

Collagen fibres with improved strength for the repair of soft tissue injuries

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Our laboratory has developed a process for self-assembly of high strength collagen fibres *in vitro* which exhibit the characteristic D period. These fibres can be cross-linked by severe dehydration (dehydrothermal cross-linking) at elevated temperature and formulated into devices used to repair soft tissues. This study was conducted to evaluate the effects of dehydrothermal cross-linking time and temperature on the tensile mechanical properties of collagen fibres. The results discussed indicate that the tensile strength of reconstituted collagen fibres is optimized by cross-linking for 5 d at 110°C. Tensile strength and modulus values of 91.8 and 896 MPa are reported for fibres cross-linked in this manner. High tensile strength and modulus values are especially important in developing biodegradable materials that promote healing of orthopaedic structures.

Keywords: Collagen fibres, extracellular matrix, self-assembly, dehydrothermal cross-linking

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Collagen is one of the primary scaffolding materials utilized in vertebrates. It is found in at least 16 different molecular forms in fibrous and non-fibrous forms¹. The structural hierarchy and mechanical properties of collagen vary from tissue to tissue². In dense regular connective tissue it forms almost parallel arrays of collagen fibrils that are separated into structural units including fibril bundles and fascicles by cells and extracellular matrix². In skin, dura mater and cornea, arrays of collagen fibres form sheet-like structures which can be modelled as either woven or composite plied structures. The common feature of all these structures is the presence of high strength collagen fibres that limit tissue expansion and prevent premature mechanical failure³.

Our laboratory has developed a process for extrusion of high strength collagen fibres⁴ by self-assembling collagen solutions under optimum conditions^{5,6}. These fibres exhibit the D period characteristic of collagen and can be cross-linked using either chemical or physical techniques, as described previously⁷. Cross-linked collagen fibres have been formulated into devices used to repair tendon/ligament^{8,9} and peripheral nerve¹⁰.

Results of implantation studies in a variety of animal models demonstrate that the repair rate of tissue replacements composed of collagen scaffolds is maximized if the scaffold degradation rate parallels the rate of wound healing in a particular anatomical site¹¹. In most anatomical sites optimum biodegradation of the implant occurs within 20 wk of implantation¹¹,

with loss of about 75% of the initial tensile strength occurring in about 4 wk⁹. Therefore, in the design of devices to replace structural tissues, it is necessary to maximize the initial collagen fibre tensile strength to compensate for the rapid loss of tensile strength of collagenous implants. Formation of collagen cross-links is associated with increased load-bearing capacity. This is particularly important in orthopaedic applications, i.e. tendon or ligament replacement.

Dehydrothermal (DHT) cross-linking of collagen involves removal of residual water and formation of synthetic peptide bonds^{12,13}. After DHT treatment, the helix-to-coil transition temperature is increased, enhancing the thermal stability of collagen without altering its triple helical structure¹³. Results of pH titration studies indicate that the number of positively and negatively charged amino acids decreases after DHT treatment¹⁴.

The purpose of this paper is to report the results of a study designed to maximize the tensile strength of collagen fibres cross-linked by severe dehydration at elevated temperatures (DHT cross-linking).

MATERIALS AND METHODS

Preparation of collagen

Rat tail tendon collagen was extracted from rat tail tendons as described previously¹⁵. The tails of Sprague-Dawley rats were obtained on killing. They were stripped of skin and then the tendons were removed by clamping a haemostat on the thin free end applying a tensile force along the tendon axis. Tendon

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fibres were then immersed in HCl, pH 2.0 (10 tendons per 100 ml), for 48 h and at 4°C. The soluble material was separated from the tendons by centrifugation for 90 min at 30 000 × g. Supernatants were sequentially filtered through 0.65- and 0.45- μ m filters yielding an ultrafiltered collagen solution. As reported previously¹⁵, sodium dodecyl sulphate gel electrophoresis and amino acid analyses of this preparation showed that it contained predominately type I collagen α chains and β and γ components.

