Reconstituted Collagen Fibres for Tissue Engineering Applications.

A thesis submitted for the degree of Doctor of Philosophy (PhD).

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Disclaimer

The content of this dissertation describes the original work by the candidate, except where specific reference is made to the work of others. No part of this dissertation has been submitted for a higher degree to any other university.

Northampton, February 2006

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Abstract

In vitro, under appropriate conditions collagen molecules spontaneously form fibrils (fibrillogenesis) with the characteristic D-periodic banding similar to native fibrils. Recently, reconstituted collagen fibres have been of interest. Due to their unique advantageous properties, such as high surface area, softness, absorbency and ease of fabrication into many product forms, biomaterial based on reformed collagen fibres have been used as a substrate for nerve regeneration, tendon and ligament replacement, wound dressing applications and suture materials. Although some work has been done, still there is little understanding of the factors affecting the physical properties of such fibres. Therefore, the aim of the work presented in this thesis was to study and better understand the influence of factors which control the structure and physical properties of extruded reconstituted collagen fibres.

Acid and pepsin soluble collagen was extracted from different animal species (bovine Achilles tendon, rat tail tendon and pig tail tendon) and was extruded, using different internal diameter laboratory tubes into a series of neutral buffers, comprised of different co-agents such as sodium chloride, different molecular weight of polyethylene glycols and poly vinyl alcohols at 37^{0}C, where fibrillogenesis occurred. After 15 minutes incubation, the fibres were washed and air-dried under the tension of their own weight. In addition, an extensive study of the effects of a wide range of cross-linking techniques (chemical, physical, biological and natural) was undertaken. The structural, mechanical (dry and wet) and thermal properties of the reconstituted collagen fibres were evaluated using optical and scanning electron microscopy, tensile mechanical tests and differential scanning calorimetry respectively.

In general, it was found that the properties of the reformed collagen fibres were dependent on pre- (animal species, extraction method, collagen concentration) during- (fibre formation medium) and post- (cross-linking) fibre formation variables. A strong correlation between fibre diameter and stress at break was observed throughout this work; by increasing the diameter of the fibre either by increasing the collagen concentration, the tube internal diameter, the amount of the co-agent or via cross-linking, a decrease of the tensile strength at break was observed. The thermal properties of the matrices appeared to be dependent on the cross-linking technique utilized. Overall, it was demonstrated that reconstituted collagen fibres can be tailor made to suit a diversity of surgical needs with properties similar to or even superior to native tissues or other synthetic materials that are already in practice.

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Abbreviations

Abbreviation	Definition
AGEs	Advance Glycation End Products
APS	Ammonium Persulfate
AS	Acid Soluble
ASBAT	Acid Soluble Bovine Achilles Tendon
ASRTT	Acid Soluble Rat Tail Tendon
BAT	Bovine Achilles Tendon
BCS	Basic Chromium Sulphate
BDDGE	1,4-butanediol diglycidyl ether
C-terminal / group / propeptide	Carboxyl terminal / group / propeptide
Dehydro-DHLNL	Dehydro di-hydroxylysino-norleucine
Dehydro-HLNL	Dehydro hydroxylysino-norleucine
DHT	Dehydrothermal
DPPA	Diphenyl-phosphorylazide
DSC	Differential Scanning Calorimetry
DMF	N, N-Dimethyl Formamide
DW	Distilled Water
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
FACIT	Fibril Associated Collagens with Interrupted Triple-helices
FDA	Food and Drug Administration
FFB	Fibre Formation Buffer
FIB	Fibre Incubation Buffer
Gly	Glycine
Gly-X-Y	Glycine-Proline (often) - Hydroxyproline (sometimes)
GTA	Glutaraldehyde
HMDC	Hexamethylene-diisocyanate
Hyp	Hydroxyproline
MES Buffer	4-Morpholineethanesulfonic Acid Buffer
mRNA	Messenger Ribonucleic Acid
Mw	Molecular Weight
N-terminal / group / propeptide	Amino terminal / group / propeptide
NDGA	Nordi-hydroguaiaretic acid
NHS	N-hydroxysuccinimide
PBS	Phosphate Buffer Saline
PEG	Polyethylene Glycol
Pro	Proline
PS	Pepsin Soluble
PSBAT	Pepsin Soluble Bovine Achilles Tendon
PSPTT	Pepsin Soluble Pig Tail Tendon
PTT	Pig Tail Tendon
PVA	Poly Vinyl Alcohol
RH	Relative Humidity
RTT	Rat Tail Tendon
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
T_D	Denaturation Temperature
TEMED	N, N, N, N-tetra-methyl-ethylene-di-amine
Tg	Trans-glutaminase
T_S	Shrinkage Temperature
UV	Ultra Violet
Vol	Volume
VPSEM	Variable Pressure Scanning Electron Microscopy
Vs	Versus

Thesis Structure

- Chapter 1 provides a general background of collagen and collagenous materials. Furthermore, this chapter deals with the techniques that were used in the following chapters.

- Chapter 2 is concerned with the evaluation of different tissues and extraction methods for the production of reconstituted collagen fibres and investigation of their structural and physical properties.

- Chapter 3 is concerned with the optimisation of the established method of Chapter 2.

- Chapter 4 is concerned with the examination of the influence of various cross-linking methods on the physical characteristics of the reformed collagen-based fibres.

- Chapter 5 summarises the findings and the impact of this work.

- Chapter 6 provides recommendations for future work so that reconstituted collagen fibres will become an available material for tissue engineering applications.

- Chapter 7 lists all the references used in alphabetical order.

- Chapter 8 is the appendix, where materials and methods are provided.

- Chapter 9 provides the scientific contribution of this work up to the point of submission (February 2006).

Chapter 1. Literature Review

1.0. Preface: Tissue Engineering and Suture Materials

Tissue engineering, in a sense, began with the use of bioactive materials, that is, materials designed to interact with the body to encourage tissue repair. In many cases, the objective was to use materials that were as inert as possible, and therefore less harmful to the host. The basis of this idea springs from the natural ability of the living cells to regenerate. Currently, the principles of tissue engineering have been applied to virtually every organ system in the body. Considerable attention has been focused on orthopaedic and maxillofacial applications, including engineering of bone, cartilage, tendon, ligament and skin. Most tissue-engineered constructs are composed of at least two important components: a group of cells and a material scaffold on which they can grow. The scaffold material should be biocompatible and biodegradable, should provide mechanical stability of the construct in the short term and a template for the three-dimensional organisation for the developing tissue, should interact with extra-cellular matrix components, growth factors or cell surface receptors and available in desirable shapes (fibres, sponges, films, hydro-gels). A number of synthetic [poly(glycolic acid), poly(lactic acid)] and natural (collagen and inorganic hydroxyapatite) biodegradable polymers are currently in use as tissue scaffolds (Bonassar and Vacanti, 1998; Chen *et al.*, 2000; Senuma *et al.*, 2000; Whang *et al.*, 2000; Chen *et al.*, 2001; Hillmann *et al.*, 2002). The ideal cell-carrier substance should be the one, which most closely mimics the naturally occurring environment in the host tissue matrix (Suh and Matthew, 2000). The synthetic biodegradable polymers used for tissue engineering are easily formed into required shapes depending on the application, with good mechanical strength and manipulate-able degradation periods. Despite these advantages, the scaffolds derived from synthetic polymers lack cell-recognition signals, and their hydrophobic property hinders smooth cell seeding. In contrast, naturally derived collagen has the potential advantages of specific cell interactions and hydrophilicity (Chen *et al.*, 2000; Chen *et al.*, 2001).

One of the first tissue engineering applications was the utilisation of sutures. First description of sutures dates back as far 2000BC. Written references have been found describing the use of strings and animal sinews for suturing. Through the centuries, a wide variety of materials like silk, linen, cotton, horsehair, collagen, and intestines, as well as precious metals have been used for operative procedures. Advances in operative techniques and evolution of suturing material have changed suture designs to become more specific to a surgical procedure or to tissue characteristics (Huber *et*

al., 2003; Blaker *et al.*, 2004). In the selection of an appropriate suture for a given situation the main characteristics that should be considered are tensile strength, strength retention, flexibility, ease of handling, knot security, diameter and tissue reactivity (Goupil, 1996; Muftuoglu *et al.*, 2004). A number of different natural and synthetic surgical suture materials are available, including absorbable and non-absorbable types and have provided satisfactory performance for many years in surgical practice (Makela *et al.*, 2002; Wada *et al.*, 2002; Huber *et al.*, 2003). Based on recent studies, Vicryl and Polydioxanone appeared to be ideal suture materials for short- and medium-term apposition respectively. Panacryl appeared to have more durable mechanical features and may well be suited to long-term tissue apposition, such as tendon repair or arthroplasty (Field and Stanley, 2004; Singh *et al.*, 2004). Polytetrafluoroethylene and Dacron vascular grafts continue to be utilized in order to bypass a segment of diseased artery (Chandy *et al.*, 2000; Li *et al.*, 2001; Phaneuf *et al.*, 2001). Tailor made reconstituted collagen fibres have been utilised for soft and hard tissue replacement with properties similar to the native tissue, avoiding problems associated with long-term foreign body implantation, such as excessive fibrosis and stitch granuloma, that could interfere with the smooth gliding of tendons repairs (Goldstein *et al.*, 1989; Kato *et al.*, 1989; Kato and Silver, 1990; Cavallaro *et al.*, 1994; Wang *et al.*, 1994; Pins *et al.*, 1997b). While significant strides have been made, many challenges still exist in the biology and engineering that must be addressed to bring these technologies to clinical practice.

1.1. Introduction / Definition

Collagen derives its name from two Greek words: "kolla" meaning "glue" and "gonno" meaning "to produce". It was first recognised as the component of tissue that when boiled produced glue and was known to the Romans as early as 50AD (Gorham, 1991). The word "collagen" was coined in the 19th century to designate "the constituent of connective tissue, which yields gelatine on boiling". Some aspects of collagen chemistry were obviously known for a much longer time for uses like the production of animal glue from hides and bones or the tanning of leather. The more modern view of collagen is a "substance" composed of individual constituent molecules assembled into cross-linked fibrils that give connective tissues their resistance to tension (van der Rest *et al.*, 1993).

Collagen is the major fibrillar component of the extra-cellular matrix and has a dominant position in the molecular structure of higher animals. It has been characterised as the "biological glue" that holds cells in place (Parenteau, 1999), and provides excellent support for cell adhesion and growth (Matsumoto *et al.*, 1999). It is found in all animal phyla except arthropods and certain classes of coelenterate and its amount varies greatly between species and organs. It constitutes about 20-30% of the total body protein and among skin proteins it is quantitatively first, accounting for 60-80% of its dry matter (Eastoe *et al.*, 1963; Pikkarainen, 1968; Bienkiewicz, 1983; Schoof *et al.*, 2001). Collagen is considered to be the main load-bearing structure of connective tissues such as skin, bone and dentin, blood vessels, cartilage, tendons and ligaments and, as such, performs many mechanical functions in the body, most of which require a tough, durable material and demonstrates biological properties such as the activation of the blood-clotting cascade (Smith and McEwan, 1990; Gorham, 1991; Rault *et al.*, 1996; Holmes *et al.*, 1998; Schoof *et al.*, 2001; Driessen *et al.*, 2003).

1.2. Collagen Diversity / Collagen Types

The name "collagen" is often used as a generic term to cover a wide range of protein molecules that all share the same characteristic triple helical configuration as a major structural motif (Ramshaw *et al.*, 1995). The soluble pre-cursors of collagen, the pro-collagens, are described as either type () pro-collagen or pro-type () collagen, where the parentheses contain Roman numerals specifying the type of the collagen. All collagens are composed of polypeptide subunits called α-chains. Three α-chains make up each molecule (γ-component) and the presence of intra-molecular covalent cross-links leads to the presence of dimers (β-components) in denaturated collagen. Type I collagen is a hetero-polymer containing two α1(I) chains and one α2(I) chain. The Roman numeral in parentheses denotes the type of collagen from which the chain originates. Types II and III are homo-polymers made up of three α1(II) and three α1(III) chains respectively (Light, 1985).

To date, 40 vertebrate collagen genes have been described, the products of which combine to form 27 distinct homo- and/or hetero– trimeric molecules (van der Rest *et al.*, 1993; Pace *et al.*, 2003; Jenkins *et al.*, 2005). Their common feature being the presence of at least one tri-peptide helical sequence of repeated $(Gly–X–Y)_{100\text{-}400}$, where X is often proline (Pro) and Y sometimes hydroxyproline (Hyp) (Rehn and Pihlajaniemi, 1996; Elamaa *et al.*, 2003; Ylikarppa *et al.*, 2003; Vaananen *et al.*, 2004). These distinct collagens have been reported differentiated by minor alterations in the properties of the collagen molecule which include changes in the length of the triple helix, the Mw, the charge profile along the helix, interruptions in the integrity of the triple helix, the size and shape of the terminal globular domains, the cleavage or retention of the latter in the supramolecular aggregate, and variation in the post-translation modifications (Bailey, 1992; Bailey and Paul, 1998).

The identification of these aggregates and their functional determination has at last allowed the collagen chemist to begin to account for the biological diversity of collagenous tissues. Classifications have been based on the genetic structure, the molecular weight, the length of the triple helix or on the fact that non-helical domains interrupt the triple helix. As more collagens are being characterised, the most useful approach is probably to categorise on the basis of the aggregated structure. This allows classification of the twenty-seven types identified to date (Pace *et al.*, 2003; Jenkins *et al.*, 2005) into 4 main groups as has been described extensively (Bailey, 1992; Hulmes, 1992; Kielty *et al.*, 1993; van der Rest *et al.*, 1993; Ramshaw *et al.*, 1995; Bailey and Paul, 1998; Bailey *et al.*, 1998; Hulmes, 2002; Kielty and Grant, 2002):

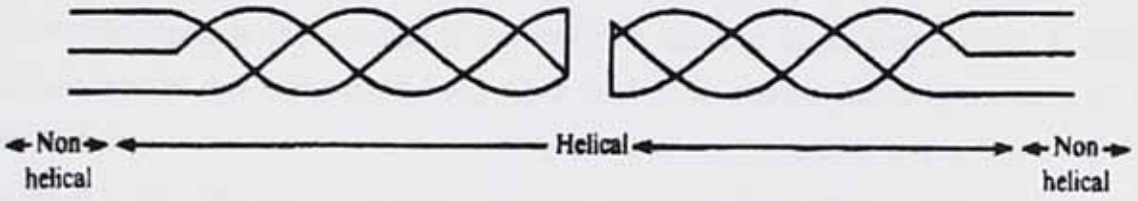
- Fibrous collagens, e.g. types I, II, III and (V and XI). The subfamily of collagens forming quarter-staggered fibrils. The nine constituent chains all share the same general features: contain a main triple-helical domain of approximately 1000 amino acids per chain; their COOH-terminal propeptides are highly homologous between themselves; and their modes of aggregation also appear to be very similar.
- Non-fibrous collagens, e.g. types IV, VIII and X. They are referred as non-fibrillar collagens, because of their inabilities to form quarter-staggered fibrils by themselves. Some of them are nevertheless able to form other types of fibrils or to participate in the quarter-staggered aggregates as essential constituents.
- Filamentous collagens, e.g. types VI and VII. Type VI collagen is characterised by the presence of very large multi-domain amino and carboxyl terminal non-triple-helical regions and assembles into beaded filaments that are formed by a complex aggregation process. Type VII collagen is characterised by a very long triple-helical region and by a large N-terminal domain that appears as a three-finger structure by rotary shadowing electron microscopy. This collagen aggregates into structures known as anchoring fibrils, which contribute to the cohesiveness of the junction between epithelial basement membranes and the underlying stroma.
- Fibril associated collagens with interrupted triple-helices (FACIT) (Shaw and Olsen, 1991), e.g. types IX, XII, XIV, XV and XVIII. These collagens are closely associated with the quarter-staggered fibrils and are characterised by the presence of a short triple-helical domain located near the carboxyl end of the molecule. This domain is homologous in this group of collagen types and may play a role in the interaction with the fibrils (Rehn *et al.*, 1996; Rehn and Pihlajaniemi, 1996; Elamaa *et al.*, 2003; Pace *et al.*, 2003; Jenkins *et al.*, 2005).

Although this quick survey of the collagen family shows how diverse these molecules are, still the presence of the triple-helical domains in their structures and the fact that they are constituents of the architecture of the extra-cellular matrix, justify grouping them into a unique protein family (van der Rest *et al.*, 1993). Of the range of collagen types, type I collagen, which is the predominant component of most collagenous tissues, is the most studied, and therefore much of the following details refer to this type of collagen.

1.3. Collagen Structure / Conformation

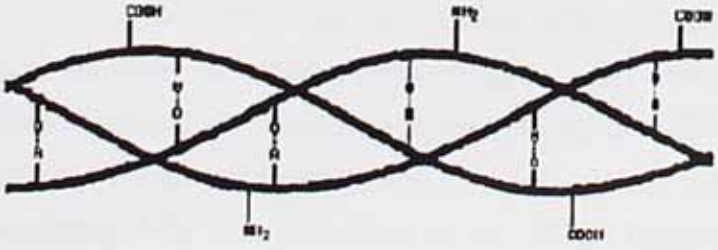
The triple helical conformation is the defining structural element of all collagens, which has been deduced from high angle X-ray fibre diffraction studies on the collagen in tendon (Ramshaw *et al.*, 1995). The collagen triple helix (tertiary structure) has a coiled-coil structure made of three parallel polypeptide chains (Figure 1).

Figure 1. The stiff triple-helical collagen structure. Source: (Gorham, 1991).



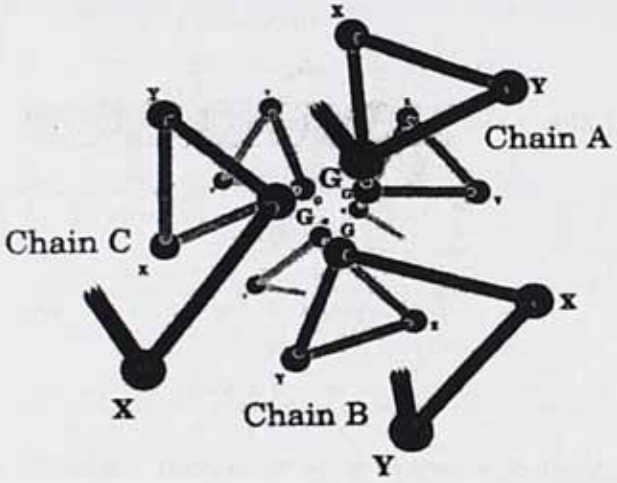
Three collagen polypeptides, or α-chains (secondary structure), are wound around each other in a regular helix to generate a rope-like structure approximately 300,000g/mol molecular weight and 280nm in length and 1.4nm in diameter. The stability factor of the helix is considered to be intra-molecular hydrogen bonds between glycines in adjacent chains. The hydroxyl groups of hydroxyproline residues are involved in hydrogen bonding and are important for stabilising the triple helix structure and two hydrogen bonds per triplet are found. The two hydrogen bonds formed are: one between the NH-group of a glycyl residue with the COO-group of the residue in the second position of the triplet in the adjacent chain and one via the water molecule participating in the formation of additional hydrogen bonds with the help of the hydroxyl group of hydroxyproline in the third position (Figure 2) (Miles *et al.*, 1995; Sasaki and Odajima, 1996b; Bailey and Paul, 1998; Paul and Bailey, 2003).

Figure 2. Hydrogen bonds within the triple helix. Source: (Paul and Bailey, 2003).



Each α-chain is a left-handed helix and the three chains are staggered by one residue relative to each other and are super-coiled around a central axis and form a right-handed super-helix (Figure 3) (van der Rest *et al.*, 1993; Wess *et al.*, 1998a; Melacini *et al.*, 2000; Holmes *et al.*, 2001a; Madhan *et al.*, 2001). Cotterill et al. (Cotterill *et al.*, 1993) were the first to observe the left-handed arrangements between filaments within collagen I fibrils, using tunnelling scanning microscopy.

Figure 3. Cross-section of a collagen triple helix. The α carbons of the amino-acyl residues are represented by balls and the peptide bonds by sticks. Note that only one glycine can occupy the centre of the helix. It can be seen that all the X and Y residues are at the surface and will have their side chains pointing outward of the helix. Each pro-peptide forms a left handed helix and assemble with the other two chains in a right handed super-helix. Source: (van der Rest *et al.*, 1993).



This super-helix is due to the twisting of the chain helices around the central axis by about $+30^0$ at every turn. Every third amino acid is thus in the centre of the helix and, for steric reasons, only glycine, with a side chain limited to a single hydrogen atom, can occupy this position without altering the triple-helical conformation. The presence of another amino acid in the glycine position or the presence of imperfections in this repetitive structure seriously alters the stability or the conformation of the helix. Hence the primary structure of a collagen triple helix can be written as $(Gly\text{-}X\text{-}Y)_n$, where a large proportion of X-residues are proline and a large proportion of Y-residues are hydroxyproline. In collagen, sequences of Gly-Pro-Hyp are the most common tri-peptides (about 12%), while sequences of the form Gly-Pro-Y and Gly-X-Hyp represent about 44% of tri-peptides and Gly-X-Y triplets with no imino acids constitute the remaining 44% (van der Rest *et al.*, 1993; Ramshaw *et al.*, 1995). Each collagen I polypeptide chain contains a helical domain with 338 repetitions of

the sequence (Gly-X-Y) (Roveri *et al.*, 2003). Proline and hydroxyproline (Figure 4) stabilise the collagen molecule. Also, because of their alicyclic nature, they stiffen the α-chain where they occur by preventing rotation around the C-N bond (Figure 5), again conferring unique properties on the molecule (Gorham, 1991).

Figure 4. Structures of (a) proline and (b) 4-hydroxyproline. Source: (Gorham, 1991).

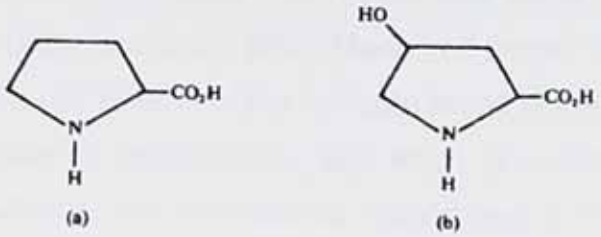
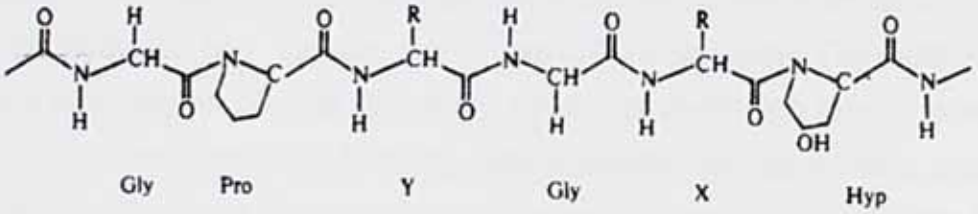


Figure 5. Incorporation of proline and hydroxyproline into collagen α-chains. Source: (Gorham, 1991).



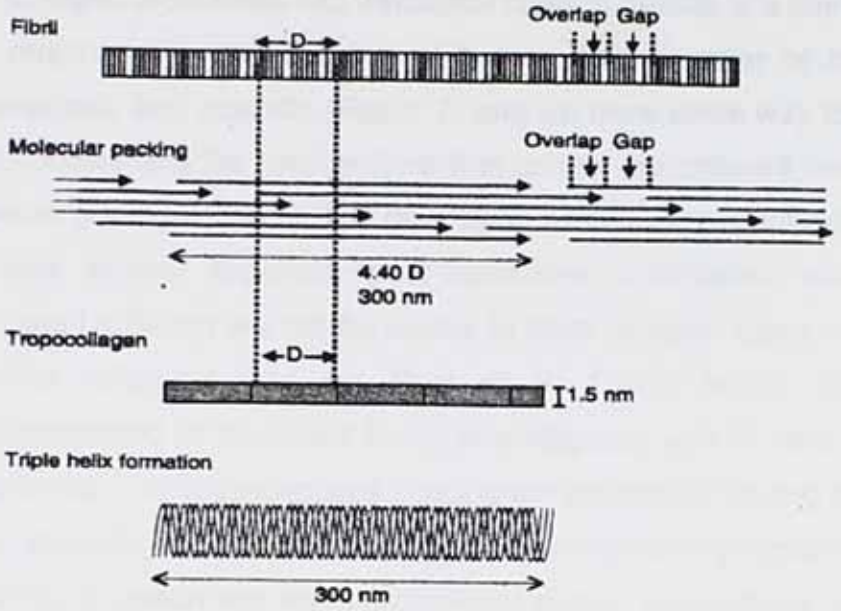
At each end of the collagen molecule is a short non-helical region known as telopeptide. These telopeptide domains are crucial for the fibril formation (Wess *et al.*, 1998b; Holmes *et al.*, 2001b), are involved in the collagen cross-linking process (Woodley *et al.*, 1991) and are responsible for the inflammatory processes (Roveri *et al.*, 2003). The simple triple-helical collagen molecule was originally known as tropocollagen. Fibroblasts synthesise the monomeric collagen unit tropocollagen and secrete it into the extra-cellular space where the tropocollagen molecules can aggregate to form long fibrils that are further stabilised by inter- and intra-molecular cross-links. Fibrils can associate to form fibres, which in turn can form much larger fibre bundles. The fibres and fibre bundles may also be stabilised by inter-molecular cross-links. Hence, through specific aggregation and cross-linking, types I, II and III collagen can form fibres of high strength and stability (Pikkarainen, 1968; Bienkiewicz, 1983; Gorham, 1991; Fratzl *et al.*, 1997).

1.4. Collagen Type I

Collagen fibrils are the primary structural elements of all connective tissues, providing a structural scaffold for other components. Furthermore, they are associated with cell interaction, migration, attachment, differentiation and organisation (Hung *et al.*, 2004). Most of the research into the assembly and molecular organisation of these fibrils has concerned type I collagen, which is abundant in skin, bone, cornea, dentin, ligament and tendon (Birk and Lande, 1981; Pins and Silver, 1995; Holmes *et al.*, 1998; Hofman *et al.*, 1999; Bos *et al.*, 2001; Meek and Boote, 2004). Although type I collagen accounts for up to 70-90% of the collagen found in the body and is normally encountered in biomaterial applications, still when it comes to evaluating the performance of biomaterials, and the potential replacement of the collagenous device by new functional tissue, then the distribution and roles of other less abundant collagen types can be important (Ramshaw *et al.*, 1995; Kielty and Grant, 2002).

Collagen type I is present in the form of elongated fibres (termed fibrils) in tissue such as skin, bone and tendon. Individual fibrils can be greater than 500μm in length, 500nm in diameter and contain more than 10^7 molecules. The collagen fibrils possess a high degree of axial alignment, which results in high tensile strength and they exhibit a characteristic D banding, which results from alternating overlap and gap zones, produced by the specific packing arrangement of the 300nm long and 1.5nm wide collagen molecules (molecular weight 300,000) on the surface of the fibril. This produces an average periodicity of 67nm in the native hydrated state (Figure 6) (Birk *et al.*, 1990; Rosenblatt *et al.*, 1994; Parkinson *et al.*, 1997; El Feninat *et al.*, 1998), although dehydration and shrinkage during conventional sample preparation for electron microscopy results in lower values of around 55 to 65nm (Chapman *et al.*, 1990; Kielty and Grant, 2002).

Figure 6. Schematic representation of the arrangement of collagen molecules within fibrils. Source: (Kielty and Grant, 2002).



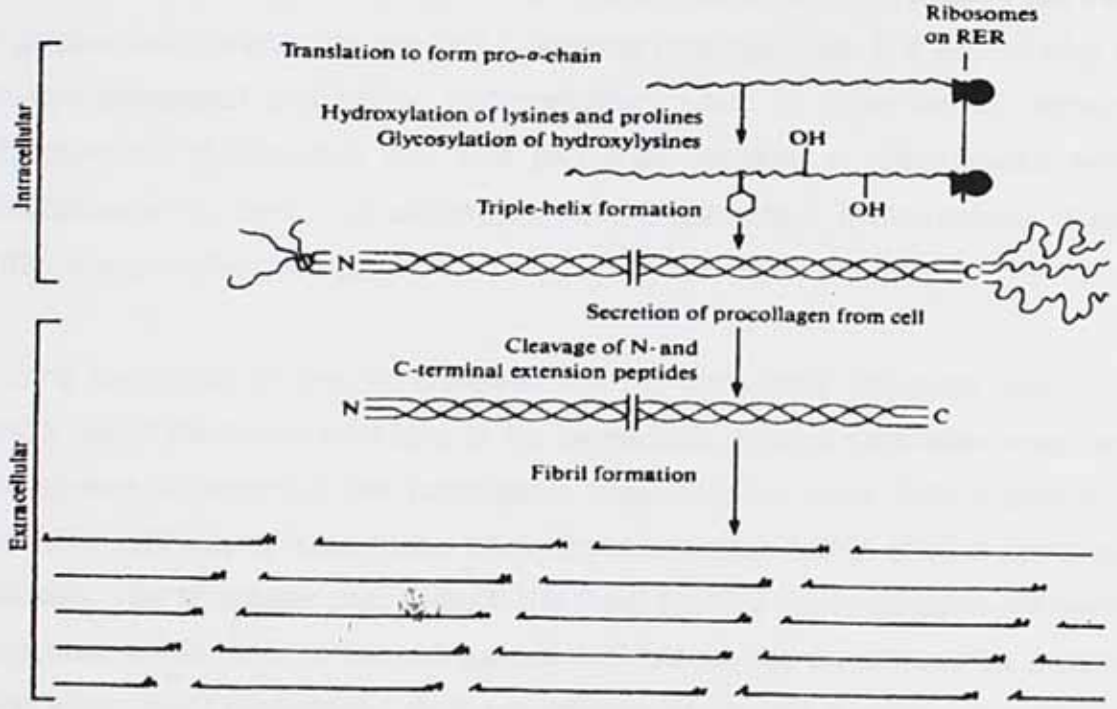
Each molecule is a co-polymer (hetero-polymer) of two $\alpha 1$ chains and one $\alpha 2$ chain. Type I collagen contains one-third glycine, is composed of a triple helix of approximately 1050 amino acids in length, has short non-triple helical sequences at either end of the triple helix, contains no tryptophan, very low tyrosine and histidine and no cysteine. Collagen type III, unlike type I, is a monomer (homo-polymer) composed of three identical $\alpha 1$ chains and it contains cysteine (involved in disulfide bonds) (Bailey and Light, 1989; Kielty and Grant, 2002).

The evolution of the simpler regular viruses to the multi-cellular animals depends on the ordered self-assembly of macromolecules into spatially extended structures that form the basis of an extra-cellular matrix (Holmes *et al.*, 1998; Graham *et al.*, 2000). Self-assembly implies that no external direction is required and that the information, which determines the final structure is built into the building block itself. A similar situation might be expected to apply in the case of collagen as well (Chapman, 1965). Individual collagen molecules will spontaneously self-assemble into ordered fibrillar structures. This process can occur *in vivo* as well as *in vitro* and previous studies have shown the similarity between the structure and mechanical properties of native and self-assembled fibres (Pins *et al.*, 1997b). Thus, the amino acid sequence of the collagen defines all the information to enable collagen to form into its native, fibrillar structure (Ramshaw *et al.*, 1995).

1.4.1. Biosynthesis / *In Vivo* Self Assembly

The pathway of collagen synthesis from gene transcription to secretion and aggregation of collagen monomers into functional fibrils in tissues is a complex multi-step process, requiring the co-ordinating of a very large number of biochemical events, both temporally and spatially (Figure 7) and we have some way to go before we can claim to understand the mechanisms that control the different levels of self-assembly in tissues (Gorham, 1991). The discussion here relates specifically to type I collagen, however several important post-translation modification steps in the biosynthesis of type I collagen are not applicable to other collagen types, in particular to the non-fibrillar collagens (van der Rest *et al.*, 1993). Briefly, fibril-forming collagens are synthesized in precursor form, procollagens, with N- and C-terminal propeptide extensions. The C-propeptides direct chain association during intracellular assembly of the procollagen molecule from its three constituent polypeptide chains. Following or during secretion into the extra-cellular matrix, propeptides are cleaved by specific procollagen proteinases, thereby triggering fibril formation (Ramshaw *et al.*, 1995; Holmes *et al.*, 2001b; Canty and Kadler, 2002; Hulmes, 2002).

Figure 7. Biosynthesis and processing of pro-collagen chains. Source: (Gorham, 1991).



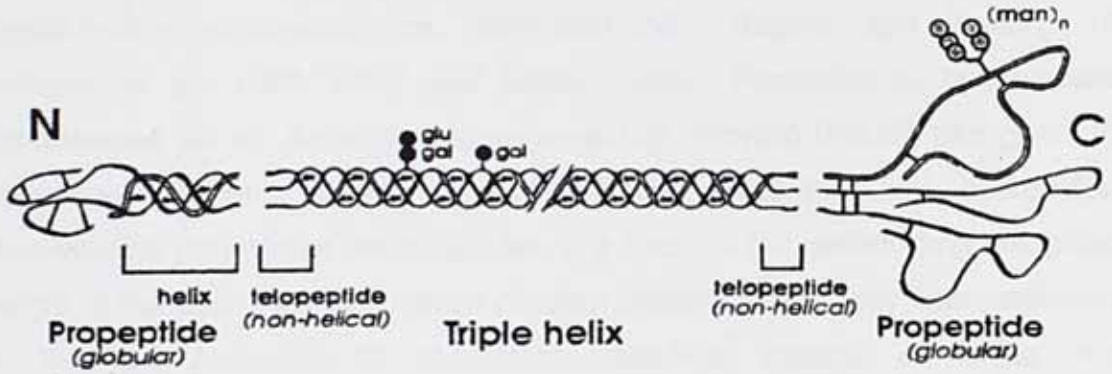
The regulation of fibril formation also involves a series of cellular and extra-cellular steps. The fibroblast is responsible for the synthesis of different collagen types; post-translational modifications of procollagen molecules such as glycosylation; synthesis of the procollagen proteinases; the synthesis of other matrix macromolecules and the organisation of collagen fibrils into bundles with tissue specific organisation. In addition, the fibroblasts influence extra-cellular events through the spatial and temporal control of secretion of the different macromolecular components (Lapiere *et al.*, 1977; Birk and Lande, 1981; Birk and Silver, 1984; Fleischmajer *et al.*, 1990; Ploetz *et al.*, 1991).

Before secretion from the cell, collagen is synthesised (mainly in fibroblasts) in a precursor form known as pro-collagen, which contains both amino- and carboxyl-terminal extension peptides. During translation of the corresponding mRNA to form the pro-α-chains, three important events take place in the rough endoplasmic reticulum. First, many of the proline residues are hydroxylated by the enzyme 4-prolylhydroxylase to form 4-hydroxyproline. Second, some of the lysine residues are hydroxylated by lysyl hydroxylases, with only a few of those at the Y position of the (Gly-X-Y)-sequence becoming so. Third, some hydroxylated lysine residues are glycosylated in a process involving two glycosyl-transferase enzymes. The first enzyme transfers a galactose residue to hydroxylysine, while the second can transfer a glucose residue onto the resulting galactosyl-hydroxylysine. The second step is not always completed and hence hydroxylysine occurs in three forms, namely un-glycosylated, glycosylated with one galactose residue, or glycosylated with the disaccharide to form 1-O-α-D-glycosyl-O-β-D-galactosyl hydroxylysine (Gorham, 1991; Canty and Kadler, 2002).

During translation of the pro-α-chains, mRNA, triple helix formation occurs. Inter-chain disulphide bonds that form in the propeptide regions have been implicated in the correct alignment of the polypeptide chains before triple helix formation. The secretion pathway of triple helical procollagen is similar to that of other extra-cellular proteins. The molecules pass through the Golgi and are then packaged into secretory vacuoles, which move to the cell surface and release the procollagen by exocytosis. The initial type I procollagen molecule after folding is still a precursor in that it has additional globular domains at each end of the molecule, the N- and C-propeptides (Figure 8). Pro-collagen aggregates have been observed within the secretory vacuoles suggesting that pro-collagen is probably secreted in an aggregated form

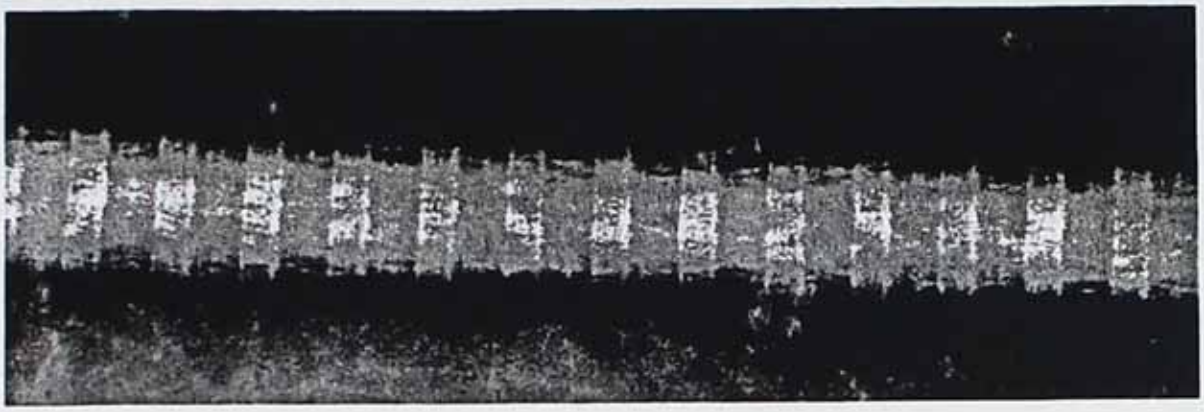
and not as monomeric molecules. Following (or possibly) during secretion the N- and C-propeptides are cleaved by specific endo-peptidases, a procollagen N-proteinase and the procollagen C-proteinase. These cleavage reactions leave short, non-triple-helical domains, called telopeptides, at each end of the collagen molecule. The telopeptides play an essential role in the assembly and maturation of the collagen matrix by inter-molecular cross-link formation (Grassmann, 1965; Gorham, 1991; Ramshaw *et al.*, 1995). Loss of diameter uniformity, induction of anti-parallel packing and changes in the fibril assembly pathway have been observed depending on the extent of loss of the N- and C-telopeptides. Even with preservation of the telopeptides using protease inhibitors during extraction/purification, alternative pathways for fibril assembly have been observed, using type I collagen (Holmes *et al.*, 1996; Holmes *et al.*, 2001b).

Figure 8. The structure of type I collagen and the relationship of the triple helix to the telopeptides and the pro-peptide domains. Source: (Ramshaw *et al.*, 1995).



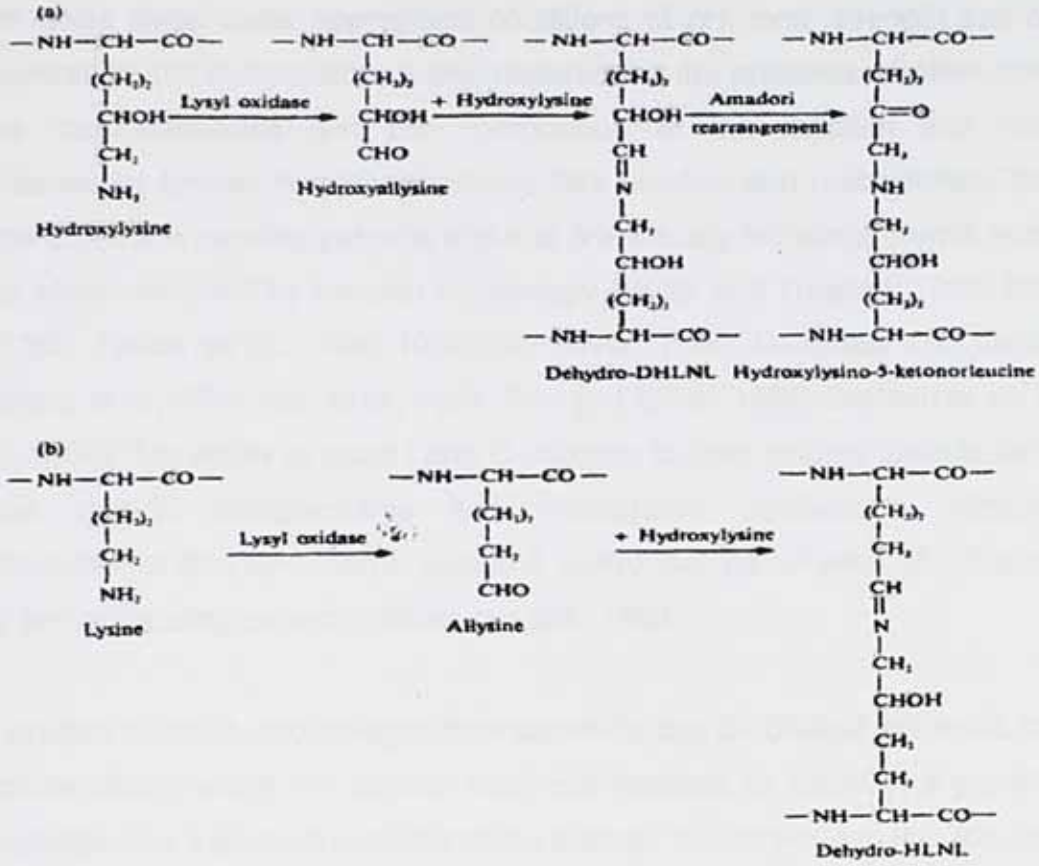
Individual collagen molecules then aggregate spontaneously in quarter-staggered arrangement in head-to-tail parallel bundles to form fibrils. The heads of the collagen molecules are staggered along the length of the fibres, accounting for the 64nm spacing of the cross-striations in the regular banding pattern seen under the transmission electron microscope (Figure 9) (Birk *et al.*, 1990; Orgel *et al.*, 2001). Reconstituted collagen fibrils prepared from acid solutions of purified collagen by warming the collagen solution (from 4^0C to 34^0C) and then neutralizing it (from pH 3.4 to pH 7.4) with a phosphate buffer gave more clearly defined staining patterns than those from native fibrils extracted directly from tissues (Chapman *et al.*, 1990).

Figure 9. An electron micrograph of a collagen fibril from bovine cornea. Source: (Holmes *et al.*, 2001b).



After fibril formation, cross-linking occurs (Figure 10) (Gorham, 1991; Bailey *et al.*, 1998). First hydroxylysines or lysines are oxidatively de-aminated by the enzyme lysyl oxidase to form hydroxyallysine or allysine respectively (Canty and Kadler, 2002). The resulting aldehydic side chains can then react with another hydroxylysine residue to form either dehydro-di-hydroxylysino-norleucine (dehydro-DHLNL) or dehydro-hydroxylysino-norleucine (dehydro-HLNL) (Bailey and Peach, 1968; Heathcote *et al.*, 1980; Knott and Bailey, 1998). Formation of hydroxylisino-5-ketonorleucine via an Amadori rearrangement of dehydro-DHLNL can give further stabilisation (Gorham, 1991). The keto-imine cross-link and hydroxylysino-ketonorleucine polymerise the molecules in a head to tail fashion and this provides strength to the fibre. However, these divalent cross-links are only intermediates and with time are converted to multivalent cross-links capable of linking several molecules. For such a reaction to occur the molecules would have to be in register. It has been therefore proposed that cross-linking takes place in two stages. Firstly longitudinal cross-linking of the end-overlapped molecules, and secondly by interaction of these cross-links between two micro-fibrils in register (Danielsen, 1981; Bailey, 1992; Bailey and Paul, 1998).

Figure 10. Formation of the naturally occurring reducible cross-links for (a) hydroxylysine and (b) lysine. Source: (Gorham, 1991).



1.4.2. *In Vitro* Self Assembly

The *in vitro* fibrillogenesis of type I collagen is a temperature dependent process, which takes place under appropriate conditions of pH, ionic strength and collagen concentration and composition. It also depends on the presence of other connective tissue macromolecules and the composition of the medium and results in spontaneously formed microscopic fibrils, fibril bundles and macroscopic fibres that exhibit D periodic banding patterns and that are virtually indistinguishable from native fibres when examined by electron microscopy (Silver and Trelstad, 1980; Brokaw *et al.*, 1985; Farber *et al.*, 1986; Kato and Silver, 1990; Delorenzi and Gatti, 1993; Cavallaro *et al.*, 1994; Hsu *et al.*, 1994; Pins and Silver, 1995; Graham *et al.*, 2000; Li *et al.*, 2004). The ability of types I and III collagen to form striated fibrils is believed to involve specific charge-charge and hydrophobic interactions. Although the mechanisms for fibril formation *in vitro* and *in vivo* may be different, the final products have similar banding patterns (Silver and Birk, 1984).

The kinetics of the *in vitro* collagen fibril assembly can be divided into three stages: a lag phase during which the solution does not increase its turbidity; a growth period characterised by a sigmoid increase of the solution turbidity due to the appearance of collagen fibrils; and a plateau phase in which turbidity again remains constant. It has been shown that the first-formed aggregates are dimeric, with the most prevalent dimer having a maximal stagger (D = 67nm) between constituent molecules (Ward *et al.*, 1986). It has been shown that (a) fibres were present at the end of the lag phase, (b) the growth phase represented increased fibre density and not additional fibre diameter growth and (c) fibre growth occurred rapidly after it was initiated (Brokaw *et al.*, 1985). The self-assembly of pepsin-extracted types I, II, and III collagen was studied *in vitro* using a turbidity-time assay and it was found that the rates and extent of collagen self-assembly were related to collagen type; types II and III collagen have higher rate constants for the lag and growth phases than does type I collagen, suggesting that the interactions that drive linear and lateral assembly of fibrils and fibril bundles form more rapidly with types II and III collagen. The final diameters of fibrils formed from types II and III collagen are consistently smaller, as indicated by the significantly lower intrinsic turbidity values for these types, than for type I collagen. These results also suggest that differences in the collagen fibril diameters are not due entirely to interactions involving intact telopeptides, and may involve helix-helix interactions (Birk and Silver, 1984; Farber *et al.*, 1986).

It is believed that type I collagen contains all the structural information that is necessary for its self-assembly into fibres, except maybe for some tissue-specific factors. However, the location of the "coding" regions, the nature of this information and how it is "translated" into intermolecular forces responsible for fibrillogenesis are still poorly understood (Bailey and Light, 1989; McBride *et al.*, 1997; Kuznetsova and Leikin, 1999; Graham *et al.*, 2000; Li *et al.*, 2004). The molecule contains three structural domains, the amino- and the carboxyl-terminal extra helical regions (the telopeptides) and the major triple helical rod-like domain. Comparison of the behaviour of protease-modified collagen and native collagen suggests that both the N-terminal and the distal region of the C-terminal telopeptides are important in the initial nucleation, while the C-terminal proximal moiety plays a relevant role in the growth phase (Helseth and Veis, 1981). Collagen fibril formation is an endothermic process involving hydrophobic and electrostatic interactions between adjacent molecules, with accompanying release of associated structured water.

The fibril formation process is promoted by agents that increase the disorder of the bulk water, such as solution temperature increments or the addition of ions that "break" water structure. By contrast, fibril formation is retarded by factors that increase the order of the bulk water, such as solution temperature decreases or addition of ions that "make" water structure. It is known that fibril formation is inhibited by molecules with long hydrocarbon chains (Delorenzi and Gatti, 1993). Similarly, it has been shown for purified chick procollagen that intact propeptide domains hinder, but do not prevent, the formation of D-periodic assemblies. The presence of the propeptide domains on the surface of a growing assembly could restrict its lateral growth and limit its final thickness (Mould *et al.*, 1990). Furthermore, it has been shown that the propeptides limit association of collagen molecules in solution and may therefore play an important role in modulating collagen fibrillogenesis until they are proteolytically removed (Berg *et al.*, 1986).

1.4.3. Mechanical Characteristics

Tensile testing has been performed to analyse the biomechanical properties of the collagen matrices. Tendons have both viscoelastic and plastic properties which are characteristics that are essential in transmitting muscle contraction-induced tensile strains into movements of the extremities while simultaneously maintaining the structural integrity of the tendon. The primary mechanical strength of individual collagen molecules is dependent upon the extra-cellular formation of the triple helix molecule that self-assembles into a collagen fibril with stabilising intra- and inter-molecular cross-links between the adjacent helical molecules (Jarvinen *et al.*, 2004).

The collagen network is primarily responsible for the mechanical properties of collagenous tissues. The fibrils and the fibril bundles are the major source of the mechanical strength of skin, tendon, cornea and blood vessels, as well as other tissues that are exposed to repeated tensile forces (Damink *et al.*, 1995; Holmes *et al.*, 1998; Osborne *et al.*, 1998; Canty and Kadler, 2002; Boote *et al.*, 2005). Other non-collagenous components are, however, believed to play important roles either through their unique viscoelastic properties, e.g. elastin, or via their interaction with collagen fibres, e.g. proteoglycans, and allow the tissue to withstand compressive and tensile forces (Hsu *et al.*, 1994; Reichenberger and Olsen, 1996). Age, length and diameter of the collagen fibres, sampling position, collagen content and strain rate were found to play important roles in the study of the collagenous tissue (Rajaram *et al.*, 1978; Arumugam *et al.*, 1992; Dombi *et al.*, 1993; Pins *et al.*, 1997a; Pins *et al.*, 1997b).

Tendons are comprised of parallel fibrils, which are assemblies of parallel collagen molecules. Although there is still little understanding of the relation between stress-induced changes in the structure and the specific shape of the stress / strain curve of collagen, it is believed that in the dry state, S-shape stress-strain curves are similar to those of crystalline polymers that yield and undergo plastic flow (Figure 11). In general, the slope of the stress-strain curve increased with strain and this is characteristic of connective tissue (Arumugam *et al.*, 1992; Wang *et al.*, 1994; Fratzl *et al.*, 1997).

Figure 11. Typical S-shape stress-strain curve of dry collagen fibres.

