete denaturation of the collagen samples, and the enthalpy of fusion (J/g). Ponceau SS (Aldrich, CI 27 190, #AS 6226-78-4; M 556.49, Fig. 1) was prepared in bidistilled water (final concentration 1 nmol dm^{-3}). The optical anisotropies were studied using polarized light microscopy to allow comparison with data for the Ponceau SS-collagen complex obtained by DSC. This analysis was done with pure, fixed, non-fixed and lyophilized collagen bundles.

Tendons were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 3 h under vacuum and subsequently for 24 h in a refrigerator. After washing in distilled water, the tendons were processed for inclusion in Histosec (Merck KgaA, Darmstadt-Germany) and sections 7 µm thick were obtained with a Microm HM 315 microtome (Walldorf, Germany). After deparaffinization and hydration, the sections were treated with Ponceau SS solution for 3 min followed by washing in 3% acetic acid for 2 min and then in water. The slides were subsequently air dried, cleared in xylene and mounted in natural Canada balsam (refractive index = 1.54).

Anisotropic optical phenomena, linear dichroism (LD) and birefringence were studied in tendon sections and in extended collagen fibers. Linear dichroism was measured by the image analysis method [18,21]. A 100 W halogen lamp was used as the light source. Monochromatic light was obtained using a narrow bandpass interference filter, wavelength of 546 nm (Edmund Industrial Optics, Barrington, NJ, USA).LD was observed and measured by removing the analyzer from the light path and rotating the microscope stage until the collagen bundles were oriented parallel or perpendicular to the electric vector of polarized light (EVPL) [21]. To examine whether the Ponceau SS-collagen complex had polarizing properties, the polarizer was removed from the light path and the sections were illuminated by non-polarized light with the analyzer in place. The microscope stage was then rotated until the collagen bundles were oriented parallel or perpendicular to the EVPL of the analyzer [21]. This procedure allowed the detection of LD resulting from light polarization caused by the oriented binding of Ponceaus SS to collagen bundles. LD was expressed as the difference betwee the relative absorption at the polarizer, where ΔLD = A□ – A□, A□ is the absorption with the collagen fibers parallel to the EVPL and A□ is the absorption perpendicular to the EVPL, respectively. Statistical treatments were based on the dichroic ratio where DR = A□/A□.

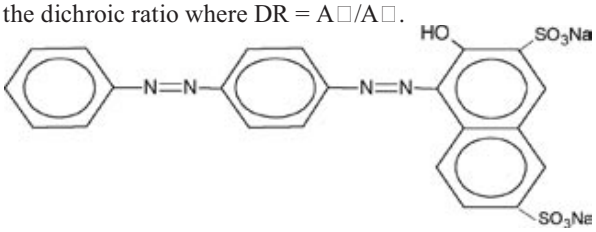

Figure 1. Ponceau SS formula (Aldrich, CI 27,190).

RESULTS

Under the conditions used here, an increase in the temperature of denaturation (T_m) resulted in a marked reduction of the thermogram area (the thermogram areas for pure collagen fibers were larger) and a drop in the corresponding values of enthalpy for the dye-collagen complex as compared to pure collagen fibers. Table I and Figure 2 illustrate the differences in the thermograms of pure collagen and the dye-collagen complex. Fixation and lyophilization reduced the enthalpy and increased the T_m. The complexation of fixed and lyophilized collagen fibers with Ponceau SS yielded similar results to those indicated above, i.e. a decreased thermogram area and increased peak denaturation temperatures (Fig. 2).

Table I. Enthalpy of fusion and maximum temperature of denaturation (Tm) for collagen in different states. The samples were used *in natura.* The values correspond to the average of three measurements each.

Experiment	Enthalpy (J/g)	Tm (^{0}C)
Pure collagen	212.7	98.8
Pure, lyophilized collagen	39.9	20.3
Pure fixed colagen	32.6	18.0
Dye-Collagen complexes	39.9	22.8

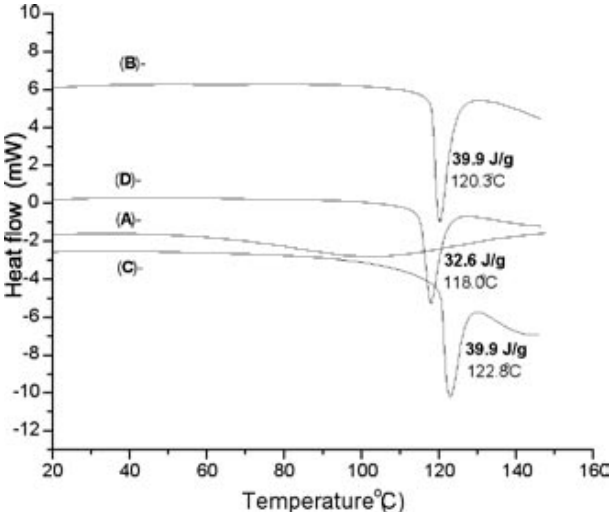

Figure 2. Differential scanning calorimetric curves for collagen samples. (A) Pure collagen, (B) Lyophilized collagen, (C) Collagen treated with Ponceau SS, (D) Collagen fixed with paraformaldehyde. These curves are the mean of three experiments for each collagen preparation.