Fibre production

Soluble collagen in HCl, pH 2.0, was concentrated by placing approximately 100 ml in a dialysis bag (molecular weight cut-off of 12 000–14 000) (Spectrapor, Los Angeles, CA) and then placing the dialysis bag in contact with about 50 g of poly(ethylene)glycol (molecular weight 15 000–20 000) (Sigma Inc., St. Louis, MO). Collagen solution was then diluted to a concentration of 0.33% (w/v) using HCl, pH 2.0. Collagen solutions were stored in disposable 30 cm³ syringes at 4°C.

Collagen fibres were produced by extruding the collagen dispersion through poly(ethylene) tubing with an inner diameter of 0.28 mm into a 37°C bath of aqueous fibre formation buffer composed of 135 mM NaCl, 30 mM *N*-tris(hydroxymethyl-2-aminoethane sulphuric acid) and 30 mM sodium phosphate dibasic. The final bath pH was adjusted to 7.5 by adding 5.0 N NaOH dropwise. Fibres were allowed to remain in the buffer for 45 min, and then placed in 500 ml isopropyl alcohol for at least 4 h. Fibres were immersed in distilled water for 15–20 min then air dried under tension⁴.

Collagen fibres were cross-linked by severe dehydration for 1, 3 or 5 d at 60, 80, 110 or 140°C in an oven at a vacuum of 50–100 mtorr following the protocol described previously⁷. Cross-linked fibres were placed on a shelf in a sealed desiccator and stored at room temperature until tested.

Mechanical tests

Air-dried fibres were mounted on a paper frame by gluing the ends of the fibres to a vertical line drawn on the frame using an epoxy adhesive, as discussed previously⁴.

Stress–strain curves for reconstituted collagen fibres were determined in tension using an Instron Tester Model 1122. Fibres were tested using a gauge length of 2.0 cm and a strain rate of 50%/min either dry or after rehydration for 45 min in phosphate-buffered saline, pH 7.5. Load–extension curves obtained from the chart recorder, as well as original cross-sectional areas determined by light microscopy, were used to calculate stress–strain behaviour, ultimate tensile strength (UTS) and low and high strain moduli. Modulus values were determined as best-fit tangents to the low and high strain regions of the load–extension curve. Cross-sectional area calculations were made assuming a circular cross-section and by measuring the diameter at three places along the fibre using a Leitz 12 Pol microscope fitted with a calibrated eye-piece. Swelling ratio was determined by dividing the differ-

ence between the wet and dry fibre diameter by the dry fibre diameter.

RESULTS

Stress–strain curves for wet and dry DHT cross-linked collagen fibres were observed to be dependent upon the cross-linking temperature and time. In the dry state, stress–strain curves were similar to that of a crystalline polymer that yields and undergoes plastic flow. At low strains, the stress–strain curve of dry fibres (Figure 1) was observed to be almost linear and have a pre-yield slope that was higher than that following yielding. The low strain modulus maximized at about 8000 MPa at a temperature of 110°C (Figure 2), while the maximum value of the ultimate tensile strength was observed to be 600 MPa at 110°C (Figure 3; see Table 1). At 140°C, the modulus at 5 d increased to 6939 MPa and was not significantly different from the value observed at 110°C (Figure 2). High strain moduli appeared to maximize at 110°C (Figure 4). Low strain moduli values observed at 60 and 80°C were

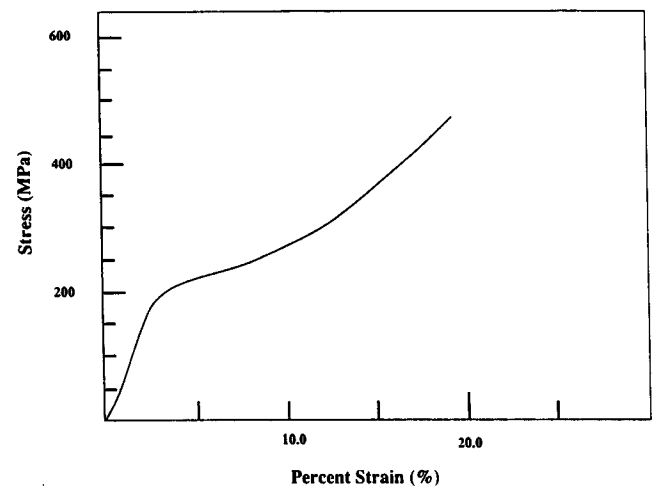


Figure 1 Tensile stress versus percent strain for a dry self-assembled collagen fibre dehydrothermal (DHT) cross-linked for 5 d at 110°C. Note the almost linear relationship between stress and strain in the low strain region.