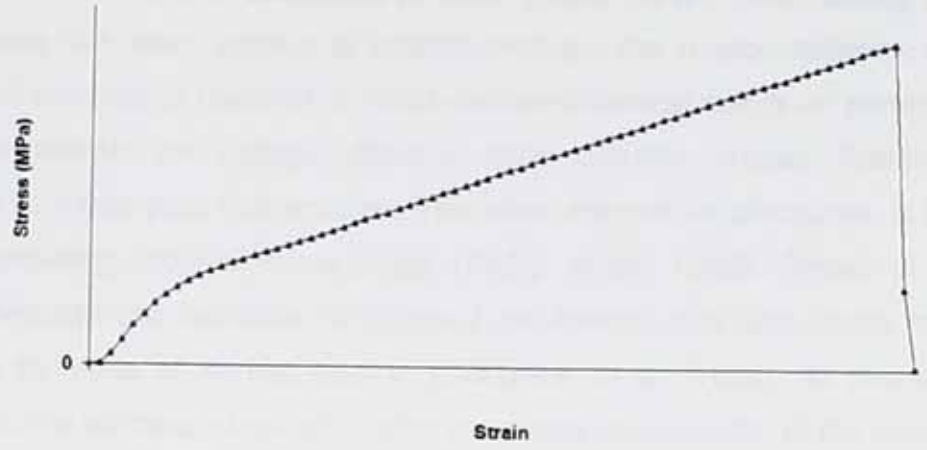
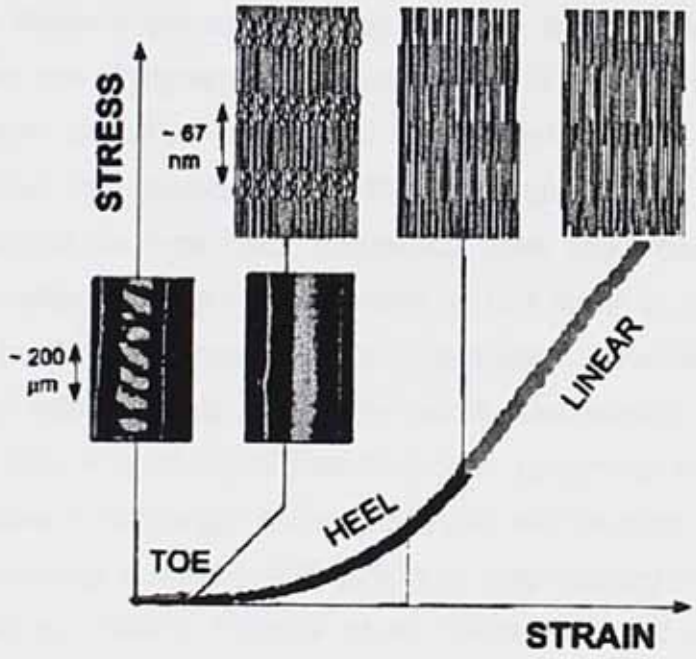


Figure 12. A typical J-shape stress-strain curve of rat-tail tendon indicating deformation mechanism associated with different regions of the curve. Source: (Fratzl *et al.*, 1997).



Typically, the J-shape stress-strain curve of rat-tail tendon can be subdivided into three regions as shown in Figure 12. The region of low strain (toe region) corresponds to the gradual removal of a macroscopic crimp in the collagen fibrils and this is visible in the light microscope. The crimp has been shown to act as a buffer or a shock absorber within the tendon, permitting small longitudinal elongation of individual fibrils without damage to the tissue (Jarvinen *et al.*, 2004). In real life, when, for example, the tail of a living rat, for example, is bent, the tendons on the

outside of the bend are stretched until the wave pattern disappears, and thus, provide a highly efficient "safety measure" for tendons to resist sudden, possibly hazardous tensile strains subjected to them (Rigby *et al.*, 1959; Bailey and Light, 1989). Once this wave pattern is straightened out the tendon behaves like a stiff spring until a stress is reached at which certain chemical bonds or perhaps ground substance between the collagen fibres or other subunits, breaks. This might allow sub-fibres to move past one another. The other alternative, of course, is that some kind of unfolding process takes place (Rigby *et al.*, 1959). Repair of damaged tendons requires the formation of crimped neo-tendon that has similar mechanical properties to those of normal tendon (Goldstein *et al.*, 1989). At strains typically beyond 2% the stiffness of rat-tail tendon increases considerably in the so-called heel region. X-ray studies have demonstrated lateral molecular packing of collagen molecules within fibrils, occurring as a result of the straightening of kinks. The straightening of the kinks allows an elongation of the fibrils and a resulting reduction in entropy, which provides the force acting against the elongation. The entropic forces increase as the number of kinks decreases leading to the typical upwards curvature of the stress-strain curve. When collagen is stretched beyond the heel region, most kinks are straightened out and no further extension is possible by the entropic mechanism described above. The exact mechanism by which mechanical energy is translated into molecular and fibrillar deformation is still unclear; most possibly, stretching of the triple helix, non-helical ends and cross-links takes place. Molecular level studies on tendon indicate that up to a strain of 2% stretching of the triple helix is the predominant mechanism of deformation. In tendons, where collagen fibres are aligned with the long axis of the tissue, viscoelastic behaviour at small strains (beyond 2%), it is believed that molecular stretching and slippage occur, resulting in increases in the length of the gap region with respect to the length of the overlap region, implying a side-by-side gliding of neighbouring molecules (Fratzl *et al.*, 1997; Pins *et al.*, 1997b; Purslow *et al.*, 1998; Silver *et al.*, 2001a). During loading, collagen molecules, fibrils, and fibril bundles deform and finally fail by a process termed defibrillation. The exact mechanism by which mechanical energy is translated into molecular and fibrillar deformation is still unclear. However, up to a macroscopic deformation of 2% molecular stretching predominates. Beyond 2%, increases in the D-period, increases in the gap region and relative slippage of laterally adjoining molecules along the fibre axis are observed (Kato *et al.*, 1989; Kato and Silver, 1990; Sasaki and Odajima, 1996a).

In leather, the structural origins of J-shaped stress-strain curves have been attributed to the progressive orientation of the collagen fibres during straining. At low strains resistance to deformation is low since the fibres themselves are not being stretched but are being aligned along the strain axis. At higher strains, as the fibres become aligned along the strain axis, further deformation can only occur by straining the fibres themselves and this requires increasingly greater stresses. Another explanation has been based on the reasonable premise that between their junction points the collagen fibre bundles may have differing degrees tautness and so, the fibrous network is extended, more and more fibres become taut and the stress increases (Attenburrow, 1993).

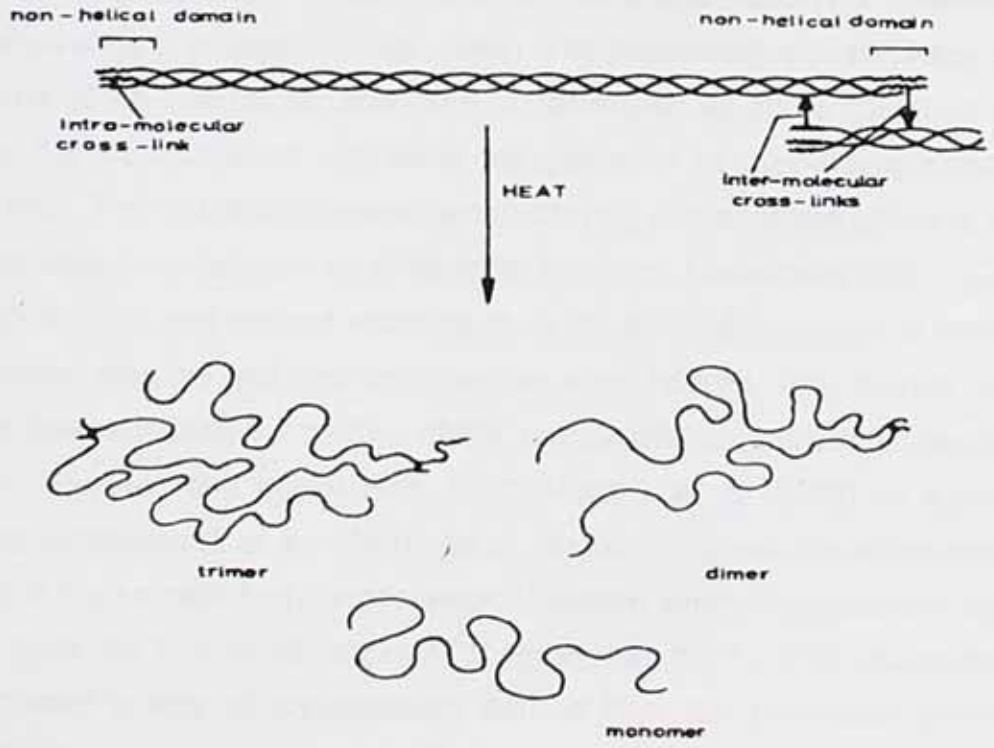
1.4.4. Thermal Properties of Collagen

Research in regards to the thermal properties of collagens has been divided into two categories. First, has been the investigation of the collagen molecule in solution, and second, has been the investigation of the properties of insoluble collagen matrices.

1.4.4.1. Collagen in Solution

When heated in solutions, the rigid triple helical molecules denature over a narrow range of temperatures, the mid-point of which is referred to as denaturation temperature (T_D). Denaturation of a protein starts with the destruction of its tertiary structure, with which also the biological function is destroyed (Verzar, 1965). The long rod-like helical molecules produce a highly viscous solution possessing a high optical rotation, but at the denaturation temperature all structure is lost and there is a dramatic decrease in viscosity and optical rotation. The denaturation of the triple helix has been shown to be a two-stage process with separation of the polypeptides as a first stage and the denaturation of their helical form as the second. Generally, the solution produced, known as gelatine, will contain not only free α-chains but also dimer, trimer and higher molecular weight components (Figure 13) (Bailey and Light, 1989).

Figure 13. Denaturation of collagen to gelatine. Source: (Bailey and Light, 1989)



The collagen-gelatine transition is a melting process in which collagen changes into a disorganised random coil. This transition may be detected by the determination of the denaturation temperature of dissolved collagen. It has been suggested that the hydrothermal stability depends on the total imino acid (proline and hydroxyproline) content rather than on the inter-chain hydrogen bonding of hydroxyl groups of hydroxyproline (Pikkarainen, 1968). Additionally, it has been found that the age and therefore the composition of the tissue, especially the fractions of proline and hydroxyl proline that contribute to the thermal stability of the tissue (Bailey and Light, 1989; Lawrie, 1998), have a profound effect on the temperature-time history required to initiate denaturation as well as on the kinetic parameters that describe this denaturation (Sankaran and Walsh, 1998). The hydrogen bonded water bridges model (as described at section 1.3) of the triple helix has been confirmed by physiological studies of the collagen molecule in solution and is supported by the observation that the thermal stability of the helix is dependent on the content of hydroxyproline and not of proline (Miles *et al.*, 1995).

1.4.4.2. Fibrous Collagen

Shrinkage is a prelude to the denaturation of the collagen fibrillar structure. It is an irreversible change that occurs on collagen fibres on heating above a certain temperature and is due to the breaking of hydrogen bonds, allowing the collagen to adopt a more stable shrunken configuration. This temperature (T_s), however, varies for different sorts of collagen (Banga, 1966). The phenomenon of shrinkage provides a measure of the thermal transition and is determined as the temperature at which the fibre, if it is unrestrained, shrinks to one-quarter of its original length (Bailey and Light, 1989). The endothermic event accompanying this transition allows a measure of the shrinkage temperature by Differential Scanning Calorimetry (DSC) (Collighan *et al.*, 2004). DSC is a method widely to study the thermal behaviour of materials as they undergo physical and chemical changes upon heating. This method measures the heat flow necessary for heating of the sample with a constant temperature rate (^{0}C/min) (Hormann and Schlebusch, 1971; Mentink *et al.*, 2002). In solution, the unfolding temperatures of a wide range of fibrous collagens are within only a few degrees of the animal's body temperature. However, when the molecules aggregate to form fibres, the T_s is usually about 27^{0}C higher than the T_D of the molecules due to the increased energy of crystallisation derived from the interaction between the closed packed molecules in the fibre (Bailey and Light, 1989; Sionkowska, 2005).

1.5. Sources of Collagen

Hides, skins and tendons, as well as fins, scales and bones are a waste material from the meat and fish industry respectively. Since these materials are a rich source of the fibrous protein collagen, they have great potential for use in the biomedical field (scaffolds for tissue culture, wound healing, implants) as well as finding application in the food industry (edible coatings, emulsifying, foaming and gelling agent) (Montero *et al.*, 1995). Investigations of molecular weights and molecular dimensions of native tropocollagen have not revealed any differences in the tropocollagens of different species (Pikkarainen, 1968). Recently, aquatic animal collagen has been investigated since it has the unique features of containing fewer imino acid residues and having a lower denaturation temperature than collagen from land vertebrates (Nagai and Suzuki, 2000; Nomura *et al.*, 2001b; Nomura *et al.*, 2001a).

Type I collagens from land animals have been extensively utilised in food, medical treatment and cosmetic materials. Collagen is used in the form of natural collagen, chemically modified collagen, telopeptide digested collagen, gelatine and physiological active peptides. The sources of these collagens are mainly bovine- or pig- skins, although much of the work has been done using rat-tail tendon collagen, almost entirely composed of type I collagen, with parallel orientation of the fibrils and a well known ultra-structure (Kato *et al.*, 1989; Arumugam *et al.*, 1992; McBride *et al.*, 1997; Raspanti *et al.*, 1997; Sankaran and Walsh, 1998).

The recent concern about the transmission of Bovine Spongiform Encephalopathy across species barriers has proven a significant drawback for manufacturing bovine-derived-biomaterials. However, the transmission of this disease to humans, if it occurs at all, requires an effective dose for a sufficiently prolonged period of time (Narotam *et al.*, 1993). Additionally, bovine collagen antigenicity may be reduced by treatments with pepsin and strong alkaline solutions, and physiochemical agents that induce cross-linking of collagen (Rodrigues *et al.*, 2003). The use of pig tendon collagen would serve as an alternative, free of the potential risk of this infection. Porcine atelocollagen has already been used clinically as a haemostatic substance and organ replacement and reported to be inert in the human body (Yamamoto *et al.*, 1999; Adams *et al.*, 2001). Furthermore, recent studies demonstrated the interaction between equine collagen fibres and hydroxyapatite composite for the production of artificial bio-mimetic for natural bone tissue (Roveri *et al.*, 2003).

1.6. Isolation, Precipitation, Purification and Characterisation of Collagen

Although collagen is ubiquitous in the mammalian body, those tissues rich in fibrous collagen such as skin and tendon are generally used as starting materials to generate collagen for use in implants, wound dressings, drug delivery and food casings. Different methods have been introduced over the years to generate different types and amounts of collagen from different species, such as neutral salt soluble collagen, alkali treated collagen, acid soluble collagen, enzyme treated collagen and insoluble collagen (Light, 1985; Friess, 1998). Among these, the most widely used methods for the extraction of collagen from tendons are the acid and/or enzyme digestion. The latter one is expected to become more widely used in clinical settings, since an inert, more bio-absorbable, with very low antigenicity and superior biocompatibility natural material is produced (Friess and Lee, 1996; Bonassar and Vacanti, 1998; Matsumoto *et al.*, 1999).

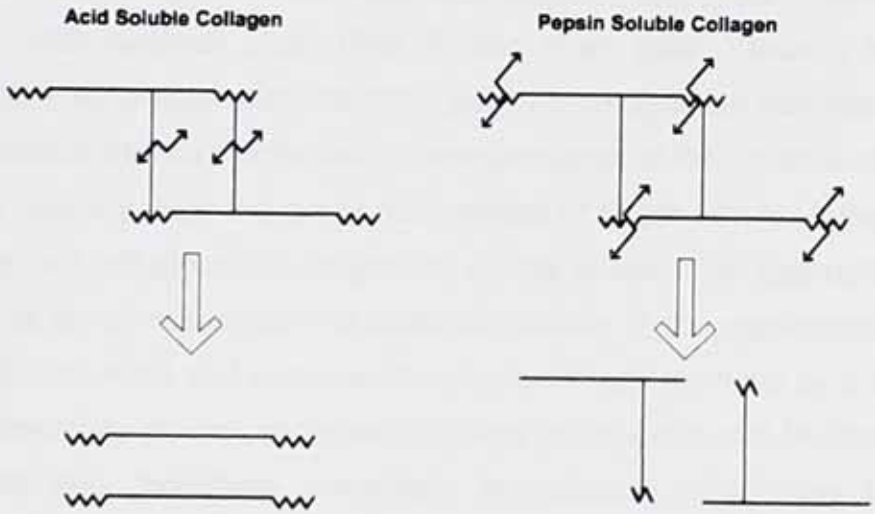
1.6.1. Acid Soluble Collagen Isolation

Dilute acidic solvents, e.g. 0.5M acetic acid, citrate buffer, or hydrochloric acid pH 2-3, are more efficient than neutral salt solutions. The inter-molecular cross-links of the aldimine type are dissociated by the dilute acids and the repulsive repelling charges on the triple-helices lead to swelling of fibrillar structures. Dilute acids will not disassociate less labile cross-links such as keto-imine bonds. Therefore collagen from tissues containing higher percentages of keto-imine bonds, i.e. bone, cartilage or material from older animals has a lower solubility in dilute acid solvents. In order to extract acid soluble collagen, generally, the tissue is ground whilst cold, washed with neutral saline to remove soluble proteins and polysaccharides and the collagen extracted with a low ionic strength acidic solution. Collagen molecules derived from dilute salt or acid solutions can be reconstituted into large fibrils with similar properties as native fibrils by adjusting the pH or temperature of the solution. The non-soluble portion under dilute salt or acid conditions, is referred to as insoluble collagen although this dominant collagen material is not absolutely insoluble and can be further disintegrated using, more frequently, enzymes to cleave additional cross-links and suspend or dissolve at first acid-insoluble structures without major damage to the triple helical structures (Rigby, 1967; Danielsen, 1981; Bailey and Light, 1989; Friess, 1998).

1.6.2. Enzyme Soluble Collagen Isolation

The individual peptide chains of tropocollagen can be broken down at various levels: cleaving the telopeptides from the intact helical part, breaking the chains into two parts with proteolytic enzymes, or to a mixture of fragments of various sizes by pepsin, collagenase, cyanogens bromide or hydroxylamine and finally to small fragments with collagenase, trypsin and pepsin (Lampiaho *et al.*, 1968). Much higher yields compared with acidic extraction can be achieved. Collagen from different sources shows different degrees of susceptibility to the enzyme; more than 90% of skin can be solubilised with this treatment (Light, 1985; Bailey and Light, 1989; Friess, 1998). The sensitivity of the various linkages to pepsin is very variable and there is evidence that the first cleavage occurs adjacent to aspartic and glutamic acids (Lampiaho *et al.*, 1968; Campos and Sancho, 2003). The action of pepsin, to release whole, soluble collagen molecules from the insoluble matrix, is diagrammatically illustrated in Figure 14.

Figure 14. Diagrammatic representation of the action of acid or pepsin on native collagen at low temperature.



The efficacy of enzymatic treatment arises from selective cleavage in the non-helical N- terminal and the distal end of the C- terminal regions. Thus, almost whole molecules are "clipped" out of the matrix. Some cross-links presumably remain, attaching small peptide remnants to the solubilised molecules. The telopeptide ends of the polymer chains are dissected but the helices remain essentially intact (Light, 1985; Na *et al.*, 1986). These telopeptide domains are involved in the collagen cross-linking process (Woodley *et al.*, 1991). The resulting material so-called monomolecular collagen or atelocollagen benefits from the removal of the antigenic

P-determinant located on the non-helical protein sections. It is conceivable that the host immune response can be greatly reduced and as a result antigenicity can be further reduced and a more competitive biomaterial can be produced (Rosenblatt *et al.*, 1989; Rosenblatt *et al.*, 1994; Yamada *et al.*, 1995; Friess, 1998; Yin Hsu *et al.*, 1999; Alam *et al.*, 2001; Ishikawa *et al.*, 2001; Rodrigues *et al.*, 2003). Pepsin at a 1:10 weight ratio of enzyme to dry weight tissue in dilute organic acid (0.5M acetic acid) at temperatures of 4-15^{0}C, pH of 1-3, for times of 4-72h provides a propitious medium in which collagen can be swollen and solubilised (Light, 1985).

1.6.3. Collagen Precipitation

Precipitation has long been used as an early step in the process of purifying proteins from complex solutions. Phase separation is achieved through addition of precipitating agents to aqueous protein solutions. Commonly used precipitating agents include inorganic salts, non-ionic polymers, polyelectrolytes and organic solvents; the different precipitation techniques have different modes of precipitation (Zeppezauer and Brishammar, 1965; Mahadevan and Hall, 1992; Iyer and Przybycien, 1996; Kuehner *et al.*, 1996; Polson *et al.*, 2003; Yasueda *et al.*, 2004). Among the different precipitation methods, polymer precipitation has received a lot of research attention because of its particular advantages of low additive consumption, high protein recovery, high degree of fractionation of target enzymes, the uncharged polymers do not influence the migration of the proteins in electrophoresis, the precipitation is strictly reversible, the biological activity of the precipitated proteins is quantitatively recovered and some neutral polymers may even act as protective, the rigorous temperature control necessary for organic solvents can be eliminated and the polymers also precipitate low-density lipoproteins, which may float in salt precipitations (Zeppezauer and Brishammar, 1965; Kim *et al.*, 2001).

Protein precipitation has been described as equilibrium between a "pure" protein phase (precipitate) and a saturated liquid phase (supernatant), with the degree of separation quantified by the apparent protein "solubility", i.e. the protein concentration in the supernatant equilibrium liquid phase (Kuehner *et al.*, 1996). It has been reported that protein precipitates were produced through a two-stage process: the formation of protein-polyelectrolyte complexes and the formation of particles from these complexes (Kim *et al.*, 2001), whilst the driving forces are found

to be mainly electrostatic attraction plus hydrogen and hydrophobic bonding (Chen and Berg, 1993). At low concentration of the additive, the protein solubility is increased and this is called the salting-in zone. At higher concentrations, solubility decreases giving rise to the salting-out effect. In the case of salts, the salting-in action reflects mainly non-specific electrostatic interactions, while the salting-out phenomenon is driven by other forces, specific for each particular precipitant (Timasheff and Arakawa, 1988). The interaction between polymers and the proteins appears to be controlled by many variables such as the pH, the ionic strength, the protein concentration and the types of ions present, the protein size, initial concentration and surface chemistry and electrolyte identity, Mw and concentration (Zeppezauer and Brishammar, 1965; Kuehner *et al.*, 1996).

1.6.4. Collagen Purification

All methods of purification are a compromise between removing as much impurity as possible without denaturing the collagen, particularly by heat, from its native form (Eastoe *et al.*, 1963). Soluble collagen is purified mainly by salt/polyelectrolyte precipitation, followed by centrifugation and dialysis. The various collagen types can be precipitated from the clear supernatant by differential salt/polyelectrolyte fractionation. By increasing the salt/polyelectrolyte concentration the various types of collagen can be salted out of solution and collected as precipitates. Intractable precipitates can be re-dissolved in 0.5M acetic acid and then dialysed against neutral salt solution if further fractionation is necessary. Collagen solutions contain varying proportions of monomer and higher molecular weight covalently linked aggregates, depending on the source and method of preparation. Truly monomeric solutions are difficult if not impossible to obtain. Pepsin-solubilised collagen usually contains higher proportions of monomer than salt- or acid- extracted material. Soluble collagen can be stored frozen or lyophilised (Light, 1985; Friess, 1998). It has been found that the freeze-drying process does not alter the structure of the collagen molecule, at least with regard to its poly-peptide composition: so the lyophilisation process is able to guarantee the formation of a spongy tablet suitable for tissue healing (Menicagli and Giorgi, 1990).

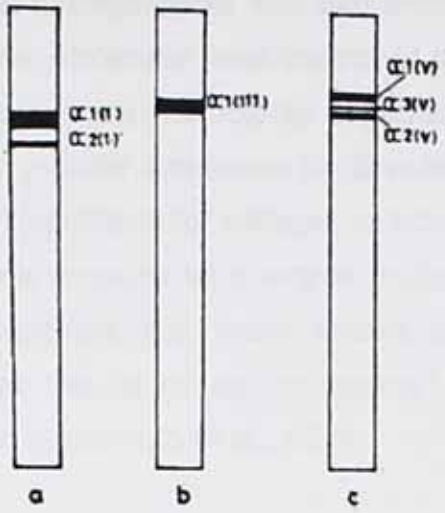
1.6.5. Collagen Analysis / Characterisation

Collagen preparations are generally analysed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and for quantification by hydroxyproline assay.

1.6.5.1. SDS-PAGE Analysis

The electrophoresis is based on the principle that charged molecules migrate through a liquid or semi-solid medium (gel) when subjected to an electric field. The most common type of electrophoresis performed with proteins is zonal electrophoresis, during which, proteins are separated from a complex mixture into distinct bands at a characteristic rate depending on its charge, size and other physical characteristics. Protein bands are visualised by staining with Coomassie Brilliant Blue and de-staining. More sensitive stains (20-50 fold increased sensitivity) have been developed using silver for both proteins and carbohydrate as well as for specific saccharides attached to collagenous chains separated by SDS-PAGE. Proteins electrophoresis may be carried out under denaturating or non-denaturating conditions. In the first case, the negatively charged detergent (SDS) is added to the sample and also incorporated into the gel. The sample is denaturated by heating. The SDS molecule binds to the denatured protein polypeptide and confers on each molecule a net negative charge, so that the polypeptide migration in the polyacrylamide gel is proportional to the molecular weight of the protein. The negatively charged molecules migrate through the gel matrix toward the anode at a velocity that is roughly proportional to the log of the molecular weight of each polypeptide. Since the denatured proteins migrate through the gel on the basis of size, it is possible to estimate the molecular weight of the individual polypeptides (Light, 1985; Bailey and Light, 1989; Smith, 1998). A typical profile of types I, III and V is shown in Figure 15.

Figure 15. Separation of molecular components of (a) Type I, (b) Type III and (c) Type V collagens by SDS-polyacrylamide gel electrophoresis. Source: (Light, 1985).



1.6.5.2. Hydroxyproline Estimation

Collagen contains the unusual amino acid hydroxylysine. Similarly, collagen contains the unique imino acid hydroxyproline. Similar to the formation of hydroxyproline from proline, hydroxylysine is formed from lysine in the endoplasmic reticulum via enzymatic hydroxylation by lysyl hydroxylase (Popenoe *et al.*, 1966; Friess, 1998). Because the percentage of the molecule made up by hydroxyproline remains relatively constant throughout the various genetic forms, the level of this compound in acid hydrolysates of tissue samples may be used to calculate the total collagen content. Using a figure of 14% hydroxyproline for skin collagen the total amount of collagen can then be easily extrapolated with a less than 10-15% error. The most frequent method for collagen content estimation involves the oxidation of free hydroxyproline with chloramine-T and reaction of the resulting hydroxyproline chromogen with p-di-methylamino-benzaldehyde. A pink colour develops and can be estimated at 550nm (Light, 1985; Avery *et al.*, 1996; Komsa-Penkova *et al.*, 1996).

1.7. Collagen Interactions

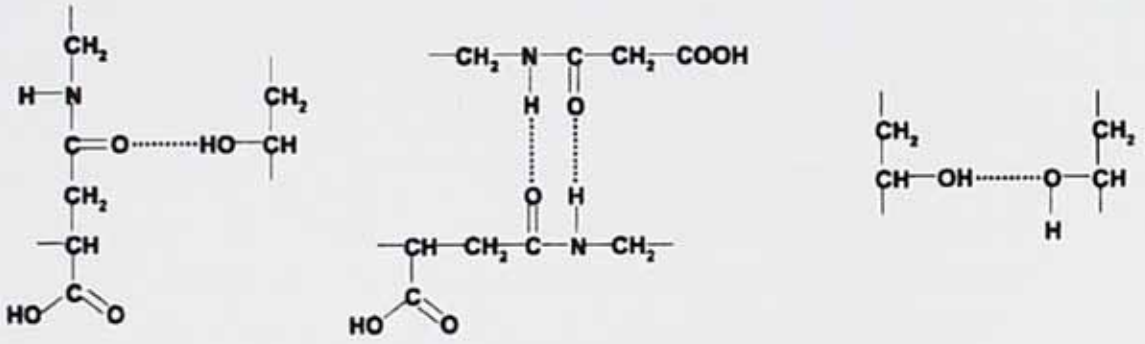
The expanding field of tissue engineering has accelerated the demand for materials, which are tissue compatible, biodegradable and with mechanical properties closely matched to the target tissues. Molecular level control of biological activity is also a highly desirable feature. Porous microstructures are also required to either allow tissue in-growth *in vivo* or to provide a template for directed tissue assembly *in vitro* (Chupa *et al.*, 2000). Since it is difficult for collagen to fabricate some prostheses on its own because it is too fragile to suture as a surgical material, it is usually necessary to combine it with other materials, e.g. water soluble polymers, polyelectrolytes, polysaccharides, referred in this study as "co-agents" (Friess and Lee, 1996; Bonassar and Vacanti, 1998; Matsumoto *et al.*, 1999).

1.7.1. Collagen Interaction with Water-Soluble Polymers

Recently, many water-soluble polymers have become an attractive field of study, due to their good processing characteristics and variable degradation rates (Jimenez-Regalado *et al.*, 2004). The main requirements to determine which synthetic polymer should be used in a blend with collagen, are its good solubility in water and good interaction with living cells (Sionkowska, 2006) . The modification of proteins and peptides by covalent attachment of polymeric modifiers (bio-conjugation) can eliminate some drawbacks of native proteins and peptides to improve their physicochemical, biomedical and pharmacological characteristics. These benefits of bio-conjugation lead to the production of many chemically modified drugs and dramatic therapeutic effects have been reported (Kaneda *et al.*, 2004). Polyethylene glycol (PEG), for instance, a low toxic and low antigenic poly-ether-diol of general structure $HO\text{-}(CH_2CH_2O)_n\text{-}H$, has been FDA approved for several medical and food industry applications (Mallikarjunan *et al.*, 1997; Fu *et al.*, 2002). It has been reported that PEG could stabilise the porous structure inside collagen sponges, facilitating cell infiltration, tissue in-growth, significant increase in the mechanical stability and enzyme degradation with improved blood compatibility (Vasudev and Chandy, 1997). PEG has been considered as an attractive non-adhesive biomaterial because of its ability to resist protein adsorption (Deible *et al.*, 1998). Furthermore, for the drug modification, polyethylene glycol has been most widely used because of its long half-life in the blood and its low interaction with organs and tissues. However, a PEG molecule cannot couple with more than one or two drug molecules since PEG has

only one or two functional groups per molecule. In contrast, other polymeric carriers, such as dextran, pollulan, gelatin and poly-vinyl alcohol (PVA) have numerous functional groups, which are capable of covalently coupling drug molecules (Yamaoka *et al.*, 1995). PVA mainly has been used as a sizing agent or stabiliser of dispersion systems and has demonstrated good solubility and biodegradability. Collagen, which is a hydrogen donor, forms hydrogen bonds with the hydroxyl group from the water-soluble polymers (Figure 16). For higher concentrations, the formation of hydrogen bonds between two different macromolecules competes with the formation of hydrogen bonds between molecules of the same polymer as well (Figure 16) (Sionkowska *et al.*, 2004).

Figure 16. Hydrogen bonds between collagen-PVA (left), collagen-collagen (middle) and PVA-PVA (right). Source: (Sionkowska *et al.*, 2004).



1.7.2. Collagen Interaction with Polysaccharides

Polysaccharide-protein interactions are involved in a wide variety of biological functions including cellular growth, recognition, adhesion and cancer metastasis (Mislovicova *et al.*, 2002). In the presence of polysaccharides some proteins change their denaturation temperature or modify their ability to form gels, aggregates or fibres. Depending on their nature, pH, ionic strength, temperature, concentration and molecular weight, the proteins can attract or repel the polysaccharides in solution. In the former case, supramolecular soluble complexes are often formed. In the latter case, thermodynamic incompatibility and hence phase separation can arise (Delben and Stefancich, 1997; Tavares and Lopes da Silva, 2003). It has been shown that electrostatic or hydrogen-bonding type complex is formed between chitosan and collagen (Taravel and Domard, 1995; Taravel and Domard, 1996). Carrageenans may be used in processing to block bacterial attachment or detach the food

pathogens attached to the carcass surfaces during slaughter operations (Medina, 2001). Different approaches have been made to use starch for the production of tailored biodegradable materials (Averous *et al.*, 2001). Colloid dispersions of proteins and gums contribute to the structure, texture and stability of the final product (Galazka *et al.*, 2001; Leong *et al.*, 2001; Akhtar *et al.*, 2002; Ibanoglu, 2002; Tsaliki *et al.*, 2003; van de Velde *et al.*, 2003). Films made of protein, starch and gum retain water and resist oxygen penetration (Shih, 1999). Use of chitosan as a drug delivery agent or chitosan/collagen composites as biomaterial (hydro-gel, film, fibre, sponge) has recently drawn a considerable attention in the applications for the repair of articular cartilage and skin tissue engineering. Good biodegradability, low immunogenicity, cell attachment and excellent ability to promote tissue regeneration were observed (Chupa *et al.*, 2000; Suh and Matthew, 2000; Ma *et al.*, 2001).

1.8. Cross-linking of Collagenous Matrices

Natural cross-linking, the formation of covalent inter- and intra- molecular bonds between proteins, is utilised by nature to create new entities with properties completely different from the original monomeric form (Bailey, 1991). The primary function of native cross-linking is to impart desired mechanical characteristics and proteolytic resistance on the collagen fibres in connective tissue. Covalent links between the molecules will undoubtedly reduce the extensibility of the fibre by preventing longitudinal slippage of one molecule against another, and increase stiffness by developing a lateral network of linkages (Friess, 1998; Canty and Kadler, 2002; Miles *et al.*, 2005). However, the lysyl oxidase mediated cross-linking would not occur *in vitro* and consequently reconstituted forms of collagen can lack sufficient strength and may disintegrate upon handling or collapse under the pressure from surrounding tissue *in vivo*. Furthermore, the rate of biodegradation has to be customised based on the specific application (Friess, 1998; Koob and Hernandez, 2002). Thus, it is often necessary to introduce exogenous cross-links (chemical, biological or physical) into the molecular structure, in order to control mechanical properties, biological stability, the residence time in the body and to some extent the immunogenicity and antigenicity of the device (Cote *et al.*, 1992; Rault *et al.*, 1996; Chen *et al.*, 1997; Osborne *et al.*, 1998; McKegney *et al.*, 2001; Paul and Bailey, 2003), resulting in insoluble structures of higher molecular weight (Friess and Lee, 1996). Furthermore, when atelocollagen is used additional cross-linking is necessary to prevent the devices from dissolving at 37^0C (Friess and Lee, 1996).

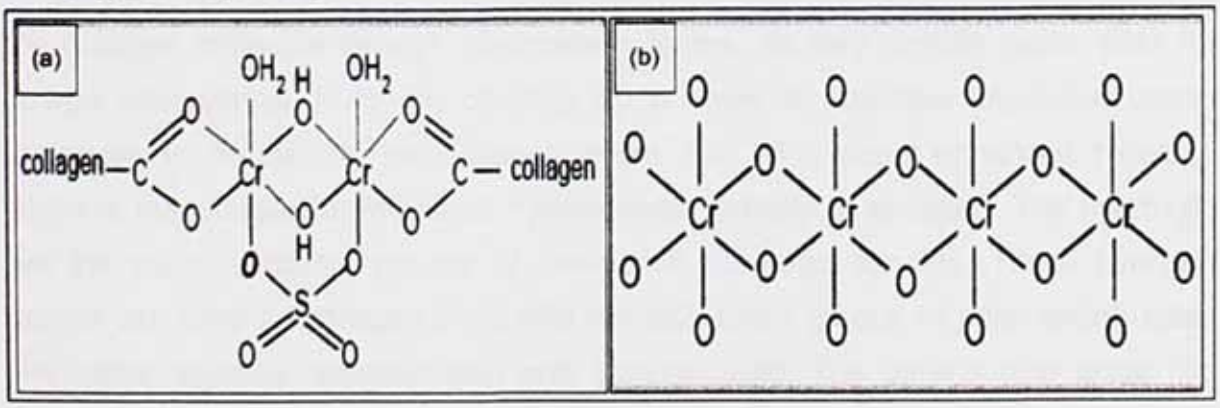
The specific objective of fabricating cross-linked collagen fibres is to produce *in vitro* a material with mechanical properties equivalent to native fibres. Therefore, the mechanical properties of the fibrous construct must be equivalent to the properties of normal tissue and be able to withstand the repetitive loads associated with muscle contraction during rehabilitation and beyond the immediate post-surgical period. The material must be biocompatible, eliciting little if any foreign body or antigenic response. Cytotoxicity of the material and its by-products must be eliminated. The material must be an optimal substrate for cell adhesion, locomotion and function and to serve as a temporary porous scaffold for new connective tissue growth (Chvapil *et al.*, 1993; Koob and Hernandez, 2002). Controlling the rate of degradation by cross-linking, not only determines the lifetime of the implant after application, but may also determine the rate of tissue regeneration (Anselme *et al.*, 1992; van Wachem *et al.*, 1994a).

1.8.1. Tanning Agents

1.8.1.1. Basic Chromium Sulphate (BCS)

Chromium forms stable basic oligomeric complexes with acidic residues on the collagen molecule at pH 3-4 (Friess, 1998). More specifically, chrome tanning involves interaction of chromium with the carboxyl groups in the side chains of aspartic acid and glutamic acid residues of collagen molecules (Suzuki *et al.*, 2000; Rajini *et al.*, 2001), bringing about irreversible matrix changes and long range ordering, thus imparting higher hydrothermal stability (Madhan *et al.*, 2002). It may as well react with protein hydrolysates to form insoluble macromolecular metal complexes (Mu *et al.*, 2003). The di-chromium complexes, as indicated by Figure 17(a), were believed to bind via multi point cross-linking. However, it was demonstrated that the participation of sulphate in the tanning species and particularly its inclusion in cross-linking species is untrue and was suggested that the role of sulphate in stabilising the collagen and in chrome tanning is to contribute to the stabilising effect of the supra-molecular water (Covington, 2001). It was concluded that the chromium species involved in tanning reactions are linear chains of four chromium ions, linked by oxy-bridges as indicated in Figure 17(b) and may bind with collagen by single point binding (Covington, 2001). In a recent work, it was proposed that the tanning mechanism of chromium salts is due to a matrix formation with collagen and water rather than cross-linking of two collagen molecules, without however ruling out the multi point cross-linking (Covington and Song, 2003).

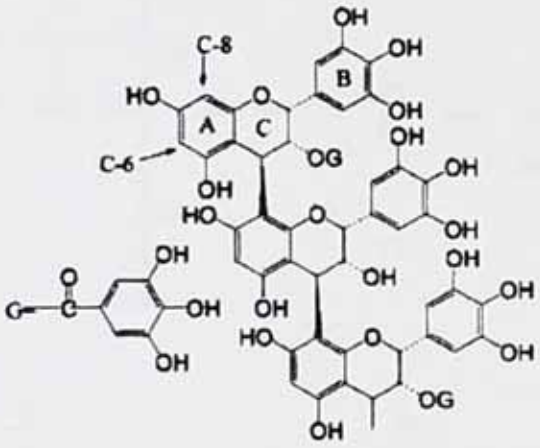
Figure 17. Possible mechanisms of covalent cross-linking of collagen with chromium salt (Jeyapalina, 2004).



1.8.1.2. Vegetable Tanning

Vegetable tannins are polyphenolic compounds of plant origin, which are of two distinct types, hydrolysable tannins (polyesters of gallic acid and polysaccharides) and condensed tannins (polymerised products of flavan-3-ols and flavan-3,4-diols, or a mixture of the two), although other tannins occur which are combinations of these two basic structures (Liao *et al.*, 2003). *Myrica rubra* tannin, for example, (Figure 18) is often attached with a galloyl group on a pyrane ring (C-ring). Because the interaction between tannins and collagen fibres is through hydrogen bond and hydrophobic bond associations and can be easily broken by organic solvent and urea solution, tannin will be easily leaked out in practical application. The C-6 and C-8 positions of A-ring of a *Myrica rubra* tannin molecule have highly nucleophilic reaction activity; therefore, it can be covalently bonded to amino groups of collagen molecules by reaction with aldehyde (Liao *et al.*, 2004a; Liao *et al.*, 2004b).

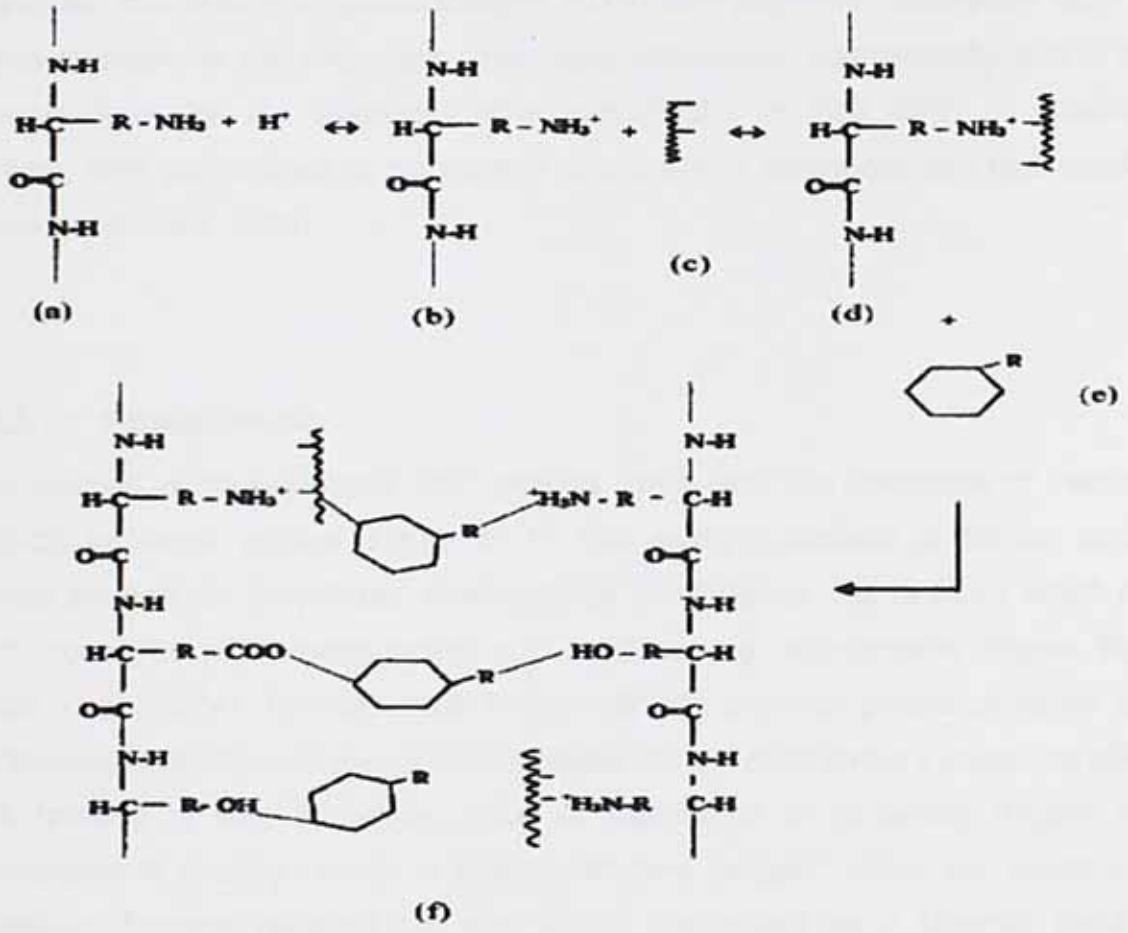
Figure 18. Myrica Vegetable Tanning. Source: (Liao *et al.*, 2004a).



Acrylic polymers are utilised for the treatment of collagen, which are likely to open up the collagen molecule through electrostatic forces, as they contain active sites for charge interactions. Such an opening up is likely to facilitate vegetable tannin molecules to diffuse into the collagen matrix and bring about enhanced ordering, which is responsible for improved hydrothermal stability of collagen. The hydroxyls are the main functional groups of interaction for these tannins. These hydroxyl groups can form a hydrogen bond with the side chain groups of polar amino acids like lysine, arginine, aspartic acid and glutamic acid. The other amino acids like serine and threonine can also involve in the hydrogen bond formation with the polyphenolic molecules (Madhan *et al.*, 2001). All these amino acid residues can act as hydrogen bond donors and acceptors. Since these polyphenolics have several

hydroxyl and carboxyl groups, they can form hydrogen bonds at multiple points, imparting additional stability to the fibre matrix (Madhan *et al.*, 2002).

Figure 19. Schematic representation of the interaction of collagen with acrylic and vegetable tannins: (a) polypeptide chain of collagen especially representing the basic amino acid residues; (b) side chains of basic amino residues charged positively at weakly acidic conditions; (c) acrylic polymer negatively charged because of the presence of carboxylic groups; (d) complex of acrylic polymer and polypeptide chain of collagen, exhibiting charged interactions; (e) representative plant polyphenolic molecule; and (f) complex of collagen–acrylic–polyphenolic indicating the multipoint electrostatic interactions between hydroxyl group of the polyphenolics and the polar amino acid residue between neighbouring polypeptide chains of collagen at the inter-fibrillar level; a similar dipole–dipole interaction is exhibited between polyphenolics of vegetable tannins and acrylic polymer. Source: (Madhan *et al.*, 2002).



1.8.2. Reactions with ε-amino Groups

1.8.2.1. Aldehydes

Aldehydes are widely used as fixatives in the preparation of specimens for light and electron microscopy and are generally considered as the most effective cross-linking agents (Meek and Chapman, 1985; Kadler and Chapman, 1988). Primary aldehydes react with the ε-amino groups of lysine residues in collagen and therefore readily form cross-links. Indeed, the reaction of all aldehydes as cross-linking agents is primarily with the ε-amino group of lysine and hydroxylysine and to a lesser extent guanidine, phenolic and thiol groups, virtually no reaction occurring with acid groups at slightly alkaline pH (Paul and Bailey, 2003). Evidence suggests that a di-pyridine structure may be involved accounting for the great stability of the cross-links formed (Kato and Silver, 1990). Although many aldehydes and di-aldehydes have been employed, formaldehyde, glutaraldehyde (GTA) and polymeric aldehydes such as starch di-aldehyde are the major ones used extensively commercially and it has become clear that the aldehydes differ considerably in their ability to cross-link collagen both with respect to the number of cross-links introduced and their stability (Bowes and Cater, 1968).

1.8.2.1.1. Formaldehyde

The reaction of formaldehyde with proteins starts with the formation of methylol adducts on amino groups (Figure 20-1). The methylol adducts of primary amino groups are partially dehydrated, yielding labile Schiff-bases (Figure 20-2), which can form cross-links with several amino acid residues, e.g. with tyrosine (Figure 20-3) (Metz *et al.*, 2004). Formaldehyde reacts with the ε-amino groups of lysine and hydroxylysine residues to give an intermediate imine, which forms a cross-link either with tyrosine or with an amide group of asparagine or glutamine (Figure 21). Formaldehyde tanning results in short methylene bridges, which are covalent in character. Tyrosine residues can also add to the adduct via a Mannich reaction (Chapman *et al.*, 1990; Gorham, 1991; Friess, 1998; Rajini *et al.*, 2001). With formaldehyde it has been shown that interaction with amine groups is followed by further condensation with amide and guanidyl groups (Bowes and Cater, 1968).

Formaldehyde treatment results in inter-chain cross-links that have been shown to be reversible. Formaldehyde vapours do not induce the co-precipitation and/or reorganisation of collagen into fibrils as observed with GTA. Inter-fibrillar cross-links have not been described for formaldehyde and it does not co-polymerise as demonstrated with GTA and subsequently cannot bind side-by-side fibrils (Cote *et al.*, 1992). Therefore, formaldehyde does not introduce bulky polymeric adducts into the fibril structure, as has been shown for GTA (Meek and Chapman, 1985), and the conspicuous stain-excluding bands seen in negative staining patterns following glutaraldehyde fixation are absent after exposure to formaldehyde. Furthermore, it has been shown that the reaction of formaldehyde with reconstituted collagen gels occurs within 10min; however further cross-linking continues for some while. For these reasons, where chemical fixation is used to stabilize macromolecules and supramolecular aggregates prior to negative staining and high resolution electron optical imaging, formaldehyde would seem to be preferable to glutaraldehyde (Kadler and Chapman, 1988).

Figure 20. Reaction of formaldehyde with proteins. Source: (Metz *et al.*, 2004).