Collagen fibers complexed with Ponceau SS showed LD (Fig. 3), with an average dichroic ratio (DR) of 1.842; the extended collagen fibers from rat tendon collagen bundles showed a marked LD (DR = 2.2). However, completely parallel collagen fibers were not obtained when mechanical strain to produce the parallel alignment of the fibers was applied (Fig. 4). The straightest bundles of fibers had the highest DR (up to 5.23) when compared to non-strained fibers in sections 7-µm-thick sections.

The Ponceau SS-collagen fiber complexes showed intense birefringence with the interference colors red, yellow and green depending on the thickness of the section or the extended fibers (Figs. 3C and 4C).

Figure 3. A 7 µm thick section of rat tendon. (A, B) LD images, (C) interference colors of birefringence. (A) Tendon axis parallel to the azimuth of polarized light (APOL) to give higher absorption. (B) Tendon axis perpendicular to the APOL. (C) Image obtained with the tendon axis oriented at 45 degrees with respect to the crossed polarizers. Bar = 21 µm.

DISCUSSION

There is a general agreement that crosslinking promotes the thermal stability of collagen fibers [3,10-11,15,17]. In the present study, the increase in denaturation temperature (T_m) was a consequence of the increase in intermolecular crosslinking produced by the presence of rod-like dye molecules. Similar interpretations were reported by Melling *et al.* [11] for collagen glycation, which agrees with the finding that collagen glycation *in vitro* increases the intrinsic and textural birefringence of these fibers.

The rod-like Ponceau SS molecules bind to collagen molecules with their long axis parallel to that of collagen, thereby allowing multi-point

Figure 4. Extended collagen fibers. (A,B) LD images. (C) Interference colors of birefringence. (A) Tendon axis parallel to the azimuth of polarized light (APOL) to give higher absorption. (B) Tendon axis perpendicular to the APOL. (C) Image obtained with the tendon axis oriented at 45 degrees with respect to the crossed polarizers, interference colors of birefringence. Bar = 300 µm.

attachments via electrostatic, hydrophobic and van der Waals forces, and hydrogen bridges, all of which contribute to the thermal stability of collagen [11]. An increased crosslinking density among the collagen molecules could promote higher chain reaggregation, thereby protecting against denaturation [6]. The Ponceau SS molecules could have a similar effect since they bind to collagen in an ordered multi-point manner that introduces a higher ordered aggregational state and crystallinity, as demonstrated by dichroism and birefringence. On the other hand, the complexation of Ponceau SS to collagen fibers may replace water, thereby contributing to the decrease in enthalpy and to the oriented aggregation of the collagen. The importance of water in the stabilization of the collagen fibers is recognized [6].

Lyophilized collagen bundles studied by DSC had a T_m = 120.3ºC, similar to that of fixed and Ponceau SS-treated fibers. The fixation of collagen fibers in paraformaldehyde increased the T_m, an observation that was expected since this fixation creates crosslinks (methylene bridges) between the collagen chains; a simultaneous decrease in the enthalpy of fusion values was seen. A decrease in entropy reflects stabilization by crosslinking or by rigid matrices [6,9], which agrees with the higher crystallinity of the fiber–Ponceau SS complex.

The alterations in the thermograms induced by the complexation of Ponceau SS to collagen fibers supported the inference that the Ponceau SS molecules became highly oriented when bound to collagen molecules. This was proven by the linear dichroism (LD) measurements and detection of the birefringence. LD is dependent on the selective absorption of light photons and is caused (in the case of Ponceau SS) by electronic transitions along the long axis of the dye, which agrees with Nordén concept of LD for the geometry of the molecule [13] (see Fig. 1). Dichroism and the dispersion of birefringence contain the same information in terms of molecular order; birefringence is an algebraic sum of all electronic transitions while, in the case of Ponceau SS, LD is caused by electronic transitions along the major axis of the rod-like dye, where the chromophores –N=N- are aligned. This finding supports the conclusion that the dye molecules are parallel to the principal axis of the collagen molecules.

In terms of functionality, the anisotropic optical properties reported here fit the reported characteristics and properties of the dichroic sulfo azo-dyes complexed with liquid crystals, multilayer films of polycations and/or cast films [1,2-4,5,7,8,22]. The commom denominator in all of these cases is the importance of molecular order as one of the factors that produces optical properties. Further, collagen fibers themselves have structural conditions to determine optical non-linearity and photonic characteristics when complexed with Ponceau SS.

In conclusion, the binding of Ponceau SS to collagen fibers increased the denaturation temperature (T_m) and decreased the area under the DSC curve and the enthalpy of fusion, all of which indicate increased crystallinity. The optical anisotropic properties of the collagen fibers were also increased after complexation with Ponceau SS. The LD of these materials indicated that collagen fibers caused the oriented binding of dye molecules. These findings support the hypothesis that the collagen-dye complexes display nonlinear optical properties.