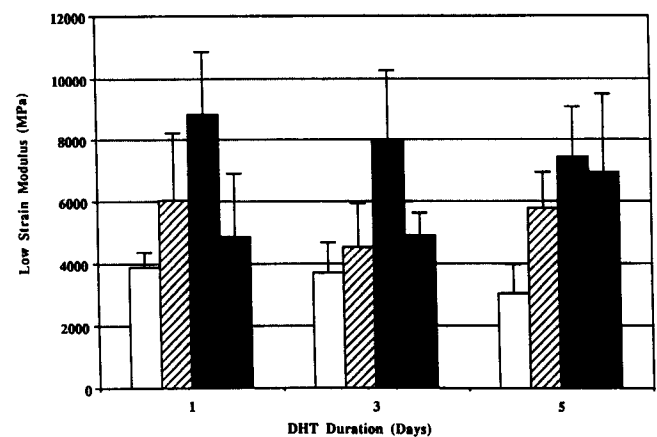


Figure 2 Low strain modulus versus DHT cross-linking time for self-assembled dry collagen fibres at 60°C, □; 80°C, ▨; 110°C, ■; and 140°C, ■.

significantly lower. Maximum strain values were about 20% at all time and temperature points studied (Figure 5). Optimum mechanical properties of dry collagen fibres were obtained at 110°C for cross-linking periods of 5 d.

Stress-strain curves for wet DHT cross-linked collagen fibres were observed to be similar in shape to curves reported previously for rat tail tendon and reconstituted collagen⁴ (Figure 6). In general, the slope of the stress-strain curve increased with strain, characteristic of connective tissue. Maximum moduli (about 800 MPa) were observed at a temperature of 110°C (Figure 7). The tensile strength was maximum at 5 d and was observed to be 91.8 MPa, which was significantly higher than the value found at other temperatures (Figure 8). Strains ranged between 10 and 20% (Figure 9) at all time and temperature points. At 110°C,

the UTS plateaued after 5 d, while the swelling ratio required about 7 d to plateau (Figure 10). For wet collagen fibres, the maximum strength was observed after DHT cross-linking at 110°C for 5 d.

DISCUSSION

Previously, we have reported^{4,11} that the UTS of collagen fibres formed from insoluble and soluble type I collagen ranged between 24 and 80.7 MPa (wet) and 168 and 442 MPa (dry). In order to optimize the UTS and high strain modulus, we have conducted a study of the effects of DHT cross-linking time and temperature on fibre mechanical properties. Of interest are the findings that the maximum strengths (both wet and dry) were observed for DHT cross-linking at 110°C.

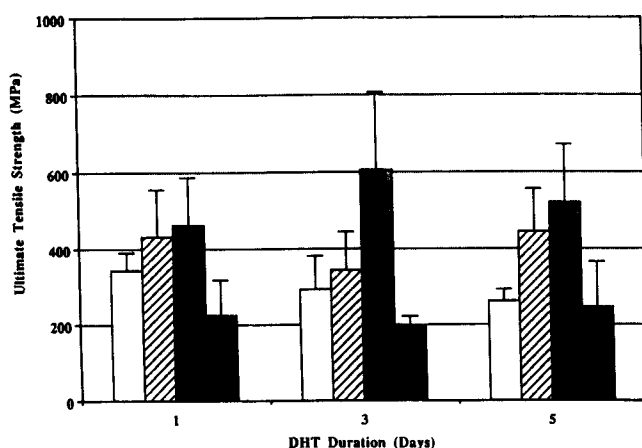


Figure 3 Ultimate tensile strength of self-assembled dry collagen fibres DHT cross-linked for different durations. □, 60°C; ▨, 80°C; ■, 110°C; ■, 140°C.