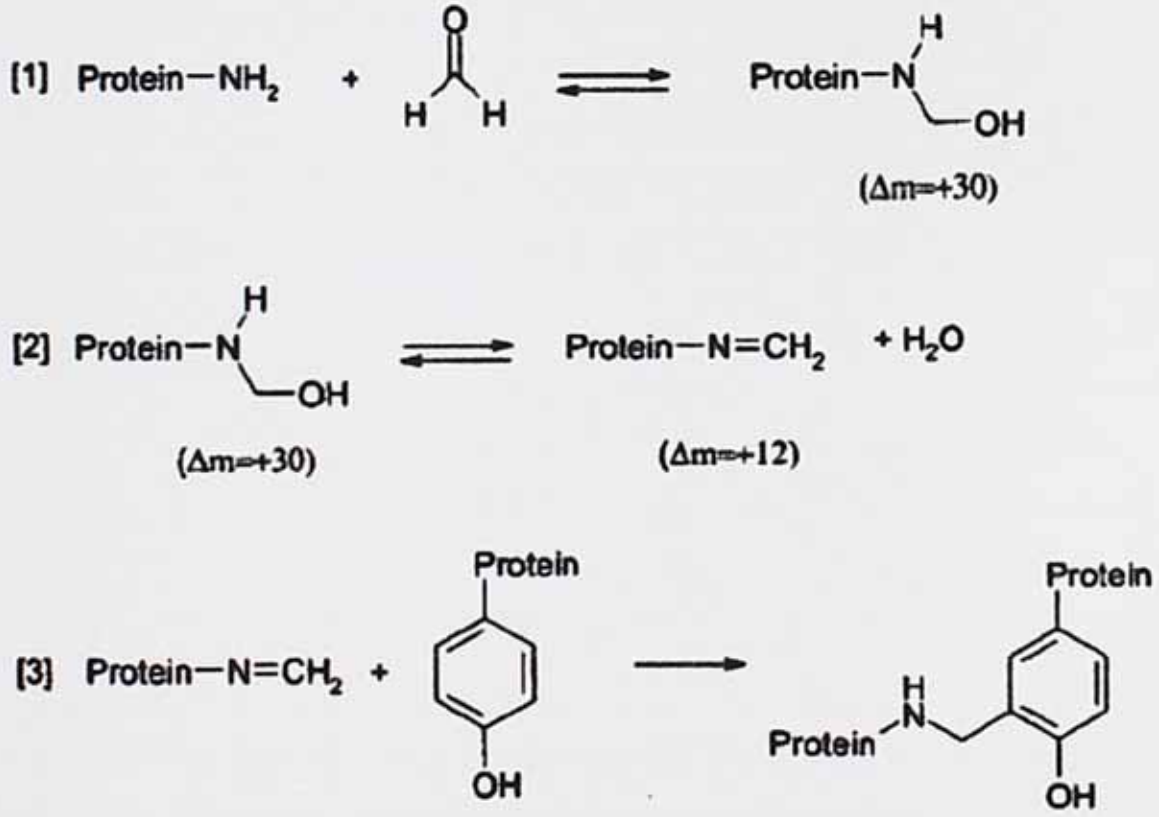
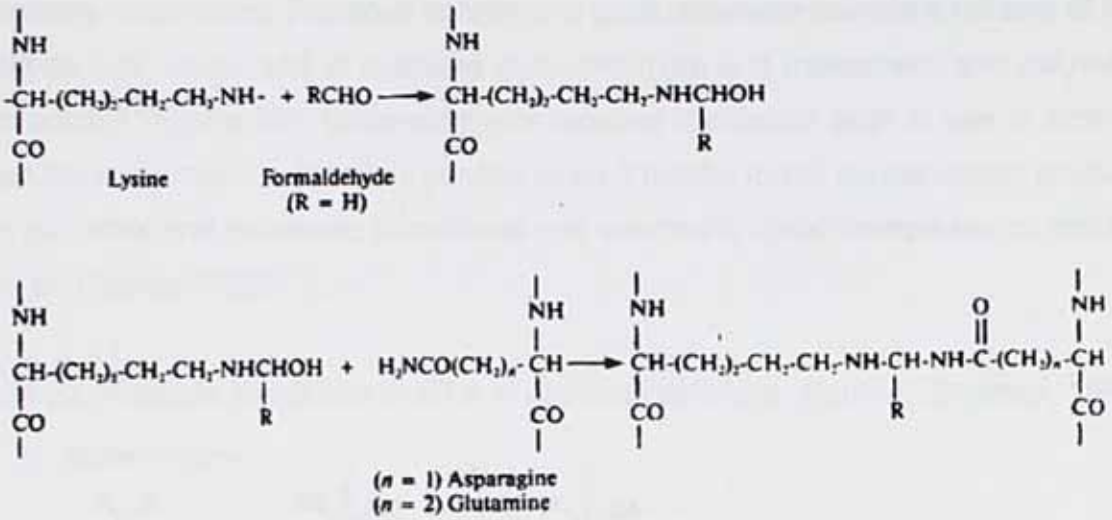


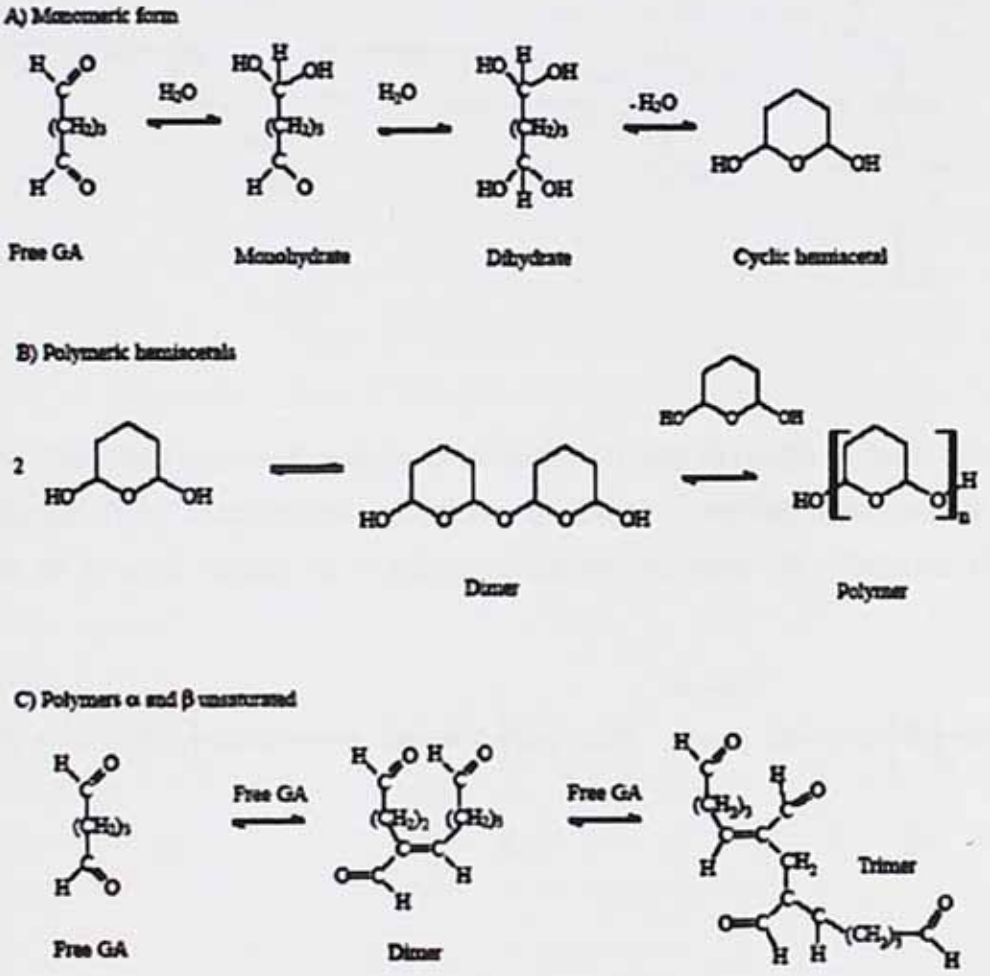
Figure 21. Cross-linking of peptide chains with mono-aldehydes. Source: (Gorham, 1991).



1.8.2.1.2. Glutaraldehyde (GTA)

GTA is an active reagent commonly used in the cross-linking of protein and polysaccharides polymers. The reaction mechanism is very complex and still not completely understood. Aqueous solutions of glutaraldehyde contain a mixture of free aldehyde and mono- and di-hydrated glutaraldehyde and monomeric and polymeric hemi-acetals (Figure 22). Glutaraldehyde requires distillation prior to use in order to obtain the monomer for chemical studies since it readily forms condensation products such as aldols and polymeric complexes and eventually cyclic complexes on storage (Paul and Bailey, 2003).

Figure 22. Possible structures of GTA in aqueous solutions. Source: (Zeeman, 1998).



Aldehyde groups of the reagent can react with either hydroxyl groups and then condenses to form a heterocyclic compound, which subsequently undergoes oxidation to a pyridinium ring (Figure 23) or with amine groups to form Schiff bases (Figure 24) (Damink *et al.*, 1995; Damink *et al.*, 1996a; Damink *et al.*, 1996b; Sung *et al.*, 1998; Paul and Bailey, 2003). The reactivity of aldehydes with amine groups is

considered to be higher than that with hydroxyl groups in physiological conditions (Liu *et al.*, 1999). It has been shown that amino acid residues in biological tissues reacted with GTA were lysine, hydroxylysine, histidine, arginine and tyrosine (Bowes and Cater, 1968; Meek and Chapman, 1985; Chachra *et al.*, 1996; Sung *et al.*, 1998).

Figure 23. Reactions of GTA with the ε-amino groups of collagen to form stable cyclic pyridinium ring cross-links. Source: (Paul and Bailey, 2003).

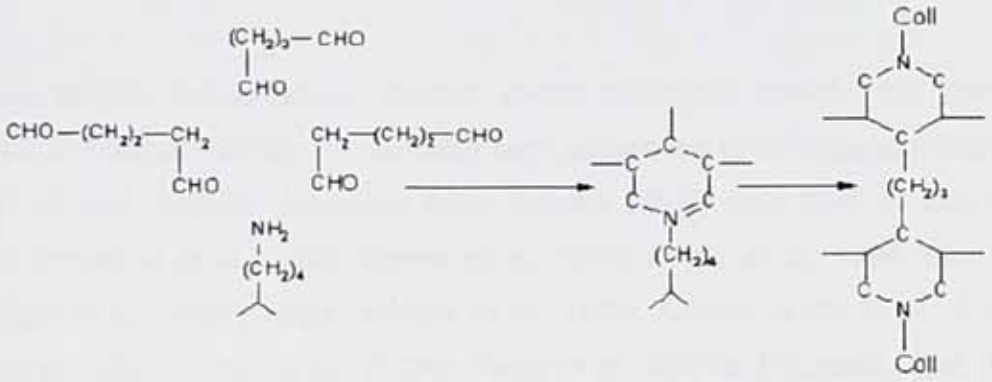
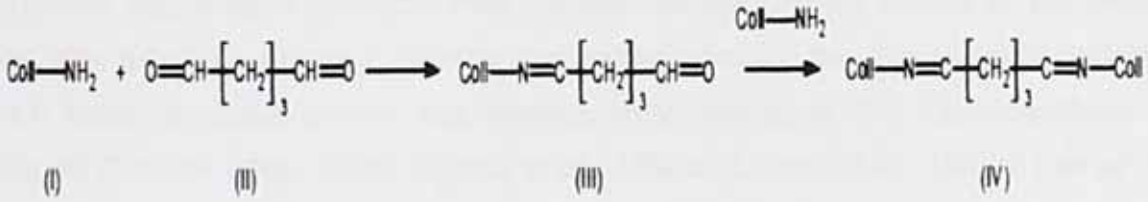
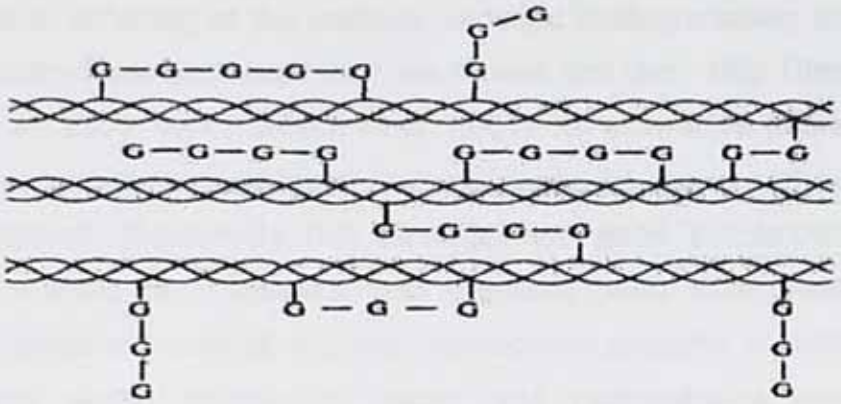


Figure 24. Reaction of amino groups of collagen (I) with GTA (II) to form Schiff base intermediates (III). Subsequent reactions of these intermediates results in the formation of a large variety of possible cross-link-moieties (IV). Source: (Wissink, 1999).



Cross-linking with GTA involves a heterogenous cross-linking distribution that occurs only on the surface of the fibrils and fibres, which leads predominantly to inter-molecular cross-links connecting distant collagen molecules (Figure 25) and prevents further penetration of reagent into the fibre and fibril structures and results in a stiffer collagen matrix (Gorham, 1991; Cote *et al.*, 1992; Mechanic, 1992; Friess, 1998).

Figure 25. Cross-linking of collagen with GTA. Intra- and inter- molecular cross-links are shown. G is the GTA monomer. Source: (Gorham, 1991).



GTA-fixed tissues (pericardium, dermal sheep collagen) exhibit high mechanical strength and thermal stability, remarkably high resistance to collagenase degradation and high *in vivo* stability. However such tissues calcify with time *in vivo* and are cytotoxic (Anselme *et al.*, 1992; Moore *et al.*, 1994; Petite *et al.*, 1994; Moore *et al.*, 1996; Chen *et al.*, 1997; Jorge-Herrero *et al.*, 1999; Adams *et al.*, 2001; Charulatha and Rajaram, 2001; Koob *et al.*, 2001a; Koob *et al.*, 2001b; Rousseau and Gagnieu, 2002). The ability of GTA to auto-polymerise probably accounts for its effectiveness as a cross-linker in protein matrices, although this process is reversible (with the subsequent hydrolysis of monomeric units) and so is also responsible for its cytotoxicity (Hey *et al.*, 1990). For formaldehyde, it has been suggested that its effectiveness as a fixative depends on its abundance rather than on its individual lengths of the cross-links (Chapman *et al.*, 1990). Un-reacted GTA or hydrolytic or enzymatic degradation products may remain non-specifically bound to the matrix even after exhaustive rinsing; thereby introducing cytotoxic non-biological derivatives, which limits fibroblast growth and consequently decreases the biocompatibility of collagen (Damink *et al.*, 1995; Zahedi *et al.*, 1998a; Zahedi *et al.*, 1998b; Lee *et al.*, 2001). Exposure to GTA vapour may limit polymerisation (Kato *et al.*, 1989). Glycine has been utilised in aqueous solution to block non-reacted aldehyde groups after cross-linking with glutaraldehyde (Tabata and Ikada, 1999; Chen *et al.*, 2000).

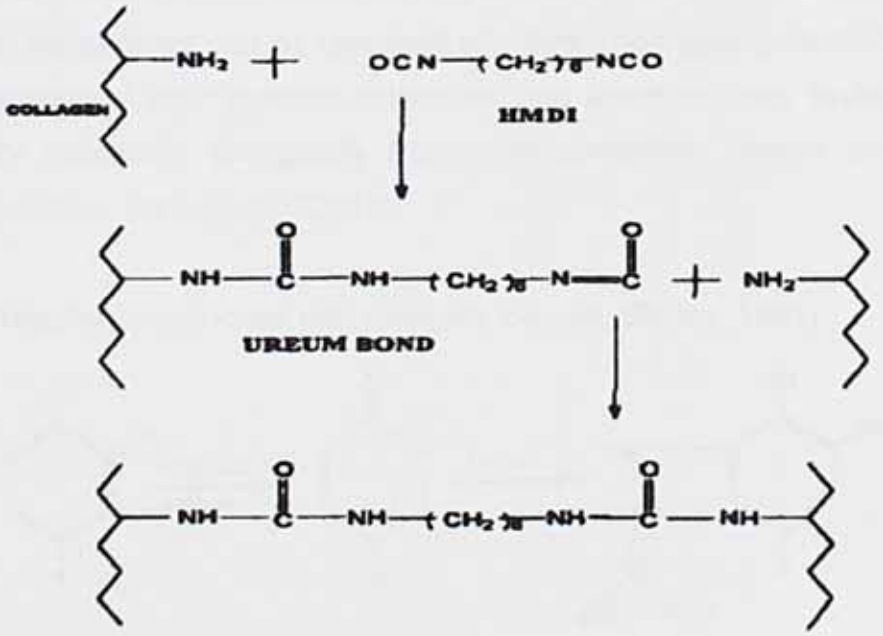
1.8.2.1.3. Starch Di-aldehyde

Formaldehyde and GTA have been used routinely to cross-link collagen, but their cytotoxic effect, stiffening of the material, reduced biodegradability and hampering endothelialisation have decreased their use (Friess and Lee, 1996; Chen *et al.*, 1997; Vaissiere *et al.*, 2000; van Wachem *et al.*, 2001). An alternative to their use for the preparation of long-life-time collagen implants would be the use of polymeric poly-aldehydes. Starch di-aldehyde has demonstrated good biocompatibility for the preparation of hydro-gels (Rousseau and Gagnieu, 2002). With di-aldehyde starch there were extensive losses of arginine. Appreciable amounts of ninhydrin positive material were eluted overlapping lysine and hydroxylysine and the peaks corresponding to proline, alanine and to a lesser extent, glycine were increased (Bowes and Cater, 1968).

1.8.2.2. Isocyanates / Diisocyanates

The isocyanate functional group reacts with amine groups on proteins and forms urea linkages. This bond is common in many molecules within the body and can undergo hydrolysis *in vivo*. The alternative reaction, if it occurs at all, would be for the terminal isocyanate to react with hydroxyl groups on proteins to form urethane link, which is relatively stable *in vivo*. However, the isocyanate-end groups react very rapidly with amines under mild conditions. The proteins (and thus lysine residues) are the targets for modification. Isocyanate exhibits two major advantages over other amine-reactive derivatives for the purpose of covalently modifying living cells. First, in the reaction of isocyanates with amines, there are no potentially toxic side products formed. Second, isocyanate functional groups react with amines at physiological pH in aqueous solutions, which is essential for the health of the cells. Although isocyanate groups are hydrolysed to amines by water, the reaction with amines is much faster than with water. Finally, the short half-life of the isocyanate group in water ensures that reactive group will not be released from the treated surface over extended time periods (Deible *et al.*, 1998; Panza *et al.*, 2000). The bi-functional reagent hexamethylene-diisocyanate (HMDC) has been used as an alternative for GTA cross-linking. HMDC will introduce aliphatic chains containing urea bonds between two adjacent amine groups (Damink *et al.*, 1995; Damink *et al.*, 1996a; Damink *et al.*, 1996b; Rault *et al.*, 1996; Sung *et al.*, 1998).

Figure 26. Reaction of isocyanates with collagen. Source: (Naimark *et al.*, 1995).



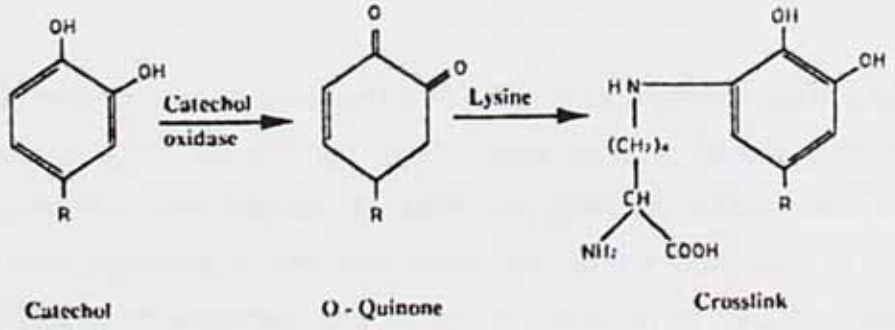
1.8.3. Natural Derived Cross-linking

Various chemical cross-linking agents (aldehydes, epoxides, isocyanates) have been used in fixing biological tissues. However, these chemicals are all highly (or relatively highly) cytotoxic, which may impair the biocompatibility of biological tissues. It is therefore desirable to provide cross-linking agents suitable for biomedical applications that are of low cytotoxicity and form stable and biocompatible end-products. Naturally occurring cross-linking agents such as nordi-hydroguaiaretic acid (NDGA) and genipin have been introduced to fix tissues (Chang *et al.*, 2002).

1.8.3.1. Quinones (NDGA)

Quinones or quininoid complexes react readily with the ε-amino lysine group of collagen. Stabilisation of some natural biological matrices can also be occurred by quinones *in vivo*, where ortho-catechols are enzymatically oxidised to o-quinones, which reacts with primary amines and forms covalent bonds that stabilise the matrices (Bailey, 1991; Paul and Bailey, 2003). Ortho-catechols of biological origin are widely used to stabilise natural biomaterials. NDGA, a plant compound with antioxidant properties, has been introduced for collagen cross-linking. It is believed that NDGA does not chemically cross-link the collagen fibrils, but it forms an NDGA polymer in which the collagen fibrils are embedded, i.e. a fibre reinforced composite (Koob and Hernandez, 2002). This process produces fibres with material properties similar to those of native tendon. Although it was found that NDGA and the residual products of the cross-linking reaction were cytotoxic to cells *in vitro*, it was shown that NDGA-cross-linked fibres can be rendered effectively non-toxic to tendon fibroblasts without diminution of their material properties, and therefore, may provide a viable approach for producing biologically based, biocompatible tendon bio-prosthesis (Koob *et al.*, 2001a; Koob *et al.*, 2001b).

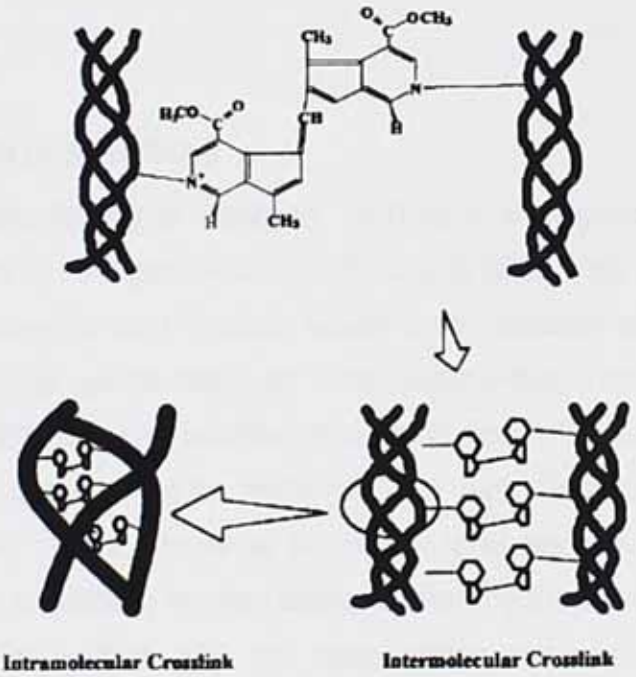
Figure 27. Reaction of quinones with collagen. Source: (Bailey, 1991).



1.8.3.2. Iridoid Glycosides (Genipin)

Genipin, an aglycone of geniposide, can be obtained from its parent compound geniposide, which may be isolated from gardenia (*Gardenia jasminoides Ellis*) plants and has demonstrated similar utility to aldehyde-fixation with significant lower toxic effects and superior biocompatibility than GTA or epoxy treated tissues at the optimum concentration (Sung *et al.*, 1998; Koob *et al.*, 2001a; Koob *et al.*, 2001b) attributed to the lower cytotoxicity of their remaining residues (Chang *et al.*, 2002; Liang *et al.*, 2004). In addition the optimal conditions for activity are pH 7.4-8.5 and temperature 25-45^{0}C, which are reasonable ranges for working with tendon and other orthopaedic tissues, and makes this substance a potential candidate for use in the development of bio-scaffolds (van Kleunen and Elliott, 2003). Genipin causes cross-linking of free amino groups, including lysine, hydroxylysine and arginine and forms intra- and inter-molecular cross-links with collagen (Figure 28). Of the reacted amino acid residues, lysine was the most reactive (Sung *et al.*, 1998).

Figure 28. Reaction of genipin with collagen. Source: (Sung *et al.*, 2000).



Genipin, has been proven to be effective at cross-linking collagenous tissues such as pericardium and aortic valves; has been widely used in herbal medicine and for various inflammatory and hepatic diseases and upon its reaction with amino acids forms dark blue pigments, which have been used in the fabrication of food dyes, in the immobilisation of enzymes and in the preparation of gelatine microcapsules (Huang *et al.*, 1998; Sung *et al.*, 1998).

1.8.4. Reactions with Carboxyl Groups

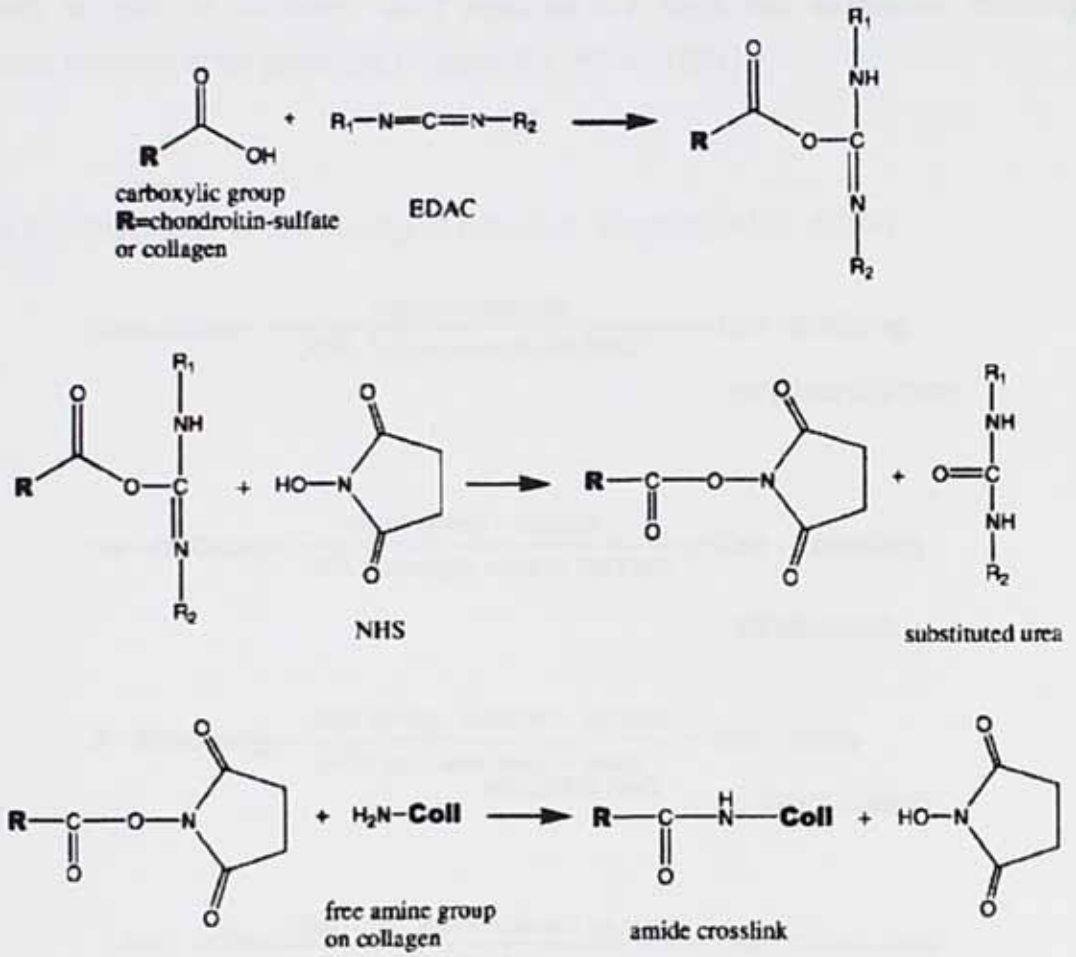
It has been reported that GTA and HMDC cross-linking of dermal sheep collagen was found to be cytotoxic with fast degradation rates. To overcome such problems, targeting of carboxyl groups as the location of inter-molecular cross-links has been introduced with carbodiimides or acyl azide methods to be representatives of this kind of cross-linkers (Paul and Bailey, 2003). Both *in vitro* and *in vivo*, it was shown to be biocompatible, and it induced regeneration of a new collagen matrix during a slow degradation rate. These reagents, therefore, could be suitable for applications such as tendon or ligament replacement (van Wachem *et al.*, 1994a; Damink *et al.*, 1996a). 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)/N-hydroxysuccinimide (NHS) and diphenyl-phosphorylazide (DPPA) appeared to be attractive alternatives to GTA fixation in biological cardiac prostheses because tissues treated with these reagents are less resistant to proteolytic attack and potencies calcification. Furthermore, EDC/NHS and DPPA do not introduce foreign cross-linking molecules (Jorge-Herrero *et al.*, 1999).

1.8.4.1. Carbodiimide (Cyanamide)

During EDC/NHS cross-linking of collagen, carboxylic acid groups of aspartic and glutamic acid residues in collagen react with EDC and NHS. This results in formation of NHS-activated carboxylic acid groups, which upon reaction with ε-amino groups from lysine and hydroxyl lysine residues form peptide-like cross-links and release NHS (Wissink *et al.*, 2001a). The addition of nucleophile NHS increases the rate and degree of cross-linking, resulting in materials with high Ts and lower free amine groups (Damink *et al.*, 1996a; Pieper *et al.*, 1999). NHS also had a beneficial effect on the immobilisation procedure as was demonstrated by the increase in the amount of attached chondroitin sulfate and the denaturation temperature (Pieper *et al.*, 2000a). These reagents are not incorporated into the amide cross-links that form (Lee *et al.*, 2001) because they act as a catalyst forming urea as a by-product (Kato and Silver, 1990); unbound and excess chemicals can be easily washed away and therefore are considered as non-toxic tanning agents (McKegney *et al.*, 2001). However, they have limited cross-linking ability because of their short length structure and inability to polymerise (Osborne *et al.*, 1998). Furthermore, it has been shown to be non-cytotoxic, biocompatible and to improve the mechanical and thermal

properties of the matrix (Damink *et al.*, 1995; Damink *et al.*, 1996a; Damink *et al.*, 1996b; Sung *et al.*, 1998; Zeeman *et al.*, 1999b; Koob *et al.*, 2001a; Koob *et al.*, 2001b; van Wachem *et al.*, 2001; Wissink *et al.*, 2001b); in combination with DHT provides optimum cross-linking (Rault *et al.*, 1996).

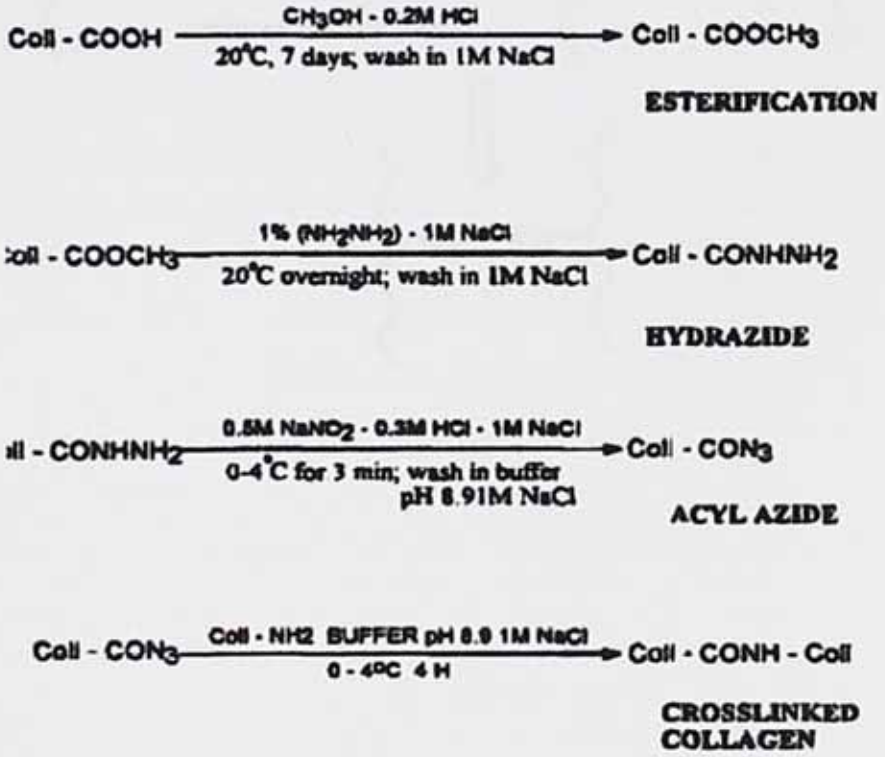
Figure 29. Collagen reaction with EDC / NHS. Source: (Lee *et al.*, 2001).



1.8.4.2. Acyl Azide

The hydrazine method has been introduced, which consists in transforming lateral carboxyl groups of collagen chains in acyl azide via formation of hydrazides, the acyl azide reacting with the amino groups of adjacent chains of collagen. The acyl azide method has been introduced as an alternative to GTA cross-linking; thermal stability, resistance to collagenase digestion and delayed calcification were observed (Anselme *et al.*, 1992; Petite *et al.*, 1994). However, the hydrazine method is unwieldy to perform routinely, as it requires 5-7 days and extensive washing for complete removal of by-products (Figure 30) (Khor, 1997).

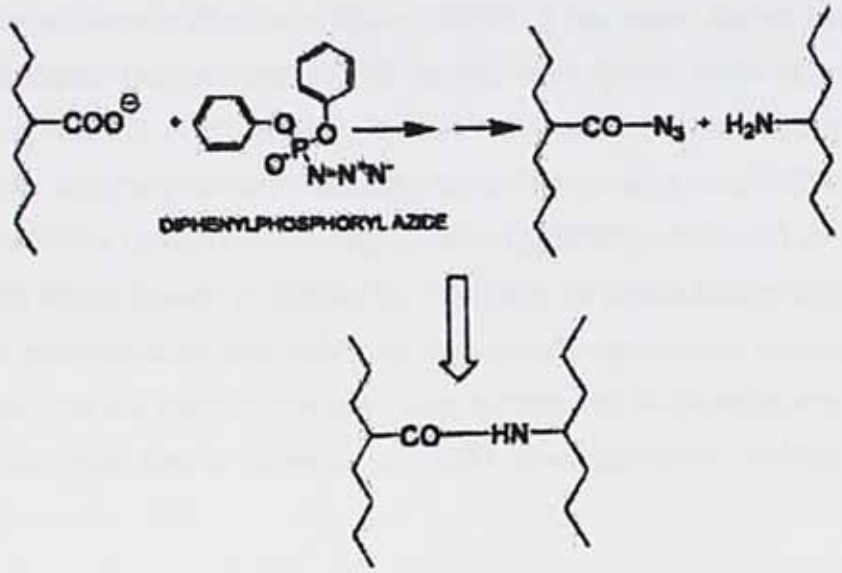
Figure 30. Acyl Azide cross-linking of collagen. Source: (Khor, 1997).



An alternative is the use of diphenyl-phosphorylazide (DPPA) to synthesise acryl azide on lateral carboxyl groups of collagen chains (Figure 31) (Jorge Herrero *et al.*, 2005). DPPA has been used previously in peptide synthesis to convert carboxylic acids into acyl azide in one step. Coupling between amino acids proceeds directly, without isolation of the intermediary acyl azide. Thus, DPPA can react with carboxylic groups of aspartic and glutamic acid residues on collagen lateral chains to form an acyl azide, which would then react with the lateral amino groups of collagen (mainly

lysine and hydroxylysine residues (Petite *et al.*, 1994; Jorge Herrero *et al.*, 2005). Furthermore, with the DPPA technique, natural cross-links between peptides chains is achieved, leaving no foreign product in the collagen protein, demonstrating biocompatibility, good handling characteristics and a resorption rate compatible with the requirements for guided tissue regeneration (Zahedi *et al.*, 1998a; Zahedi *et al.*, 1998b).

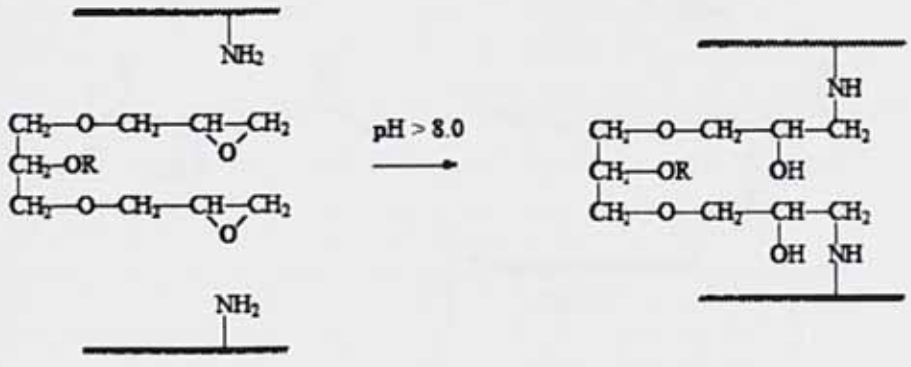
Figure 31. DPPA cross-linking of collagen. Source: (Jorge Herrero *et al.*, 2005).



1.8.5. Epoxides

Epoxy compounds have been extensively used in the past decade for the stabilization of collagen-based materials including porcine aortic heart valves (Chen *et al.*, 1997; van Wachem *et al.*, 1999). Generally mixtures of bi- and tri-functional glycidyl ethers based on glycerol are applied. The family of poly-(glycidyl ether) reagents of different molecular sizes and functionalities have been used to cross-link collagen based materials, with cross-linking occurring between a number of amino acid residues (Khor, 1997; Sung *et al.*, 1998; Courtman *et al.*, 2001). Due to its highly strained three-membered ring, epoxide groups are susceptible to a nucleophilic attack of bases and acids (Paul and Bailey, 2003). It has been shown that amino acid residues in biological tissues reacted with epoxy were lysine, hydroxylysine, histidine and arginine (Chachra *et al.*, 1996). It has been reported that epoxy compounds (ethylene glycol diglycidyl ether) exhibited lower cytotoxicity than GTA (Sung *et al.*, 1998), a remarkable resistance to degradation (probably attributed to the cleavage site of collagen being hidden or altered by the action of cross-linking agents, resulting in inhibition of enzyme-substrate interaction) markedly reduced or eliminated immune cell penetration into the body of the graft and increase of the mechanical and thermal properties of the fixed tissue (Chen *et al.*, 1997; Zeeman *et al.*, 1999b; Courtman *et al.*, 2001).

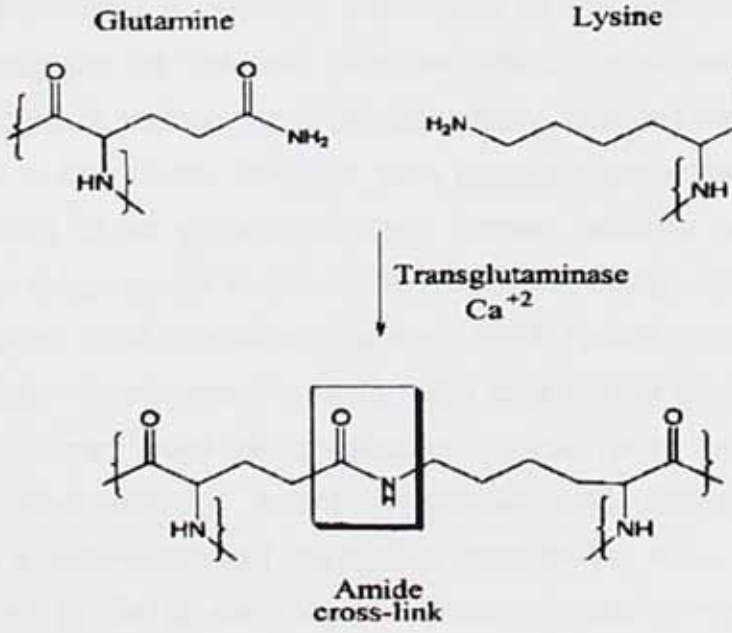
Figure 32. Reaction of epoxy compound with collagen. Source: (Zeeman, 1998).



1.8.6. Enzyme Generated Cross-linking

The enzyme trans-glutaminase (Tg) cross-links and stabilises proteins in the extra cellular matrix. It is present in most cells and tissues and therefore has the potential to be an important bioactive molecule in bone repair. It is a cell adhesion molecule, it is a calcium dependent and guanosine tri-phosphate binding enzyme that catalyses several post-translation modification of proteins by forming inter- and intra-molecular bonds between an ε-amino group of a peptide-bound lysine residue and a γ-carboxyl-amide group of a peptide-bound glutamyl residue (Bailey, 1991; Raghunath *et al.*, 1999; Babin and Dickinson, 2001; Orban *et al.*, 2004; Chen *et al.*, 2005). The isopeptide cross-link formed is common in nature, e.g., fibrin and keratin, but not in native collagen, although the extracted enzyme can be used *in vitro* to cross-link collagen and elastin (Paul and Bailey, 2003; Orban *et al.*, 2004). The formation of the Tg cross-link appears to be irreversible because it seems that nature has not provided an enzyme for the cleavage of this cross-link in proteins (Schittny *et al.*, 1997). The bonds that form are covalent and resistant to proteolysis thereby increasing the resistance of tissues to chemical, enzymatic and physical disruption. The introduction of cross-linkages with microbial Tg into protein molecules is reported to improve the strength of the concerned protein (Nomura *et al.*, 2001b). The cross-linking of extra-cellular proteins is thought to enhance cellular responses such as cell attachment, spreading and differentiation at the biomaterial interface (Heath *et al.*, 2002; Collighan *et al.*, 2004; Hung *et al.*, 2004).

Figure 33. Reaction of Tg with collagen. Source: (Orban *et al.*, 2004).



1.8.7. Carbohydrate Mediated Cross-linking

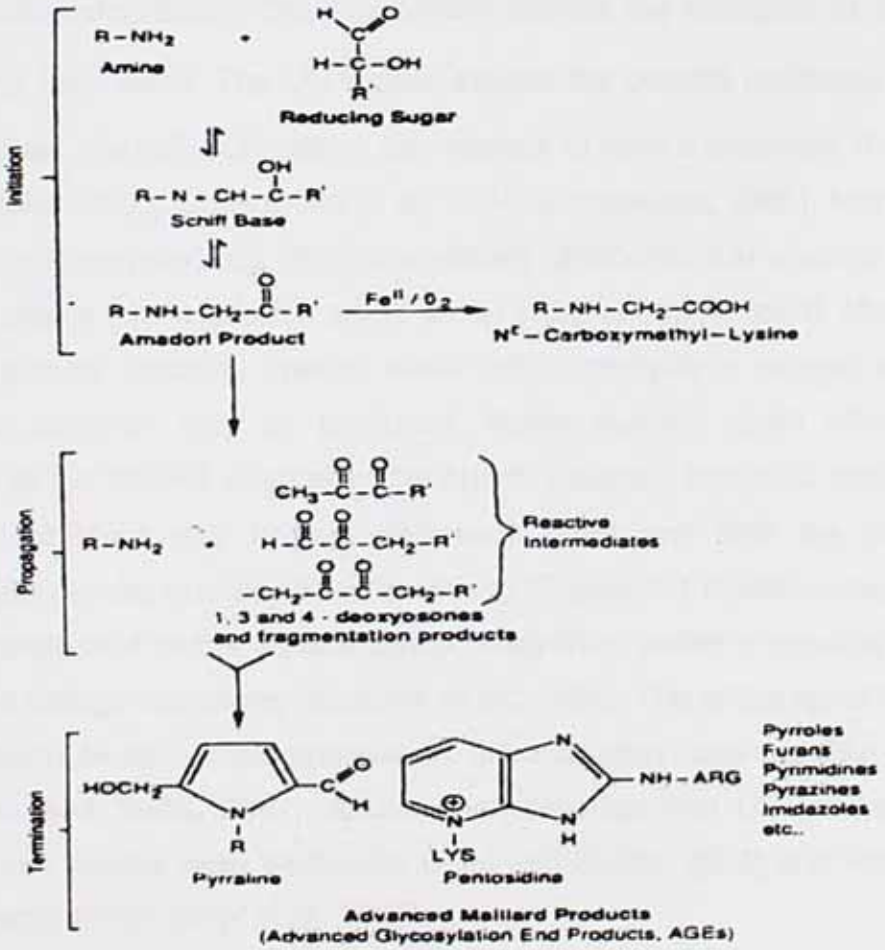
When the aldehyde / carbonyl group of reducing sugars react with the free amino groups of lysine molecules, a reversible Schiff's base is formed followed by a fairly stable ketoamine (the Amadori rearrangement product). This reactive species may then undergo further reaction to form stable covalent cross-links or degradation to glyoxal, which, as a di-aldehyde, readily forms cross-links with collagen. A complex heterocyclic product has been isolated from glycated collagen, termed pentosidine, and it arises in a reaction between free ε-groups of lysine and arginine residues in a peptide chain with free oxo-groups of pentoses or hexoses (glucose / ribose) (Monnier *et al.*, 1991; Miksik *et al.*, 1997; Mentink *et al.*, 2002). After a complex series of reactions, such as rearrangement, cyclization, fragmentation, dehydration and oxidative modification, a class of permanently modified proteins known as advanced glycation end products (AGEs) is formed (Brownlee *et al.*, 1988; Nagaraj and Monnier, 1995; Kielty and Grant, 2002; Mentink *et al.*, 2002; Wu and Monnier, 2003). This chemical reaction is termed non-enzymatic glycosylation, or "Maillard reaction" (Figure 34) (Monnier *et al.*, 1991; van der Rest *et al.*, 1993) after Louis Camille Maillard, who first described it and it is the major factor responsible for the modification in long lived proteins, such as collagen and crystallins and for the complications of diabetes mellitus, aging, Alzheimer, Parkinson, Alexander, arteriosclerosis and diffuse Lewy body disease (Paul and Bailey, 1996; Castellani *et al.*, 1997; Bailey *et al.*, 1998; Hong *et al.*, 2000; Bailey, 2001; Mentink *et al.*, 2002).

Initially glucose affects the collagen side-chains, which in turn affect the cell-collagen interactions through the integrins and collagen-matrix component interactions, but the most deleterious effects of glycation are caused by the formation of intermolecular cross-links throughout the molecule, which increase stiffness, decrease solubility and total collagen content, reduce proteolytic digestibility, increase heat denaturation time and results in a loss of the ability to form precise supramolecular aggregates in tissues, including blood vessels, tendons, bones, cartilage and other musculo-skeletal tissues (Nagaraj and Monnier, 1995; Paul and Bailey, 1996; Paul and Bailey, 1999; Reddy *et al.*, 2002; Fodil-Bourahla *et al.*, 2003; Reddy, 2003; Aronson, 2004).

Glycated collagen fibres were shown to resist degradation by collagenases, whilst carbohydrate induced cross-linking (glucose / ribose) were found to be relatively ineffective cross-linkers for sclera (Wollensak and Spoerl, 2004). Glucose demonstrated a dose-dependent increase in cross-linking, while ribose and fructose has been shown to give an even faster increase in cross-linking (Benazzoug *et al.*, 1998; Mentink *et al.*, 2002). Thus, the biomechanical changes of the tendons

glycated with glucose may be less obvious than those glycated with ribose (Bailey, 2001; Reddy, 2003). To conclude, inhibition of the toxic carbonyl compounds can be achieved through anti-oxidants, enzymatic inactivation and free radical scavengers (Monnier *et al.*, 1991; Fu *et al.*, 1992; van der Rest *et al.*, 1993; Bailey, 2001; Monnier, 2003). Collagen incubated with *finger millet* and *kobo millet* extracts (antioxidant activity / high nutritional value) inhibited glycation (Hegde *et al.*, 2002). Furthermore, aspirin is thought to act as an anti-glycation agent by acetylating free amino groups on proteins, and limiting the sugar-induced formation of Amadori products AGEs. Aspirin also inhibits the increase in the collagen molecular spacing seen with glycation (Malik and Meek, 1994). However, incubation of sugars with aspirin showed that sugars were still able to bind to collagen, suggesting that the inhibitory functions of aspirin are more likely to be post-Amadori; supporting the theory that aspirin acts as a free radical scavenger. Furthermore, it has been shown that in the presence of aspirin neither bovine nor human cornea swelled (Hadley *et al.*, 2001).

Figure 34. General scheme of Maillard reaction. Source: (van der Rest *et al.*, 1993).



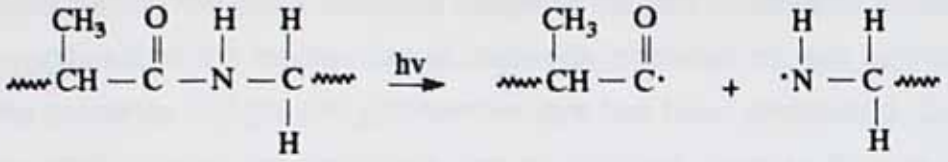
1.8.8. Physical Treatments

Physical and chemical cross-linking techniques are used in order to control the rate of biodegradation of collagen and to improve its thermal and mechanical properties. Physical methods of cross-linking have the advantage of avoiding the inclusion of foreign substances either as residual molecules or as compounds formed during *in vivo* degradation. However, they generally provide a low density of cross-links (Friess, 1998; Zahedi *et al.*, 1998a; Zahedi *et al.*, 1998b).

1.8.8.1. Ultra Violet (UV) Irradiation

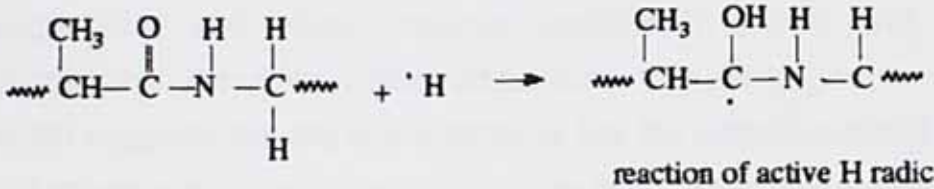
UV irradiation is a good method of stabilisation of biomedical materials. Formation of cross-links during UV-irradiation is thought to be initiated by free radicals formed on aromatic amino acid residues, which indicates a rather limited maximum degree of cross-linking due to the small number of tyrosine and phenylalanine residues in collagen. These amino acids are the sensitive chromophores, which may initiate the photo-degradation of collagen chains and photo-destruction via the cleavage of side groups (Sionkowska, 2006). The mechanism causes the formation of the hydroxyl radical ($OH^{\bullet}$) from water. The OH radical attacks the peptide backbone to produce peptide radicals (-NH-$C^{\bullet}$-CO-), which can interact to form a cross-link (Friess, 1998; Paul and Bailey, 2003; Sionkowska *et al.*, 2004; Sionkowska, 2005). In the presence of water-soluble polymer, e.g. PEG, the primary photochemical reaction is breaking of polymer chains (chain scission arises at the weak chemical bond). Macro-radicals formed in primary process, interact easily with atmospheric oxygen and various products of oxidation may be produced. Active radicals again initiate polymer destruction of the second polymer in the blend. Collagen and PEG radicals formed during UV exposure may interact with each other and alter the properties of collagen/PEG blends, resulting in cross-linking (Figure 35) (Sionkowska, 2006). UV irradiation is efficient for the introduction of cross-links, which is especially useful for treatment of collagen solutions (Vizarova *et al.*, 1994). The efficiency of the reaction depends mainly on the sample preparation, the irradiation dose and time of exposure (Sionkowska and Wess, 2004). It has been reported that UV irradiation of wet collagen fibres causes rapid insolubility (Paul and Bailey, 2003) and increases their tensile strength (Bellincampi *et al.*, 1998).