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REFERENCES

1. Advincula RC (2001) Molecularly ordered low molecular weight azobenzene dyes and polycation alternate multilayer film: aggregation, layer order, and photoalignment. *Chem. Mat*. **13**, 2870–2878.
2. Bauman D, Moryson H (1997) Guest-host interaction in dichroic dye-liquid crystal mixtures in smectic phases. *J. Mol. Struct.* 404, 113–120.
3. Budrugeac P, Trandafir V, Albu MG (2003) The effect of the hydration degree on the hydrothermal and thermo-oxidative stability of some collagenous matrices. *J. Therm. Anal. Calorim.* **72**, 581–585.
4. das Neves UM, dos Santos Jr DS, Giacometti JA, Zílio SC, Misoguti L, Balogh DT, de Oliveira Jr ON, Mendonça CR (2003) Optical storage properties in cast films of an azopolymer. *Mat. Res*. **6**, 409–414.
5. Dragan S, Schwartz S, Eichhorn KJ, Lunkwitz K (2001) Electrostatic self-assembled nanostructures between polycations of integral type and azo dyes. *Colloids Surf.* **195,** 243–251.
6. Flandin F, Buffevant C, Herbage D (1984) A differential scanning calorimetry analysis of the age-related changes in the thermal stability of rat skin collagen. *Biochim. Biophys. Acta* **791**, 205–211.
7. Grabtchev IK, Bojinov VB, Moneva IT (1998) Functional properties of azomethine substituted benzanthrone dyes for use in nematic liquid crystals. *J. Mol. Struct.* 47, 119 –25.

8. Komitov L, Ruslim C, Matsuzawa Y, Ichimura K (2000) Photoinduced anchoring transition in a nematic doped with azo dyes. *Liq. Cryst.* **27**, 1011–1016.
9. Kronick PL, Cooke P (1996) Thermal stabilization of collagen fibers by calcification. *Connec. Tissue Res*. 33, 275-282.
10. les Lous M, Flandin F, Herbage D, Allain JC (1982) Influence of collagen denaturatuion on the chemorheological properties of skin, assessed by differential scanning calorimetry and hydrothermal isometric tension measurements. *Biochim. Biophys. Acta* **717**, 295–300.
11. Melling M, Pfeiler W, Karimian-Teherian D, Schllaninger M, Sobal G, Zangeler C, Menzel EJ (2000) Differential scanning calorimetry, biochemical, and biomechanical analysis of human skin from individuals with diabetes mellitus *Anat. Rec*. **259**, 327–333.
12. Miles CA, Burjanadze TV, Bailey A (1995) The kinetics of the thermal denaturation of collagen in unrestrained rat tail tendon determined by differential scanning calorimetry. *J. Mol. Biol***. 245**, 437–446.
13. Nordén B (1978) Applications of linear dichroism spectroscopy. *Appl. Spectr. Rev.* **14**, 157–248.
14. Silver D, Miller F, Harrison R, Prockop DJ (1992) Helical model of nucleation and propagation to account for the growth of type I collagen fibers from symmetrical pointed tips: a special example of self-assembly of rod-like monomers. *Proc. Natl. Acad. Sci. USA* **89,** 9860–9864.
15. Slatter DA, Miles CA, Bailey AJ (2003) Asymetry in the triple helix of collagen-like heterotrimers confirms that external bonds stabilize collagen structure. *J. Mol. Biol*. **329**, 175–183.
16. Snowden JM, Eyre DR, Swann DA (1982) Vitreous structure VI. Age-related changes in the thermal stability and crosslinks of vitreous, articular and tendon collagens. *Biochim. Biophys. Acta* **706**, 153–157.
17. Usha R, Ramasami T (1999) Influence of hydrogen bond, hydrophobic and electrovalent salt linkages on the transition temperature, enthalpy and activation energy in rat tail tendon (RTT) collagen fibre. *Thermochimica Acta* **338,** 17–25.
18. Vidal BC (1995) Crimp as part of a helical structure. *C. R. Acad. Sci. Paris, Science de la Vie* **318,** 173–178.
19. Vidal BC (1995) From collagen type I solution to fibers with a helical pattern: a self-assembly phenomenon. *C. R. Acad Sci. Paris, Science de la Vie* **318**, 831–836.
20. Vidal BC (2003) Image analysis of tendons helical superstructure using interference and polarized light microscopy. *Micron* **34**, 423–432.
21. Vidal BC (2003) Image analysis of linear dichroism in collagen nano-silver complexes. *Microscopy* **97,** 21–23.
22. Yoo D, Wu AP, Lee J, Rubner MF (1997) New electroactive self-assembled multilayer thin film based on alternately absorbed layers of polyelectrolytes and functional dye molecules. *Syn. Mat.* **85,** 1425–1426.

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