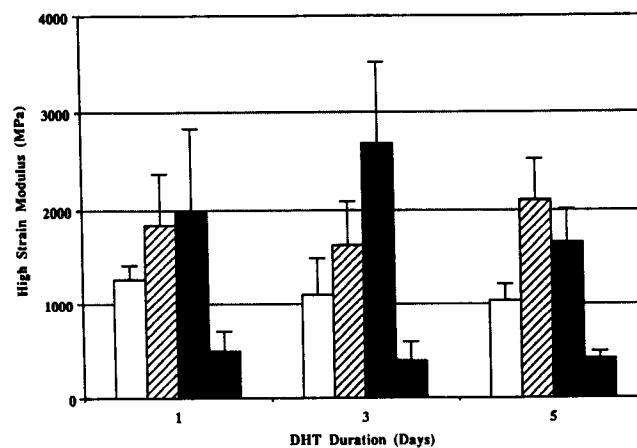


Figure 4 High strain modulus of self-assembled dry collagen fibres DHT cross-linked for different durations. □, 60°C; ▨, 80°C; ■, 110°C; ■, 140°C.

Table 1 Mechanical properties of fibres extruded using ultrafiltered soluble rat tail tendon collagen

Type of collagen fibre (n)	Ultimate tensile stress (MPa)	Ultimate strain (%)	Low strain modulus (MPa)	High strain modulus (MPa)
DHT 1 at 60°C Dry (12)	343.5 ± 49.2	18.72 ± 2.38	3875 ± 479	1261.0 ± 151.4
Wet (17)	7.9 ± 2.6	16.48 ± 3.88	—	58.3 ± 26.1
DHT 1 at 80°C Dry (12)	432.4 ± 122.3	17.63 ± 4.62	6053 ± 2185	1837.0 ± 537.5
Wet (10)	19.9 ± 6.2	7.40 ± 1.69	—	280.4 ± 115.7
DHT 1 at 110°C Dry (12)	463.8 ± 122.6	15.65 ± 6.71	8843 ± 2003	1966.2 ± 869.8
Wet (11)	43.0 ± 13.5	8.50 ± 2.74	—	608.2 ± 103.7
DHT 1 at 140°C Dry (9)	224.9 ± 92.7	26.74 ± 4.55	4860 ± 2066	492.5 ± 208.7
Wet (11)	18.0 ± 7.8	20.57 ± 8.34	—	127.8 ± 51.7
DHT 3 at 60°C Dry (9)	293.5 ± 86.7	21.25 ± 5.14	3676 ± 1004	1098.0 ± 386.1
Wet (22)	17.4 ± 7.1	18.45 ± 7.15	—	129.5 ± 68.0
DHT 3 at 80°C Dry (12)	342.3 ± 102.0	17.30 ± 2.00	4517 ± 1394	1629.0 ± 450.8
Wet (11)	24.8 ± 12.4	8.70 ± 2.46	—	248.3 ± 69.6
DHT 3 at 110°C Dry (11)	605.2 ± 202.3	22.51 ± 1.84	8012 ± 2252	2688.0 ± 833.0
Wet (10)	46.7 ± 10.1	8.47 ± 2.34	—	627.6 ± 103.8
DHT 3 at 140°C Dry (11)	194.9 ± 25.7	22.36 ± 4.34	4890 ± 738	396.3 ± 199.4
Wet (8)	26.4 ± 5.4	9.83 ± 3.44	—	280.5 ± 87.9
DHT 5 at 60°C Dry (11)	260.3 ± 31.9	22.09 ± 4.45	3072 ± 914	1031.0 ± 185.8
Wet (21)	8.8 ± 2.7	15.89 ± 3.20	—	70.2 ± 26.7
DHT 5 at 80°C Dry (11)	446.2 ± 109.0	17.45 ± 3.34	5790 ± 1158	2096.0 ± 433.0
Wet (23)	56.4 ± 22.1	8.94 ± 2.94	—	732.3 ± 220.6
DHT 5 at 110°C Dry (11)	521.4 ± 149.1	22.43 ± 4.97	7431 ± 1657	1650.0 ± 356.0
Wet (12)	91.8 ± 31.4	11.87 ± 1.92	—	895.8 ± 205.9
DHT 5 at 140°C Dry (7)	247.3 ± 117.6	17.38 ± 8.27	6939 ± 2566	411.9 ± 84.1
Wet (5)	15.0 ± 5.5	15.69 ± 2.32	—	95.8 ± 40.6