Figure 35. Possible mechanisms of transformations in collagen/PEG blend induced by UV irradiation. Source: (Sionkowska, 2006).

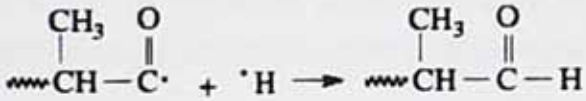


chain scission of collagen

formation of H radical from PEG



reaction of active H radical with collagen



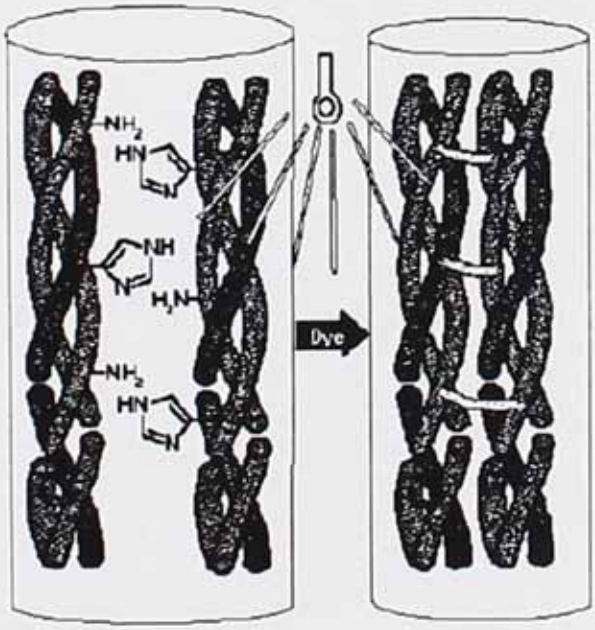
reaction of new formed radicals

1.8.8.2. Dye-Mediated Photo-oxidation

Dye-mediated photo-oxidation has been used to explore the role of specific amino acids in proteins. This process involves the use of a photo-oxidative dye (methylene blue, methylene green, rose Bengal, riboflavin, proflavin, eosin, etc), which acts as a cross-linking oxidation catalyst or promoter and which can be removed from the cross-linked product after catalysing the formation of inter- and intra-molecular cross-links. The intensity of the light employed and the length of time required to cross-link a given tissue will vary depending upon the type and the amount of the tissue, the thickness of the sample, the distance between the sample and the irradiation source, the catalyst employed, the concentration of the catalyst and the type and the intensity of the light source (Mechanic, 1992). This treatment will result in modification of

histidine, tryptophan, tyrosine and methionine residues, with possible additional changes as well. New cross-links may be formed and increase the bio-stability of the fixed tissue. The exact chemical nature of the new cross-links is unknown; however a hypothesis for the mechanism of cross-link formation caused by the tissue reaction in the presence of light and photoactive dye has been introduced. Oxidation has been reported to alter the imidazole ring of histidine, leading to side chains containing either aldehyde end groups or an imidazole peroxide. These activated histidine-derived side chains may then react with lysine to form stable, intermolecular cross-links (Figure 36). It has been noted that photo-oxidation of a collagen solution resulted in the formation of a protein mass "no longer soluble under the most extreme denaturing conditions" (Moore *et al.*, 1994; Adams *et al.*, 2001). Photo-oxidation of pericardium tissue yields a material with a relatively small shrinkage temperature change, yet with resistance to protein extraction, cyanogen bromide digestion, proteolytic enzyme degradation, *in vivo* calcification, high tensile strength, *in vivo* degradation and which supports endothelial growth and is biocompatible. Remarkably, the denaturation temperature was similar to the untreated material, which suggests that the tissue behaves like the original and that the cross-links did not influence the tissue character (Moore *et al.*, 1994; Moore *et al.*, 1996; Moore and Adams, 2001). However, cross-linking by rose Bengal was found to be a relatively ineffective cross-linkers for sclera (Wollensak and Spoerl, 2004).

Figure 36. Cross-linking using dye mediated photo-oxidation. Source: (Adams *et al.*, 2001).



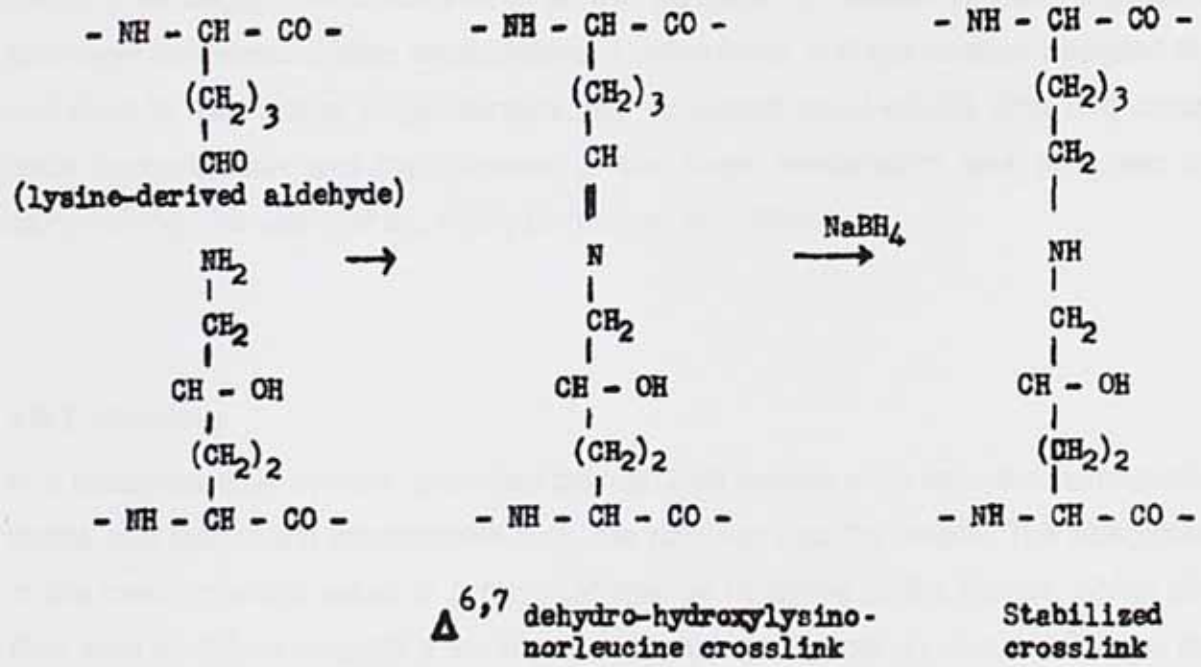
1.8.8.3. Dehydrothermal (DHT)

DHT cross-linking is a physical method, which is applied to stabilise collagen and to prevent matrix collapse while no cytotoxic reagents are introduced (Matsumoto *et al.*, 1999). The number and types of cross-links formed by DHT treatment remain unclear. Stabilisation is believed to be due to the formation of inter-chain cross-links as a result of condensation reactions either by amide formation or esterification between carboxyl and free amino and hydroxyl groups respectively (Cote *et al.*, 1992; Friess, 1998; Pieper *et al.*, 1999). Possible reactive amino acid residues involved in DHT cross-linking include lysine, aspartic acid, glutamic acid, arginine, serine, threonine and alanine. 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 improve the tensile strength of dry collagen fibres by limiting inter-fibrillar slippage; in the wet state it is likely that cross-linking also limits swelling of the fibres. DHT cross-linking of collagen involves removal of residual water. It has been shown that these structurally important water molecules are not removed during air-drying. However, DHT removes irreversibly the water trapped within collagen molecules and therefore enhances chemical bonds within collagen (Cote and Doillon, 1992; El Feninat *et al.*, 1998). After DHT treatment, the helix-to-coil transition temperature is increased, enhancing the thermal stability of collagen without altering its triple helical structure (Wang *et al.*, 1994).

1.8.9. Chemical Modification of Collagen (Reduction)

The major intermolecular covalent cross-links in the tissues of immature animals formed through lysyl oxidase as aldimine or oxoimines (formerly known as ketoimines) and may be chemically reduced with agents such as sodium borohydride (Figure 37) (Bailey and Peach, 1968; Bailey *et al.*, 1998). The resulting compounds are acid and heat labile (Bailey and Light, 1989). Mild reduction with sodium borohydride stabilises these labile imine bonds into stable secondary amines rendering the collagen insoluble, increasing the mechanical strength and the denaturation temperature. Furthermore, reduction with sodium borohydride allows cell adhesion and proliferation (Rousseau and Gagnieu, 2002; Paul and Bailey, 2003). Furthermore, borohydride reduction has been shown to decrease fluorescence background during carbohydrate mediated cross-linking (Miksik *et al.*, 1997).

Figure 37. The formation, rearrangement and reduction chemistry of the oxoimine reducible cross-links of collagen. Source: (Bailey and Peach, 1968).



1.9. Cross-linking Efficiency

While there are a number of methods for examining the physical state of collagen in solution, many of these, e.g. polarimetry, spectrophotometry, and viscosity are not applicable to measurement of the fibrous tissue itself. Differential Scanning Calorimetry (DSC) provides a potential method for examining changes in connective tissue collagen *in situ*, sensitive to addition of covalent cross-links and to reductions in triple-helical content (Hormann and Schlebusch, 1971; Miles *et al.*, 1995; Mentink *et al.*, 2002). The effect of cross-linking and/or stabilising the collagen structures can be indirectly evaluated from the swelling measurements (Vizarova *et al.*, 1994).

1.9.1. Differential Scanning Calorimetry (DSC)

Calorimetric analysis by DSC allows measurement of the thermal stability of the collagen triple helical structure. In evaluating the fixation of biological tissue, denaturation temperature is often used as an indicator of the degree of cross-linking of the fixed tissue (van Wachem *et al.*, 1994a; Chachra *et al.*, 1996; Chen *et al.*, 2005). The degree of cross-linking of the samples is related to the increase in shrinkage temperature after cross-linking. Furthermore, a linear relation between the decrease in free amine group content, the increased cross-linking time and cross-linker concentration and the increase in shrinkage temperature was observed for cross-linking (Cavallaro *et al.*, 1994; Damink *et al.*, 1995).

1.9.2. Swelling

In a biological environment, polymers both absorb components from the surrounding media and also leach components from the polymer into the media. The absorption of the chemicals can result in a physical change in shape of the device, which can then lead to failure through a variety of mechanisms. Under *in vivo* conditions, the leaching of plasticisers, stabilisers, antioxidants, pigments, lubricants, fillers, residual monomers, polymerisation catalysts and other chemical additives from plastics or rubbers both change the properties of the polymer and cause local or synthetic toxicological problems (McMillin, 1996).

An integral part of the physical behaviour of hydro-gels is their swelling behaviour in water, since upon preparation they must be brought in contact with water to yield the

final, solvated network structure. Knowledge of the swelling characteristics of a polymer is of paramount importance in biomedical and pharmaceuticals applications since the equilibrium degree of swelling influences the solute diffusion co-efficient through these hydro-gels, the surface properties and surface mobility, the optical properties, especially in relation to contact lens applications and the mechanical properties (Peppas, 1996). Furthermore, the wet-ability / swelling ratio of a polymer scaffold is considered very important for homogenous and sufficient cell seeding in three dimensions (Chen *et al.*, 2000; Chen *et al.*, 2001; John *et al.*, 2001).

Much experimental evidence has established the important role of water in stabilising the triple helical structure of collagen. Using X-ray diffraction it has been shown that the characteristic collagen structure is dependent on the water content of the protein. Furthermore, it has been shown that a limited amount of water is firmly bound to the helix backbone (probably to amide groups) and collagen configuration is stabilised by water bridges between adjacent peptide carbonyl groups (Luescher *et al.*, 1974). Since cross-linking could affect the water binding sides, cross-linking efficiency could be evaluated by swelling studies.

1.10. Applications of Collagenous Materials

The use of collagen-based materials for a variety of clinical, cosmetic, biomedical and food applications has been greatly increased (Smith and McEwan, 1990; Yarat *et al.*, 1996). The attractiveness of collagen as a biomaterial rests largely on the view that is a natural material and is therefore seen by the body as a normal constituent rather than a foreign matter (Friess, 1998), although it has been reported that potential allergic reactions to collagen as a foreign protein can be induced (Wells *et al.*, 1997). Impurities and endo-toxins in commercially available preparations can potentially transmit disease or act as an adjuvant in eliciting an immune response upon implantation, advancements in purification methods and analytical assays have essentially assured minimal immunogenicity (Liu *et al.*, 1999). Additionally, collagen possesses many desirable features making in an excellent choice as a biomaterial, among which are its high tensile strength, controllable cross-linking, inexpensive and easy to prepare in large reproducible quantities, biodegradability, haemostatic properties, low antigenicity and extensibility, high degree of resistance to proteolysis, bio-stability, ease of handling, low inflammatory and cytotoxic properties and its ability to promote cellular attachment and growth and consequently tissue healing and regeneration (Hey *et al.*, 1990; Yarat *et al.*, 1996; Wells *et al.*, 1997; Matsumoto *et al.*, 1999; Charulatha and Rajaram, 2001; McKegney *et al.*, 2001). Furthermore, its association with growth factors or other extra-cellular components, such as proteoglycans, seeding of osteogenic cells and chemical modifications could enhance its applicability as a biomaterial (Wells *et al.*, 1997; Rocha *et al.*, 2002).

Furthermore, it is easy available in large quantities and purification procedures and fabrication processes have made it possible to make collagen products of different textures and shapes (Rault *et al.*, 1996). Collagen based biomaterials in different physical forms (fibres, films, sponges, hydro-gels, powder) are widely used for tissue engineering, for soft and hard tissue repair (Hofman *et al.*, 1999; Schoof *et al.*, 2001). The use of collagen sponges from porcine tendon (John *et al.*, 2001), bovine corium (Wallace *et al.*, 1992) or combinations of collagen and silicone or polyurethanes (Yamamoto *et al.*, 1999; Kawai *et al.*, 2000; Ulubayram *et al.*, 2001) has been proposed for the treatment of severe burns, drug delivery and haemostatic applications, scaffold for tissue-guided regeneration, cerebral, peripheral nerve, pulmonary, vascular and hepatic implants and biodegradable composites (Anselme *et al.*, 1992; Cote *et al.*, 1992; Ueda *et al.*, 2002). Collagen films have been developed for clinical use as burn and wound dressings, abdominal wall repair, dura matter replacement, haemostatic/control bleeding agent, enhancer of healing of open

dermal wounds, organ replacement, surgical tampons and bone/skin reinforcements (Collins *et al.*, 1991; Cote and Doillon, 1992; Pachence, 1996; Yarat *et al.*, 1996; Peterkova and Lapcik, 2000; Tiller *et al.*, 2001). Collagen hydro-gels have a long history in biomedical applications (corneal shields, hard and soft tissue implants, drug delivery matrices, injectable suspensions) (Rosenblatt *et al.*, 1994; Blanco *et al.*, 1999; Evans *et al.*, 2001; Hutcheon *et al.*, 2001; Hunter *et al.*, 2002). Collagen fibres have been extensively used as substrate for nerve regeneration, bone, tendon and ligament replacement, wound dressing applications, suture materials and knitted into meshes for vascular applications because their unique/advantageous properties, such as high surface area, softness, absorbency and easy of fabrication into many product forms. Fibres made from natural sources (collagen, celluloses, alginates, chitin and chitosan) have been considered the most promising due to their excellent biocompatibility, non-toxicity, and potential bioactivity at the wound surface and beyond (Kato and Silver, 1990; Cavallaro *et al.*, 1994; Wang *et al.*, 1994; Pins and Silver, 1995; Kojima *et al.*, 1998; Ueno *et al.*, 1999; Comut *et al.*, 2000; Menard *et al.*, 2000; Rhee *et al.*, 2001; Yamada *et al.*, 2001; Knill *et al.*, 2004). Such biodegradable polymers have been extensively used in tissue engineering to fabricate three-dimensional scaffolds for the regeneration of tissues and must have sufficient strength, biocompatibility and controllable biodegradability and must have a pore structure that allows cell colonisation and growth of both skin fibroblasts and keratinocytes (Narotam *et al.*, 1993; Hanthamrongwit *et al.*, 1994; van Wachem *et al.*, 1994a; Lee *et al.*, 1998; Chevallay *et al.*, 2000; Roche *et al.*, 2001). It is essential that the physical properties of these materials must be similar to the tissue that is being replaced, apart from being biologically acceptable (Charulatha and Rajaram, 2003).

1.11. Overall Aim of the Study Based on Previous Work

The aim of the study presented in this thesis was to study factors which control the structure and properties of reconstituted collagen fibres. Whilst there is some literature in the field there is relatively little understanding of what controls the biophysical characteristics of such materials.

Most studies prior to this research were based either on acid soluble rat-tail tendon (Wang *et al.*, 1994; Pins and Silver, 1995; Christiansen *et al.*, 2000) or acid soluble bovine collagen (Kato *et al.*, 1991; Dunn *et al.*, 1993; Cavallaro *et al.*, 1994). For these materials the telopeptides remain intact; therefore a less biocompatible material is produced (Section 1.6.2.). It was apparent that no work on reformed collagen fibres had directly compared different extraction routes (acid and pepsin solubilisation) and it was a further aim of the present research to make such a comparison and to develop our knowledge about fibres produced from pepsin solubilised collagen. Also in the case of rat tails the material is not likely to find practical application due to ethical considerations. There is a need to look at other collagen sources and this is addressed in the work reported in this thesis.

In an attempt to optimise the properties and investigate the applications of reconstituted collagen fibres, different experiments were carried out comparing continuous with non-continuous formed fibres (Kato and Silver, 1990), using different diameter extrusion tubes (Dunn *et al.*, 1993), investigating the biocompatibility of the fibres (Kato and Silver, 1990; Dunn *et al.*, 1992; Chvapil *et al.*, 1993; Cavallaro *et al.*, 1994), their interaction with proteoglycans (Pins *et al.*, 1997b) or even the mineralization of the fibres (Christiansen and Silver, 1993). Furthermore, based on the fact that the *in vitro* self-assembly of collagen is dependent on pH, temperature, presence of electrolyte and ionic strength (Section 1.4.2.), the effect of pH and temperature on the formation of collagen fibres have been investigated (Christiansen *et al.*, 2000). Although some work has been carried out investigating the influence of sodium chloride (Christiansen and Silver, 1993; Dunn *et al.*, 1993; Wang *et al.*, 1994; Pins and Silver, 1995; Christiansen *et al.*, 2000) or polyethylene glycol (Cavallaro *et al.*, 1994; Laude *et al.*, 2000) on the properties of reconstituted collagen fibres, there is no study available either directly comparing these agents or indeed examining the effects of replacing them with other compounds.

Furthermore, since the physical properties of collagenous materials are dependent on the cross-linking method adopted, it is important to determine the alteration of physical properties that result from cross-linking (Charulatha and Rajaram, 2003). At present, there is no commonly accepted ideal cross-linking treatment for collagen-derived bio-prostheses. Thus, it is necessary to evaluate different cross-linking reagents and compare their ability to provide suitable characteristics for different applications. Indeed, several cross-linking methods have been studied and evaluated for collagenous materials (Section 1.8.), but only a few for reconstituted collagen fibres; GTA (Kato and Silver, 1990; Dunn *et al.*, 1992; Dunn *et al.*, 1993), formaldehyde (Rajini *et al.*, 2001), chromium (Rajini *et al.*, 2001), DHT (Dunn *et al.*, 1993; Wang *et al.*, 1994; Pins and Silver, 1995), cyanamide (Kato and Silver, 1990; Dunn *et al.*, 1993), EDC (Dunn *et al.*, 1992), NDGA (Koob *et al.*, 2001a; Koob *et al.*, 2001b; Koob and Hernandez, 2002). However, there appears to be no literature, which has compared experimentally all the different cross-linking methods in one type of any collagenous structure. Therefore it was felt that it would be of much value to investigate the effects of a wide range of different cross-linking techniques on the properties of reconstituted collagen fibres and at the same time evaluate the influence of the cross-linking method on the structural, thermal and mechanical characteristics of the reconstituted fibre.

1.12. Aims and Objectives

Main Aim: The overall aim of this work was to study and better understand the influence of factors which control the structural characteristics and the mechanical properties of reconstituted collagen fibres prepared by extruding collagen solutions into a fibre formation bath.

Specific Aims: To better understand how to optimise the properties of such fibres, the process was divided into three broad areas of investigation:

- Pre-fibre formation:
  - To compare and evaluate the influence of different collagen tissues, extraction methods and collagen concentrations.
  - To establish the influence of extrusion tube diameter.

- During-fibre formation:
  - To investigate and compare the effects of different co-agents and different amounts of co-agents present in the fibre formation bath.

- Post-fibre formation:
  - To investigate and compare the effects of different fibre washing baths.
  - To compare the effects of different cross linking methods on fibre properties in both wet and dry conditions

Chapter 2. Choice of Raw Material

2.1. Aim

The aim of the work described in this chapter was to compare structural and mechanical properties of reconstituted collagen fibres derived from rat-tail tendon, bovine Achilles tendon and pigtail tendon. Furthermore, a preliminary comparison between acid and pepsin soluble collagen based on mechanical characteristics was carried out.

2.2. Materials and Methods

All suppliers are listed in Appendix.

2.2.1. Raw Materials

For this study three different animal tissues were utilised:

- Rat-tail tendon.
- Bovine Achilles tendon.
- Pigtail tendon.

The procedure for the isolation and purification of collagen, as well as the reconstitution of the collagen fibres, described below, was the same, independent of the tissue utilised, unless otherwise stated and was based on previous research work with slight modifications (Cavallaro *et al.*, 1994; Pins *et al.*, 1997a).

2.2.2. Rat- and Pig- Tail Tendon-Collagenous Tissue Isolation

Rat- and pig- tails were transferred in a thermo-protected container in ice. Tail tendon collagen fibres were obtained after manually dissecting them out from the surrounding fascia of Sprague-Dawley rats and Large-White piglets. A haemostatic ring was clamped on the thin end of the tail, a tensile force was applied on the clamped end, and the tendon fibres were pulled out of the tendon. The process was repeated until no additional fibres were removed upon subsequent clamping and application of force. Tendon fibres were then repeatedly washed with distilled water and kept frozen until use.

2.2.3. Bovine Achilles Tendon-Collagenous Tissue Isolation

Bovine Achilles tendons were collected from a slaughterhouse, transferred in ice and manually dissected out from the surrounding fascia. They were extensively washed with distilled water and frozen. The frozen tendons were minced through a 0.75cm grinder plate with ice, in order to avoid heat denaturation and then kept frozen until use.

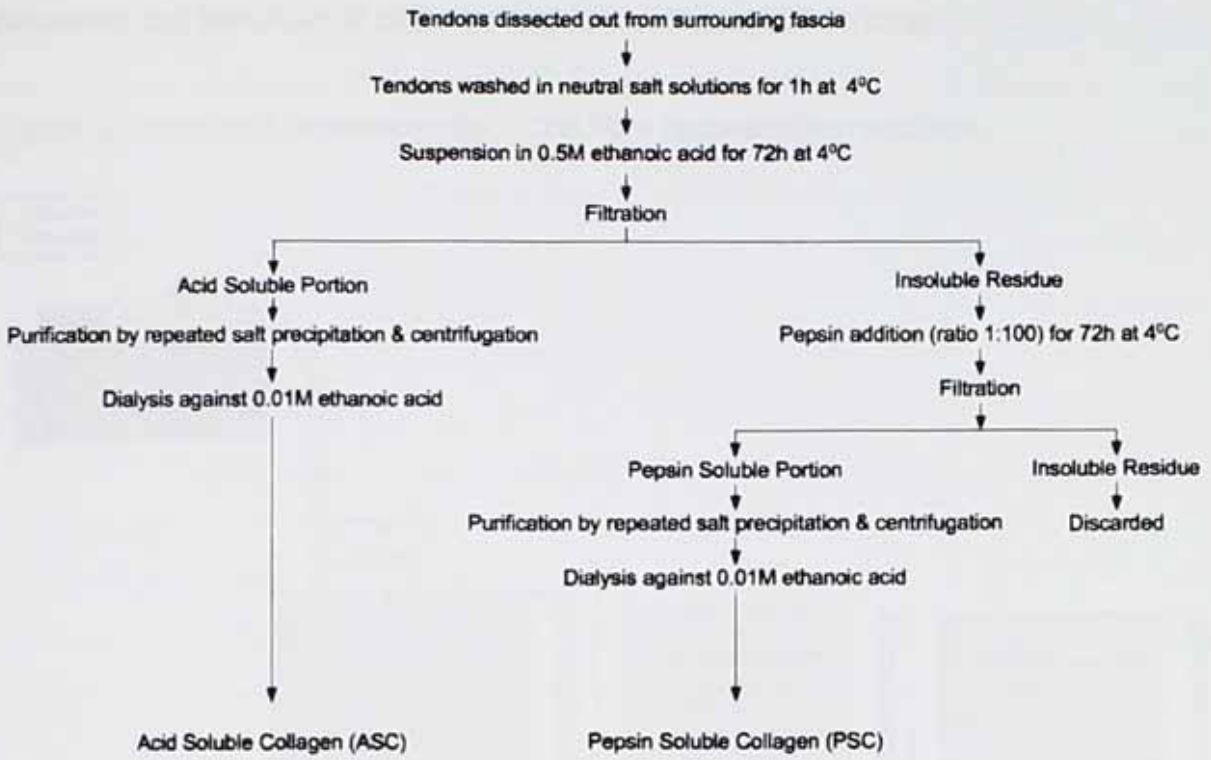
2.2.4. Acid Extraction of Collagen

The frozen tendons were washed at 4^0C for an hour in three changes of 50vol (vol/wt) of 3.7mM and 0.35mM Phosphate Buffer and 51mM sodium chloride, at pH 7.5 and subsequently were suspended in 0.5M ethanoic acid [50vol (vol/wt)] at 4^0C for 72 hours in order to extract soluble collagen. The suspension was filtered through a 1mm nylon screen to separate the solubilised collagen from the insoluble residue. The acid soluble collagen was purified by repeated precipitation in 0.9M sodium chloride, centrifugation at 12,000g, at 4^0C for 45min and re-dissolution in 1M ethanoic acid. The collagen solution was dialysed (8,000 Mw cut off) against 0.01M ethanoic acid and kept either refrigerated or frozen until used (Figure 1).

2.2.5. Pepsin Extraction of Collagen

The insoluble residue from the acid extraction (Section 2.2.4.) was re-suspended in 50vol (vol/initial wt) of 0.5M ethanoic acid and pepsin [enzyme to initial (tendon) wet weight ratio 1:100 (Steven *et al.*, 1969; Kopp *et al.*, 1989)] for 72h at 4^0C. The suspension was filtered through a 1mm nylon screen and the pepsin soluble collagen purified by repeated salt precipitation, centrifugation and re-dissolution in ethanoic acid as described above (Section 2.2.4) (Figure 1). The final collagen solutions were either kept refrigerated or frozen until used. The collagen concentration was determined by hydroxyproline assay (Appendix) and the purity by SDS-Page Electrophoresis assay (Appendix).

Figure 1. Flow diagram of the collagen solutions purification.



2.2.6. Fibre Formation

A 5ml syringe containing the collagen solution was loaded onto a syringe pump system and set to infuse at 0.4ml/min. One end of a silicone laboratory tube (20cm in length and 1.5mm internal diameter) was connected to the syringe with the other end placed at the bottom of a container, which was connected to a trough. Collagen solution was extruded into a "Fibre Formation Buffer" (FFB) comprising of 118mM Phosphate Buffer and either 20% of polyethylene glycol (PEG), Mw 8,000, or 20% Salt at pH 7.55 and 37^{0}C. The collagen instantaneously gelled on contact with the neutral pH solution and the fibre began to form. Since the free end of the silicone tubing was placed at the bottom of the container, the fibres produced could rise due to buoyancy. When they reached the surface, a stream of air was applied in order to direct them through the trough. Fibres were allowed to remain in this buffer for a maximum period of 10min and then, using an aluminium wire (Figure 3), were transferred and immersed for a further 10min in a "Fibre Incubation Buffer" (FIB) comprising of 6.0mM Phosphate Buffer and 75mM sodium chloride at pH 7.10 and 37^{0}C. Finally, the fibres were transferred in a distilled water bath for further 10min at room temperature and then air-dried under the tension of their own weight at room

temperature. The length of fibres produced was limited by the length of the FFB container and therefore all of them were approximately 30cm long.

Figure 2. Schematic representation of the fibre reconstitution process.

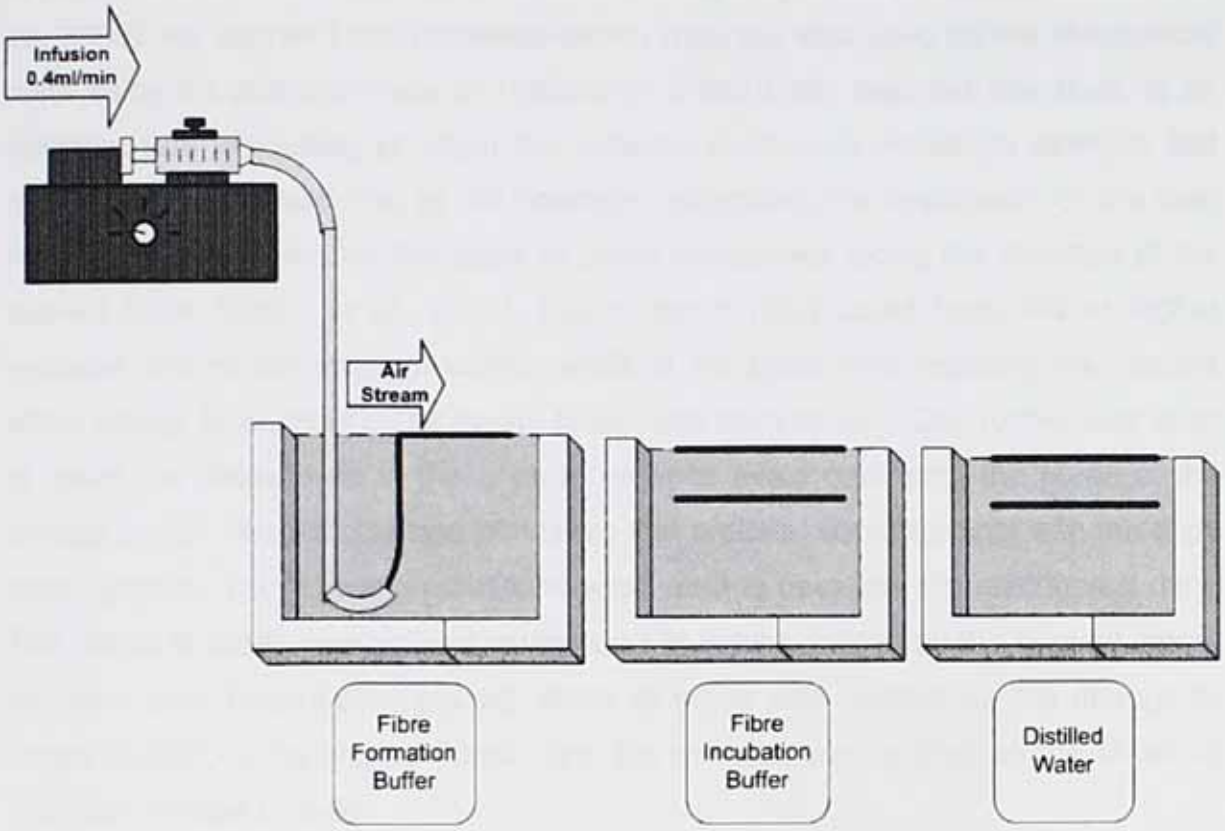


Figure 3. Transferring of fibres through the various containers.

2.2.7. Mechanical Testing

There are many different methods available for the determination of the mechanical properties of materials. However, in the present study a uni-axial tensile test was used due to the nature of the material and the simplicity of the procedure (Osborne *et al.*, 1998). An Instron 1122 Universal testing machine was used for the mechanical tests using an extension rate of 10mm/min. It has been reported that there is an optimum rate of loading at which the collagen fibres give maximum strength and extension. It is argued that at the optimum rate during the application of the load there is sufficient time for the fibrils to orient themselves along the direction of the applied force (Rajini *et al.*, 2001). Higher strain rates could have led to higher modulus and tensile strength values, while at the same time reducing the fracture strain (Wang *et al.*, 1998). The gauge length was fixed at 5cm. Soft rubber was used to cover the inside area of the grips in order to avoid damaging the fibres at the contact points. Results obtained with fibres that broke at contact points with the grips were rejected. The following definitions were used to calculate the mechanical data. The stress at break was defined as the load at failure divided by the original cross-sectional area (engineering-stress); strain at break was defined as the change in length divided by the original length; and the modulus was defined as the stress at 2% strain divided by 0.02.

2.2.8. Microscopy

A Nikon Eclipse E600 optical microscope fitted with a calibrated eyepiece and a Hitachi S3000 Variable Pressure Scanning Electron Microscope (VPSEM) were used to carry out observations of the microstructure of the fibres. The cross sectional area of each fibre was calculated by measuring the diameter at five places (every 1cm) along the fibre longitudinal axis using the optical microscope. It was assumed that the fibres were circular in making cross-sectional area determinations. Fracture surfaces of collagen fibres that had been extended to failure during mechanical testing were examined by SEM.

2.2.9. Viscosity Measurements

Viscosity measurements were made using a Brookfield Viscometer (DV-III Model). The temperature (9.6-9.9^{0}C) of the collagen solutions was maintained during the measurements by means of a thermostatically controlled tank. A fixed volume of collagen solution was measured each time. Readings were taken, using the spindle No CP42, at rotational speeds of 0.10, 0.20, 0.30, 0.40, 0.50 and 0.60rpm. Scale values were read every 5sec under shear. For each collagen solution the measurements were an average of three replicates (Ibanoglu, 2002).

2.3. Results and Discussion

It has been suggested that the characteristics of collagen scaffolds are affected by polymer concentration and molecular weight or inherent viscosity, water phase %, as well as any surfactants or electrolytes added to the water phase (Whang *et al.*, 2000). In this work, all of these variables were kept at constant levels and the effect of animal species, the extraction method and the protein concentration on the structural and mechanical properties of reconstituted collagen fibres was investigated.

2.3.1. Initial Comparison of the Starting Materials

The rat-tail tendon appeared to be readily digestible under acidic conditions and therefore enzyme digestion of this material was not utilised in this study. An outstanding 90% yield was found for the pepsin soluble bovine Achilles tendon (PSBAT), whilst only 10% was the yield of the acid soluble bovine Achilles tendon (ASBAT). Pigtail solubilisation under acidic condition was minimal, and as a consequence only pepsin soluble pigtail tendon (PSPTT) collagen was used. SDS-PAGE results (not shown) revealed that the relative amount of collagen types present is tissue and species dependent. The rat-tail tendon was almost entirely composed of type I collagen (>95%), followed by bovine Achilles tendon (90%), with the pigtail tendon having the lowest (75%); the reverse was the case regarding type III.

Different species appeared to be more or less digestible under different conditions. It has been reported that rat-tail tendon is as homogenous as possible (more than 95% Type I) and an easily isolated tissue, which makes it an ideal starting material for collagen extraction (Silver and Trelstad, 1980; Raspanti *et al.*, 1997; Mentink *et al.*, 2002). In contrast, an insignificant amount was extracted under acidic conditions from pig tail tendon. Similar to our results, it has been reported that porcine skin atelocollagen is comprised of 70-80% type I and 20-30% type III collagen (Yamamoto *et al.*, 1999; Ueda *et al.*, 2002). An intermediate situation was observed for bovine Achilles tendons. In regards to the homogenicity of the bovine collagen, it has been shown that the type III collagen content in calf-skin is about 8% (Tajima, 1996; Li *et al.*, 2003). The collagen yield of the pepsin extraction from bovine Achilles tendon was much higher than that of the acid extraction, which seems to agree with previous observations (Light, 1985; Bailey and Light, 1989; Friess, 1998; Li *et al.*, 2003). However, these results opposed to those found for acid and pepsin soluble fish skin,

where the acid soluble portion was higher than the pepsin one; the authors suggested that fish skin might be different in terms of domain or cross-links and totally different from calf skin collagen type I in terms of sequence and composition of amino acids (Jongjareonrak *et al.*, 2005). In addition to the high yield benefit of the pepsin soluble collagen, it has been mentioned that the so-called monomolecular collagen or atelocollagen benefits from the removal of the antigenic P-determinant located on the non-helical protein sections. It is conceivable that the host immune response can be greatly reduced and as a result antigenicity can be further reduced and a more competitive biomaterial can be produced (Rosenblatt *et al.*, 1989; Rosenblatt *et al.*, 1994; Yamada *et al.*, 1995; Friess, 1998; Yin Hsu *et al.*, 1999; Alam *et al.*, 2001; Ishikawa *et al.*, 2001; Rodrigues *et al.*, 2003). To summarise, the rat-tail tendon had the highest type I collagen content followed by bovine Achilles tendon, with the pigtail tendon having the lowest; the reverse was the case regarding type III. It was shown that the relative amount of collagen types present is tissue and species dependent.

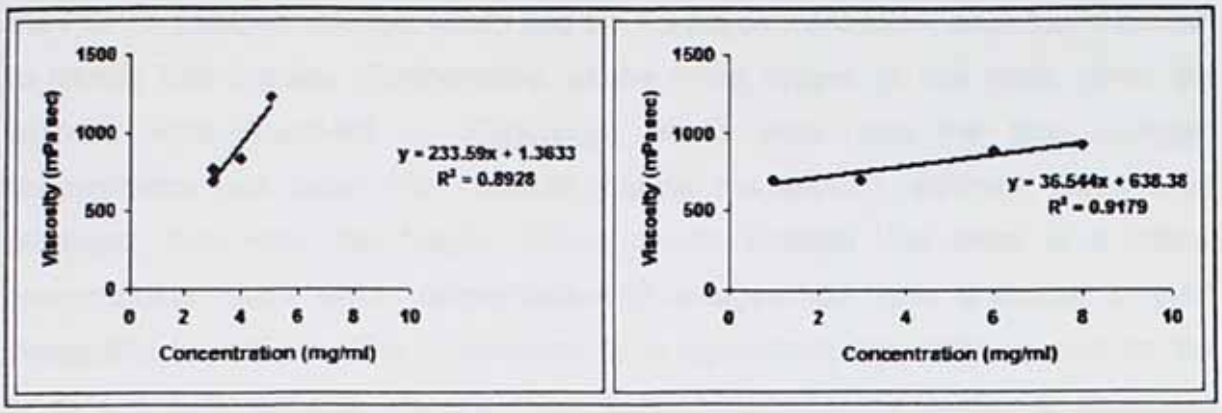
Table 1 shows the collagen concentrations as revealed by hydroxyproline analysis and the corresponding viscosities of the collagen solutions. It was found that with increasing the collagen concentration, an increase in viscosity was observed. A high correlation between the concentration and the viscosity, (R^2) of 0.89 and 0.92 for the acid and pepsin soluble treatments respectively was observed (Figure 4).

Table 1. Collagen Treatments Concentration and Viscosity.

Treatment	Collagen Concentration (mg/ml)	Viscosity [mPa (x) sec]*
ASRTT Batch 1	3	695
ASRTT Batch 2	4	829
ASBAT Batch 1	3	763
ASBAT Batch 2	5	1223
PSBAT Batch 1	3	704
PSBAT Batch 2	6	888
PSBAT Batch 3	8	922
PSPTT Batch 1	1	698

*: Viscosity measurements given here were carried out at a shear rate of 1.15.

Figure 4. Correlation between Viscosity and Concentration for the Acid (left) and Pepsin (right) Soluble Treatments.



It can be observed that for the acid treatments the viscosity appeared to increase rapidly as the concentration was increased, in comparison to the pepsin soluble treatments. The removal of the non-helical telopeptides by pepsin digestion could be the reason for the difference in viscosity between the acid and the pepsin soluble treatments of same concentration. Additionally, it can be observed that the viscosity of the ASRTT collagen solution was lower than that of the equal concentration ASBAT and the viscosity of the ASBAT was lower than that of the equal concentration PSBAT collagen. The difference between the acid derived solutions can be attributed to the different collagen type contents related to the different species.

Visual observations indicated different degree of transparency between the treatments. It was observed that the RTT derived solution was the most transparent and decrease of transparency was achieved as the concentration was increased. Similar to our work, different degrees of transparency have been reported for different extraction methods (Li *et al.*, 2004) and different species (Fernandez-Diaz *et al.*, 2001). Additionally, it has been mentioned that the loss of transparency is reflected in the increase of light scattering, which is proportional to the mass per unit length of the observed material (Brokaw *et al.*, 1985). The mass per unit length of molecules with a homogenous electron density is itself proportional to the diameter and concentration of the macromolecule in solution (Notbohm *et al.*, 1993), which can explain the decrease in transparency by increasing the collagen concentration. Previous work has demonstrated that the rate of change in turbidity is linearly dependent on collagen concentration (Farber *et al.*, 1986).

During the fibre manufacture process, it was found that the higher the collagen concentration, the easier it was to handle the derived fibres. The fibres derived from the PSPTT collagen solution, which had the lowest concentration, were found difficult to handle and transfer. Furthermore, at the initial stages of this work, when the tendons were dissolved in ≥75%vol/wt acetic acid, and the final collagen concentration was lower than 1mg/ml (results not shown), although fibres were produced, they were too fragile. These results indicate that there is a critical concentration, below which, reconstitution of collagen into fibres is difficult or even impossible to achieve. This observation is in agreement with other studies on the kinetics of fibrillo-genesis, where a critical concentration for the reaction to occur was demonstrated (Bailey and Light, 1989). Additionally, it had been shown that the reconstitution of collagen fibrils depends upon the concentration of collagen (Woodley *et al.*, 1991). Furthermore, it has been shown for collagen sponges, that there is a minimum effective collagen concentration [0.1% (w/v)] (Hanthamrongwit *et al.*, 1994; Chen *et al.*, 2000).

2.3.2. Structural Observations

In order to reveal the microstructure and better understand the fracture mechanisms of the reconstituted collagen fibres both light and scanning electron microscopy were employed. As was discussed in the previous section, by increasing the collagen concentration, the viscosity of the solutions was increased (Table 1) and not even prolonged centrifugation (2h at 4^0C at 12,000g) was able to degas the PSBAT Batch 3 (8mg/ml) and consequently, on occasion, air bubbles were trapped within the fibres produced from this collagen solution (Figure 5 & 6a). When fibres containing trapped air bubbles were tensile tested, fracture tended to occur at the side of the bubble (Figure 6b).

Figure 5. An air bubble trapped within a reformed collagen fibre (Optical Microscope).

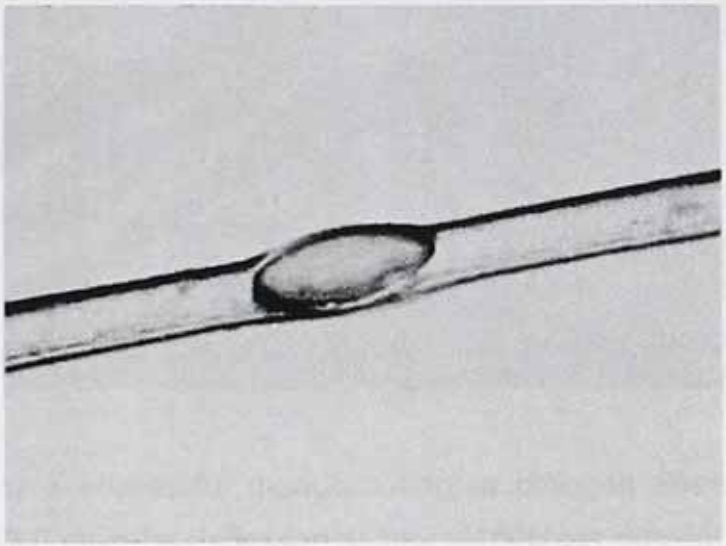
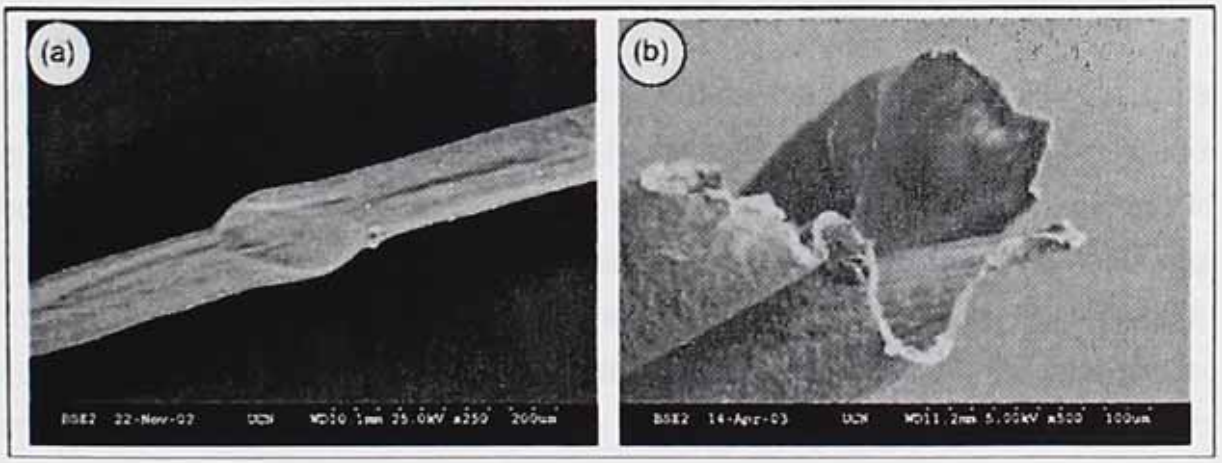
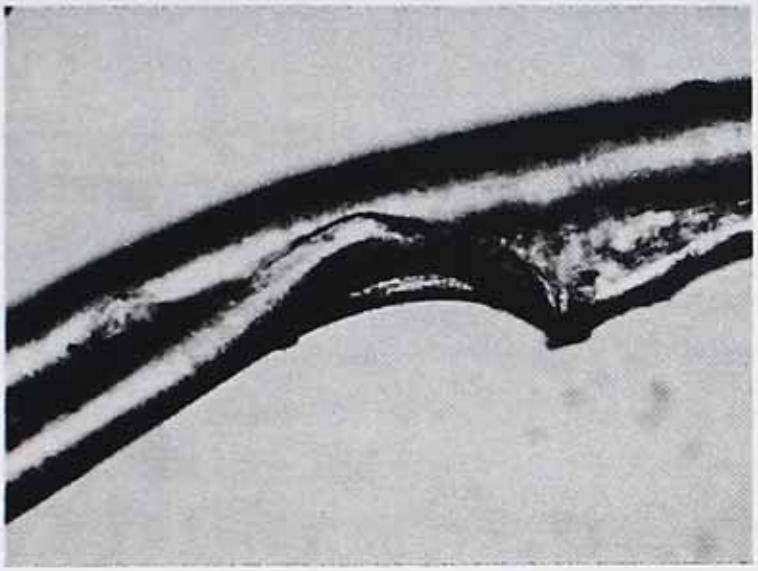


Figure 6: An air bubble trapped within a reformed collagen fibre; (a) whole fibre and (b) break surface (SEM).



The fibres in the wet state were soft and fragile, especially those produced from a low concentration collagen solution. Additionally the laboratory system for fibre manufacture was not a continuous one. As a result, the operator frequently had to interfere with the process, providing more opportunities to cause damage. For example, even a slight increase or decrease in the flow rate of the air steam that was utilised to drive forward the fibres within the FFB could cause an irreversible tangle of the fibre (Figure 7).

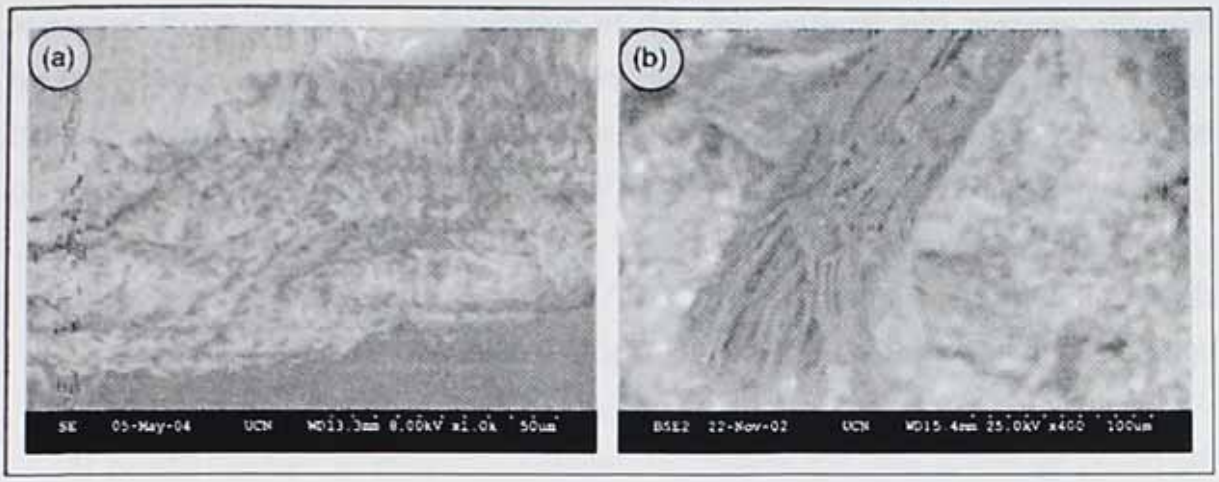
Figure 7. An irreversible tangle of a reformed collagen fibre.



To summarise, for a successful manufacturing of collagen threads, the collagen solution and the FFB must be degassed as any air bubbles present could form weak spots and breaks in the fibre. The flow of the FFB must be steady, or it can dramatically affect the structure of the produced fibres as has been demonstrated previously (Cavallaro *et al.*, 1994).

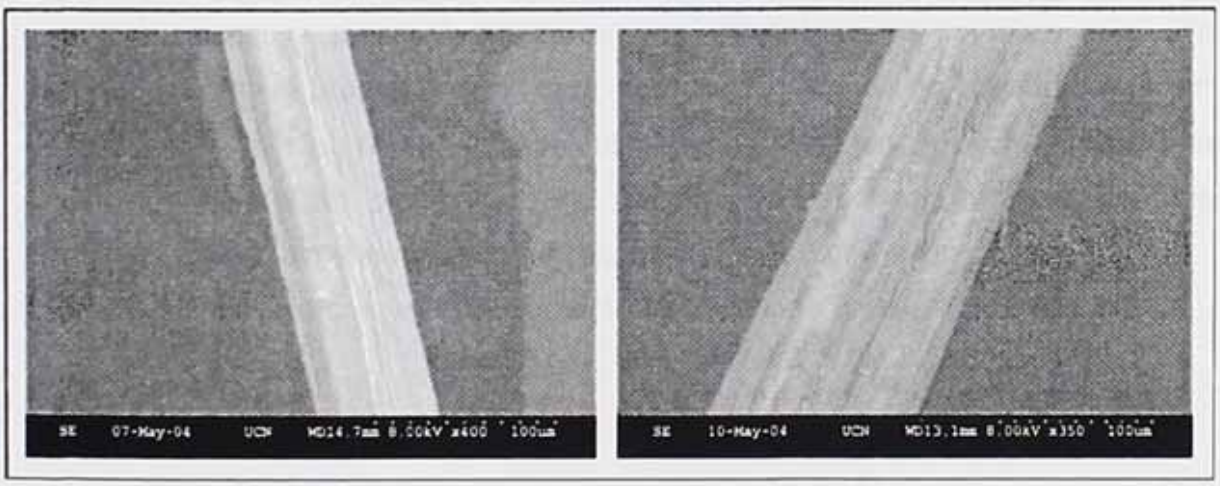
The break surfaces of all reconstituted fibres showed a similar pattern, with the same apparent internal structure independent of the treatment. The inter-fibrillar space appeared to be completely filled for the reformed fibres (Figure 8a) in contrast to leather fibres, where clearly separated fibrils could be observed (Figure 8b). This was not a surprise, since it has been mentioned that very little free inter-fibrillar space occurs in fibres formed *in vitro* (Brokaw *et al.*, 1985). Furthermore, the inter-fibrillar space has been reported to be filled when polymers were utilised for the production of composite fibres (Hepworth and Smith, 2002) and elastin based materials (Dutoya *et al.*, 2000).

Figure 8. Inter-fibrillar space of a reconstituted (a) and a leather (b) fibre.



The surface morphology of the reformed collagen fibres exhibited a rough exterior with undulations in the fibre diameter along its length. Ridges and crevices were also apparent running roughly parallel to the axis of the fibre (Figure 9) as has been described previously (Christiansen and Silver, 1993). The air stream that was utilised to forward the fibres within the FFB as well as transferring the fibres from one buffer to the other might have been responsible for the formation of the crevices along the longitudinal axis. Additionally, it has been suggested that fibril alignment occurring during transferring (Shao *et al.*, 2001) and the substructure (Christiansen and Silver, 1993) of the reconstituted collagen fibre could also be related to an uneven surface morphology.

Figure 9. Typical surface morphology of reconstituted collagen fibres.



Although the internal structure revealed from examination of the failure surfaces appeared to be the same independent of the raw material and process, different types of fracture pattern were distinguished following an extensive SEM study. These fracture patterns could be classified into four categories as has been described previously (Arumugam *et al.*, 1992; Pins *et al.*, 1997a; Rajini *et al.*, 2001):

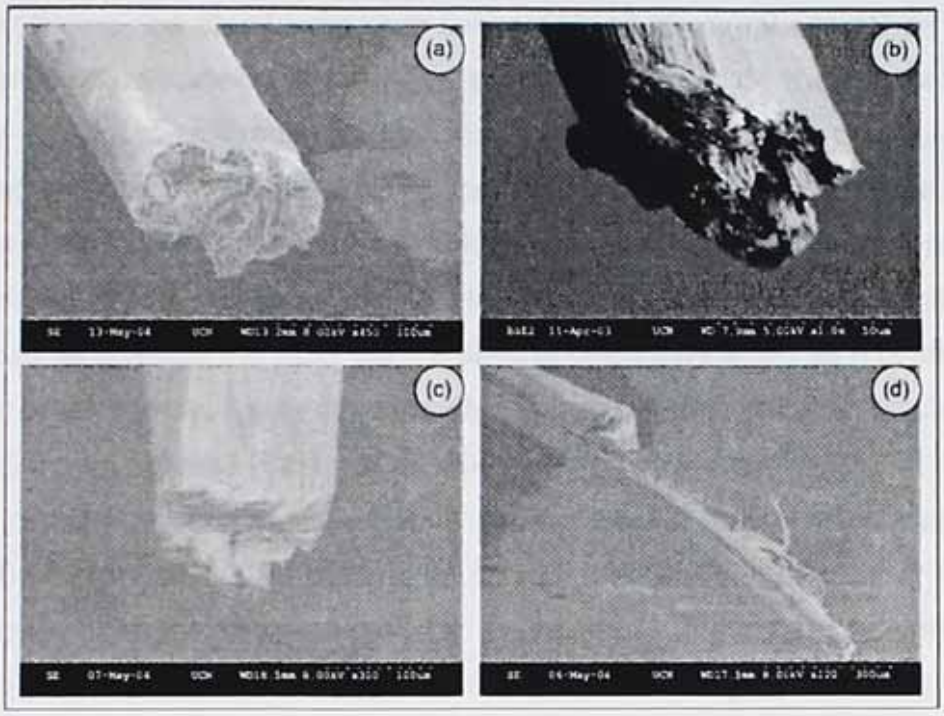
(a) Relatively smooth fracture surface, in which fracture has occurred in a single plane perpendicular to the fibre axis (Figure 10a). It has been suggested that when the application of load is slow, the fracture occurs in this smooth way.

(b) Rough fracture, in which the internal structure of the fibre appeared drawn out (Figure 10b). It was proposed that stretching (e.g. during lifting, transferring and vertically drying the fibres) generated an outer surface with more highly orientated collagen fibrils that appeared to be more extendible and maybe stronger than the inner core. Fibre stretching prior to mechanical testing appears to increase the difference between the degree of fibril alignment of the outer and the inner portions. On mechanical testing, failure occurs first within the inner core with the outer aligned layer failing later. This leaves the end appearing drawn out (Pins *et al.*, 1997a).

(c) Split fracture surface, where fracture occurred not only in planes perpendicular to the fibre axis but also along the fibre axis, resulting in a splitting of the fibre along its axis (Figure 10c). Occasionally fibrils may act like flaws in the fibres at which type fracture probably can initiate. Incipient step fracture results from stress concentrations at the cracks, which are then propagated in between the hierarchical planes along the fibre axis. The simultaneous propagation of more than one crack along the fibre axis can result in splitting of the fibre before breaking. The length of the splinter has been found to be proportional to the degree of stretching in production. This may be explained by the outer fibrils being more highly aligned.

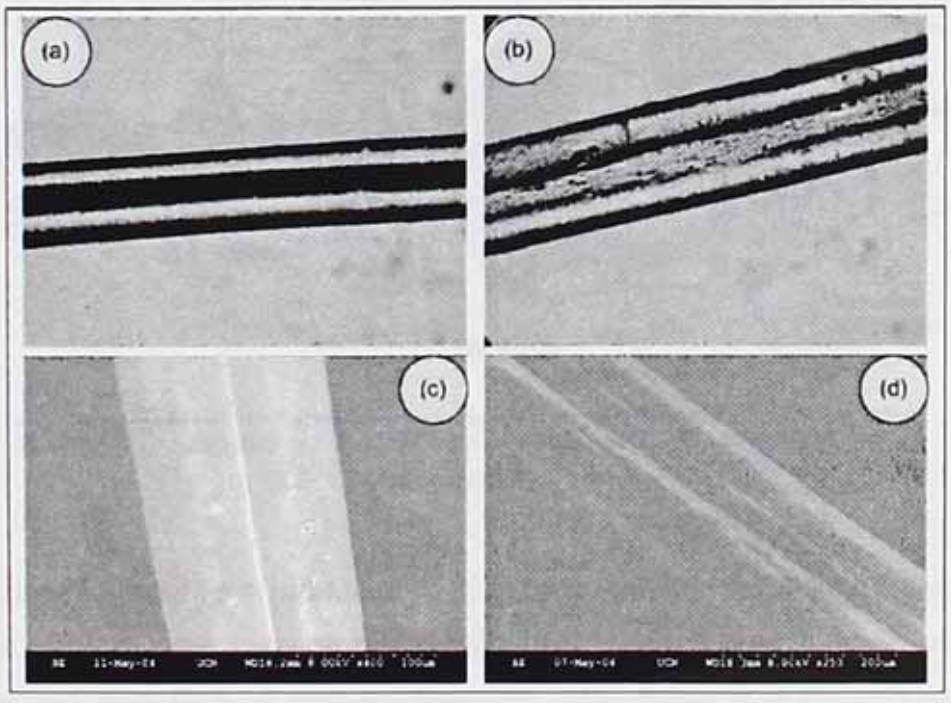
(d) Fibrillation fracture, with the fractured end split into smaller fibrils (Figure 10d).

Figure 10. The effect of stretching [lower (a), higher (d)] on the breaking mechanism of reconstituted collagen fibres.



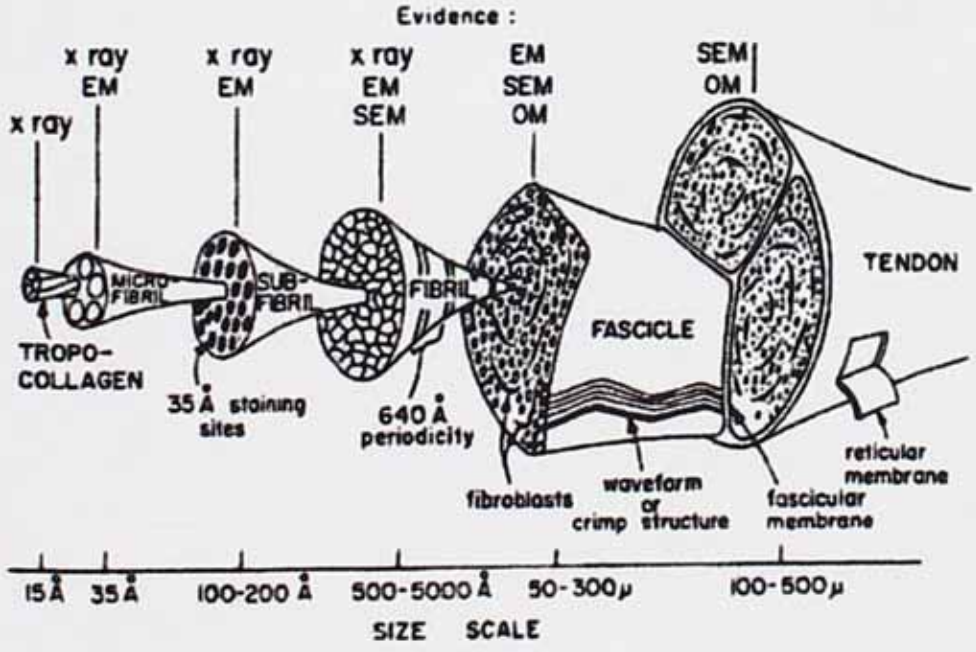
As can be seen from Figure 11, some fibres appeared to have a "cavity" lying along their longitudinal axis. This characteristic was observed throughout this study and was more obvious in fibres with larger diameter.

Figure 11. Low (a, c) and high (b, d) in diameter reconstituted collagen fibres with a cavity lying along their longitudinal axis (a, b) optical and (c, d) SEM pictures.



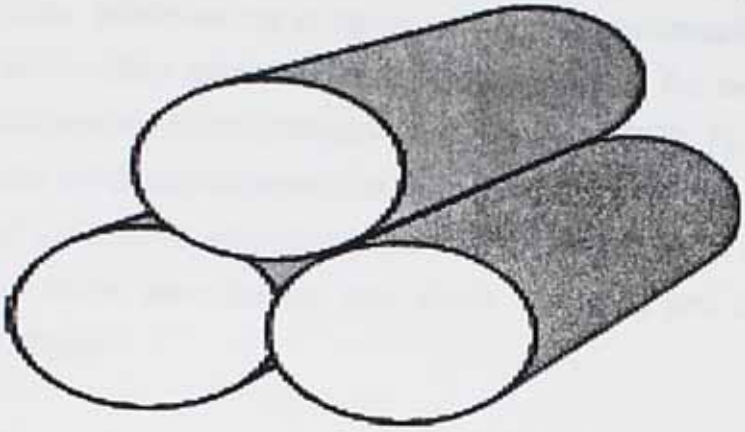
This phenomenon can be most likely attributed to the air steam that was utilised to move the fibres within the FFB. During the fibre formation procedure, as has been mentioned before, the fibres were very susceptible to structural alterations and as a consequence the air steam could have tangled the fibres in the middle. Another possibility is that a microfilm rather than a macro-fibre was produced during transferring the fibres from the FFB to the FIB and that the sides of the microfilm came closer and formed the long cylindrical-like shape which appeared as fibre. A third possibility is that the fibres did not remain for a sufficient period of time within the FFB and as a result, the PEG did not have enough time to act as a coating agent. In support of this theory, it may be noted that the penetration of PEG into clay films is a time dependent process (Baker *et al.*, 2004). It has been mentioned that the *in vivo* diameter of a tropocollagen molecule is around 1.5nm, that of a collagen fibril is around 80-100nm and that of a collagen fibre is around 1-4μm (Rhee *et al.*, 2001); human Achilles tendon fibre ranges from 26 to 30μm (Figure 12) (Jarvinen *et al.*, 2004), whilst human skin is composed of collagen fibrils ranging from 20nm to 40μm in diameter (Silver *et al.*, 2001b).

Figure 12. Hierarchical structure of human Achilles tendon. Source: (Baer *et al.*, 1991).



The smallest diameter fibre produced in this study derived from PSBAT (3mg/ml) and had value of 52μm, which is larger than either the *in vivo* human Achilles tendon fibre or the human skin fibre. It can be therefore speculated that the reformed fibres produced contained multi-fibres attached to one another (Figure 13).

Figure 13. Possible *in vitro* fibre conformation (multi-fibre attachment).



Finally, similar void formation has been seen in composite fibres. It may be supposed that such voids can alter the mechanical properties of the fibres, since cracks can more easily propagate through regions containing voids (Bleach *et al.*, 2002).

2.3.3. The Effect of Collagen Solution Concentration on Fibre Diameter

From Table 2, it can be seen that fibres derived from a lower collagen concentration had a lower diameter, which seems to agree with a previous investigation (Laude *et al.*, 2000). This observation appeared to be independent of the extraction method used and also independent of the utilisation of either salt or PEG. Furthermore, fibres derived form pepsin extracted collagen had lower diameter than those produced from the acid extraction (compared within the same species). It has been shown that alkali extracted collagen fibrils were thicker than those from acid and pepsin extracted collagen (Li *et al.*, 2004).

Table 2. Collagen concentration and dry fibre diameter (1.5mm diameter tube).

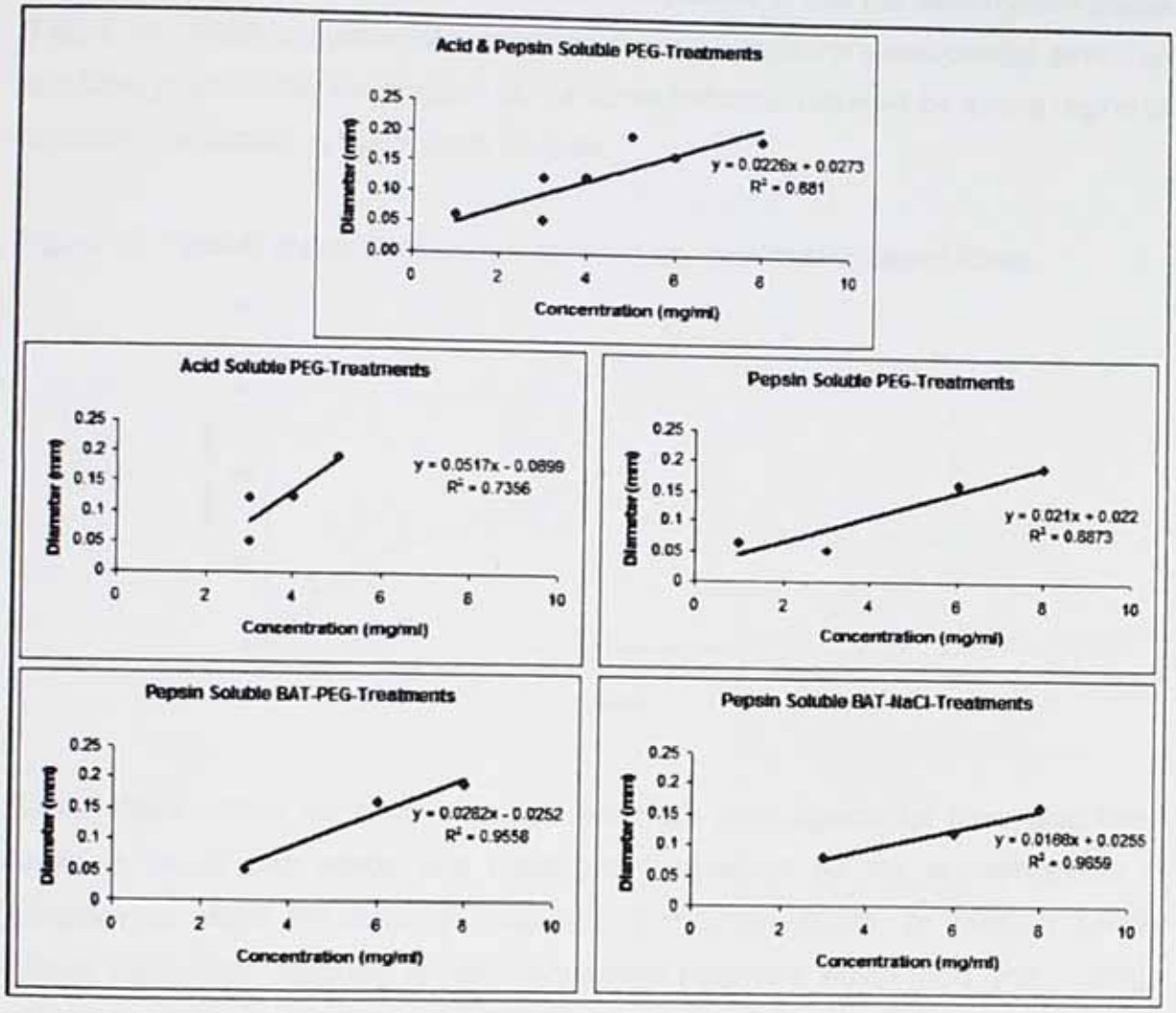
Treatment		Collagen Concentration (mg/ml)	Dry Fibre Diameter (mm)
ASRTT Batch 1 (PEG)		3	0.05 ± 0.01
ASRTT Batch 2 (PEG)		4	0.13 ± 0.02
ASBAT Batch 1 (PEG)		3	0.12 ± 0.02
ASBAT Batch 2 (PEG)		5	0.19 ± 0.02
PSBAT Batch 1	PEG	3	0.05 ± 0.01
	Salt		0.08 ± 0.01
PSBAT Batch 2	PEG	6	0.16 ± 0.05
	Salt		0.12 ± 0.01
PSBAT Batch 3	PEG	8	0.19 ± 0.01
	Salt		0.16 ± 0.02
PSPTT Batch 1 (PEG)		1	0.06 ± 0.01

Fibres produced from ASRTT collagen had a lower diameter than the fibres produced by ASBAT collagen at equal concentration. It has been mentioned that in some tissues collagen fibres show a remarkable uniformity in diameter. For example, in human cornea the collagen fibres have a diameter of 32.5nm, while the diameter of cornea fibres from rabbit are 29.7nm (Bailey and Light, 1989; Huang *et al.*, 2001). Also, PSBAT collagen fibres had a lower diameter than PSPTT collagen fibres, even though they were derived from a higher concentration collagen solution. Although, this appears to be a reliable result, it is surprising, since, as has been mentioned, type III collagen produces fibrils with a smaller diameter than type I collagen (Birk and Silver, 1984; Silver and Birk, 1984; Birk *et al.*, 1990; Tajima, 1996; Jarvinen *et al.*, 2004), and as already explained (Section 2.4.1), rat-tail tendon contains lower amounts of type III collagen than bovine Achilles tendon and bovine Achilles tendon contain lower amounts of type III than pigtail tendon. This contradiction could possibly be attributed to the interaction of the different collagen types that regulate the fibril structure, tissue architecture and function (Birk and Silver, 1984). It has been

shown for type I and type III collagen extracted from skin that type I forms thick bundles, type III thin bundles, whilst intermediate sized bundles were observed whose size depended on the proportion of type III (Lapiere *et al.*, 1977). Similarly, it has been demonstrated that the co-polymerisation of type V collagen with type I collagen resulted in fibrils with smaller diameters; increasing the amounts of type V collagen leads to a progressive decrease in the mean fibril diameter (Birk *et al.*, 1990). In addition, it has been shown that different collagen types form heterotypic fibrils in a variety of tissues; for example types I and III have been found in the dermis and tendon (Keene *et al.*, 1987; Fleischmajer *et al.*, 1990). During dermal development there is a decrease in the type III collagen content and an increase in fibril diameter as the tissue matures (Smith *et al.*, 1982). These observations indicate that heterotypic fibril assembly in vitro is an important parameter that should be considered since could affect the diameter of the reformed collagen fibres. Furthermore, it has been shown that the thickest collagen fibres were identified with the tendons exposed to heavy mechanical loading (Achilles), whereas tendons that bear lighter loadings (*Extensor pollicis longus*) but carry out functions of high specificity have substantially smaller collagen fibrils and fibres (Jarvinen *et al.*, 2004), which can explain the difference in diameter between the rat and the bovine originated fibres. PSBAT collagen produced fibres of lower diameter than those produced from an equal concentration ASBAT collagen, which might be attributed to the enzymatic digestion of the telopeptide region. However, it has been found that pepsin solubilisation of collagen produces thicker aggregates (Notbohm *et al.*, 1993).

Fitting a linear regression model to the overall concentration Vs fibre diameter graph, a low correlation (R^2=0.68) was obtained. However, the correlation increased when acid and pepsin derived collagen results were separated giving R^2 values of 0.74 and 0.89 respectively. The correlation becomes even stronger when results separated by species; the PSBAT derived fibres in the presence of salt gave an R^2 value of 0.99 and in the presence of PEG Mw 8,000 an R^2 value of 0.96 was obtained (Figure 14). These observations demonstrate that reconstitution of collagen in fibre form is a complicated process, since several factors could affect their physical properties. In this instance, species, pepsin or acid digestion and collagen concentration all appeared to affect the dry fibre diameter.

Figure 14. Correlation between Concentration and dry reconstituted collagen fibres diameter as derived from Table 2.

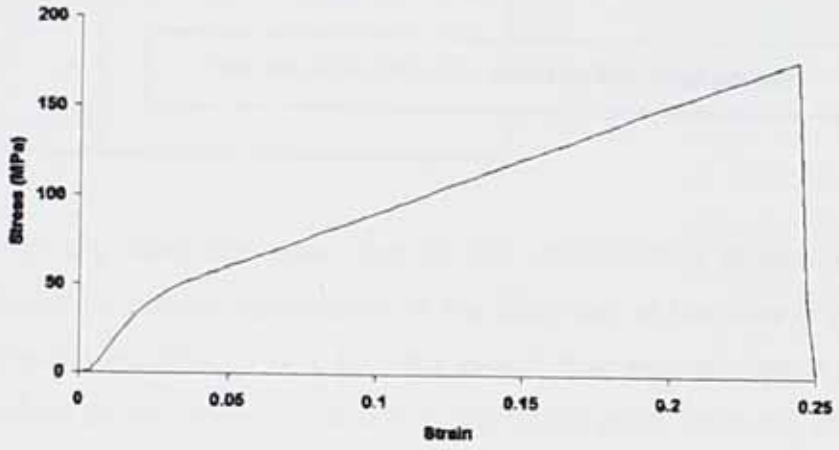


Finally, it has been mentioned that two factors are involved in producing a strong mechanical repair: the technique used and the suture material. A number of multi-strand methods have been described to increase the strength of suture repair. However, the increased amount of suture material causes thickening of the tendon at the repair site, which, in some cases, restricts the gliding of the tendon in the narrow tendon sheath. The development of suture materials with minimal cross-sectional diameter and sufficient strength to withstand the range of forces generated in the early rehabilitation protocol might provide a solution to the problem (Kujala *et al.*, 2004). In this study, it has been shown that by controlling the concentration of the collagen solution, the fibre diameter can be controlled and this may form the basis to a competitive material for tissue repair.

2.3.4. Observations on Mechanical Properties of Reformed Collagen Fibres

The stress-strain curves obtained from the dried fibres all had the same typical shape (Figure 15), which consisted of a small toe region, a region of steeply rising stress up to a knee point where the gradient of the curve reduced, followed by a long region of constant gradient up to the point of fracture.

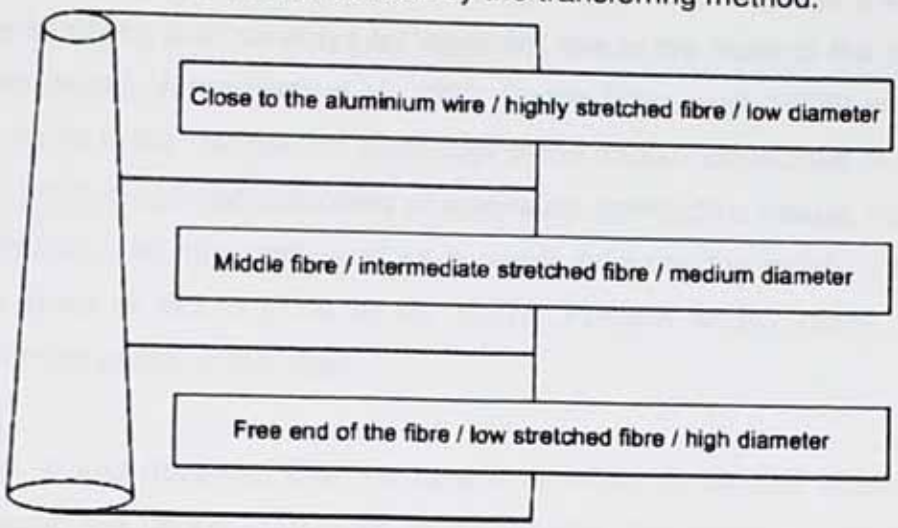
Figure 15. Typical shape stress-strain curve of dry reformed collagen fibres.



Stress-strain curves for dried biological materials (e.g. ligaments) have also been found to be of this shape and have been explained by the re-orientations of components within the material (Hepworth and Smith, 2002). In addition, similar curves have been reported for semi-crystalline polymers which yield and undergo plastic flow (Pins and Silver, 1995). The curves exhibited non-linear toe regions followed by regions where the stress increased linearly with the strain, which persisted until failure, independent of the collagen origin. Similar curves for dry reconstituted collagen fibres have been reported (Rigby *et al.*, 1959; Cavallaro *et al.*, 1994; Pins *et al.*, 1997b; Gentleman *et al.*, 2003).

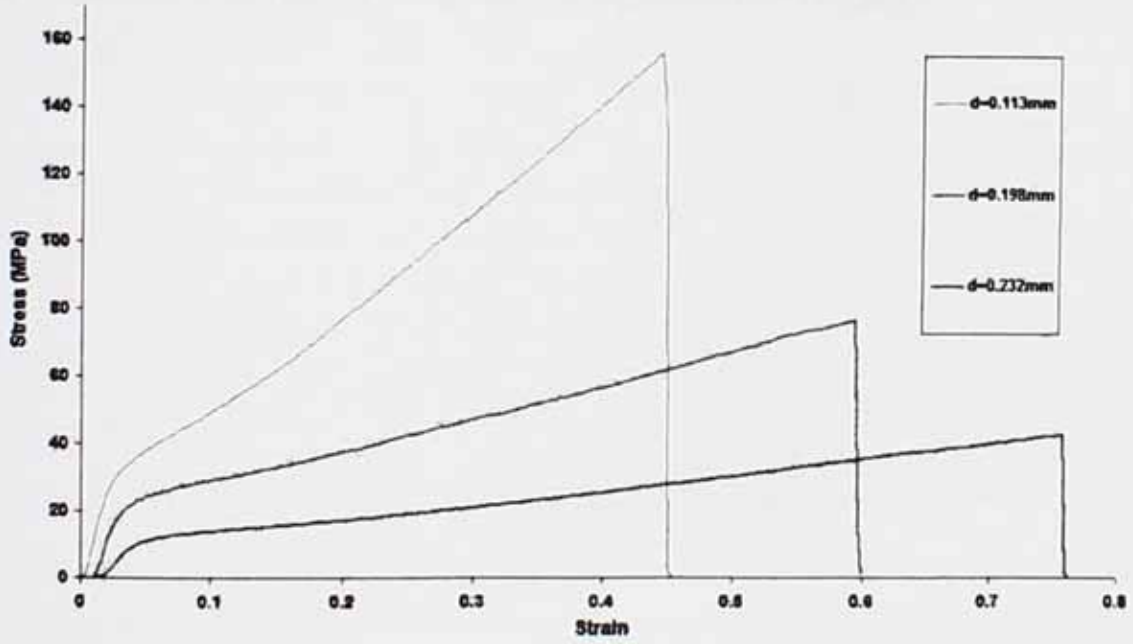
As has been mentioned previously, a non-continuous fibre production system was used in this study. As a result, after approximately every 30cm of fibre produced it was necessary to stop the process, lift the fibre vertically and transfer it, using an aluminium wire, from the one buffer to the other. This transferring process, led to uneven stretching of the fibres and as a result some variation in the diameter of the fibre produced which depended on proximity to the aluminium wire. Those regions that were closer to the aluminium wire stretched more than those in the middle regions and these were stretched more than those at the free end (Figure 16).

Figure 16. Uneven fibre diameter caused by the transferring method.



The variation in dry fibre diameter due to this unavoidable stretching resulted in different stress-strain curves depending on the diameter of the fibre (Figure 17). The regions close to the aluminium wire had the lowest diameter and were characterised by high stress/low strain values at failure, while fibres away from the aluminium wire had the highest diameter and were characterised by lower stress/higher strain at failure values.

Figure 17. Stress Vs Strain curves for PEG Mw 8,000 PSBAT collagen (6mg/ml) variation with diameter (d).



These results appear to be in agreement with previous reports where it was pointed out that the sampling position plays an important role in the study of the collagenous tissue / biomaterials (Arumugam *et al.*, 1992; Garcia Paez *et al.*, 2003) and may play an important role in the mechanical behaviour of the implant developed. Furthermore, it has been pointed out that stretching of extensible connective tissues may serve to generate fibrillar axial alignment and as a result increase the tensile strength and reduce the strain at failure (Pins *et al.*, 1997a; Purslow *et al.*, 1998), which was observed in most cases in this study.

Furthermore, it was observed that the largest diameter fibres (low stress at break) exhibited longer toe region in contrast to the smaller diameter fibres (high stress at break), which had lower toe regions. The low modulus of the non-linear toe region is believed to reflect the un-crimping of the collagen fibrils in the fibre, as well as the initiation of stretching of the triple helix, non-helical ends and cross-links. The initial linear region of the curve, where the tensile modulus of the fibres was measured, represents the state where the collagen molecules and fibrils in the fibre have straightened and are in tension. This process eventually leads to defibrillation and finally failure (Pins and Silver, 1995; Silver *et al.*, 2000; Silver *et al.*, 2001a). Bearing that in mind, we can conclude that the observed difference in toe region between the low and high in diameter fibres can be attributed to the initiation of stretching of the low in diameter fibres during lifting, transferring and drying process.

2.3.5. Mechanical Properties of Dry Reconstituted Collagen Fibres

Table 3 summarises the mechanical properties of the reconstituted collagen fibres produced in this study. As has already been mentioned, there is a direct relation between collagen concentration and fibre diameter depending on species and digestion method (acid or enzyme). It can also be observed that by decreasing the collagen concentration, a decrease in dry fibre diameter occurred with a consequent decrease in force required to break the fibre. In general, this decrease in dry fibre diameter was associated with a decrease in strain at break and an increase at stress at break and also modulus. The above observations were independent of the presence of either salt or PEG in the FFB. However, the fibres produced with salt showed higher stress and force at break values and lower strain at break values in comparison to the PEG derived ones. Further explanations will be discussed in the next chapter (Chapter 3).

Table 3. Overall Mechanical Characteristics; 20%PEG Mw 8,000 (or NaCl), 1.5mm tube internal diameter, 0.4ml/min extrusion rate.

Treatment		Dry Diameter (mm)	Stress at Break (MPa)	Strain at Break	Force at Break (N)	Modulus at 2% Strain (MPa)
ASRTT (3mg/ml) n=8		0.05 ± 0.01	255 ± 89	0.19 ± 0.02	0.50 ± 0.05	3310 ± 1089
ASRTT (4mg/ml) n=7		0.13 ± 0.02	134 ± 43	0.21 ± 0.05	1.56 ± 0.10	1591 ± 608
ASBAT (3mg/ml) n=6		0.12 ± 0.02	168 ± 62	0.30 ± 0.06	1.84 ± 0.15	1485 ± 568
ASBAT (5mg/ml) n=7		0.19 ± 0.02	108 ± 19	0.36 ± 0.06	3.12 ± 0.25	560 ± 455
PSBAT (3mg/ml)	PEG n=5	0.05 ± 0.01	169 ± 53	0.27 ± 0.04	0.35 ± 0.04	1659 ± 609
	NaCl n=12	0.08 ± 0.01	237 ± 104	0.23 ± 0.04	1.03 ± 0.18	1832 ± 776
PSBAT (6mg/ml)	PEG n=32	0.16 ± 0.05	99 ± 61	0.44 ± 0.17	1.57 ± 0.35	594 ± 647
	NaCl n=25	0.12 ± 0.01	208 ± 57	0.27 ± 0.06	2.23 ± 0.32	1581 ± 490
PSBAT (8mg/ml)	PEG n=14	0.19 ± 0.01	55 ± 5	0.42 ± 0.10	2.88 ± 0.28	323 ± 200
	NaCl n=15	0.16 ± 0.02	130 ± 31	0.27 ± 0.05	2.49 ± 0.18	858 ± 270
PSPTT (1mg/ml) n=8		0.06 ± 0.01	102 ± 37	0.22 ± 0.06	0.29 ± 0.03	1409 ± 324

The low concentration ASRTT produced, as judged by tensile strength, the strongest fibres (255MPa), while the high concentration of PSBAT with PEG produced the weakest fibres (55MPa). The rest of the treatments produced fibres whose tensile strength ranged between these values. Comparing the acid- and pepsin- soluble collagen derived from bovine Achilles tendon, it can be seen that the stress at break values were comparable (168 and 169MPa respectively) for fibres produced from the

same collagen concentration (3mg/ml) although they had different fibre diameter (0.123 and 0.052mm respectively). These results are in contrast to previous data, where it was stated that removal of the non-helical ends inhibited initiation of self-assembly (Christiansen *et al.*, 2000) and matrices produced from pepsin soluble collagen were 20-times weaker than similar lattices produced from acid-extracted collagen (Woodley *et al.*, 1991; Cavallaro *et al.*, 1994).

Fitting a linear regression model to the mean overall Stress at break Vs fibre diameter values from Table 3, a low correlation (R^2=0.48) was obtained. However, the correlation appeared to increase by separating the treatments to digestion method (acid and pepsin digestion: R^2 values of 0.89 and 0.65 respectively) and co-agent present (PEG and NaCl: R^2 values of 0.96 and 0.93 respectively) (Figure 18A). In general, all treatments gave a strong correlation with R^2 values ranging from 0.72 to 0.97 (Figure 18B).

Figure 18A. Correlation between Stress at break and Dry reconstituted collagen fibres diameter as derived from Table 3.

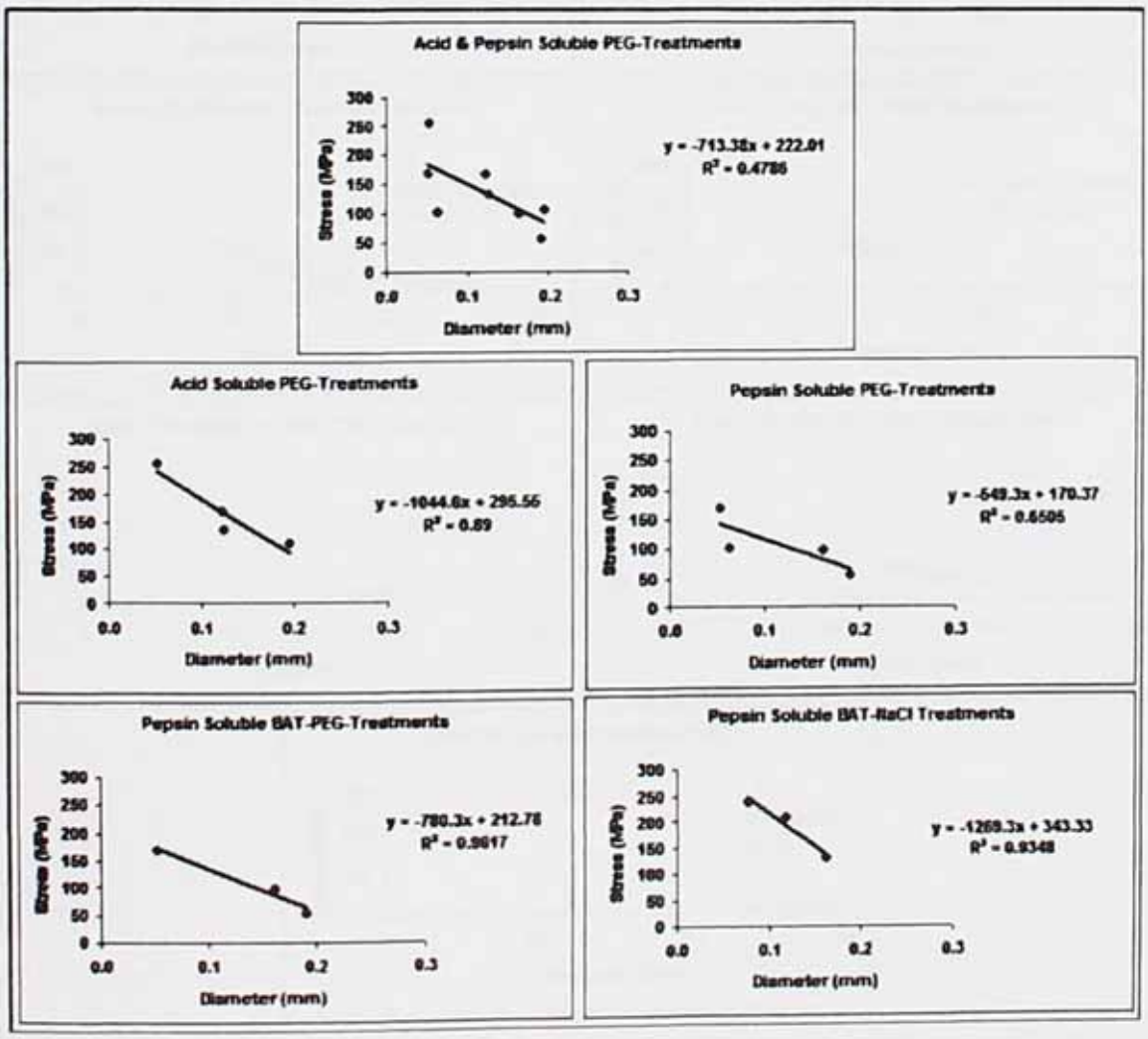
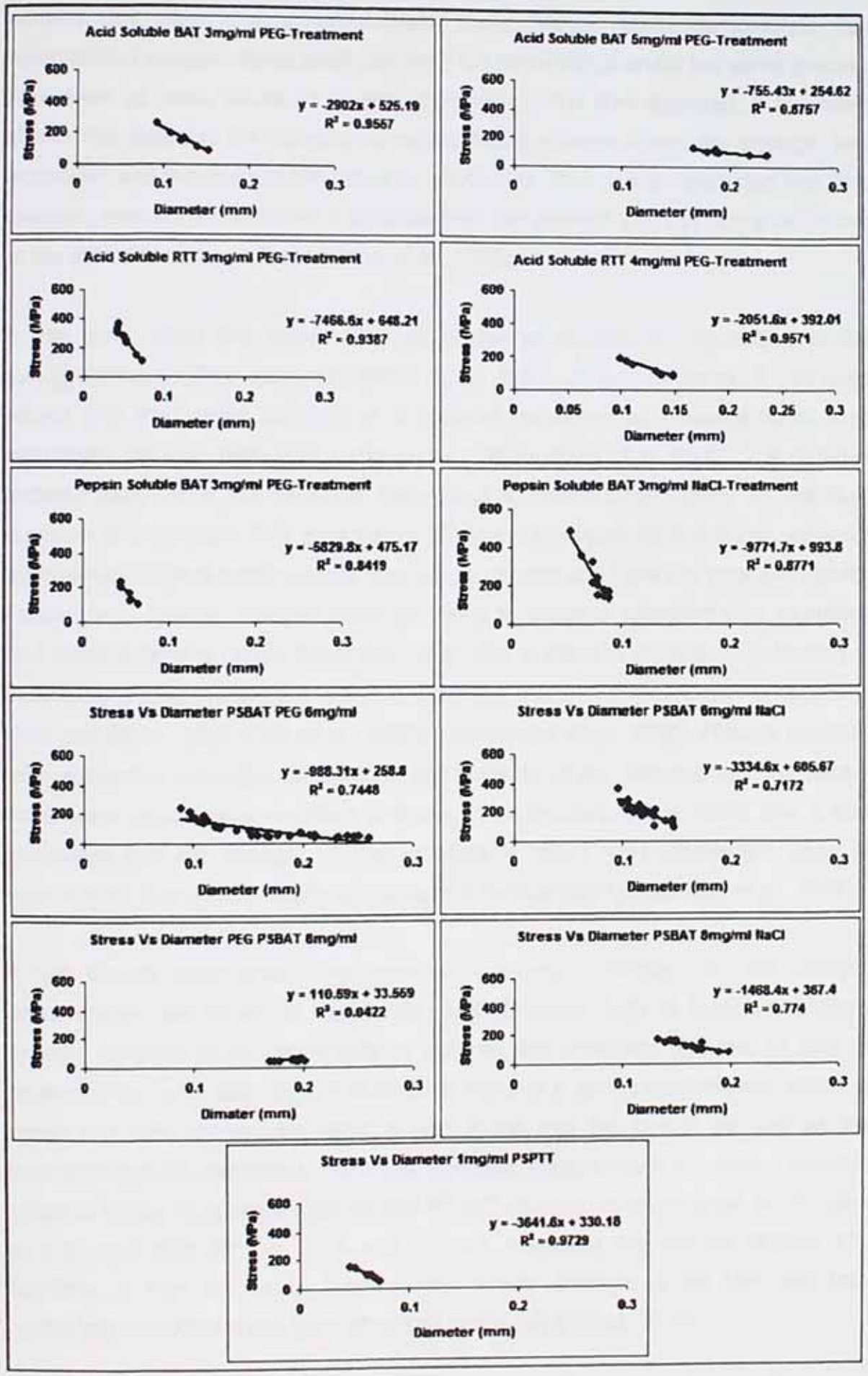


Table 18B (continue).

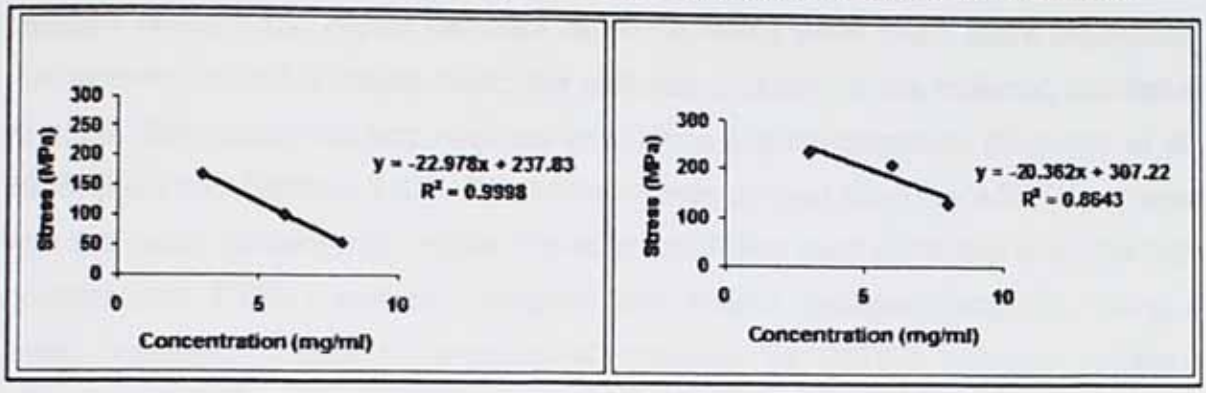


The only exception appeared to be the PSBAT Batch 3, presumably due to the air-bubbles that were trapped within these fibres. These results demonstrate that reconstituted collagen fibres produced from the same tissue under the same process had stress at break values in reverse proportion to the fibre diameter. It has been shown that reducing the diameter of reconstituted collagen fibres, the strength was increased, and the degradation rate was unaffected. Thus, it was concluded that fibre diameter should be minimized to optimise both the strength and the degradation rate of the extruded collagen fibres (Dunn *et al.*, 1993).

It has been noted that tensile strength of dermal wounds is proportional to the average collagen fibre diameter (Marks *et al.*, 1991). In general terms, it has been shown that the tensile strength of a material increases as cross-sectional area decreases, because there is less chance for defects (Saito *et al.*, 2002). The collagen substructures within the extruded fibres tend to orient longitudinally as the fibre diameter is decreased. This longitudinal alignment increases fibre strength, whereas transversely aligned substructures may act as defects and have the opposite results. Furthermore, smaller collagen fibres are likely to possess improved fibril alignment and packing density (larger fibres have a smaller surface to volume ratio) leading to increased "strong interactions within or between collagen fibrils" (Dunn *et al.*, 1993; Pins and Silver, 1995; Pins *et al.*, 1997b; Gentleman *et al.*, 2003). Results obtained from subjecting reconstituted collagen structures to stress revealed that the denser fibrils were much more resistant to stress than the less dense fibrils and it was concluded that the strength of the structure is much more dependent upon its architecture, than upon the strength of each individual bond (Parkinson *et al.*, 1997).

It has already been shown that there is a strong correlation of both collagen concentration and stress at break with fibre diameter. Both of these correlations become stronger by limiting results to species and treatment (Figures 14 and 18 respectively). In an attempt to find whether there is a correlation between stress at break and collagen concentration, it was found that the overall as well as the treatment specific correlations were low. However, fitting a linear regression model to stress at break Vs concentration for the PSBAT collagen solutions gave an R^2 value of 0.99 and 0.86 for the PEG and the salt treatment respectively (Figure 19). Similarly, a high correlation between the tensile strength of rat skin and total hydroxyproline content has been observed previously (Vogel, 1974).

Figure 19. Correlation between stress at break and concentration of PSBAT collagen in the presence of PEG Mw 8,000 (left) and NaCl (right) as derived from Table 3.

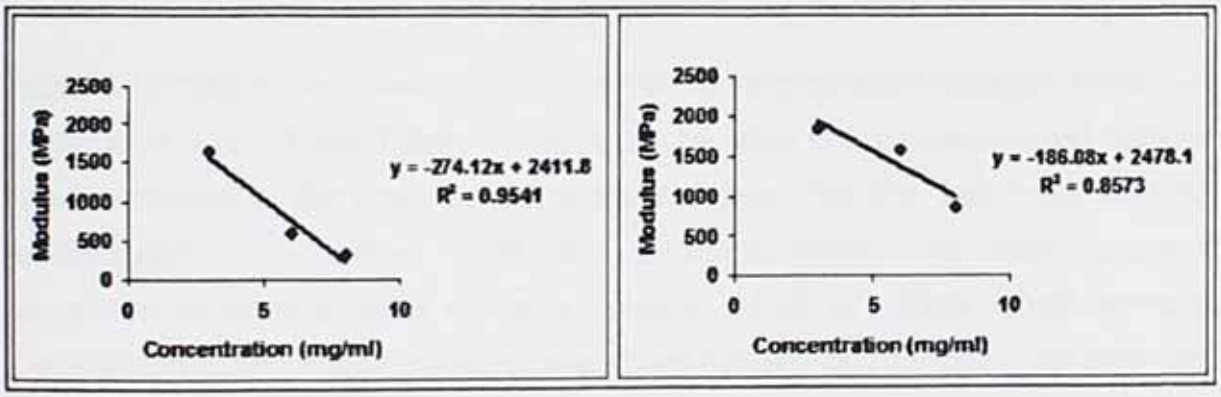


These results indicate that the collagen concentration can affect the dry fibre diameter, which as a consequence can affect the stress at break of the reconstituted collagen fibres. It has been implied that collagen content can influence the mechanical properties of collagen fibres (Danielsen and Andreassen, 1988), soluble collagen content had a negative correlation to tensile strength of rat skin (Dombi *et al.*, 1993) and the strength of gelatine gels is dependent on the concentration of gelatine (Babin and Dickinson, 2001). In addition, previous studies have demonstrated that no significant contraction of collagen sponges (Cote *et al.*, 1992) is observed as has been described with hydrated collagen gels composed of low initial collagen concentrations that contract more than gels with higher initial collagen concentrations (Zhu *et al.*, 2001). Therefore it can be concluded that lower initial collagen concentrations can result in higher final collagen densities and as a consequence in higher fibril alignment. Fibres with higher fibril alignment, have smaller diameter and as a result lower force at break and higher tensile strength and low porosity as has been shown (Pins *et al.*, 1997a; Bleach *et al.*, 2002).