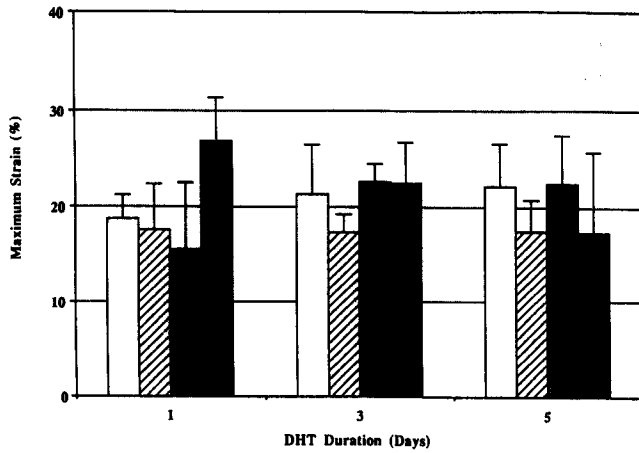


Figure 5 Maximum tensile strain for self-assembled dry collagen fibres DHT cross-linked for different durations. □, 60°C; ▨, 80°C; ■, 110°C; ■, 140°C.

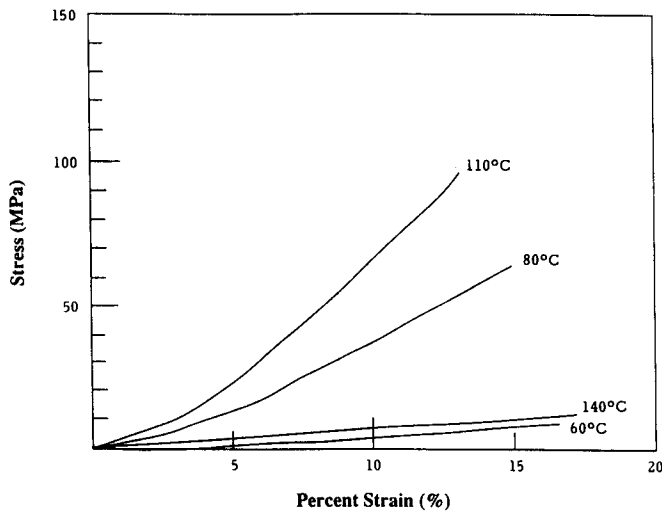


Figure 6 Tensile stress-strain curves as a function of wet DHT temperature of self-assembled dry collagen fibres cross-linked for 5 d.

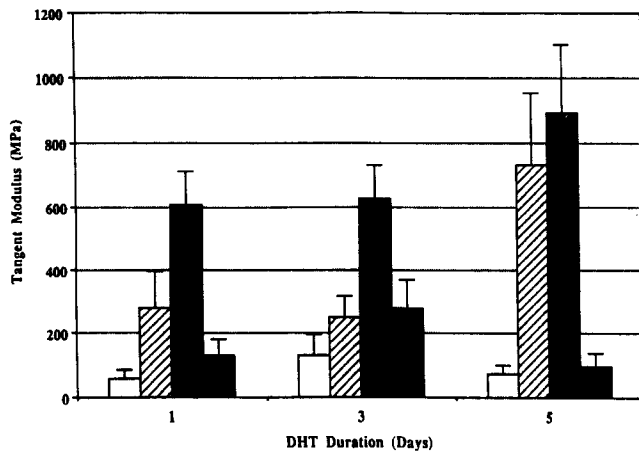


Figure 7 Tangent modulus versus DHT cross-linking duration for wet self-assembled collagen fibres. □, 60°C; ▨, 80°C; ■, 110°C; ■, 140°C.

Optimum tensile properties were observed for a cross-linking time of 5 d; at that time point the UTS was 91.8 MPa (wet) and 521 MPa (dry) with moduli of 7431 MPa (dry) and 896 MPa (wet). At temperatures below and above 110°C, DHT cross-linking is less effective in increasing the tensile mechanical properties. Below 110°C, the degree of cross-linking has been shown to be lower than at 110°C¹³, while above 110°C we hypothesize that some chain cleavage may occur.