The 5mg/ml ASRTT and the 8mg/ml PSBAT collagen produced the largest diameter fibres and consequently higher forces were required to break them. As noted earlier (Table 3) the strain at break appeared to increase and the modulus values to decrease with increasing collagen concentration and/or collagen fibre diameter, within the same species. These results seem to be similar to those obtained with synthetic materials; thicker sutures had lower values of tensile strength than their thinner counterparts, while thicker sutures could sustain higher force before they broke (Makela *et al.*, 2002). Even in glass fibres, tensile strength is reduced as the fibre diameter is increased (Thomason, 1999). However, these results are in disagreement with previous work (Rigby *et al.*, 1959), where it was pointed out that

structures containing a high percentage of collagen were found difficult to stretch and usually broke before they elongated appreciably, while structures with a lower collagen content and higher per cent of elastic fibres were much more extensible. Furthermore, modulus values reflect the stiffness or rigidity of the material; the higher its value, the greater the load required to produce a given extension (Osborne *et al.*, 1998; Field and Stanley, 2004). The stiffest fibres derived from the ASRTT collagen with the lower concentration, whilst the least stiff fibres were produced from the high concentration PSBAT collagen (8mg/ml) and ASBAT collagen (5mg/ml). Fitting a linear regression model to modulus at 2%strain Vs PSBAT collagen solutions concentration R^2 values of 0.95 and 0.86 for the PEG and the salt treatment were obtained respectively (Figure 20). In either case, a negative correlation between collagen concentration and modulus was observed; by increasing the concentration a decrease in modulus values was followed.

Figure 20. Correlation between modulus at 2%strain and concentration of PSBAT collagen in the presence of PEG Mw 8,000 (left) and NaCl (right) as derived from Table 3.



It has been reported that the ultimate tensile strength of different types of connective tissue is directly related to the collagen fibril diameter, suggesting that fibre structure may play an important role in dictating the mechanical properties of different tissues (Pins *et al.*, 1997a). Recent observations suggested that types II and III collagen molecules, apart from forming thinner fibrils, might be more flexible than type I collagen; type I collagen fibres may be designed to more efficiently store and then transmit elastic energy to accomplish additional work, while fibres rich in types II and III collagen may be designed to store and then redistribute this energy in low strain dissipation. For instance, tissues rich in type I collagen (tendons) have limited extensibility, in contrast to tissues with a high content of type II (cartilage) and III (fetal skin, aortic arch), which are more extensible and tend to undergo greater

deformation (Silver *et al.*, 2002). Similarly, it has been noted that fibril diameters are reduced if collagen III is incubated with collagen I (Notbohm *et al.*, 1993) or if collagen type V is incubated with collagen type I (Birk *et al.*, 1990). Also type III collagen forms smaller fibrils and does not have the same strength as type I collagen (Eriksen *et al.*, 2002). The mechanical properties of tendons are also related to the diameter of the collagen fibres, the larger fibres being able to withstand higher tensile forces than smaller diameter fibres. In fact, it has been shown that the structural organisation of the tendon can be used as a surrogate of its biomechanical properties (Jarvinen *et al.*, 2004). These observations appeared to agree with our results; fibres richer in type III (bovine and pig derived) elongated more than fibres with higher type I collagen content (rat derived). Furthermore, if these type I collagen fibres find application as sutures, a low percentage elongation will be a disadvantage since it may cause such sutures to be difficult to tie (Makela *et al.*, 2002). Additionally, it can be concluded from this *in vitro* study that heterotypic assembly could function to regulate fibril diameter and as a result the mechanical properties of the final material, which seems to agree with previous observations (Lapiere *et al.*, 1977; Birk *et al.*, 1990).

Table 4 compares the mechanical properties of reconstituted collagen fibres with many other fibres. From Tables 3 and 4 can be seen that self-assembled collagen fibres produced in this study have mechanical properties that are similar to other reconstituted fibres, native fibres and synthetic fibres. The basic structural component of tendons is the collagen molecule, which is a triple helical molecule. The strength of the tendon matrix is determined not only by changes in the inter- and intra-molecular binding of collagen fibres, but also by alterations in the supra-molecular and macroscopical organisation of collagen fibres within the matrix (Jarvinen *et al.*, 2004). It has also been shown with materials (polymers, ceramics, reinforced metals and composites) that the testing conditions affect the mechanical properties obtained and therefore, during *in vitro* studies, the same testing conditions must be maintained in order to compare results (Wang *et al.*, 1998). Although, consistency was achieved throughout this work, nevertheless reference values for other fibre types are given in Table 4 for comparison reasons. It can be observed that the collagen fibres produced in this study had comparable mechanical characteristics to those produced in other works; however reformed collagen fibres appeared to have lower stress and strain at break values than some of the synthetic/natural fibres available.

Table 4. Mechanical properties of dry synthetic and natural fibres.

	Dry Diameter (mm)	Stress at Break (MPa)	Strain at Break (%)	Force at Break (N)	Modulus (MPa)	Reference
RTT		363-366	15.60-13.80		2130-2690	(Kato *et al.*, 1989)
Native RTT Insoluble corium AS RTT (1mg/ml)	0.110 0.051 0.059	312.1 193.3 355	13.3 25.7 23.2	2.89 0.40 0.93	4747 4189 4272	(Pins and Silver, 1995)
Bovine Corium	0.024-0.088	75-160		0.08-0.46		(Dunn *et al.*, 1993)
1% w/v Bov. Cor. 1% w/v + CS-PG 1% w/v +PG	0.050 0.049	58.1 7.4 90.4	14.6 12.4 15.1		432 141 621	(Garg *et al.*, 1989)
RTT		120.02	31			(Rajini *et al.*, 2001)
ASBAT Fibre (5mg/ml)		224				(Cavallaro *et al.*, 1994)
Polyolefin Polyethylene terephthalate		275 50-100	2.8 3			(Shao *et al.*, 2001)
Pearl glue Peal glue-collagen		31.8 100	2.9 20.6			(Hepworth and Smith, 2002)
PDS PLLA		600 300	62 35			(Makela *et al.*, 2002)
Synthetic rubber Silks		50 500-972	850 4-20			(Altman *et al.*, 2003)
Catgut Nylon 66 Steel Poly(glycolic Acid) Polypropylene		370 585 660 840 435	25 41 45 22 43			(Goupil, 1996)

2.4. Conclusions

Repair and regeneration of tissues by tissue engineering is dependent on the use of biodegradable polymer scaffolds, which support, reinforce and organise the regenerating tissue. Reconstituted collagen fibres have been shown to be a competitive matrix for tissue engineering applications and have been the subject of recent research. This chapter demonstrated that reconstituted collagen fibres have the same surface morphology and filled inter-fibrillar space, independent of the tissue origin, digestion method or collagen concentration. A strong correlation between collagen concentration, fibre diameter and stress at break was found. Higher diameter fibres had higher strain at break values, higher forces were required to break them and exhibited lower modulus at 2%strain values. Finally, since bovine Achilles tendon is an abundant tissue and collagen with reduced immunogenicity and high biocompatibility can be extracted at high yields, for these reasons, pepsin soluble bovine Achilles tendon was the material that was chosen for this study.

Chapter 3. Optimisation

3.1. Aim

The aim of the work described in this chapter was to investigate factors that could affect the structural and mechanical properties of reconstituted bovine collagen derived fibres. The procedures investigated could be separated into pre-, during- and post- fibre formation.

3.2. Materials and Methods

All suppliers are listed in Appendix.

3.2.1. Raw Material

Bovine Achilles tendon collagen derived from pepsin digestion was used as described previously (Chapter 2). Briefly, bovine Achilles tendons were collected from a slaughterhouse, manually dissected out from the surrounding fascia, washed with distilled water, minced with ice in order to avoid heat denaturation, and then further washed at 4^0C for an hour in three changes of neutral salt solutions. Consequently, the minced tendons were suspended in 0.5M ethanoic acid and pepsin (1:100 of the initial wet weight) for 72h at 4^0C. The suspension was centrifuged (12,000g for 45min at 4^0C) and the Pepsin Soluble Collagen (PSC) purified by repeated acid-salt precipitation (0.9M NaCl) followed by dialysis. The final collagen solution was either kept refrigerated or frozen until used. The collagen content was determined by hydroxyproline assay and the concentration subsequently adjusted to 6 and 8mg/ml.

3.2.2. Fibre formation

The fibre formation procedure was based on the one described previously (Chapter 2). The effect of the following factors was investigated:

3.2.2.1. Pre- Fibre Formation / Effect of Diameter of Extrusion Tube

A 5ml syringe containing collagen solution (6mg/ml) was loaded into the syringe pump system connected to silicone tubing. This tubing was 30cm in length and either 1.0 or 1.5 or 2.5mm in internal diameter. The pump was set to infuse at 0.4ml/min or at 1.2ml/min when the 2.5mm tube was used. The collagen solution was extruded into the Fibre Formation Buffer (FFB) comprising of 118mM Phosphate Buffer and 20% (w/v) PEG Mw 8,000 at pH 7.35 and temperature of 37^0C. The fibres remained in this solution for 10min. Fibres were then transferred and immersed for a further 10min in a Fibre Incubation Buffer (FIB) comprising of 6.0mM Phosphate Buffer and 75mM sodium chloride at pH 7.32 at 37^0C followed by distilled water wash for further 10min. Finally, they were air-dried under the tension of their own weight.

3.2.2.2. During- Fibre Formation / Amount and Type of Co-agent

A 5ml syringe containing collagen solution (6mg/ml) was loaded onto a syringe pump system connected to silicone tubing (30cm in length and 1.5mm internal diameter) and set to infuse at 0.4ml/min.

3.2.2.2.1. PEG / Salt Concentration Effect

Collagen solution was extruded in phosphate FFB that contained either:

- 5, 20 and 40% (w/v) PEG, Mw 8,000 at pH 7.55 and 37^0C, and
- 5, 20 and 40% (w/v) Sodium Chloride at pH 7.14 and 37^0C.

3.2.2.2.2. Different Co-agents Effect

A series of experiments was then carried out to compare different co-agents at 20% addition:

- 20% PEG, Mw 8,000 at pH 7.55 and 37^{0}C.
- 20% Sodium Chloride at pH 7.14 and 37^{0}C.
- 20% Sodium Sulfate at pH 7.14 and 37^{0}C.
- 20% PEG, Mw 200 at pH 7.53 and 37^{0}C.
- 20% PEG, Mw 1,000 at pH 7.41 and 37^{0}C.
- 20% PVA, Mw 9-10,000 at pH 7.55 and 37^{0}C.
- 20% PVA, Mw 20,000 at pH 7.55 and 37^{0}C.
- 20% Soluble Starch at pH 7.31 and 37^{0}C.
- 20% Gum Arabic at pH 7.40 and 37^{0}C.
- 20% Glycerol at pH 7.20 and 37^{0}C.
- 20% D(+) Glucose at pH 7.40 and 37^{0}C.
- 20% Casein at pH 7.55 and 37^{0}C.

The fibres were allowed to remain in the above buffers for a period of 10min and then were immersed as previously in the FIB, followed by a distilled water bath and finally air-dried under the tension of their own weight.

3.2.2.3. Post- Fibre Formation / Final Bath Composition

3.2.2.3.1. Comparison of Distilled Water and 70% Isopropanol

A 5ml syringe containing collagen solution (6mg/ml) was loaded onto the syringe pump system connected to silicone tubing (30cm in length and 1.5mm internal diameter) and set to infuse at 0.4ml/min. The collagen solution was extruded into a FFB comprising of 118mM Phosphate Buffer and 20% (w/v) PEG Mw 8,000 at pH 7.35 and temperature of 37^{0}C. The fibres remained in this solution for 10min. Fibres were then transferred and immersed for further 10min in a FIB comprising of 6.0mM Phosphate Buffer and 75mM sodium chloride at pH 7.32 at 37^{0}C followed by immersion in either distilled water or 70% (v/v) isopropanol solution in water for further 10min. Finally, they were air-dried under the tension of their own weight.

3.2.2.3.2. Comparison of Distilled Water for 15 min and DW, PBS, Isopropanol, Tris-HCl and Vegetable Oil Overnight

A second set of experiments was carried out as above. However, in this case, 8mg/ml collagen solution was used, and the fibres, at the end of the process, were left overnight, at room temperature into either of the following solutions: DW, 0.01M PBS pH 7.4, 100% isopropanol, 50mM Tris-HCl pH 7.4 or 100% vegetable oil. Control fibres were those that were made and dried as before.

3.2.3. Mechanical Testing and Microscopy

The mechanical characteristics as well as the microstructure of the reformed collagen fibres were analysed as described previously (Chapter 2). Briefly, an Instron 1122 Universal machine was used for the mechanical tests at an extension rate of 10mm/min, the gauge length was fixed at 5cm (and 3cm for the 3.3.2.3.2. set of experiments), soft rubber was used to cover the inside area of the grips in order to avoid damaging the fibres at the contact points and fibres that broke at contact points with the grips were rejected. A Nikon Eclipse E600 optical microscope and a Hitachi Variable Pressure Scanning Electron Microscope (VPSEM) were respectively used to measure the diameter of the fibres and to carry out observations of the microstructure of the fibres. Each fibre cross sectional area was calculated assuming a circular cross section and by measuring the diameter at five (and four for the 3.2.3.2. set of experiments) places along the fibre using the optical microscope fitted with a calibrated eyepiece graticule.

3.3. Results and Discussion

3.3.1. Pre-Fibre Formation / Effect of Diameter of Extrusion Tube

Fibres derived from the low and medium internal diameter extrusion tube had the same structure (Figure 1). However, it was found that the 2.5mm tube was too large to produce a reasonable / consistent fibre shape (Figure 2). What was observed looked like an infusion of collagen solution in a buffer and due to the air stream and the lifting, transferring and drying method a fibre-shaped gel with bubbles throughout its longitudinal axis was produced. Increase of the extrusion rate (from 0.4ml/min to 1.2ml/min) did not improve the situation.

Figure 1. Optical microscope pictures of fibres derived from 1.0mm (a) and 1.5mm (b) internal diameter tube.

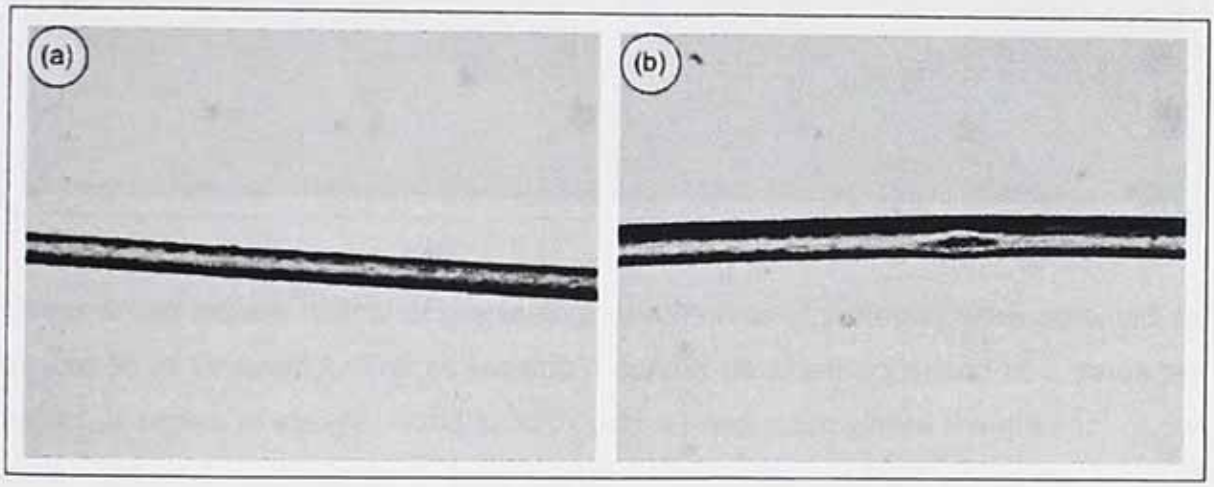
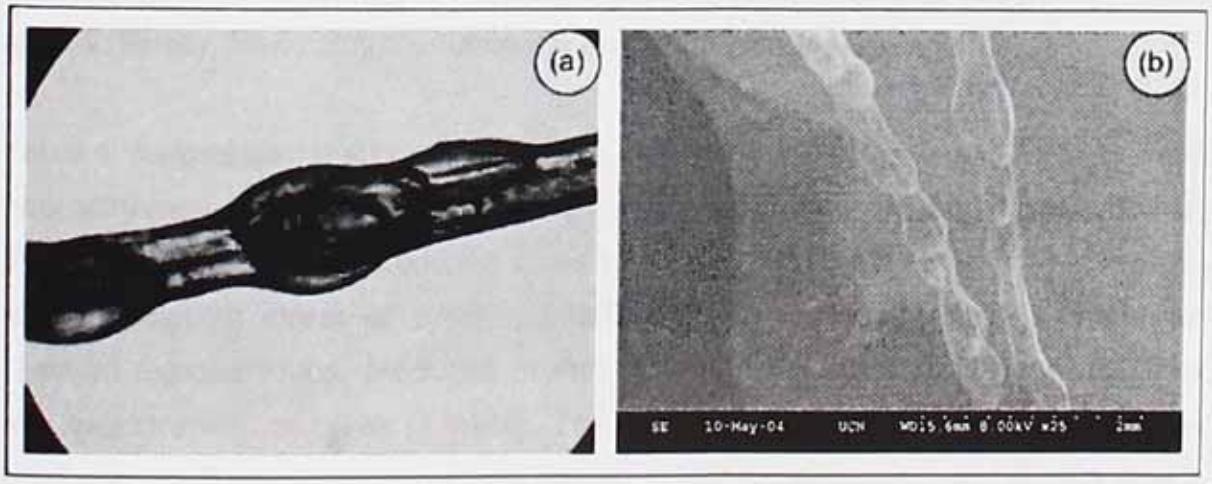
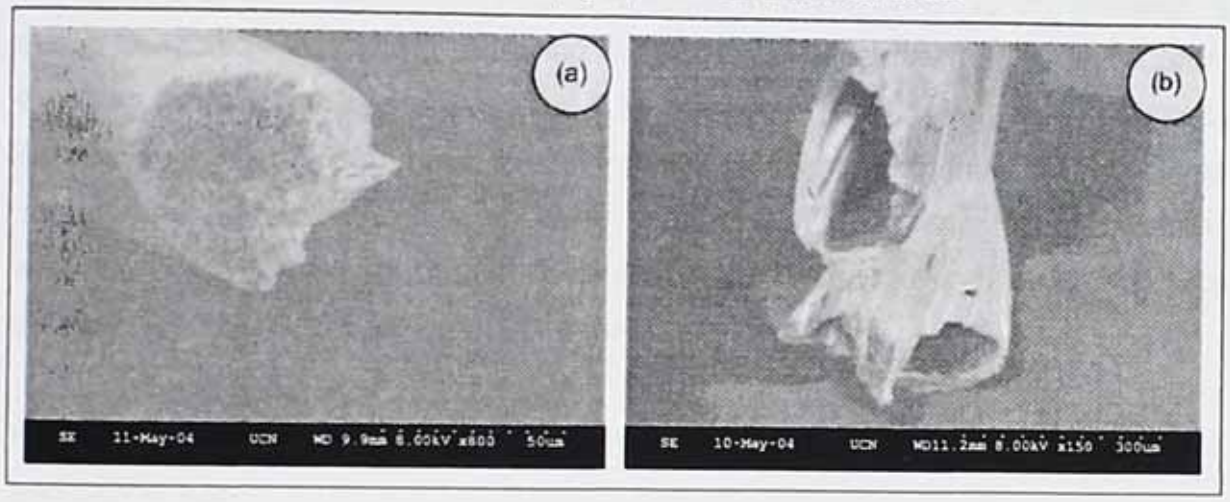


Figure 2. Optical (a) and SEM (b) pictures of fibres derived from 2.5mm internal diameter tube.



The bubbles that appeared along the axis of the 2.5mm-tube-derived-fibres, created weak points and all the fibres broke there during tensile testing. Fibres derived from the 1.0 and 1.5mm tube both showed the same breaking patterns, which have been described in detail in Chapter 2. From Figure 3, it can be observed that the low and medium diameter extrusion tube produced fibres with filled inter-fibrillar spaces, while air-bubbles were trapped within the fibres that derived from the high in diameter tube.

Figure 3. SEM pictures of the breaking surfaces of reconstituted collagen fibres derived from 1.0mm (left) and 2.5mm (right) internal diameter tube.



Stress-strain curves typical of dry biological materials (ligaments) were obtained as described in Chapter 2. The stress-strain curves obtained consisted of a small toe region, a region of steeply rising stress up to a knee point where the gradient of the curve reduced, followed by a long region of constant gradient up to the point of fracture. The stress-strain curves showed a diameter dependent variation as described in Chapter 2, Figure 17. Fibres of low diameter showed high stress/low strain graphs, while fibres of high diameter demonstrated low stress/high strain graphs. Finally, fibres of higher diameter exhibited a longer toe region.

Table 1 summarises the properties of the dry reconstituted collagen fibres derived from different internal diameter tubing. It was found that the smallest internal diameter tubing (1.0mm) produced fibres with the smallest diameter (0.084mm) and with the highest stress at break (200MPa), whilst the largest internal diameter (2.5mm) extrusion tube, produced dry fibres with the highest diameter (0.459mm) and lowest stress at break (13MPa). The middle diameter (1.5mm) extrusion tube produced dry fibres with intermediate values; 0.123mm diameter and 136MPa stress at break.

Table 1. Physical and mechanical properties of dry reconstituted collagen fibres as a function of different in internal diameter extrusion tube.

	Diameter (mm)	Stress at Break (MPa)	Strain at Break	Force at Break (N)	Modulus at 2% Strain (MPa)
1.0mm (n=21)	0.08 ± 0.01	200 ± 58	0.24 ± 0.04	1.03 ± 0.12	1841 ± 517
1.5mm (n=18)	0.12 ± 0.03	136 ± 58	0.36 ± 0.13	1.41 ± 0.17	856 ± 749
2.5mm (n=5)	0.46 ± 0.04	13 ± 3	0.34 ± 0.07	2.16 ± 0.55	17 ± 10

Fitting a linear regression model (Figure 4) to the stress at break Vs dry diameter, a strong correlation was found regarding the fibres derived from the 1.0 and 1.5mm extrusion tubes (R^2 values of 0.85 and 0.89 respectively). However, since the fibres derived from the 2.5mm tube were highly non-uniform and their diameter was irregular, the correlation was very low (R^2 value of 0.07). Similarly, high correlations were observed for overall stress Vs overall diameter and overall stress Vs tube diameter (R^2 values of 0.94 and 0.99 respectively) (Figure 5).

Figure 4. Correlation between stress at break and Dry fibre diameter for the 1.0, 1.5 and 2.5mm tube.

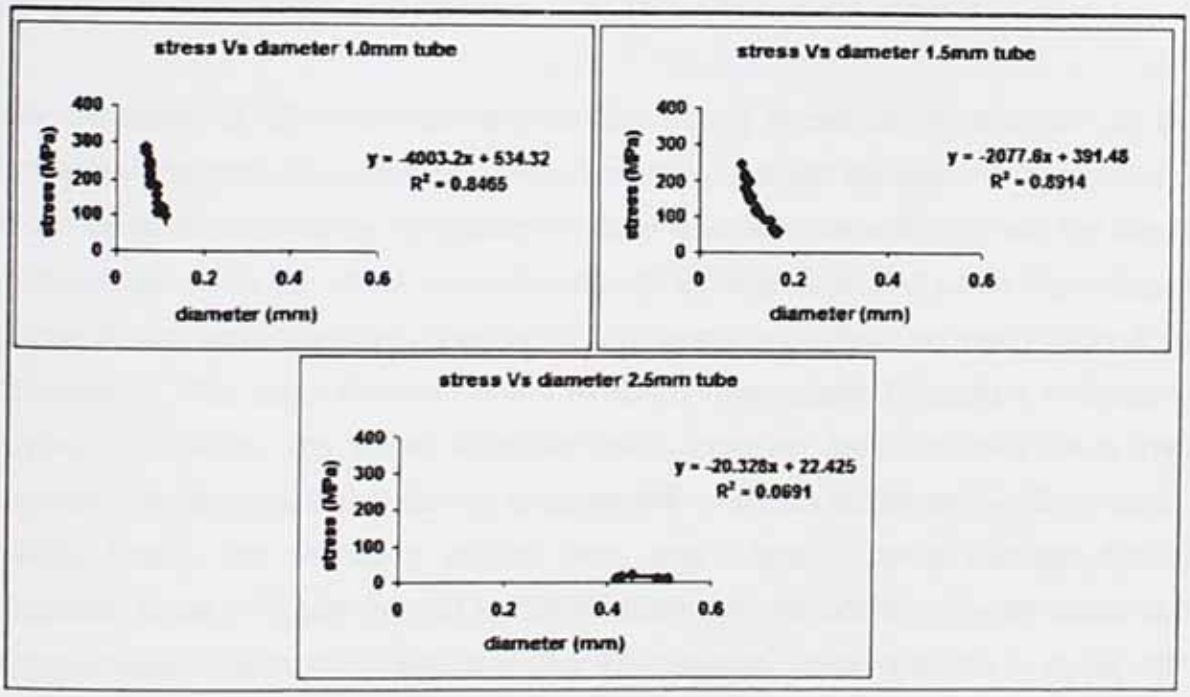
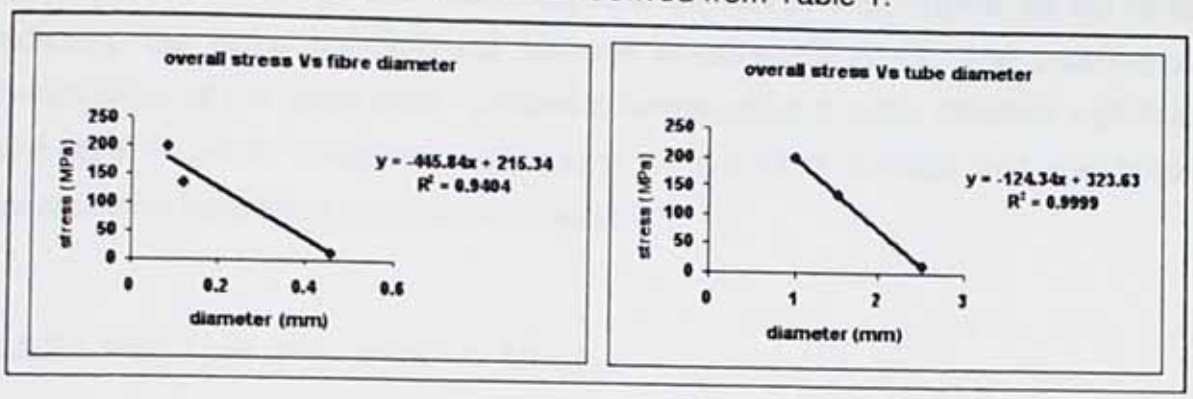


Figure 5. Correlation between overall stress at break and overall dry fibre diameter and stress at break and tube diameter as derived from Table 1.



Furthermore, it was found that by increasing the extrusion tube diameter (1.0, 1.5 and 2.5mm), a great decrease in modulus values (1841, 855 and 17MPa respectively) was obtained and an increase in force (1.0, 1.4 and 2.2N respectively) required to break the fibres was observed. An increase in strain at break values from 0.24 to 0.36 was observed between the 1.0 and 1.5mm diameter extrusion tubes. However, the difference in strain at break values appeared to slightly decrease from 0.36 to 0.34 in going from the 1.5 to the 2.5mm internal diameter tube. This could be attributed to the air-bubbles creating weak points, which did not allow the fibres to elongate as much as they would have done if they have been uniform.

The utilisation of different internal diameter tubing appeared to influence all the measured mechanical properties of the dried reconstituted collagen fibres produced. It can be observed that by increasing the fibre diameter, the stiffness and the stress at break values of the fibres were decreased, as was observed when the collagen concentration was increased (Chapter 2). That result appears to support the previous observation, that lower diameter fibres exhibit a more packed structure and higher alignment of fibrils. The lowest diameter fibres, however, failed at lower loads than the other two groups, which seems to agree with previous observations (Dunn *et al.*, 1993). Finally, the utilisation of the 1mm and 1.5mm internal diameter tubing produced fibres of 0.084mm and 0.122mm diameter respectively. These values are approximately one tenth smaller than the tube utilised, which appears to agree with previous results (Dunn *et al.*, 1993; Gentleman *et al.*, 2003). This demonstrates that the dry fibre diameter can be controlled by varying the inner diameter of the extrusion tube. However there would appear to be a critical tube diameter (somewhere between 1.5 and 2.5mm) over which the extrusion becomes disrupted and the fibre becomes ultimately non-uniform, at least under the conditions used here.

3.3.2. During- Fibre Formation / Amount and Type of Co-agent

No structural differences were observed in these sets of experiments. All the fibres exhibited the same characteristic s-shape stress-strain curve. The microscopic investigation of the failed ends revealed a homogenous internal structure with filled inter-fibrillar space. Detailed explanations of the fibre surface and the failure mechanisms have been provided in Chapter 2.

3.3.2.1. PEG / Salt Concentration Effect

In this set of experiments the effect of the concentration of the most widely used co-agents, PEG Mw 8,000 (Cavallaro *et al.*, 1994; Laude *et al.*, 2000) and NaCl (Pins and Silver, 1995) was investigated. The mechanical properties of the fibres produced are summarised in Table 2. These experiments demonstrate that the self-assembly of collagen is a process depending on the electrolyte and its concentration as has been observed previously for collagen micro-fibrillar networks (Jiang *et al.*, 2004).

An association between the increase in the co-agent concentration and the fibre diameter was not found. For the same % of co-agent, it was found that salt-induced fibres had higher force at break values. In chapter 2, it was shown that for the same collagen concentration, the salt-induced fibres required higher forces for failure compared to PEG and had higher tensile strength values. For the low co-agent concentration the tensile strength values were found to be similar between PEG and NaCl, however, by increasing the amount of the co-agent, the salt induced fibres gave higher stress at break values in comparison with those derived from PEG. The strain-at-break values were higher for the PEG-derived fibres. It has been shown that, in composite fibres, the elongation to break arises from both the collagen and the polymer (Bleach *et al.*, 2002). Since collagen molecular and fibrillar slippage plays an important role in the tensile deformation of aligned connective tissue such as tendon, it is more likely that PEG, at least in these *in vitro* studies, plays a role in promoting either molecular or collagen fibrillar slippage, as has been suggested for decorin (Pins *et al.*, 1997b; Fischer *et al.*, 2000). Furthermore, the observed decrease in tensile strength of the fibres, especially in the higher concentration of co-agent, appears to agree with previous results, where it was shown that increasing dermatan sulphate concentration, has an inhibitory effect on the mechanical strength of reconstituted collagen fibrils (Danielsen, 1982). In the presence of salt, the elongation is derived only from the collagen, since the NaCl is a much more compact molecule than PEG, and as a result, less extensible fibres were produced with

decreased displacement at break and increased modulus values in comparison with the PEG ones. Finally, the modulus values appeared to decrease, as the concentration of the co-agent was increased. This observation appears to be in agreement with previous work, where it was shown that by increasing the concentration of electrolytes, a decrease in tensile modulus was observed (Agrawal *et al.*, 2000; Loret and Simoes, 2004).

Table 2. Physical and mechanical properties of reconstituted collagen fibres as a function of the co-agent concentration (PEG, Mw 8,000 and NaCl).

	Diameter (mm)	Stress at Break (MPa)	Strain at Break	Force at Break (N)	Modulus at 2% Strain (MPa)
5% PEG (n=13)	0.13 ± 0.02	139 ± 34	0.22 ± 0.04	1.84 ± 0.20	1233 ± 526
20% PEG (n=18)	0.12 ± 0.03	136 ± 58	0.36 ± 0.13	1.41 ± 0.17	856 ± 749
40% PEG (n=10)	0.22 ± 0.07	57 ± 40	0.30 ± 0.13	1.44 ± 0.33	377 ± 466
5% NaCl (n=17)	0.14 ± 0.02	135 ± 59	0.17 ± 0.06	2.02 ± 0.60	918 ± 523
20% NaCl (n=25)	0.12 ± 0.02	208 ± 57	0.27 ± 0.06	2.23 ± 0.32	1581 ± 490
40% NaCl (n=16)	0.14 ± 0.02	142 ± 41	0.24 ± 0.07	2.23 ± 0.47	1158 ± 687

During precipitation experiments, has been shown that the mean particle size of the precipitates was generally reduced when the ionic strength was increased because of the high level of counter-ions around the protein in the solution (Kim *et al.*, 2001). However, with an overdose of the polyelectrolyte, the particle size was remarkably increased (Kim *et al.*, 2001), leading to a decrease in the effectiveness of the precipitation (Ramshaw *et al.*, 1984). These observations are in accord with other work, where it was mentioned that flocs formed under polymer overdosing conditions are larger than flocs formed under polymer under-dosing conditions (Chen and Berg, 1993). Bearing in mind that (a) both salt (Timasheff and Arakawa, 1988; Kuehner *et al.*, 1996) and PEG (Zeppezauer and Brishammar, 1965; Ramshaw *et al.*, 1984; Mahadevan and Hall, 1992) can precipitate collagen, (b) the *in vitro* self-assembly of collagen molecules into fibrils with periodic patterns (Giraud-Guille *et al.*, 1994) is analogous to the precipitation of collagen (Candlish and Tristram, 1964), (c) precipitation (bulky result) and self-assembly (structural, highly organised result) are processes driven by forces, which are mainly electrostatic attraction plus hydrogen and hydrophobic bonding (Chen and Berg, 1993), and (d) taking as particle size the diameter of the fibre produced, the same results obtained from these experiments;

the fibre diameter increased as the electrolyte concentration increased from the 5 to 20% (under-dosing conditions), but at 40% electrolyte content (overdose conditions), the fibre diameter was decreased.

For both PEG and Salt treatments an increase in elongation to break values was observed as concentration increased from 5 to 20% and a reduction from 20 to 40%. It has been mentioned that the elongation depends on the amount of filler (salt / PEG) and the polymer used (Bleach *et al.*, 2002). The observed strain at failure at 5% co-agent addition presumably could be attributed more to the polymer and less to the filler, while at 20% it can be attributed to both of them. Furthermore, it has been shown that increasing the amount of filler decreases the amount of polymer available for elongation (Bleach *et al.*, 2002), which only seems to agree with the results obtained at 40% co-agent utilisation, possibly indicating that the 20% co-agent comprises a value close to the optimum for maximum elongation.

Between the treatments, the stress at break obtained with salt was the highest for the 20% co-agent concentration while for the PEG similar values were obtained for the 5 and 20% treatment. Utilisation of 40% for either co-agent gave decreased values of tensile strength since higher diameter fibres were obtained. For the salt treatment, the increase in fibre diameter appears to decrease the force required to break the fibre, while when the fibre diameter was increased as a factor of the collagen concentration, the force at break was increased. In this instance, it can be assumed that the increase in fibre diameter is due to the increased co-agent amount rather than to higher fibril packing and a more fragile material can be produced. Therefore, the fibres can break easier.

Fitting a linear regression model for the stress at break Vs dry fibre diameter for the PEG treatments, a high correlation was found (R^2 for 5, 20 and 40% PEG was 0.85, 0.89 and 0.84 respectively) (Figure 6). It has been demonstrated that the density of micro-spheres and therefore their diameter could be controlled by the amount of PEG incorporated into the polymer backbone (Fu *et al.*, 2002). Alumina fibres produced in the presence of polymers, showed that the lower the polymer content, thinner fibres obtained and the fibre diameter distribution was narrower than when the higher polymer concentration was used (Jancic and Aleksic, 2000). However, between the salt treatments, only the 20% gave a high correlation value (R^2 value for 5, 20 and 40% NaCl was 0.56, 0.72 and 0.51 respectively) (Figure 7). It has been mentioned

that in low NaCl concentration, due to the salting in effect, protein molecules are in the right configuration to build up a rigid and more viscoelastic material (Tsaliki *et al.*, 2003). At higher salt concentrations, due to passing the optimal amount of water removable for fibre stability, this right configuration might be lost (Candlish and Tristram, 1964). These results indicate that there is an "*ideal*" concentration of co-agent for the production of reproducible reconstituted collagen fibres. Similar results have been reported in previous work, when it was mentioned that acid extracts of collagenous tissue, when neutralised or precipitated with the "*correct*" ionic strength of salt tend to form fibres with the longitudinal periodicity observable in the electron microscope (Candlish and Tristram, 1964).

Figure 6. Correlations of stress at break and fibre diameter of the PEG Mw 8,000 treatments.

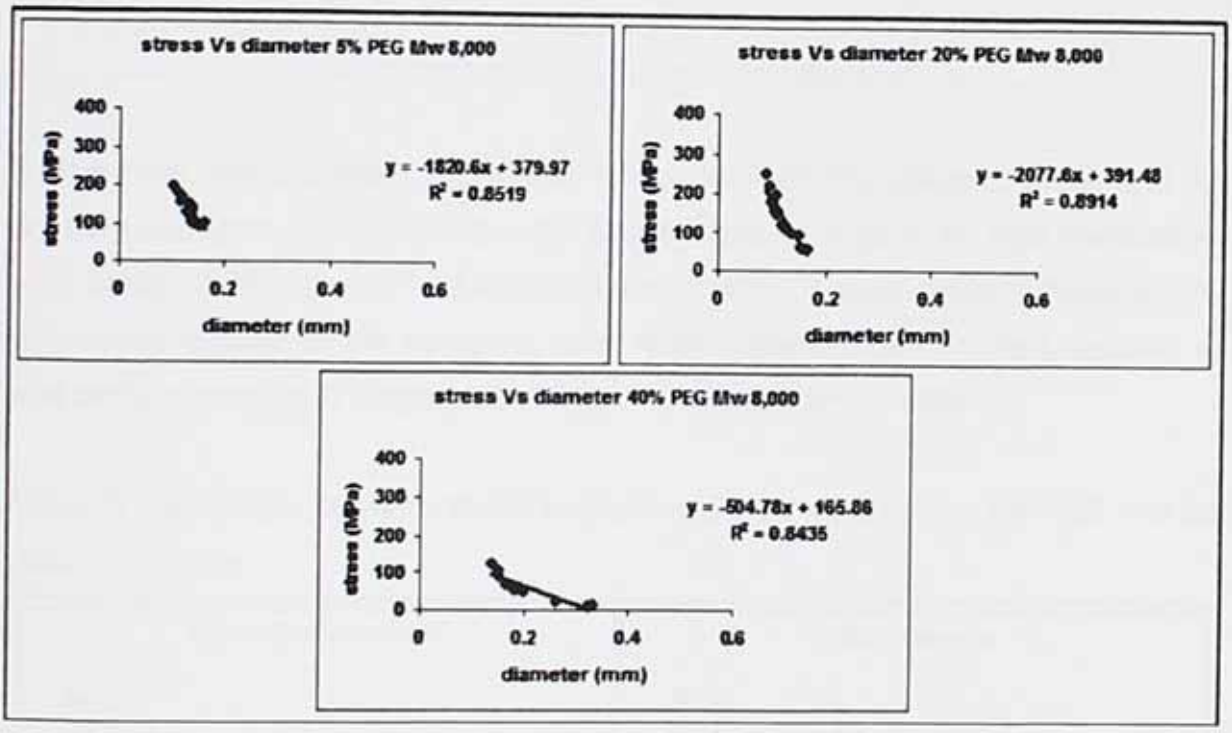
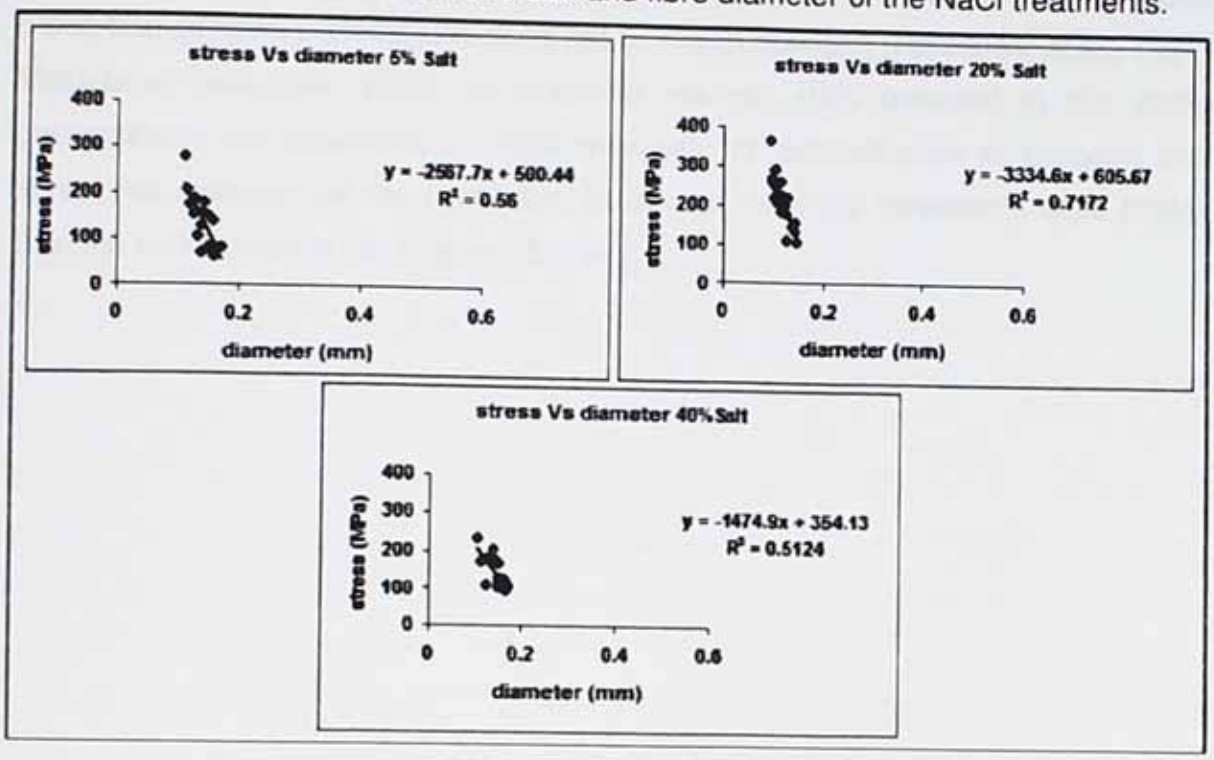
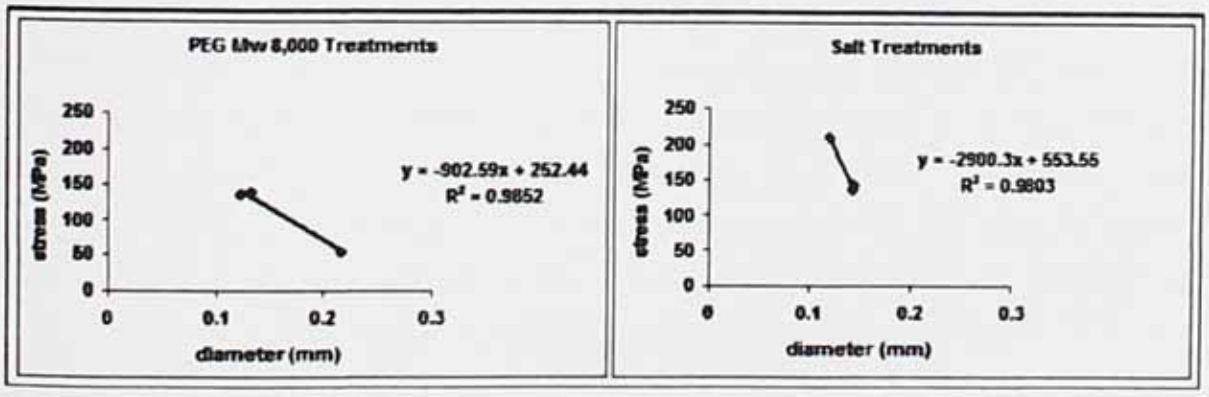


Figure 7. Correlations of stress at break and fibre diameter of the NaCl treatments.



Furthermore, fitting a linear regression model between the stress at break and the dry fibre diameter of the PEG and the Salt treatments (Figure 8), high correlations were obtained (R^2 value of 0.99 and 0.98 respectively). These results indicate that by varying the amount of the co-agent, tailor made materials can be manufactured as was demonstrated with varying the collagen concentration (Chapter 2).

Figure 8. Correlation between stress at break and fibre diameter for the PEG and the NaCl treatments.



Finally, it has been shown that the use of PEG for precipitating native collagen has a number of advantages over conventional salt precipitating methods, since low amounts of PEG and low-speed centrifugation are required, PEG has been shown to be non-denaturing and does not interfere with subsequent electrophoretic or ion

exchange chromatographic steps, appropriate PEG concentration and Mw can precipitate different proteins and does not bind with collagen (Ramshaw *et al.*, 1984; Mahadevan and Hall, 1992). In a similar manner, data provided in this study demonstrates the superiority of PEG over salt for reconstitution of collagen into fibres. For example, all the correlation values for the PEG treatments were higher than the counterpart of salt (Figures 6, 7 and 8).

3.3.2.2. Effect of Different Co-agents

The importance of combining pepsin soluble collagen (atelocollagen) with other biocompatible polymers in order to produce matrices of sufficient strength has been pointed out (Friess and Lee, 1996; Bonassar and Vacanti, 1998; Matsumoto *et al.*, 1999). Furthermore, salt has been shown to enable the production of reconstituted collagen fibres, which are comparable to those produced from PEG Mw 8,000 in terms of mechanical properties. In this section, the effect of other co-agents on the mechanical properties of the reconstituted collagen fibres will be discussed.

The glycerol, D (+) glucose, PVA Mw 20,000 and casein buffers all failed to produce fibres suitable for testing. Fibres did form in the glycerol and glucose buffers but they were extremely fragile and it was found impossible to transfer them from the FFB to the FIB, even after extended incubation in the FFB (2-hours). It has been reported that glycerol not only inhibits the fibril formation of either acid or pepsin soluble collagen type I, but also disassembles already formed fibrils (Na *et al.*, 1986; Na *et al.*, 1989), although it stabilises the collagen molecule, resulting in moderate increase in denaturation temperature. The stabilising effect of glycerol is achieved by its binding to the surface of the collagen molecule; this leads to stabilisation of every individual polypeptide α-chain without increasing the number of inter-chain stabilising hydrogen bonds (Penkova *et al.*, 1999). However, it has been shown that non-electrolytes, such as sugars and glycerol, usually increase the strength of gelatine gels and provide stabilisation through hydrogen bonding (Fernandez-Diaz *et al.*, 2001). The viscous nature of the PVA buffer (Mw 20,000) caused the fibres to break while they were being lifted out. The Casein, Gum Arabic and Starch buffers had to be filtered since they were too concentrated (saturated). The solution recovered after filtration was used as FIB. Additionally, since these solutions were not transparent, it was found difficult to observe the fibre formation. From the casein buffer, "tube-shape" material rather than a fibre was formed and was not suitable for testing. Fibres produced in the presence of Gum Arabic and Starch had to remain in the buffer for 30min in order to gain sufficient strength for transfer to the FIB.

Table 3 summarises the physical and mechanical properties of the reconstituted collagen fibres produced in this set of experiments. It can be seen that the highest tensile strength fibres were produced using salt (208MPa), while the lowest in tensile strength were produced when the sodium sulphate (34MPa) was utilised. The rest of the treatments gave values ranging between those two limits. The highest strain at

break values were obtained when the PEG Mw 8,000 (0.36) was used, while the other PEGs (0.19) gave the lowest values. The highest modulus values were produced when the salt (1581MPa) was utilised, while the sodium sulphate (293MPa) treatment gave the lowest in stiffness fibres.

Table 3. Physical and mechanical properties of reconstituted collagen fibres formed in the presence of 20% co-agent.

	Diameter (mm)	Stress at Break (MPa)	Strain at Break	Force at Break (N)	Modulus at 2% Strain (MPa)
Sodium Sulphate (n=7)	0.30 ± 0.11	34 ± 39	0.21 ± 0.08	1.21 ± 0.54	293 ± 453
Sodium Chloride (n=25)	0.12 ± 0.01	208 ± 57	0.27 ± 0.06	2.23 ± 0.32	1581 ± 490
PEG Mw 200 (n=16)	0.10 ± 0.02	158 ± 72	0.19 ± 0.06	1.18 ± 0.38	1303 ± 725
PEG Mw 1,000 (n=12)	0.10 ± 0.01	157 ± 59	0.19 ± 0.04	1.17 ± 0.49	1350 ± 664
PEG Mw 8,000 (n=18)	0.12 ± 0.03	136 ± 57	0.36 ± 0.13	1.41 ± 0.17	856 ± 749
PVA Mw 9-10,000 (n=11)	0.14 ± 0.03	153 ± 35	0.21 ± 0.06	2.31 ± 0.36	1359 ± 672
Soluble Starch (n=7)	0.12 ± 0.02	178 ± 77	0.29 ± 0.02	2.00 ± 0.31	831 ± 712
Gum Arabic (n=8)	0.14 ± 0.02	107 ± 30	0.25 ± 0.10	1.66 ± 0.20	625 ± 439

Fitting a linear regression model between overall stress at break and dry fibre diameter, a relatively high correlation was obtained (R^2 value of 0.73) (Figure 9). This correlation, between the individual treatments, appears to fluctuate, indicating that different co-agents have a different effect on the properties of the reconstituted collagen fibres. In section 3.4.2.1, it was demonstrated that there was a correlation between stress at break and the fibre diameter for the 20% PEG and NaCl treatments (R^2 value of 0.89 and 0.72 respectively) (Figure 3 and 4). Similar high correlations were obtained for the PVA Mw 9-10K, Sodium sulphate, Gum Arabic and Starch treatment (R^2 value of 0.82, 0.82, 0.89 and 0.86 respectively), whilst the PEG Mw 200 and Mw 1000 gave the lowest values (R^2 value of 0.67 and 0.52 respectively) (Figure 10). Between these correlation values, it can be seen that the highest was obtained from PEG Mw 8,000. Previous studies have demonstrated the superiority of high Mw PEG against low Mw PEGs, salts and polymers for the precipitation of proteins (Ramshaw *et al.*, 1984; Mahadevan and Hall, 1992).