The number and types of cross-links formed by DHT treatment remain unclear. It was first hypothesized that interchain amide bonds are formed by the interaction between free amino and carboxyl groups of amino acid side chains. This hypothesis was later supported by a study which demonstrated that the number of free amino and carboxyl groups decreased when DHT treatment was applied¹⁴. Possible reactive amino acid residues involved in DHT cross-linking include lysine, aspartic acid, glutamic acid^{7,16}, arginine, serine, threonine⁷ and alanine¹⁶. In rat tail tendon, the summation of these reactive residues is about one-third of the total residue amount¹⁷. The increase in wet tensile properties as a result of DHT cross-linking is likely to be attributed to interaction between charge and polar amino acid residues. DHT cross-linking may

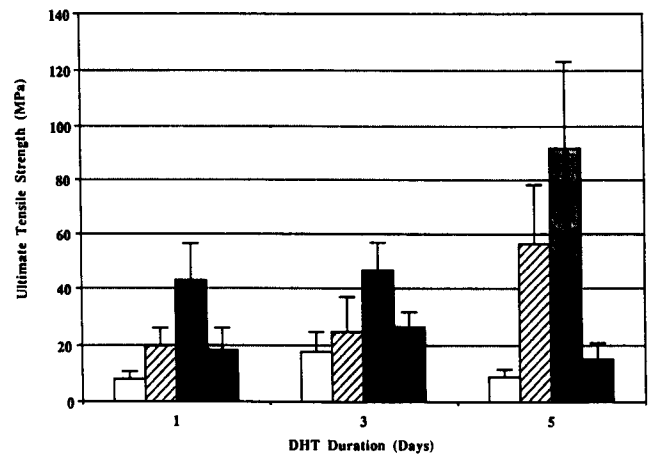


Figure 8 Ultimate tensile strength versus DHT cross-linking duration for wet self-assembled collagen fibres. □, 60°C; ▨, 80°C; ■, 110°C; ■, 140°C.

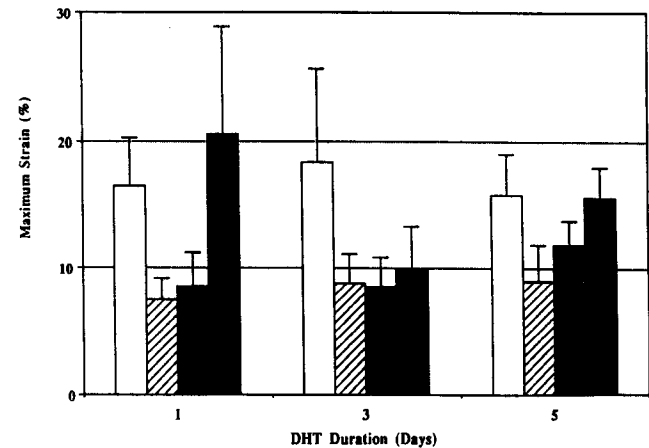


Figure 9 Maximum strain versus DHT cross-linking time for wet self-assembled collagen fibres. □, 60°C; ▨, 80°C; ■, 110°C; ■, 140°C.

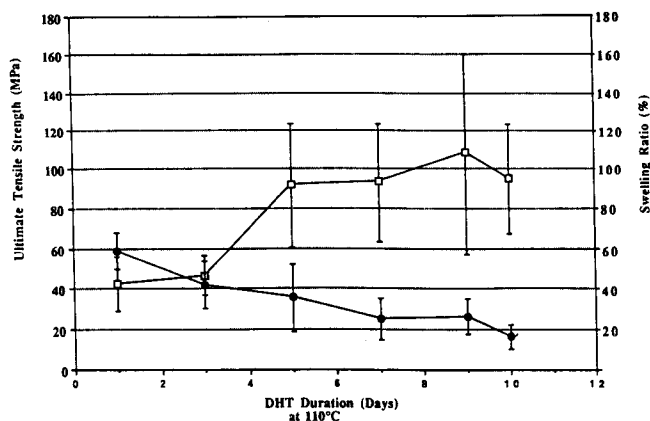


Figure 10 Ultimate tensile strength (MPa), □, and swelling ratio (%); ●, versus DHT cross-linking time for wet self-assembled collagen fibres.

improve the tensile strength of dry collagen fibres by limiting interfibrillar slippage; in the wet state it is likely that cross-linking also limits swelling of the fibres.