Figure 9. Correlation between overall stress at break and dry fibre diameter for the 20% co-agent.

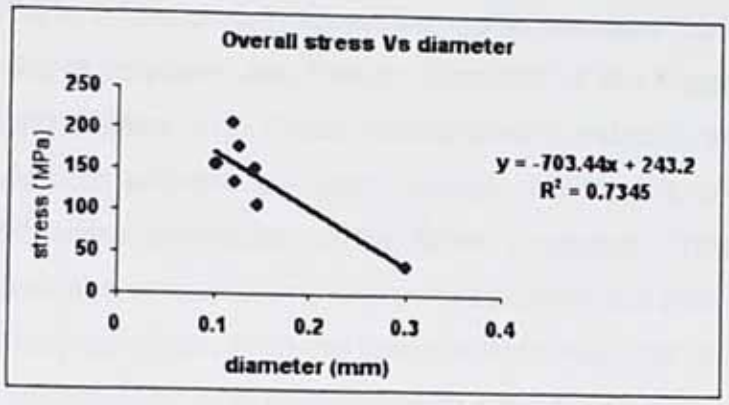
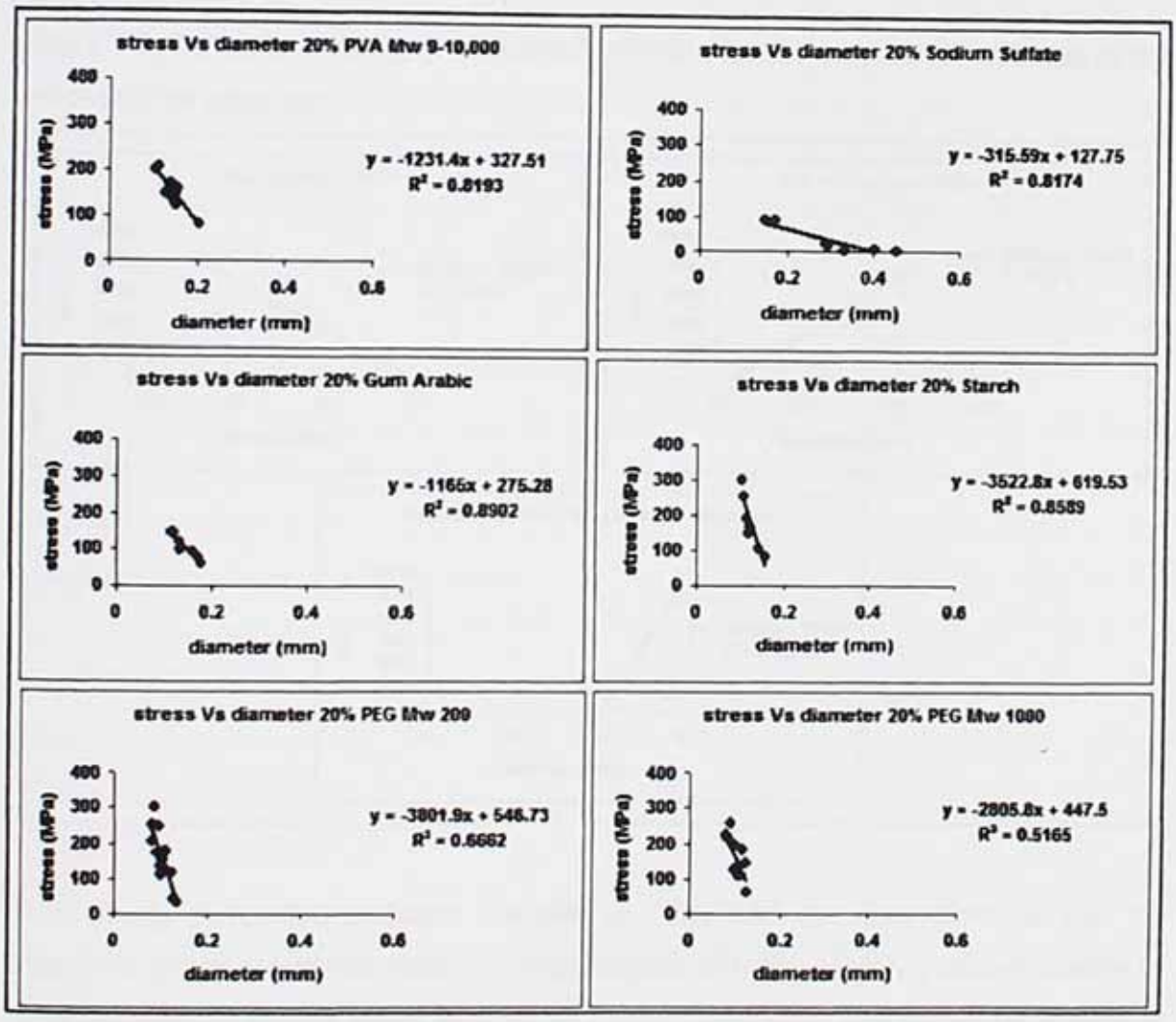


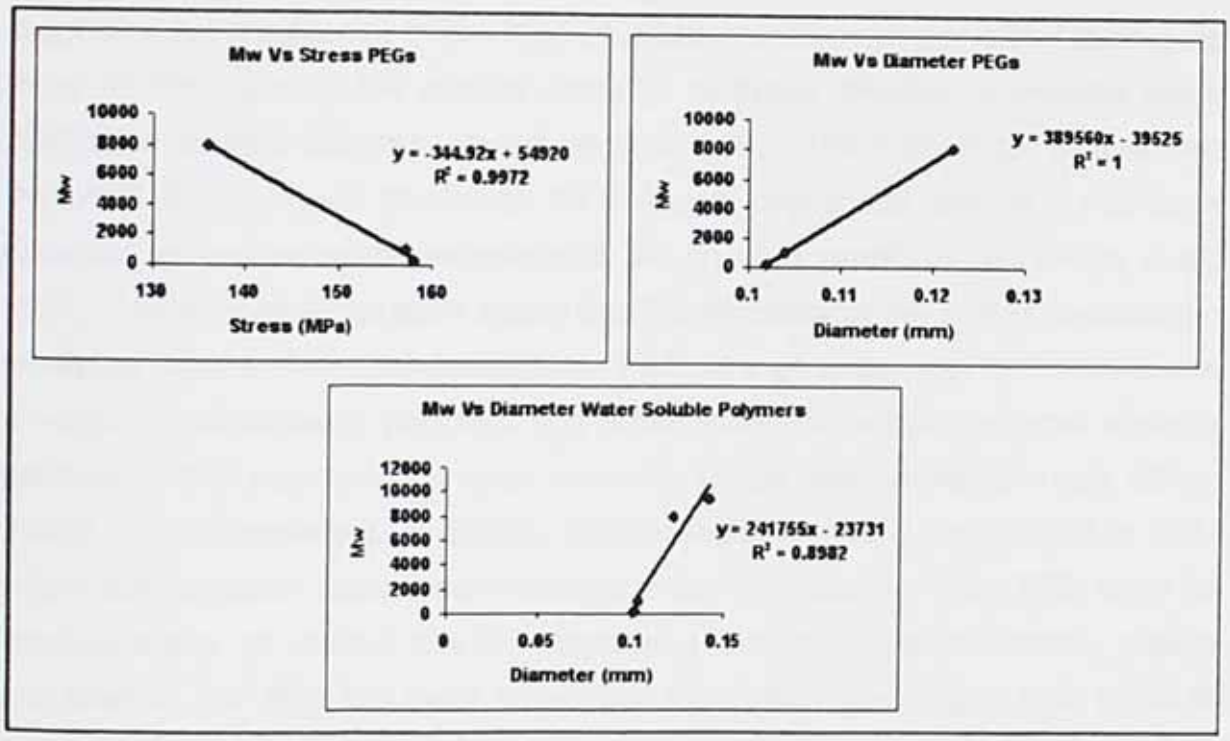
Figure 10. Correlation between stress at break and dry fibre diameter for the 20% co-agent treatments.



From this set of experiments it can be observed that the lower molecular weight PEGs (200 and 1,000) gave similar values regarding their mechanical properties while both differ from results obtained with the higher molecular weight one (8,000).

Fitting a linear regression model Mw Vs stress at break and Mw Vs diameter for the PEG treatments, the R^2 values obtained were 0.997 and 1.000 (Figure 11). Even, if the PVA Mw 9-10K is added to the regression model between the molecular weight of these water-soluble polymers and the dry diameter of the fibres, an R^2 value of 0.898 was obtained (Figure 11). These results clearly indicate that the molecular weight of water-soluble polymer co-agent strongly affects the physical and as a consequence mechanical properties of the fibres produced. These results are in agreement with previous observations where was pointed out that as the molecular weight increases (oxy-ethylene units) the material becomes less brittle and therefore can resist crack propagation when subjected to an applied stress. An increase in molecular weight increases either the number of bonds formed per surface area, or the strength of the bonds formed increases (Al-Nasassrah *et al.*, 1998).

Figure 11. Correlation between Mw and dry fibre diameter and stress at break of the water-soluble polymers.



This strong correlation between the Mw of PEG and the fibre diameter can be explained at the molecular level. At physiological pH, the slightly positive charge of collagen attracts anions so as to achieve electro-neutrality. The presence of ions in the intra-fibrillar compartment is constrained by steric considerations. Previous studies have shown that the spaces within the fibre (i.e. the gaps between the polypeptide helixes and between the fibrils) are approximately in the size range 10-500Å. In addition the inter-fibre void spaces are of sizes ranging from about 0.2μm to

several microns. Thus Cl^- and water molecules of size 3.6Å, Na^+ and Ca^{+2} of size around 2Å, can easily penetrate into the intra-fibrillar compartment whilst larger molecules cannot and as a result, they osmotically compress the fibres (Leikin *et al.*, 1994; Rao *et al.*, 1995; Kuznetsova and Leikin, 1999; Loret and Simoes, 2004). Furthermore, it has been shown that large fragments of chitosan may not be able to penetrate the alginate fibre and will therefore only interact with the fibre surface, whereas smaller fragments could partially penetrate the fibre network and become entangled (Knill *et al.*, 2004). The question arises as to how far into the collagen fibre structure the PEG molecule can penetrate. It is known that PEG penetration into clay films is a process dependent on time and molecular size. It was shown that the greater the molecular weight of the PEG, the longer time was required for it to be absorbed into the clay. Additionally, whilst lower molecular weight (PEG-200) and as a consequence smaller oligomer lengths (13Å) along the C-axis PEG could penetrate the films, the higher molecular weight PEG (PEG-10,000) was size excluded (633Å) and needed to "reptate" to intercalate into the clay (Baker *et al.*, 2004). It has been suggested that reptation (a motion that a polymer translates forwards and backwards along its own axis) is the primary mode of hindered diffusion of proteins when confined in collagen matrices (Rosenblatt *et al.*, 1989). Therefore, it can be assumed that PEG 8,000 aligned parallel to the collagen longitudinal axis as it has been observed for hydroxyapatite nano-crystals via crystallographic studies (Rhee *et al.*, 2001). Furthermore, it has been shown that the efficiency of the protein fractionation increased with the molecular weight of the PEG; at a given polymer concentration on a weight-by-weight basis, polymers of a higher molecular weight are more effective precipitants than polymers of a lower molecular weight (Mahadevan and Hall, 1992). Taking these observations, in addition with our result regarding the 20,000 Mw PVA, where fibre formation was not achieved due to the high viscosity of the FFB, it can be concluded that an optimal Mw for polymers should exist for satisfactory protein precipitation. For PEG has been shown that the optimal Mw ranges from 6,000 to 8,000 (Mahadevan and Hall, 1992).

The PVA-induced fibres appeared to have lower displacement and higher force for break and modulus values in comparison with the PEG Mw 8,000. It has been shown that the PEG molecule cannot couple with more than one or two drug molecules since PEG has only one or two functional groups per molecule, while other polymeric carriers, such as dextran, pullulan, gelatin and polyvinyl alcohol (PVA) have numerous functional groups (Yamaoka *et al.*, 1995). Similarly, it can be assumed that

the multifunctional PVA coupled with more free sides of collagen than PEG and as a result a stiffer material was produced.

The gum Arabic treatment gave relatively high strain at failure values and the second lowest modulus values. It has been shown that the addition of xanthan gum on protein systems had a positive effect on emulsification process due to network creation (Tsaliki *et al.*, 2003).

The utilisation of soluble starch appeared to improve the mechanical properties of the reconstituted collagen fibres; the stress-at-break values were higher than those produced from gum Arabic and the water-soluble polymer treatments. Also the extension was found to be the second best after the PEG Mw 8,000. Furthermore, the hydrophilic nature of starch has been utilised to improve mechanical properties and thermal stability during interaction with hydrophobic cellulose fibres (Averous *et al.*, 2001).

3.3.3. Post-Fibre Formation / Final Bath Composition

No structural differences were observed in these sets of experiments. All the fibres appeared to break in the same way and the internal structure was identical with filled the inter-fibrillar space. Detailed explanation of the structure and the failed ends of reconstituted collagen fibres has been provided on Chapter 2. The stress-strain curves again were similar to those described in previous sections.

3.3.3.1. Comparison of Distilled Water and Isopropanol

The method utilised in obtaining the results reported in this section was based on Cavallaro (Cavallaro *et al.*, 1994), who after the FIB used an isopropanol bath. However, in a series of papers from Silver and co-workers (Silver *et al.*, 2000; Silver *et al.*, 2001a) distilled water or combinations of alcohol and distilled water were utilised (Dunn *et al.*, 1992; Wang *et al.*, 1994). It was of interest therefore, to compare the effect of these liquids on the fibres produced. Table 4 summarises the mechanical properties of the dry fibres produced in this set of experiments. It can be seen that the fibres produced in the presence of alcohol had higher diameters and as a consequence lower stress at break values. The higher diameter fibres appeared to be more extendable with the fibre derived from the 1.5mm tube in the presence of alcohol giving an impressive 50% strain at break value. The modulus values appeared to decrease when alcohol was used. The force required to break the fibres appeared to be similar for the 1.0mm tube, while a slight decrease from the distilled water to alcohol was observed when the 1.5mm tube was utilised.

Table 4. Physical and mechanical properties of reconstituted collagen fibres derived from different tubing in the presence of alcohol and distilled water.

	Diameter (mm)	Stress at Break (MPa)	Strain at Break	Force at Break (N)	Modulus at 2% Strain (MPa)
1.0mm DW (n=21)	0.08 ± 0.01	200 ± 58	0.24 ± 0.04	1.03 ± 0.12	1841 ± 517
1.0mm Alcohol (n=8)	0.11 ± 0.01	121 ± 40	0.41 ± 0.09	1.04 ± 0.13	844 ± 417
1.5mm DW (n=18)	0.12 ± 0.03	136 ± 57	0.36 ± 0.13	1.41 ± 0.17	856 ± 749
1.5mm Alcohol (n=18)	0.13 ± 0.02	115 ± 38	0.49 ± 0.17	1.37 ± 0.16	725 ± 393

Greater difference was observed between results when the 1.0mm tube was used. It can be speculated that due to the smaller diameter, the alcohol could possibly more easily penetrate further into the fibre and somehow affect the mechanical properties of the reconstituted fibres. It has been reported that ethanol, by removing soluble intermediates present in cross-linked fibres, may affect the material properties of the fibres (Pieper *et al.*, 1999; Koob *et al.*, 2001a; Koob and Hernandez, 2002).

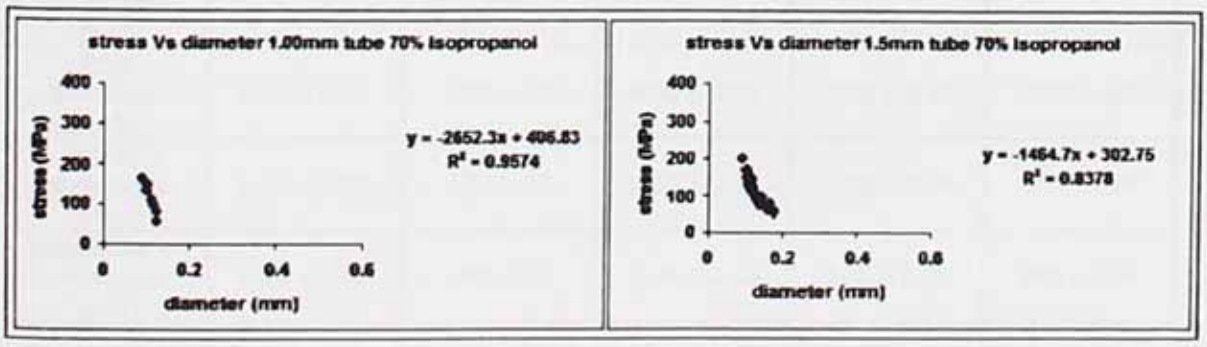
The difference in diameter between the alcohol and the distilled water treated fibres can be attributed to the temperature difference between the FIB (37^0C) and the distilled water (37^0C) and alcohol solution (18^0C). The rapid temperature change could have resulted in "shocking" the fibres and as a consequence the fibres produced had dry diameter closer to their wet ones. In contrast the distilled water bath was kept at the same temperature as the FIB and so no "shocking" occurred and the decrease in diameter was greater.

Although it would have been expected that fibres produced in the presence of alcohol would have a smaller diameter due to a dehydration effect of the alcohol, this was not observed and may be attributed to the coating effect of PEG. PEG could have created an impermeable layer on the surface of the fibre and as a consequence, the alcohol could not penetrate into the core and that subsequent led to a non-uniform drying. It has been reported that for large molecular weight PEG long chains attach to external surfaces of film, increasing diffusion of the probe and decreasing water within the film (Baker *et al.*, 2004). In the case of the distilled water, the fibres dried in a progressive pattern, while the alcohol facilitated an accelerating drying of the outer surface, whilst allowing more moisture in the core area. Water molecules are known to form an integral part of the triple-helix structure of the collagen molecule and in the assembly of collagen molecules into organised fibres. It has been shown that these structurally important water molecules are not removed during air-drying (El Feninat *et al.*, 1998). It was argued that rigidification of fibres was accomplished by a loss of water from the fibres, since the flexibility of concentrated liquid crystalline phases (such as a collagen fibre) is a very sensitive function of the volume fraction of solvent remaining in the fibre (Rosenblatt *et al.*, 1994). Bearing in mind the above observations and that water acts as a plasticizer for biopolymers (Taravel and Domard, 1996) and as a mild plasticizer for collagen (Menard *et al.*, 2000) and is similar to the influence of organic plasticizers on the mechanical behaviour of synthetic polymers (Attenburrow, 1993), this speculation can be justified by the modulus values, which were lower for the alcohol treatments and the strain at break values, which were higher for the distilled water treatments.

Although alcohol treatment produced fibres with high extension to break, special care should be paid if it is utilised in fibre for use in biomedical application, since it has been recommended to avoid the use of non-biocompatible alcohols such as ethanol, methanol and isopropanol due to their potentially deleterious effects on the body of the patient receiving them (Trollsas *et al.*, 2002). Organic solvents including alcohol have been used extensively in the study of proteins and are noted to alter the balance of electrostatic charges on the protein and change the organisation of dipolar moments. However, these effects are also shown to be minor, mild and reversible. Furthermore, the deleterious effects of alcohol treatments are more pronounced in soluble proteins as opposed insoluble proteins such as the type I collagen employed in this investigation (Christiansen and Silver, 1993). Furthermore, use of organic solvents has been shown to enable EDC cross-linking, to modulate mechanical properties of GTA-treated pericardium, to prevent calcification of GTA cross-linked aortic cusps. Additionally, the use of ethanol did not alter the physicochemical characteristics with respect to collagen stabilisation, suggesting that similar degrees of cross-linking were obtained (Pieper *et al.*, 1999). Furthermore, washing reconstituted collagen fibres in PBS and ethanol, toxic agents resulting from chemical cross-linking processes can be eliminated without reduction in material properties (Koob *et al.*, 2001a; Koob *et al.*, 2001b).

In section 3.4.1, it was demonstrated that there was a correlation between stress at break and the fibre diameter for the 1.0 and 1.5mm tube with 20% PEG treatments washed with distilled water (R^2 value of 0.85 and 0.89 respectively). Similar high correlations were obtained for the 1.0 and 1.5mm 20% PEG treatments washed with 70% isopropanol (R^2 value of 0.96 and 0.84) (Figure 12).

Figure 12. Correlation between stress at break and fibre diameter for the 70% isopropanol treatments.



3.3.3.2. Effect of Post-Fibre Formation Bath on the Physical and Mechanical Properties of Reconstituted Collagen Fibres

In this set of experiments, the effect of different liquids in the baths used after the fibre formation buffer on the properties of reconstituted collagen fibres was investigated. Table 5 summarises the findings of this work. The control fibres were found to be the thickest ones, whilst the alcohol treatment gave the thinnest ones. As previously, a high correlation (R^2=0.94) was observed between fibre diameter and stress at break. All the fibres were stronger, as judged by their tensile strength, than the control ones and those obtained from the alcohol bath were the strongest (343MPa). The alcohol treated fibres were found to be least extensible with the Tris-HCl and the control producing an impressive >50% strain at break value. The stiffer (high modulus) fibres were obtained from the alcohol treatment, whilst overnight treatment in distilled water gave fibres with the lowest in modulus values. Finally, higher forces are required to break the fibres derived from the Tris-HCl treatment, while the lowest were obtained for the distilled water overnight treatment.

Table 5. Physical and mechanical properties of reconstituted collagen fibres as a factor of different "baths".

	Diameter (mm)	Stress at Break (MPa)	Strain at Break	Force at Break (N)	Modulus at 2.2% Strain (MPa)
Made & Dried as before (control) (n=19)	0.18 ± 0.01	110 ± 17	0.51 ± 0.13	2.87 ± 0.15	377 ± 252
DW overnight (n=4)	0.17 ± 0.01	112 ± 10	0.37 ± 0.07	2.55 ± 0.12	162 ± 215
100% isopropanol overnight (n=7)	0.11 ± 0.01	343 ± 77	0.32 ± 0.07	3.08 ± 0.32	1293 ± 619
PBS overnight (n=13)	0.15 ± 0.03	170 ± 67	0.39 ± 0.08	2.73 ± 0.32	1075 ± 906
Tris-HCl overnight (n=6)	0.16 ± 0.02	199 ± 53	0.53 ± 0.07	3.76 ± 0.26	407 ± 518
Vegetable oil overnight (n=7)	0.15 ± 0.02	182 ± 47	0.44 ± 0.08	3.24 ± 0.25	905 ± 374

It can be seen that the largest diameter fibres were produced when the fibres remained in the distilled water bath for a 15min period of time. This period of time, most possibly, was sufficient to remove excessive PEG from the fibres, but was not long enough to remove all of it. As a consequence, larger fibres were produced.

A possible explanation of the effect of alcohol on the mechanical properties of reconstituted collagen fibres has been given in previous section (3.4.3.1.). However, in this instance, different results were obtained. Whilst previously, the alcohol treated fibres appeared to have larger diameter, lower stress at break, higher displacement and lower modulus values and required lower forces for failure, this time the alcohol treated fibres had lower diameter, higher stress at break, lower displacement, higher modulus values and required higher forces for failure. This time, due to the prolonged exposure to the alcohol bath, optimum dehydration was achieved. As a consequence, the fibres produced had lower diameter and higher stress at break values. As has been explained before, fibres with lower diameter are less extendable and possess higher fibrillar alignment and as a result, higher forces are required for failure. The difference in modulus values can be attributed to the effect of water on the fibre formation, which was explained above.

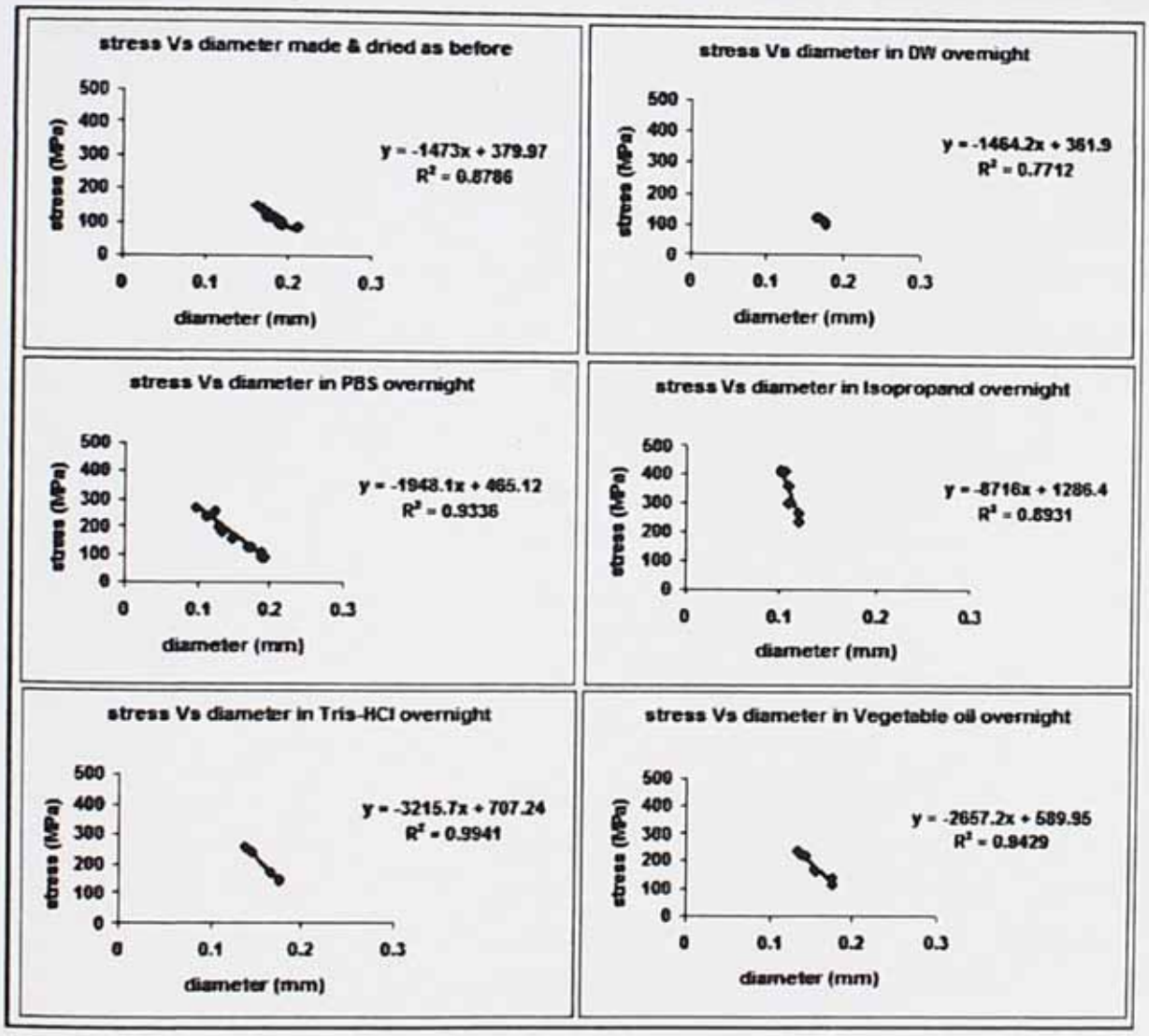
The difference between the distilled water and the neutral buffer solutions (PBS and Tris-HCl) can be attributed to electrostatic interactions between the fibres and the salt-solutions. For example, it has been shown that sodium di-hydrogen phosphate and sodium chloride are electrolytes that increase the surface electrostatic repulsion of the suspended particles (Yasueda *et al.*, 2004). Furthermore, it has been shown that the rate of precipitation is pH and salt solution dependent (Candlish and Tristram, 1964). These observations indicate that the choice of the buffer remains of paramount importance since it could influence the mechanical properties of the reconstituted collagen fibres. This result appears to be in agreement with previous work, when it was shown that suture type and size, the passage of time and the incubation media have all had a significant effect on the mechanical parameters of the sutures (Field and Stanley, 2004).

In the leather industry, oil/water emulsions have been used in order to improve the extendibility of leather products. The vegetable oil, by lubricating the fibres, achieved a strain at break value of 44%, with a stress at break value of 182MPa, making the fibres produced a competitive biomaterial. Previous studies demonstrated the

utilisation of olive oil in micro-spheres of hydroxyapatite-reconstituted collagen as supports for osteoblast cell growth. The use of olive oil, which consisted of oleic acid and triolein, the metabolites commonly found in plant and animal was found unlikely to have any harmful effects on the cells (Yin Hsu *et al.*, 1999).

Fitting a linear regression model for the stress at break values and the diameter, high correlations (R^2 values) ranging from 0.77 for the distilled water overnight treatment to 0.99 for the Tris-HCl treatment were found (Figure 13).

Figure 13. Correlation between stress at break and diameter for the fibres produced with different washing baths.



3.4. Conclusions

Reconstituted collagen fibres have been shown to be a competitive biomaterial for soft tissue repair and regeneration. In this chapter it was demonstrated that several factors could influence the mechanical properties of the produced fibres. Such factors were found to be the extrusion tube diameter, the amount and type of the co-agent utilised, the final washing buffer composition. A strong correlation between fibre diameter and stress at break was found. Higher diameter fibres had higher strain at break values, higher forces were required to break them and exhibited lower modulus at 2%strain values than the low diameter fibres. Furthermore, high correlation was found between the Mw of the co-agent and the fibre diameter. Finally, the produced fibres had mechanical properties similar to those produced in previous studies and native fibres.

Chapter 4. Cross-linking

4.1. Aim

The aim of the work described in the present chapter was to assess for the first time the effect of a wide range of cross-linking methods on the physical, mechanical and structural properties of reconstituted collagen fibres prepared in the same way from the same collagen source. This study was intended to evaluate the feasibility of using cross-linking techniques to stabilise collagen-derived biomaterials.

4.2. Materials and Methods

All suppliers are listed in Appendix.

4.2.1. Raw Material

Bovine Achilles tendon collagen derived by pepsin digestion was used as described previously (Chapter 2). Briefly, bovine Achilles tendons were collected from a slaughterhouse, manually dissected out from the surrounding fascia, washed with distilled water, minced with ice in order to avoid heat denaturation, and then further washed at 4^0C for an hour in three changes of neutral salt solutions. Consequently, the minced tendons were suspended in 0.5M-ethanoic acid and pepsin (1:100 of the initial wet weight) for 72h at 4^0C. The suspension was centrifuged (18,000g for 45min at 4^0C) and the Pepsin Soluble Collagen (PSC) purified by repeated acid-salt precipitation (0.9M NaCl) followed by dialysis. The final collagen solution was either kept refrigerated or frozen until used. The collagen content was determined by hydroxyproline assay and the concentration subsequently adjusted to 8mg/ml.

4.2.2. Fibre Formation

The fibre formation procedure was the same as described previously (Chapter 2). Briefly, a 5ml syringe containing collagen solution (8mg/ml) was loaded onto a syringe pump system connected to silicone tubing (30cm in length and 1.5mm internal diameter). The collagen solution was extruded into the FFB comprised of 118mM Phosphate Buffer and 20% (w/v) PEG Mw 8,000 at pH 7.35 and at a temperature of 37^0C. The fibres remained in this solution for 10min. Fibres were then transferred and immersed for a further 10min in the FIB comprising of 6.0mM Phosphate Buffer and 75mM sodium chloride at pH 7.32 at 37^0C. After that, the cross-linking experiments were carried out as follows.

4.2.3. Cross-linking Methods

Most available collagenous systems, like the one used in this study, for biomedical applications have been based on atelo-collagen, which can be obtained by enzymatic removal of non-helical polypeptides. However, using this soluble material implies that additional cross-linking is necessary to prevent the devices from dissolving at 37^0C, due to partial denaturation of the collagen triple helix (Friess and Lee, 1996; Chevallay *et al.*, 2000). The conditions used in fixing reconstituted collagen fibres have commonly been reported in the literature and are often referred to as optimum. The following systems were used in the present study:

Tanning agents

- 1%Myrica (pH 4.55) in DW overnight (Madhan *et al.*, 2002).
- 1% Basic Chromium Sulphate (BCS) overnight (pH 3.57) (Rajini *et al.*, 2001).

Aldehydes

- Formaldehyde
  - 1% formaldehyde in DW overnight (pH 3.80) (Rajini *et al.*, 2001; Usha and Ramasami, 2005).
  - 0.625% formaldehyde in 0.01M (pH 7.20) PBS overnight.
- Glutaraldehyde
  - 1% glutaric di-aldehyde (GTA) in distilled water (DW) (pH 3.77) overnight (Rajini *et al.*, 2001; Usha and Ramasami, 2005).
  - 0.625% GTA in 0.01M PBS (pH 7.4) overnight at room temperature. Bovine / pig / ostrich pericardia (Visser *et al.*, 1992; Chen *et al.*, 1997; Huang *et al.*, 1998; Sung *et al.*, 1998; Garcia Paez *et al.*, 2001; Chang *et al.*, 2002; Garcia Paez *et al.*, 2003).
- Starch Di-aldehyde
  - 1.3%starch di-aldehyde (suspension) in DW overnight (Rehakova *et al.*, 1996).

Isocyanates

- 5%HMDC. The fibres washed in a 100% 2-propanol solution for 30min and then immersed in a 5%HMDC solution in 100% 2-propanol overnight (Koide *et al.*, 1993; Naimark *et al.*, 1995).

Natural Derived Cross-linking

- 0.5% NDGA. 1g NDGA was dissolved in 75ml of 0.1M NaOH and 125ml of 0.01M PBS were added (pH 10.65) (Koob *et al.*, 2001a; Koob *et al.*, 2001b; Koob and Hernandez, 2002; Koob and Hernandez, 2003).
- 0.625% genipin in 0.01M PBS (pH 7.4) overnight at room temperature. Bovine pericardia (Chang *et al.*, 2002; Liang *et al.*, 2004).

Carboxyl Groups Cross-linking

- Carbodiimide. EDC: fibres transferred in a 215ml 0.05M MES buffer containing 1.731g EDC and 0.415g NHS overnight. The following day, 2 PBS washes were carried out (van Wachem *et al.*, 1994a; van Wachem *et al.*, 2001; Wissink *et al.*, 2001a).
- Acyl Azide Method. DPPA: fibres transferred in a 0.5% DPPA in DMF overnight. The following day, 2 washes in borate buffer (0.04M di-sodium tetra-borate, 0.04M boric acid) pH 8.8 were carried out (Petite *et al.*, 1994; Brunel *et al.*, 1996; Rault *et al.*, 1996; Roche *et al.*, 2001).

Epoxides

- 4% epoxy compound (ethylene glycol diglycidyl ether) in 0.025M di-sodium tetra-borate (pH 9.0) overnight at room temperature (Chen *et al.*, 1997; Huang *et al.*, 1998; Sung *et al.*, 1998).

Enzyme Generated Cross-linking

- Microbial Trans-glutaminase: fibres incubated at room temperature overnight in a 0.1mg/ml trans-glutaminase in 50mM Tris-HCl at pH 7.4 (Collighan *et al.*, 2004).

Carbohydrate Mediated Cross-linking

- Fibres transferred in a 0.25M ribose in 0.2M phosphate buffer overnight (Hegde *et al.*, 2002; Reddy *et al.*, 2002).

Physical Methods Cross-linking

- UV irradiation: The fibres were dried overnight at room temperature under the tension of their own weight. The following day, UV irradiation was carried out at 1mW/cm^2 at 1cm-distance, overnight. The fibres were conditioned at room temperature at 65% relative humidity for at least 48h. [Other authors have

used irradiation ranging from 2 to 16mW/cm^2 at 5 to 17cm distance from the sample for period of time ranging from 1 to 92h (Ryu *et al.*, 1997; Bellincampi *et al.*, 1998; Lee *et al.*, 2001; Tyan *et al.*, 2002; Yunoki *et al.*, 2003; Sionkowska *et al.*, 2004; Sionkowska, 2006).

- Dye-mediated Photo-oxidation: fibres transferred overnight in a 0.02M phosphate buffer containing 0.05% methylene blue and at 10cm distance from the container a 150W halogen lamp was placed (Mechanic, 1992; Adams *et al.*, 2001).
- De-hydro-thermal cross-linking (DHT): The fibres were dried overnight at room temperature under the tension of their own weight. Although it has been demonstrated that the strength of reconstituted collagen can be improved by varying the time and temperature of DHT cross-linking and the optimum time for cross-linking appears to be 3-5d at 110^{0}C in an oven at a vacuum of 50-100mtorr (Collins *et al.*, 1991; Dunn *et al.*, 1992; Wang *et al.*, 1994), in this study, due to experimental equipment availability the following day, DHT cross-linking was carried out at 110^{0}C, under vacuum for 6h. Subsequently, the fibres were conditioned at room temperature at 65% relative humidity for at least 48h. DHT treatment at 105^{0}C at 10Pa air pressure for 24h to induce cross-linkage of the collagen molecules has been recommended for collagen sponges (Yamamoto *et al.*, 1999).

Chemical Modification of Collagen

- Reduction: 0.05% sodium borohydride in DW. The fibres remained overnight in 0.01M PBS (Rousseau and Gagnieu, 2002).

As control, the following solutions were used:

- Made / Dried as before
- DW overnight
- PBS overnight
- 100%isopropanol overnight

The following day, all the fibres (apart from the DHT- and the UV- treated ones) were washed in deionised water to remove traces of un-reacted cross-linking agents, air-dried under the tension of their own weight and finally conditioned at room temperature at 65% relative humidity for at least 48h.

The following table summarises the cross-linking methods employed in this study according to the nature of the method and the reactive group of collagen.

	Reactive Group
Tanning Agents:	
1% BCS	carboxyl group
1% Myrica	ε-amino group
Chemical Fixatives:	
0.625% Formaldehyde	ε-amino group
1% Formaldehyde	ε-amino group
0.625% GTA	ε-amino group
1% GTA	ε-amino group
1.3% Starch Di-aldehyde	ε-amino group
5% HMDC	ε-amino group
4% epoxy compound	ε-amino group
EDC	carboxyl group
0.5% DPPA	carboxyl group
Natural Derived Chemical Agents:	
0.5% NDGA	ε-amino group
0.625% Genipin	ε-amino group
Carbohydrate Mediated:	
Ribose	ε-amino group
Biological Method:	
Trans-glutaminase	γ-glutamyl - ε-lysine
Physical Methods:	
UV	aromatic amino acid
Photo-oxidation	mainly histidine
DHT	condensation or esterification
Chemical Reduction:	
0.05% Sodium Borohydride	Schiff Base

4.2.4. Cross-linking Efficiency

The cross-linking efficiency was evaluated through thermal and swelling studies.

4.2.4.1. Denaturation Temperature Measurement

Denaturation temperatures of each group of fibres were measured using a Metter-Toledo differential scanning calorimeter. Thermal denaturation, an endothermic transition, was recorded as a typical peak, and two characteristic temperatures were measured corresponding to the onset and peak temperatures (Anselme *et al.*, 1992; Rault *et al.*, 1996). The reconstituted collagen fibres were hydrated overnight at room temperature in a PBS buffer at pH 7.4. This technique is widely used in studying the thermal transitions of collagenous tissues (Sung *et al.*, 1998; Chang *et al.*, 2002).

4.2.4.2. Swelling Ratio

Collagen fibres were swollen overnight in PBS (pH 7.4) at room temperature. The fibres then were removed and quickly blotted with filter paper to remove excess surface water (Charulatha and Rajaram, 2003). The swelling ratio was then calculated as has been described previously (Kato and Silver, 1990; Wang *et al.*, 1994):

$$\textbf{Swelling Ratio} = \frac{\textbf{(Mean Wet Fibre Diameter)} - \textbf{(Mean Dry Fibre Diameter)}}{\textbf{(Mean Dry Fibre Diameter)}} * \textbf{100}$$

4.2.5. Mechanical Tests / Microscopy

The mechanical characteristics as well as the microstructure of the reformed collagen fibres were analysed as described previously (Chapter 2). Briefly, an Instron 1122 Universal machine was used for the mechanical tests of dry and wet fibres, at an extension rate of 10mm/min, the gauge length was fixed at 3cm, soft rubber was used to cover the inside area of the grips in order to avoid damaging the fibres at the contact points and results from fibres that broke at contact points with the grips were rejected. The dried reconstituted collagen fibres were equilibrated in PBS (pH 7.4) at

room temperature overnight prior to mechanical testing (Charulatha and Rajaram, 2003). A Nikon Eclipse E600 optical microscope and a Hitachi Variable Pressure Scanning Electron Microscope (VPSEM) were used to measure the diameter of the fibres and to carry out observations of the microstructure of the fibres respectively. Each fibre cross sectional area was calculated assuming a circular cross section and by measuring the diameter at four places along the fibre using the optical microscope fitted with a calibrated eyepiece graticule.

4.3. Results and Discussion

4.3.1. General Structural Properties

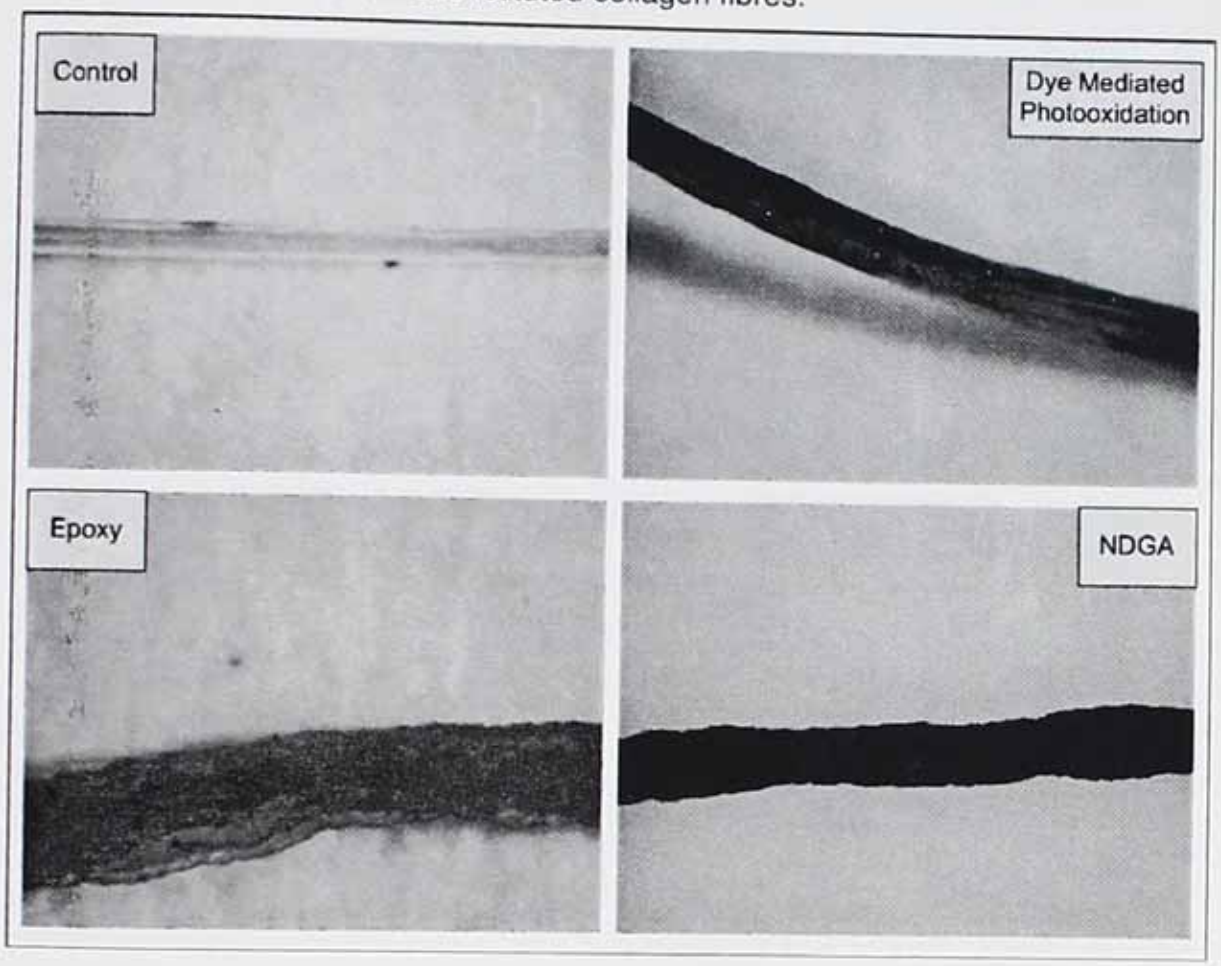
In order to reveal the microstructure and better understand the fracture mechanisms of the cross-linked reconstituted collagen fibres both light and scanning electron microscopy were employed. Table 1 indicates the colour changes that occurred due to the fixation of the fibres.

Table 1. Colour changes due to cross-linking treatments.

	Colour
Control	White
1% BCS	Greenish
1% Myrica	Brown / reddish
0.625% Formaldehyde	Lighter white than HMDC
1% Formaldehyde	Lighter white than HMDC
0.625% GTA	Yellowish / orange
1% GTA	Yellowish / orange
5% HMDC	Whiter than the control
0.5% NDGA	Brown / reddish
EDC	Similar to control
DPPA	Lighter white than the HMDC
4% epoxy compound	Dark yellow / orange-ish
Trans-glutaminase	Similar to control
Ribose	Light brownish / yellowish
UV	Yellowish / orange-ish
Photo-oxidation	Blue
DHT	Yellowish / orange-ish
0.625% Genipin	Blue
1.3% Starch Di-aldehyde	Similar to control
0.05% Sodium Borohydride	Similar to control

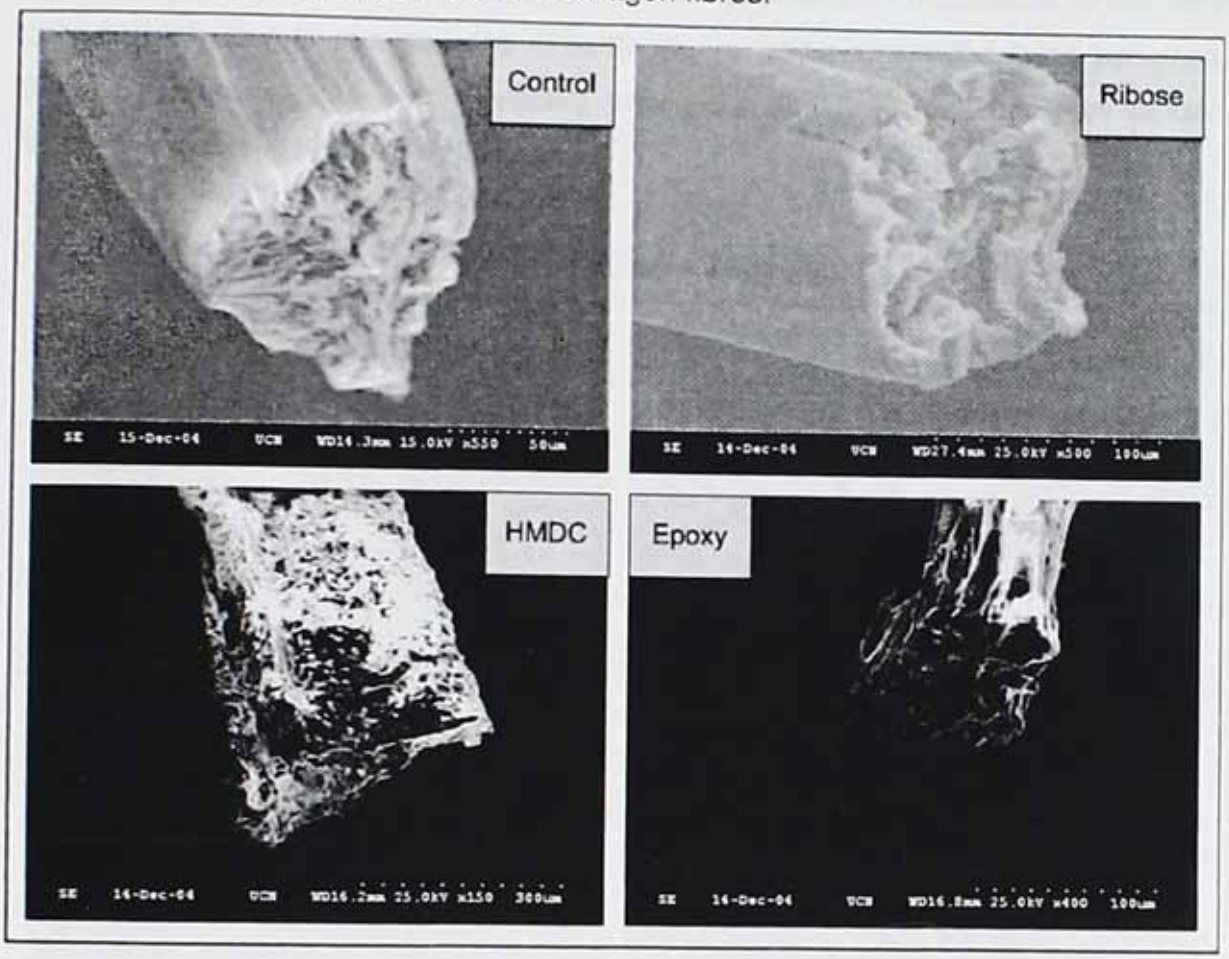
Similar results have been reported for bovine, porcine and canine pericardium and dermal sheep collagen fixed with GTA, epoxy and genipin (Chen *et al.*, 1997; Huang *et al.*, 1998; Sung *et al.*, 1998; van Wachem *et al.*, 1999; Liang *et al.*, 2004) as well as in non-enzymatic glycation of tendons and bones and human skin and lens (Nagaraj and Monnier, 1995; Hong *et al.*, 2000; Reddy, 2003). It has been speculated that the discolouration result can be attributed to the reaction of the cross-linking agent with the amino acid residues (Chen *et al.*, 1997; Huang *et al.*, 1998; Sung *et al.*, 1998; Liang *et al.*, 2004), while the blue colour of the dye mediated cross-linking can obviously attributed to the methylene blue that was utilised for the fixing process. Figure 1 demonstrates some of the discolouration, which occurred due to cross-linking.