This study demonstrates that the UTS and tangent moduli of wet collagen fibres gradually increase with temperature until they reach their peak values at 110°C. Since it is known that the increase in fibre stiffness is the result of an increase in cross-link density^{7,18}, it may be reasonable to suggest that the cross-link density increases with cross-linking temperature until 110°C is reached. When cross-link density increases, the mobility of the polypeptide chains decreases, thus augmenting the mechanical stability of the collagen fibres. However, at 140°C, the collagen may have partially denatured, resulting in the poor mechanical properties observed. A recent study suggests that the cross-linking and denaturation effects may occur simultaneously¹⁶. Consequently, it is possible that the cross-linking effect predominates below 110°C and the denaturation effect predominates at 140°C.

The strength of reconstituted collagen fibres also increases with cross-linking time until reaching a plateau after 5 d at 110°C (Figure 8). This result suggests that after 5 d a reduction of the free amino and carboxyl groups has occurred, and there are fewer free active groups available to form cross-links¹³. The plateau in UTS after 5 d also suggests that the degree of denaturation is probably a result of the cross-link temperature and not the cross-link time. If prolonged treatment at 110°C induced denaturation, a drop in mechanical strength would be observed with time. In addition to the increase in UTS, there is a decrease in the swelling ratio of the collagen fibre with prolonged DHT treatment. As the equilibrium water content of the fibre is modified by the treatment, some of the water binding sites may be occupied or removed by the formation of cross-links, which may explain the augmented mechanical properties and reduced swelling ratio (Figure 10).

Of considerable interest is the high tensile strength and modulus observed, as well as the six-fold decrease in tensile strength going from the dry to the wet state. In the dry state, collagen fibres have moduli as high as

8000 MPa and tensile strengths up to about 600 MPa. The large decreases in these properties in the hydrated state suggest that water molecules act to break down hydrogen and electrostatic bonds that hold collagen fibrils together. In addition, the UTS of uncross-linked collagen fibres in the wet state is less than 2 MPa (data not shown), suggesting that hydrogen and electrostatic bonding between molecules plays a critical role in the load-bearing capacity of this material. The role of cross-linking appears to be that of minimizing the distances between neighbouring molecules, preventing the incorporation of excess water that would prevent hydrogen and electrostatic bond formation between molecules. It has been estimated that the molecular weight between cross-links is about 55 000 (two cross-links per collagen α -chain)¹⁴, which would hardly be enough to provide a direct resistance to failure by mechanical mechanisms. Therefore, it is likely that the high tensile strength of collagen is a direct reflection of the hydrogen and electrostatic bonding that occurs between the charged pairs that are able to form intermolecularly¹⁹.

Nestler *et al.*²⁰ measured the flexibility of the collagen triple helix in solution. Based on their determinations, a value of 4000 MPa for the molecular stiffness has been calculated². The value of dry collagen fibre stiffness reported in this study is about twice (8000 MPa) the molecular stiffness, while in the wet state the value is less than one-quarter (895 MPa) of the estimated value. This suggests that the flexibility of the collagen molecule is enhanced in the presence of water molecules and that water molecules probably allow rotational and translational freedom of segments of the triple helix. It is likely that, in the absence of water molecules, these water binding sites are available to bond intermolecularly to stiffen the collagen triple helix and prevent slippage and translation to occur between neighbouring molecules. This evidence further supports the hypothesis that intermolecular hydrogen and electrostatic bonding is crucial to force transmission within DHT cross-linked collagen.

In conclusion, we have demonstrated that the strength of reconstituted collagen can be improved by varying the time and temperature of DHT cross-linking. The optimum time for cross-linking appears to be 5 d at 110°C and probably results in improved UTS by preventing interfibrillar slippage and limiting the number of water molecules that inhibit formation of hydrogen and electrostatic bonds between collagen molecules.

REFERENCES

- 1 Prockop DJ. Mutations in collagen genes as a cause of connective tissue diseases. *N Engl J Med* 1992; **26**: 540–546.
- 2 Silver FH, Kato YP, Ohno M, Wasserman AJ. Analyses of mammalian connective tissue. Relationship between hierarchical structures and mechanical properties. *J Long-Term Eff Med Impl* 1992; **2**: 165–198.
- 3 Dunn MG, Silver FH. Viscoelastic Behavior of Human Connective Tissues: Relative Contribution of Viscous and Elastic Components. *Conn Tiss Res* 1983; **12**: 59–70.

- 4 Kato YP, Christiansen DL, Hahn RA, Shieh SJ, Goldstein JD, Silver FH. Mechanical properties of collagen fibres: a comparison of reconstituted rat tail tendon fibres. *Biomaterials* 1989; **10**: 38-42.
- 5 Williams BR, Gelman RA, Poppke DC, Piez KA. Collagen Fibril Formation. *J Biol Chem* 1978; **253**(18): 6578-6585.
- 6 Silver FH, Langley KH, Trelstad RL. Type I Collagen Fibrillogenesis: Initiation via Reversible Linear and Lateral Growth Steps. *Biopolymers* 1979; **18**: 2523-2535.
- 7 Weadock K, Olson RM, Silver FH. Evaluation of Collagen Crosslinking Techniques. *Biomater Med Dev Artif Organs* 1984; **11**(4): 293-318.
- 8 Goldstein JD, Tria AJ, Zawadsky JP, Kato YP, Christiansen D, Silver FH. Development of a Reconstituted Collagen Tendon Prosthesis. *J Bone Jt Surg* 1989; **71-A**(8): 1183-1191.
- 9 Kato YP, Dunn MG, Zawadsky JP, Tria AJ, Silver FH. Regeneration of Achilles Tendon with a Collagen Tendon Prosthesis. *J Bone Jt Surg* 1991; **73-A**(4): 561-574.
- 10 Rizvi AH, Wasserman AJ, Zazanis G, Silver FH. Evaluation of Peripheral Nerve Regeneration in the Presence of Longitudinally Aligned Collagen Fibers. *Cells Mater* 1991; **1**(4): 279-289.
- 11 Christiansen DL, Pins GD, Wang M-C, Dunn MG, Silver FH. Collagenous Biocomposites for the Repair of Soft Tissue Injury. *Mater Res Soc Symp Proc* 1992; **252**: 151-158.
- 12 Yannas IV, Tobolsky AV. Cross-linking of gelatine by dehydration. *Nature* 1967; **215**: 509-510.
- 13 Silver FH. GAG inhibition of collagen-platelet interaction. PhD thesis, MIT, 1977.
- 14 Silver FH, Yannas IV, Salzman EW. *In Vitro* Blood Compatibility of Glycosaminoglycan-Precipitated Collagens. *J Biomed Mater Res* 1979; **13**: 701-716.
- 15 Silver FH, Trelstad RL. Type I Collagen in Solution: Structure and Properties of Fibril Fragments. *J Biol Chem* 1990; **255**(19): 9427-9433.
- 16 Gorham SL, Light ND, Diamond AM et al. Effect of Chemical Modifications on the Susceptibility of Collagen to Proteolysis. II. Dehydrothermal Crosslinking. *Int J Biol Macromol* 1992; **14**: 129-138.
- 17 Vincent JFV. *Structural Biomaterials*, revised ed. Princeton, NJ: Princeton University Press, 1990: 108.
- 18 Rodriguez F. *Principles of Polymer Systems*. New York: McGraw-Hill, 1982: 221.
- 19 Silver FH. A molecular model for linear and lateral growth of type I collagen fibrils. *Collagen Rel Res* 1982; **2**: 219-229.
- 20 Nestler FH, Hvidt S, Ferry JD, Veis A. Flexibility of collagen determined from dilute solution viscoelastic measurements. *Biopolymers* 1983; **22**: 1747-1758.

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