Figure 1. Discolouration of reconstituted collagen fibres.



The break surfaces of the dry cross-linked fibres showed a consistent pattern, with the same apparent internal structure independent of the treatment. The inter-fibrillar space appeared to be completely filled as can be seen from Figure 2. The only exception appeared to be the HMDC treated fibres, where small spherical particles were observed. Splitting or fibrillation fractures were not observed as in previous published data (Rajini *et al.*, 2001).

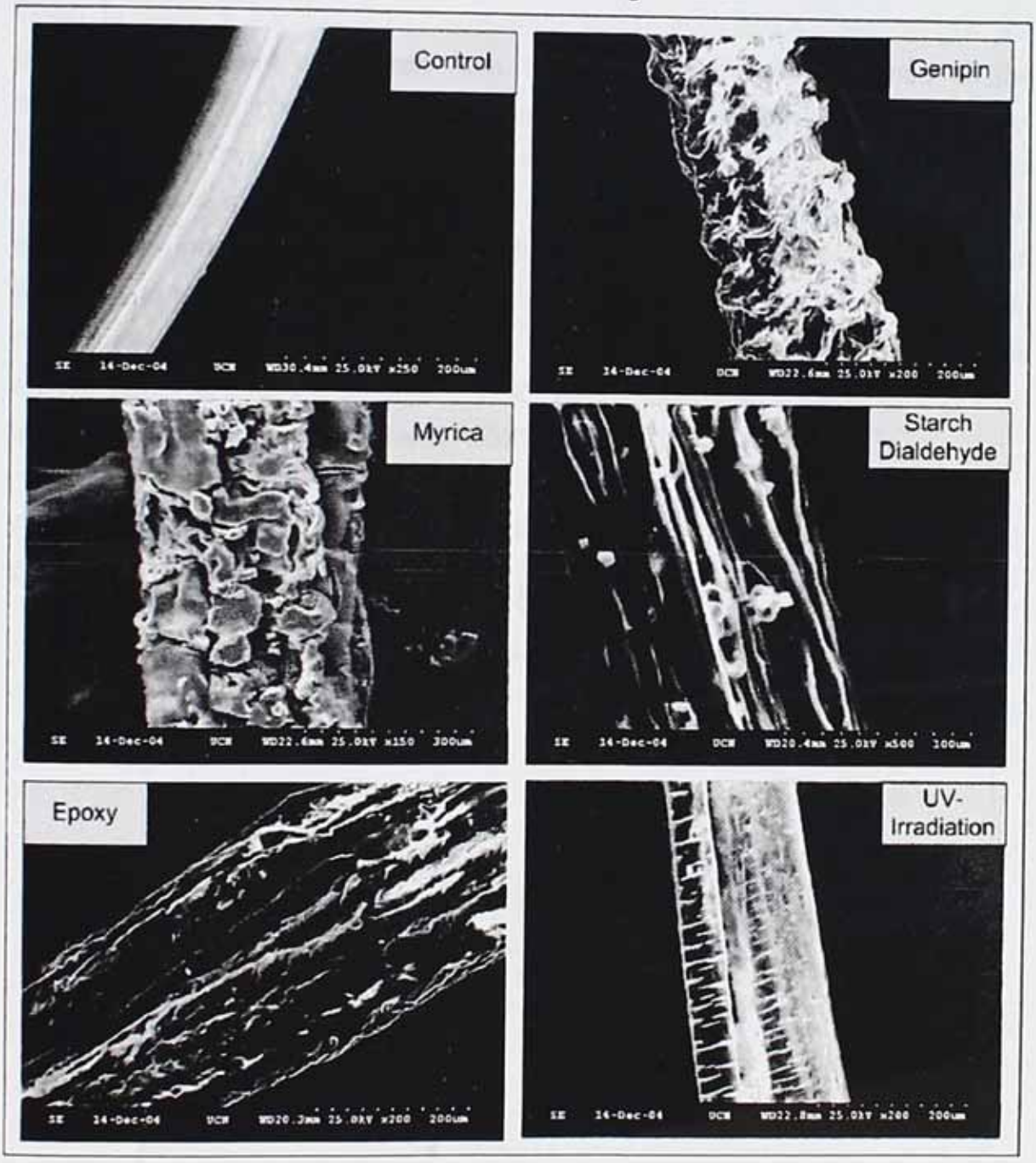
Figure 2. Failed ends of reconstituted collagen fibres.



The surface morphology of the collagen fibres appeared to deviate depending on the cross-linking method employed as can be seen in Figure 3. Similarly, it has been mentioned that different cross-linking treatments can modify the surface properties of collagenous materials and induce cell and platelet behaviours (Cote and Doillon, 1992). The control fibres, as has been described in chapters 2 and 3 demonstrated a smooth external surface. A similar surface was observed for sodium borohydride, ribose, photo-oxidation, trans-glutaminase and EDC treated fibres. Similarly, electron microscopic observation of microbial trans-glutaminase treated shark collagen showed no difference in morphological feature from the control (Nomura *et al.*, 2001a). The myrica fixed fibres appeared to have a damaged external surface. The epoxy, GTA, formaldehyde, DPPA, NDGA and chromium modified fibres exhibited a rough exterior surface with ridges and crevices running roughly parallel to the axis of the fibre. Similar results were observed with the starch di-aldehyde treatment; however, particles of starch appeared on the surface of the fibres. The genipin and HMDC fibres showed a rough external surface, with ridges and crevices running parallel to the longitudinal axis as well as wavy patterns running transversely across

the fibre length. The UV and DHT treated fibres demonstrated ridges and crevices running parallel to the axis, but also gaps appeared to form transverse to the fibre axis, possibly due to the increase of temperature under which the fixing occurred. Surface irregularity has been reported previously for UV-irradiated rat-tail tendon fibres (Sionkowska and Wess, 2004).

Figure 3. Surface morphology of reconstituted collagen fibres.

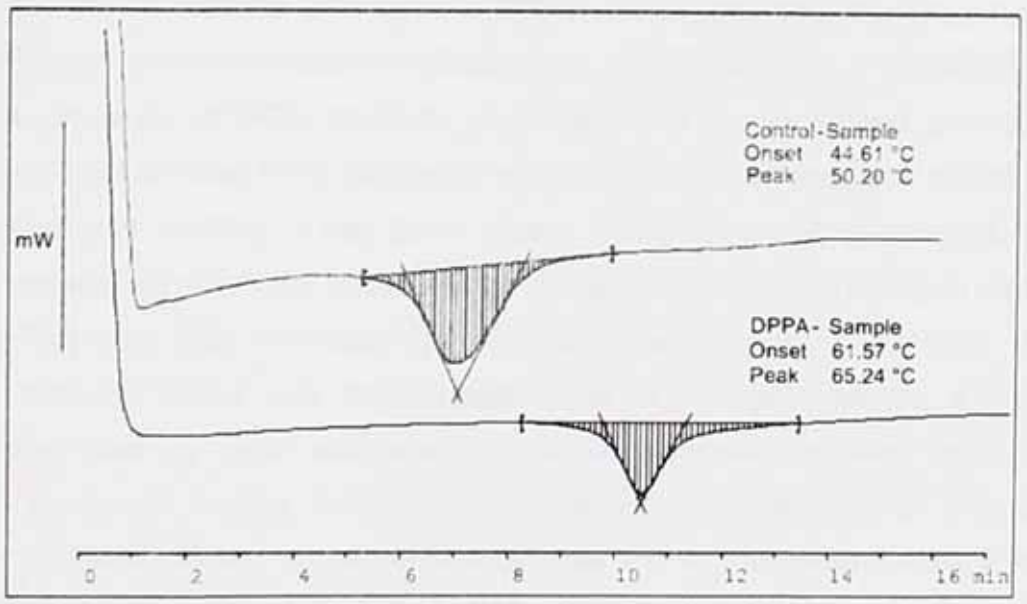


4.3.2. Cross-linking Efficiency

4.3.2.1. Thermal Properties

When collagen in a hydrated state is heated, the crystalline triple helix will be transformed into amorphous randomly coiled peptide chains which results in shrinkage of the collagen fibre (Hormann and Schlebusch, 1971; Mentink *et al.*, 2002). In Figure 4, a typical DSC thermograph can be seen and a presentation of the parameters that can be derived from it. Peak Temperature is defined as the temperature of maximum power absorption during denaturation, while Onset Temperature is the temperature at which the tangent to the initial power versus temperature line crosses the baseline. As can be seen cross-linking increases onset and peak temperatures and also causes the peak width to decrease. This decrease is thought to be due to (a) cross-linking giving a better organisation and stabilisation of the helices (Mentink *et al.*, 2002), (b) the increase in cross-linkages that break exothermically and (c) the situation in which as the number of cross-links increases, less water can be bound (Chen *et al.*, 2005).

Figure 4. Typical DSC Thermograph for the control (red) and DPPA (black) cross-linked collagen fibres.



The control samples had onset temperatures ranging from 41 to 45^{0}C (alcohol and PBS treated fibres respectively), whilst the peak temperatures were ranging from 46 to 50^{0}C (alcohol and distilled water treated fibres respectively) (Table 2).

Table 2. Denaturation temperatures of control reconstituted collagen fibres.

	Onset (^{0}C)	Peak (^{0}C)
Alcohol overnight (n=	41.50 ± 0.20	45.97 ± 0.28
DW overnight (n=	43.36 ± 0.35	49.97 ± 0.10
Tris-HCl overnight (n=2)	43.94 ± 1.11	47.29 ± 0.42
PBS overnight (n=3)	44.85 ± 1.71	47.43 ± 1.52
Made & Dried as before (n=	44.64 ± 0.65	48.78 ± 1.19

The reconstituted collagen fibres produced in this study had a denaturation temperature (46-50^{0}C) which is higher than native collagen (37-39^{0}C) because when the molecules are aggregated to form fibres, the shrinkage temperature is higher than the denaturation temperature of the molecules due to the increased energy of crystallisation derived from the interaction between the close packed molecules in the fibre (Bailey and Light, 1989; Friess and Lee, 1996; Chevallay *et al.*, 2000; Orban *et al.*, 2004; Sionkowska, 2005). In addition, the denaturation temperature of the fibres produced was higher than that of collagen sponges (43-44^{0}C) (Chevallay *et al.*, 2000; Vaissiere *et al.*, 2000; Roche *et al.*, 2001), collagen gels (38^{0}C) (Orban *et al.*, 2004) and collagen films (44^{0}C) (Petite *et al.*, 1994). It has been shown that the intermolecular cross-links responsible for increased thermal stability and resistance to collagenase digestion, may form most easily in collagen fibres followed by sponges and films; in collagen gels there is less formation of crosslinks because in gels the molecules are more dispersed (Rault *et al.*, 1996; Chevallay *et al.*, 2000). In addition, the presence of PEG could be responsible for the increased shrinkage temperature of the reformed fibres compared with that of collagen sponges, where no added polymer was present. It has been shown that polymers may increase the shrinkage temperature of tendon by increasing the volume fraction of collagen within the fibrils. Polymers may increase the shrinkage temperature by shifting the equilibrium between native and denaturated forms of collagen towards a more compact native form by steric exclusions. A similar proposal has been made to explain the increased melting temperature of DNA in the presence of polymer (Madhan *et al.*, 2002). Other experimental work has shown that neutral salts affect the thermal stability of collagen in a typical ion-specific way. The general lyotropic effects of neutral salts may alter the structure of the solvent, thereby modifying solvent-macromolecule interactions involved in the stabilisation of the native conformation (Luescher *et al.*, 1974).

The thermal stability of a protein can be intrinsically changed by alteration of the amino acids or extrinsically changed by addition of suitable stabilizing agents (cations, co-enzymes, membranes and peptides) (Argos *et al.*, 1979). To improve the thermal stability, glycosaminoglycan or chitosan has been utilised. However, the most efficient way to increase the thermal stability is to treat collagen with cross-linking agents (Danielsen, 1982; Chevallay *et al.*, 2000). In this present study, a range of widely used chemical, biological and physical cross-linking methods were compared. A great range of values were obtained for the cross-linked samples with onset temperatures ranging from 30 to 101.2^{0}C (DHT and BCS treated fibres respectively) and peak temperatures ranging from 40 to 106.1^{0}C (DHT and BCS treated fibres respectively) (Table 3). This is perhaps not surprising, since it has previously been shown that collagen-based biomaterials can be prepared with a wide range of thermal stabilities, collagenase susceptibilities and cytocompatibility by varying the formation procedures and reaction conditions (Rault *et al.*, 1996; Chevallay *et al.*, 2000).

Table 3. Denaturation temperatures of cross-linked reformed collagen fibres (n=2).

	Onset (^{0}C)	Peak (^{0}C)
1% BCS	101.20 ± 1.32	106.10 ± 0.91
1% Myrica	77.81 ± 1.98	81.91 ± 2.63
0.625% Formaldehyde	66.46 ± 0.35	70.34 ± 2.11
1% Formaldehyde	65.44 ± 0.49	68.58 ± 1.33
0.625% GTA	71.75 ± 0.93	75.41 ± 0.86
1% GTA	71.09 ± 1.27	75.44 ± 0.81
5% HMDC	61.74 ± 1.05	66.55 ± 0.21
0.5% NDGA	62.24 ± 0.06	66.18 ± 0.35
EDC	57.05 ± 1.37	61.24 ± 2.91
DPPA	60.59 ± 1.39	64.74 ± 0.71
4% epoxy compound	83.46 ± 0.71	86.49 ± 0.64
Trans-glutaminase	44.86 ± 0.11	47.69 ± 0.20
Ribose	47.70 ± 0.76	55.81 ± 0.31
UV	34.75 ± 2.83	47.60 ± 7.34
Dye Photo-oxidation	46.44 ± 0.59	51.95 ± 1.14
DHT	30.08 ± 3.14	40.24 ± 1.71
0.625% Genipin	61.47 ± 0.59	67.13 ± 0.47
1.3% Starch Di-aldehyde	41.74 ± 0.48	50.87 ± 1.43
0.05% Sodium Borohydride	41.16 ± 0.57	43.87 ± 1.58

It has been suggested that the denaturation temperature depends on the size of the co-operating units introduced in the shrinking process, the larger the unit, the slower the kinetics and the higher the shrinkage temperature (Rajini *et al.*, 2001). Furthermore, it has been shown that the nature of the bonds formed and the stability of the cross-links introduced vary with the aldehyde utilised. For example, it has been shown that the amount of formaldehyde required is four times greater than that of GTA to obtain similar changes, which was attributed to the structural changes associated with the collagen-aldehyde reaction (Usha and Ramasami, 2005). These observations can explain the difference between the shrinkage temperatures of the GTA and formaldehyde cross-linked fibres.

Comparing the cross-linking methods, sodium borohydride, UV and trans-glutaminase treatments produced denaturation temperatures which were similar to the control samples; while DHT treatment produced fibres with a denaturation temperature lower than the control and starch di-aldehyde, photo-oxidation and ribose treatments produced fibres with slightly higher shrinkage temperatures than the controls. DSC melting profiles of collagens are indicative of molecule and fibril integrity. Obtaining a melting temperature lower than the control samples, has been attributed to mode of fabrication of the material, except in cases where endotherms were below 40^0C, which would have implied denatured collagen (Wallace *et al.*, 1992). It has been suggested that cross-linking and denaturation effects may occur simultaneously. Consequently, it is possible that the cross-linking effect predominates below 110^0C and the denaturation effect predominates at 140^0C (Wang *et al.*, 1994). These observations exclude the possibility that either of the physical treatments resulted in a denaturated material since the 110^0C temperature limit was not exceeded. Furthermore, differences in shrinkage temperature obtained from chemical treatments and physical methods may be attributed to the stabilisation chemistry of the method employed. For example, cross-linking of pericardium with GTA and Denacol™ has been shown to involve extensive cross-linking as well as polymer formation within and in addition to the native pericardial matrix, leading to a rise in matrix complexity and thermal stability. Although, it was not observed in this study, DHT-cross-linked fibres should have higher shrinkage temperatures than UV-irradiated ones, since more cross-links can form during DHT treatment (condensation reactions between carboxyl and amino groups) compared with UV irradiation (bonds between free radicals generated on aromatic acid residues) (Weadock *et al.*, 1996). In contrast the dye-mediated photo-oxidation process is a catalytic process involving

the modification of and cross-link formation of existing matrix components, resulting in a material with little added matrix complexity and no significant rise in thermal stability compared with untreated tissue (Moore *et al.*, 1996). Cross-linking of rat tail tendon in the presence of sodium borohydride yielded the same denaturation temperature as the native tendon. Increasing the stability of the mature aldimine bond by borohydride reduction does not affect the length of the bond between the two molecules in the native fibre. Thus, the fact that reduction had no appreciable effect on the temperature of denaturation of rat-tail tendon compared to non-reduced tendon provides evidence that the transition is not initiated by a thermally induced breakage of cross-links (Miles *et al.*, 2005). In a similar manner, it has been shown that trans-glutaminase cross-linking had no effect on the hydrothermal stability of bovine skin collagen. The average number of tanning units per collagen monomer required to elicit a change in hydrothermal stability is far greater than the number of cross-links that trans-glutaminase could incorporate, suggesting that enzymatic cross-linking of collagen cannot produce a tanning effect (Collighan *et al.*, 2004). In another study, it was demonstrated that the addition of microbial trans-glutaminase to shark collagen accelerated the collagen fibril reconstitution, and that when added to collagen gel it raised slightly the already low denaturation temperature of shark collagen (from 40 to 42^{0}C) indicating that the cross-linking of shark collagen makes no significant contribution to the stability of helix conformation of the collagen molecule (Nomura *et al.*, 2001a).

However, it has been shown that microbial trans-glutaminase itself (Chen *et al.*, 2005) or in the presence of urea (Nomura *et al.*, 2001b) and increased amounts of porcine trans-glutaminase [collagen to enzyme 50 to 1 (wt/wt)] (Orban *et al.*, 2004) made it possible to reconstruct collagen and to raise the denaturation temperature. Additionally, it has been shown that ribose, a more reactive pentose, gives a faster increase in cross-linking than glucose. However, rat-tail tendon, comprised mainly by type I collagen is more susceptible to glycation than rat-tail skin composed of both type I and III collagens (Mentink *et al.*, 2002). These observations lead to the conclusion that a rise in shrinkage temperature may not be necessary predictor of tissue stabilisation, but rather a reflection of the particular chemical nature of the stabilisation (Moore *et al.*, 1996). Therefore, further work would be necessary in order to safely conclude whether stabilisation of the fixed fibres was achieved.

The rest of the chemical treatments gave denaturation temperatures higher than 60^0C, indicating without doubt that they were effective cross-linkers (Chen *et al.*, 1997). Denaturation of collagen is characterised by transition of the triple helix of the collagen molecule to a random coil, accompanied by a macroscopic shrinkage. Cross-linking results in stabilisation of the triple helix structure, thus increasing the shrinkage temperature (Wissink *et al.*, 2001b). It has been pointed out that highly cross-linked collagen-fibres do not release molecular collagen with known chemo-static characteristics. Therefore, the production of endogenous growth factors by activated inflammatory cells, recognised as essential for healing processes, is minimal (Chvapil *et al.*, 1993). Cross-links cause an increase in the collagen denaturation temperature because they decrease the entropy of the melting transition. It is important to point out, however, that this transition is a bulk response and does not reflect the exact number or location of the cross-links (Anselme *et al.*, 1992; Friess *et al.*, 1999). Furthermore, it has been reported that there is a decrease in collagen denaturation enthalpy change when specific agents have cleaved hydrogen bonds and its increase in the presence of a hydrophobic exothermic bond breaking agent. Therefore, these results indicate that samples with higher denaturation temperature have more hydrogen bonds and/or fewer hydrophobic bonds than samples with lower denaturation temperatures (Kopp *et al.*, 1989).

Higher denaturation temperatures suggest that samples have higher degree of cross-linking. However, care has to be taken when the degree of cross-linking of different reagents is directly related to the shrinkage temperature values. The denaturation temperature of fixed tissue may be influenced by:

- The type of cross-linking introduced (Damink *et al.*, 1995; Damink *et al.*, 1996a; Damink *et al.*, 1996b; Sung *et al.*, 1998)
- The collagenous matrix; higher denaturation temperatures expected for fibres rather than sponges, films or gels (Rault *et al.*, 1996; Chevallay *et al.*, 2000)
- The structure of the collagenous matrix; lighter (lower density) collagen sponges demonstrated consistency of cross-linking in comparison to the heavier (higher density) ones due to reduced penetration of formaldehyde atmosphere into the porous structures (Friess *et al.*, 1999).
- The time of the reaction; formaldehyde, GTA and acyl azide treatments stabilised collagen structures increasingly with increase in the amount of time of the reaction. After cross-linking, more energy and therefore higher

temperatures were necessary to break the helical structures (Anselme *et al.*, 1992; Friess *et al.*, 1999)

- The concentration of the reagents; the exothermic peak in composites increased with increased GTA concentration for sponge preparation, suggesting the requirement of higher energy for the thermal decomposition of collagen sponges with higher cross-linking extent (John *et al.*, 2001)
- The source of collagen and the preparation of the collagenous structure. From Table 4, it can be observed that different species, different extraction methods, different collagen content and different co-agent concentration and/or type result in different denaturation temperatures. It can be seen that by increasing the collagen content for the acid soluble treatments, a decrease in the denaturation temperature was observed, while an increase was observed for the pepsin soluble treatments. Furthermore, by increasing the amount of the co-agent, a slight decrease in the denaturation temperature was observed.

Table 4. Denaturation temperatures of reformed collagen fibres (n=2) produced from collagen extracted from different species and using different extraction methods, co-agents types (PEG Mw 8,000 or NaCl) and concentration.

	Onset (^{0}C)	Peak (^{0}C)
AS BAT (3mg/ml) 20% PEG	45.12 ± 0.68	47.34 ± 0.45
AS BAT (5mg/ml) 20% PEG	42.66 ± 0.59	46.70 ± 1.16
AS Rat (3mg/ml) 20% PEG	42.58 ± 0.23	45.38 ± 0.69
AS Rat (4mg/ml) 20% PEG	41.75 ± 1.06	44.75 ± 0.38
PS BAT (3mg/ml) 20% PEG	44.64 ± 0.96	46.83 ± 0.71
PS BAT (6mg/ml) 20% PEG	45.00 ± 0.74	47.93 ± 0.72
PS BAT (8mg/ml) 20% PEG	44.64 ± 0.65	48.78 ± 1.19
PS BAT (3mg/ml) 20% Salt	46.66 ± 1.20	49.06 ± 1.57
PS BAT (6mg/ml) 20% Salt	46.41 ± 1.22	50.10 ± 1.33
PS BAT (8mg/ml) 20% Salt	47.58 ± 0.14	50.72 ± 0.21
PS PTT (1mg/ml)	44.92 ± 1.27	48.29 ± 0.28
PS BAT (6mg/ml) 5% PEG	47.34 ± 0.04	50.24 ± 0.29
PS BAT (6mg/ml) 20% PEG	45.00 ± 0.74	47.93 ± 0.72
PS BAT (6mg/ml) 40% PEG	43.81 ± 2.72	47.09 ± 2.69
PS BAT (6mg/ml) 5% Salt	48.74 ± 0.82	51.79 ± 0.88
PS BAT (6mg/ml) 20% Salt	46.41 ± 1.22	50.10 ± 1.33
PS BAT (6mg/ml) 40% Salt	46.18 ± 0.05	50.53 ± 0.25

Although, all of the above factors could affect the cross-link density and as a consequence the cross-linking efficiency, which can be characterised by denaturation temperature, nevertheless it was felt helpful to provide reference values (Table 5) for comparison. As it can be seen different denaturation temperatures can be obtained depending on the cross-linking method, collagen type and species utilised as described in this study.

Table 5. Thermal properties of collagenous matrices.

	Onset (°C)	Peak (°C)	Reference
Monomolecular atelo-collagen		37	(Friess and Lee, 1996)
Pro-collagen type I		42	(Wong *et al.*, 2001)
Bovine type I sponge Collagen-GAG-Chitosan DPPA low concentration DPPA high concentration	36.2 39.9 42.1 55.1	43.6 48.6 49.1 60.6	(Vaissiere *et al.*, 2000)
Formaldehyde GTA Starch di-aldehyde		87 87 75	(Bowes and Cater, 1968)
Type I Insoluble BAT EDC/NHS		55.4 75.9 – 82.4	(van Wachem *et al.*, 2001; Wissink *et al.*, 2001b; Wissink *et al.*, 2001a)
Reconstituted RTT GTA Acyl azide		58 81 80.5	(Charulatha and Rajaram, 2003)
0.2%GTA RTT		82	(Charulatha and Rajaram, 2001)
Bovine pericardium Acyl azide Hydrazine 2h Acyl azide Hydrazine 3h Acyl azide Hydrazine 12h GTA (0.0075%) 24h GTA (0.0075%) 72h	37 41 54 61 53 60	46 48 56 69 56 68	(Anselme *et al.*, 1992)
Young calves sponges 25µl DPPA per 0.8g sponge	36.3 42.9	44 49.7	(Roche *et al.*, 2001)
Control sponge β-rays at 15Kgy 25µl/gr sponge DPPA 250µl/gr sponge DPPA	36.3 42.9 55.5	44.0 30 49.7 62.0	(Chevallay *et al.*, 2000)
Bovine Pericardium fresh Pericardium GTA Pericardium hydrazine Pericardium DPPA Film control Film GTA Film Hydrazine Film DPPA	58.6 81.8 79.2 78.5 44.4 69.6 65 70.3	62.8 85.1 83.4 81.4 52 74.6 69.9 72.6	(Petite *et al.*, 1994)
Fresh canine iliac arteries GTA Poly-glycidyl ether EDC		63.1±3 82.3±3 68±3 72±0.5	(Courtman *et al.*, 2001)
Fresh porcine pericardium GTA Epoxy		59.5-61-6 87.1-88 70.5-80.4	(Chachra *et al.*, 1996; Chen *et al.*, 1997; Sung *et al.*, 1998)
Dermal sheep collagen GTA EDC/NHS 1.5% HMDC Acyl azide		56 78 86-87 70-74 82.4	(van Wachem *et al.*, 1994b; van Wachem *et al.*, 1994a; Damink *et al.*, 1995; Damink *et al.*, 1996a; Damink *et al.*, 1996b)
Bovine pericardial GTA Photo-oxidation		65 85 65	(Moore *et al.*, 1994)
0.0075%GTA 1% EDC 1% HMDC DPPA		75.6 61 70.8 72.7	(Rault *et al.*, 1996)
Formaldehyde		55	(Friess *et al.*, 1999)

Penicillamin Borohydride Trypsin-collagen		75.4 72.5 74.9	(Kopp *et al.*, 1989)
Fibrillar collagen solution Sponge-silicone		54 50	(Wallace *et al.*, 1992)
Native collagen Sponges DHT sponges		37 37 37	(Chevallay *et al.*, 2000)
HMDC fibres		64-73	(Chvapil *et al.*, 1993)
EDC ASBAT Fibres		50-65	(Cavallaro *et al.*, 1994)
Bated hide GTA Formaldehyde Vegetable Chrome Trans-glutaminase		60.3 78.2 74.8 75.3 100.2 55.5-57.7	(Collighan *et al.*, 2004)
RTT BCS GTA Formaldehyde		63 107 92 87	(Rajini *et al.*, 2001)
BAT DHT EDC DHT / EDC		61.9 61.8 79.2 80.6	(Pieper *et al.*, 1999)
Acetic Acid Soluble Pepsin Soluble Alkali Soluble Commercial Type I		38 41.5 36 42	(Li *et al.*, 2003)
Porcine type I atelo-collagen Oxidised glycogen / $NaBH_4$	36.1 50.8-71.8	74 60.6-77.3	(Rousseau and Gagnieu, 2002)
Native RTT Acrylic polymer Mimosa Vegetable Tanning Acrylic & Mimosa		58 68 76 86	(Madhan *et al.*, 2002)
Fresh Bovine Pericardium Genipin		62.4 65.1-77.2	(Liang *et al.*, 2004)
Bovine Collagen Bovine EDC/NHS Equine Collagen Equine EDC/NHS		54.2 78.4-86.3 57.8 76.5-85.7	(Angele *et al.*, 2004)
Bovine Pericardium Photo-oxidation GTA Denacol™		65 66 78 73	(Moore *et al.*, 1996)
Dermal Sheep Collagen BD epoxy pH 4.5 BD epoxy pH 9.0 BD epoxy pH 4.5 Acylation BD epoxy and EDC pH 4.5		47.2 68.8 72.5 65.6 79.6	(van Wachem *et al.*, 1999)
BAT Collagen Collagen 33mM EDC Collagen 14mM EDC Collagen CS 33mM EDC Collagen CS 14mM EDC Collagen DS 14mM EDC Collagen Hep 33mM EDC Collagen Hep 14mM EDC Collagen HS 14mM EDC		61.0 76.7 72.1 75.2 70.8 71.0 74.2 68.3 68.6	(Pieper *et al.*, 2000a; Pieper *et al.*, 2000b)
Dermal sheep collagen 1% BDDGE 4% BDDGE		45 66 67	(Zeeman *et al.*, 1999a)
RTT 0h UV RTT 20h UV RTT 36h UV RTT 66h UV		39.3 47.3 32.9 44.6	(Sionkowska, 2005)
AS Bovine Collagen / Tg = 50000 / 1 Collagen / Tg = 50 / 1		38 54 66	(Orban *et al.*, 2004)

4.3.2.2. Swelling Ratio

Collagenous biomaterials have been extensively studied and used for skin and tendon replacement. Collagen molecules are known to bind a large amount of water and to present evenly distributed negative and positive charges on their surfaces. Cell adhesion and blood coagulation are two fundamental events that occur on the surface of implanted materials. The adhesion of cells and the induction of a thrombosis depend on numerous factors present on the biomaterial surface. Among these factors, the wet-ability and the equilibrium water content seem to be the most important (Cote and Doillon, 1992). Table 6 summarises the mean dry and wet diameter measurements as well as the swelling ratio.

Table 6. Swelling measurements of reconstituted collagen fibres.

	Dry Fibre Diameter (mm)	Wet Fibre Diameter (mm)	% Swelling
Control Samples			
Alcohol overnight	0.11 ± 0.01 (n=7)	0.27 ± 0.01 (n=5)	147.52
DW overnight			
Tris-HCl overnight	0.16 ±0.02 (n=6)	0.65 ± 0.06 (n=3)	310.79
PBS overnight	0.15 ± 0.03 (n=13)	0.28 ± 0.02 (n=5)	87.72
Made & Dried as before	0.18 ± 0.01 (n=19)	0.30 ± 0.02 (n=5)	62.59
Cross-linked Samples			
1% BCS	0.24 ± 0.03 (n=5)	0.31 ± 0.04 (n=3)	31.94
0.625% Formaldehyde	0.19 ± 0.02 (n=6)	0.31 ± 0.04 (n=5)	61.22
1% Formaldehyde	0.22 ± 0.02 (n=6)	0.34 ± 0.02 (n=4)	57.37
0.625% GTA	0.26 ± 0.03 (n=5)	0.31 ± 0.03 (n=5)	17.71
5% HMDC	0.33 ± 0.08 (n=7)	0.34 ± 0.03 (n=5)	1.09
0.5% NDGA	0.31 ± 0.03 (n=6)	0.45 ± 0.06 (n=5)	44.00
EDC/NHS	0.17 ± 0.02 (n=6)	0.37 ± 0.05 (n=4)	122.78
DPPA	0.20 ± 0.03 (n=5)	0.31 ± 0.04 (n=3)	54.35
4% epoxy compound	0.22 ± 0.04 (n=5)	0.25 ± 0.03 (n=5)	11.95
Trans-glutaminase	0.16 ± 0.02 (n=6)	0.62 ± 0.05 (n=3)	292.21
Ribose	0.15 ± 0.01 (n=6)	0.23 ± 0.02 (n=4)	52.02
UV	0.16 ± 0.01 (n=6)	0.25 ± 0.03 (n=5)	57.33
Photo-oxidation	0.18 ± 0.02 (n=6)	0.39 ± 0.02 (n=4)	119.44
DHT	0.17 ± 0.01 (n=6)	0.26 ± 0.02 (n=4)	53.98
0.625% Genipin	0.24 ± 0.03 (n=5)	0.34 ± 0.05 (n=5)	41.44
1.3% Starch Di-aldehyde	0.20 ± 0.03 (n=7)	0.31 ± 0.03 (n=5)	58.57
0.05% Sodium Borohydride	0.10 ± 0.01 (n=6)	0.31 ± 0.04 (n=5)	209.76

It has been suggested that the ability of the implanted materials to adsorb water is an important determinant of the calcification rate and extent (Golomb *et al.*, 1993). Additionally, for scaffolds for skin tissue engineering or artificial dermis substitutes, the water retention and permeation is of importance for the absorption of body fluids and for transfer of cell nutrients and metabolites through the materials (Ma *et al.*,

2001). Swelling can be defined as the process in which a macromolecular material receives liquid and simultaneously enlarges its volume (Rehakova *et al.*, 1996).

Of the control fibres, alcohol treatment produced the lowest dry fibre diameter followed by PBS and distilled water. As regards the wet diameter, alcohol treatment produced the lowest value followed by PBS and distilled water. As a consequence, the swelling ratio was higher for the alcohol treatment with PBS and distilled water to follow. This appears to be in agreement with previous observation, where it has been mentioned that the water content decreases, when the fibre diameter increases (Averous *et al.*, 2001). It is worth mentioning that the Tris-HCl treatment produced by far the largest diameter wet fibres (0.649mm) with the second largest diameter being produced from the distilled water treatment (0.298mm). Furthermore, the swelling ratio of the Tris-HCl treatment was 310% with the alcohol treatment to follow, having less than the half value of the Tris-HCl treatment (147%).

Comparing the cross-linked fibres, dry diameter increased in the following order of treatment: sodium borohydride (lowest diameter) < ribose < trans-glutaminase < UV < EDC < DHT < photo-oxidation < 0.625% formaldehyde < starch di-aldehyde < DPPA < 1% formaldehyde < epoxy < GTA < BCS < genipin < NDGA < HMDC (largest diameter). A different order was obtained when the wet diameters were measured after re-hydration in PBS overnight: ribose (lowest diameter) < epoxy < UV < DHT < 0.625% formaldehyde and GTA <starch di-aldehyde < sodium borohydride and BCS < DPPA < HMDC < genipin < 1% formaldehyde < EDC < photo-oxidation < NDGA < trans-glutaminase (largest diameter).

The swelling ratio order starting from the lowest was found to be HMDC < epoxy < GTA < BCS < genipin < NDGA < ribose < DHT < DPPA < UV < 1% formaldehyde < starch di-aldehyde < 0.625% formaldehyde < photo-oxidation < EDC < sodium borohydride < trans-glutaminase. Previous work has shown that the water content of collagen may be modified by chemical and physical cross-linking treatments; the order that was observed in collagenous matrices was GTA < formaldehyde < cyanamide (Cote and Doillon, 1992) and DHT < EDC (Pieper *et al.*, 1999). These findings are in agreement with our observations. Using X-ray diffraction, it has been shown that the characteristic collagen structure is dependant on the water content (Luescher *et al.*, 1974). The character of macromolecular cross-linking influences the degree of swelling. If the binding is physical, the swelling process is less limited; with

chemical cross-linking, the swelling is more restricted (Vizarova *et al.*, 1994; Rehakova *et al.*, 1996). In general, as has been shown before (Kato and Silver, 1990), cross-linking reduces the swelling ratio and so swelling ratio measurement can be used as an indication of effective cross-linking (Pieper *et al.*, 1999).

It was observed that on increasing the formaldehyde concentration, an increase in dry fibre diameter and a decrease in swelling were observed. Similar results have been reported in the literature (Dalton and Shoichet, 2001; John *et al.*, 2001; Angele *et al.*, 2004). In this work an increase in formaldehyde concentration did not appear to affect the shrinkage temperature, while previous studies have demonstrated that by increasing the EDC concentration higher denaturation temperatures were obtained (Cavallaro *et al.*, 1994; Pieper *et al.*, 2000a; Angele *et al.*, 2004). Previous work on dermal sheep collagen demonstrated that an increase in 1,4-butanediol diglycidyl ether (BDDGE) concentration, in solution pH and in reaction temperature would accelerate cross-link reaction. On the other hand, the cross-link efficacy was decreased if the BDDGE concentration or the reaction pH was increased (Zeeman *et al.*, 1999a).

The difference between the types of cross-links introduced can have an effect on the degree of swelling of the materials; increasing cross-linking reduces water uptake (Hutcheon *et al.*, 2001). For example, in our study as well as in GTA cross-linking of pericardium tissue demonstrated higher swelling ratio than the epoxy counterpart. This may indicate that the surface of the epoxy fixed collagenous tissue was more hydrophilic than the GTA one. This may be because the epoxy has a couple of hydrophilic ether bonds (-O-), while GTA has only hydrophobic carbon-carbon bonds (C-C) (Chen *et al.*, 1997).

It has been proposed that cross-linking decreases the degree of swelling (Charulatha and Rajaram, 2003). It has also been suggested that cross-linking will give higher shrinkage temperature values (Damink *et al.*, 1995; Damink *et al.*, 1996a; Damink *et al.*, 1996b; Sung *et al.*, 1998). However, a direct relation between the shrinkage temperature and the degree of swelling was not observed in this study. It has been suggested that comparing at a given cross-link density the chemical structure of the cross-links may also influence the degree of swelling (Damink *et al.*, 1995; Damink *et al.*, 1996a; Damink *et al.*, 1996b; Sung *et al.*, 1998). For example, although homogenous distribution can be achieved by different cross-linking agents,

differences in the solubility (HMDC-slightly-water-soluble and GTA-water-soluble) are expected to account for the differences observed in the degree of cross-linking of the resulting materials (Damink *et al.*, 1995).

The behaviour of biopolymers during swelling can be the result of the simultaneous influence of at least two antagonistic factors: the polarity of the composite material and the formation of hydrogen bonds between the macromolecular components. The polarity of the material increases and influences swelling positively, while cross-linking through hydrogen bonds influences the swelling negatively (Rehakova *et al.*, 1996).

4.3.3. General Mechanical Characteristics

During loading of collagen fibres, collagen molecules, fibrils, and fibril bundles deform and finally fail by a process termed defibrillation (Pins *et al.*, 1997b). The stress-strain curves of dry and wet reconstituted collagen fibres were obtained by loading the material in uniaxial tension, since it has been mentioned that by using tensile testing, the cross-link density may be related to tensile deformation behaviour (Lee *et al.*, 2001).

In general, three different shaped curves were obtained depending on the swelling ratio / hydrated state of the fibre. In the low swelling ratio / dry state, typical s-shaped stress-strain curves (Figure 5) were observed, similar to those for a crystalline polymer that yields and undergoes plastic flow (Kato *et al.*, 1989; Wang *et al.*, 1994; Pins and Silver, 1995). In the high swelling ratio / wet state, typical j-shaped stress-strain curves (Figure 6) were observed. Similar shaped stress-strain graphs have been reported for re-hydrated reformed fibres derived from bovine corium (Kato *et al.*, 1989; Pins and Silver, 1995) as well as pericardium tissue (Garcia Paez *et al.*, 2003) and rat tail tendon (Gentleman *et al.*, 2003). The slope of the stress-strain curve increases with strain, and is a characteristic of collagenous tissues, such as leather, skin, rat-tail tendon and reconstituted collagen (Attenburrow, 1993; Wang *et al.*, 1994; Fratzl *et al.*, 1997). Fibres with intermediate swelling ratio exhibited 'intermediate'-shape graphs (Figure 7). The intermediate shape graph can be defined as a graph obtained from a fibre that exhibited initially a toe region, followed by a small knee region and then a j-shape curve. It has been reported that extensible connective tissues contain networks of fibrous collagen in an amorphous matrix. It is the reorientation of the collagen fibres within these networks that allows large extensions of the tissues and is responsible for their non-linear stress-strain curves (Purslow *et al.*, 1998).

Figure 5. A typical S-shaped stress-strain curve of dry reformed collagen fibres.

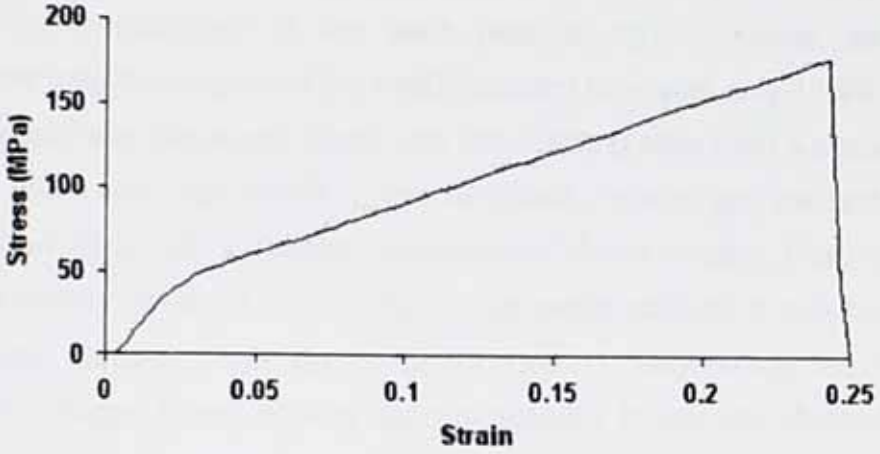


Figure 6. A typical J-shaped stress-strain curve of wet reformed collagen fibres.

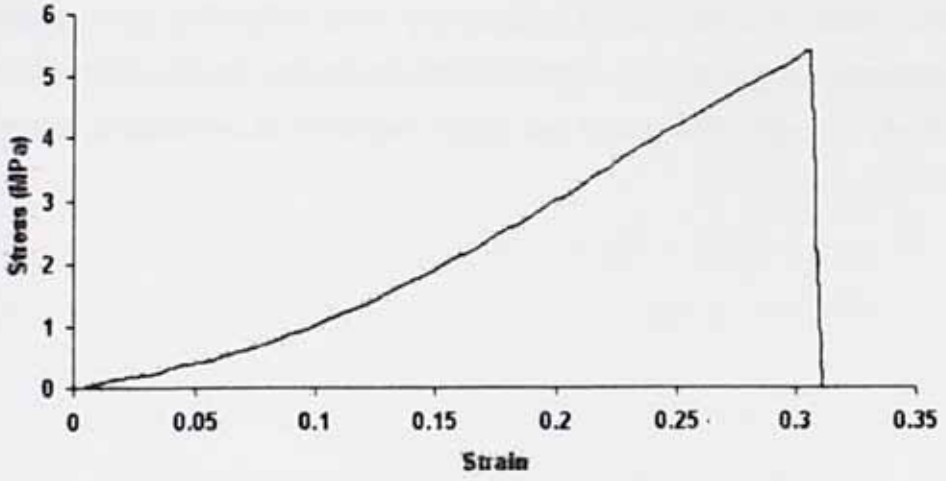
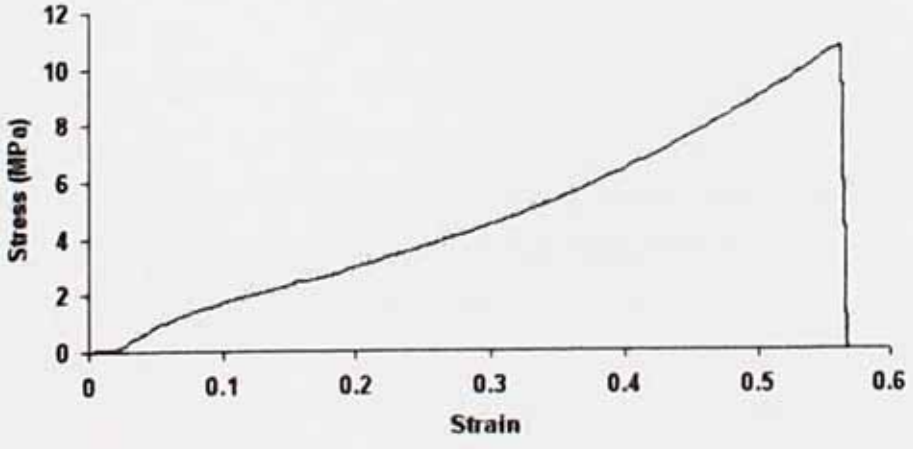


Figure 7. An example of an 'Intermediate' stress-strain curve.



It is worth pointing out that in the dry state, all treatments but myrica, which produced J-shape curves, showed the same shape graphs; a deviation though was observed between the wet ones. For example, the HMDC treated fibres exhibited the same s-shape graphs, independent of the state (wet or dry). However, as described previously, the swelling degree of the HMDC treated fibre was only 1.09%. In general the same pattern was observed; fibres with low swelling ratio yield s-shape graphs in both states; fibres with high swelling ratio exhibited J-shape graphs; and fibres with intermediate swelling ratio exhibited 'intermediate'-shape graphs. From the different stress-strain curves obtained depending on the water content, it can be concluded that the water content plays an important role in determining the mechanical properties of collagen fibres as has been suggested previously (Arumugam *et al.*, 1992). Furthermore, stress-strain curves similar to Figure 6 have been obtained on testing dry native rat tail tendon fibres, independent of the extension rate, as well as from wet native rat tail tendon fibres extended at high strain rates, while 'intermediate shape' graphs were exhibited from wet native rat tail tendon fibres stretched at medium strain rates, clearly indicating that the strain rate is of great importance when the mechanical properties of collagen fibres are under investigation (Arumugam *et al.*, 1992).

4.3.4. Mechanical Properties

In vitro, under appropriate conditions (temperature, pH, ionic strength, composition of fibre formation medium), collagen molecules spontaneously agglomerate into stable forms such as fibres and gels (fibrillo-genesis) that are similar to native fibres (Hunter *et al.*, 2002). Since the lysyl oxidase mediated cross-linking pathway would not occur *in vitro*, reconstituted forms of collagen can lack sufficient strength and may disintegrate upon handling or collapse under the pressure from surrounding tissue *in vivo*. Moreover, associations between fibres required for effective stress transfer within the discontinuous fibrillar network do not approximate those that occur *in situ*. Thus, it is often necessary to introduce exogenous cross-links (chemical or physical) into the molecular structure, in order to control mechanical properties, the resistance time in the body and to some extent the immunogenicity of the device (Koob *et al.*, 2001a; Koob *et al.*, 2001b; Koob and Hernandez, 2002). This study is concerned with the major cross-linking techniques, which were applied to extruded pepsin soluble bovine Achilles tendon derived collagen fibres and Tables 7 and 9 summarise the physical and mechanical properties of dry and wet reconstituted collagen fibres produced.

With the exception of trans-glutaminase, EDC/NHS, ribose, physical methods and sodium borohydride all the other cross-linking treatments gave fibres with a higher diameter than the control ones. The similarity, as judged by the diameter, between the physical methods and the control can be attributed to the lack of any additional chemical reagents. The sodium borohydride releases hydrogen, and as a consequence creates bubbles within the solution. When these bubbles were attached to the fibres, they created weak points and as a result the fibres tended to break at these points. To avoid this, three brief incubations of the fibres into three different borohydride solutions were carried out. It has been shown before (Chapter 2) that the transfer method used facilitated a decrease in fibre diameter due to fibres stretching under their own weight. It is proposed that this led to an increase fibril alignment and as a consequence an increase in stress at break. As a result, the highest tensile strength values were obtained from the sodium borohydride treatment. However, these results may not necessarily be due to the cross-linking efficiency, but as discussed above could most possibly be attributed to the handling of the sodium borohydride treatment. DSC results, indicated no change in denaturation temperature, which seems to support this theory, but further studies are required to positively conclude whether this cross-linking method was effective or not.

Table 7. Properties of dry reconstituted collagen fibres.

	Diameter (mm)	Stress at Break (MPa)	Strain at Break	Force at Break (N)	Modulus at 2.2% Strain (MPa)
Control					
Made & Dried as before (n=19)	0.18 ± 0.01	110 ± 17	0.51 ± 0.13	2.87 ± 0.15	377 ± 252
DW overnight (n=4)	0.17 ± 0.01	112 ± 10	0.37 ± 0.07	2.55 ± 0.12	163 ± 215
PBS overnight (n=13)	0.15 ± 0.03	170 ± 67	0.39 ± 0.08	2.73 ± 0.32	1075 ± 906
100% isopropanol overnight (n=7)	0.11 ± 0.01	343 ± 77	0.32 ± 0.07	3.08 ± 0.32	1293 ± 619
Tris-HCl (n=6)	0.16 ± 0.02	199 ± 53	0.53 ± 0.07	3.76 ± 0.26	407 ± 518
Cross-linked					
1% BCS (n=5)	0.24 ± 0.03	45 ± 16	0.57 ± 0.17	1.89 ± 0.25	22 ± 10
1% Myrica (n=5)	0.41 ± 0.02	8 ± 2	0.15 ± 0.04	1.04 ± 0.15	23 ± 9
0.625% Formaldehyde (n=6)	0.19 ± 0.02	64 ± 16	0.60 ± 0.10	1.76 ± 0.10	37 ± 59
1% Formaldehyde (n=6)	0.22 ± 0.02	54 ± 14	0.63 ± 0.13	1.97 ± 0.18	45 ± 40
0.625% GTA (n=5)	0.26 ± 0.03	32 ± 10	0.55 ± 0.05	1.62 ± 0.23	27 ± 22
1% GTA (n=7)	0.24 ± 0.03	32 ± 17	0.37 ± 0.16	1.33 ± 0.38	31 ± 14
5% HMDC (n=7)	0.33 ± 0.08	20 ± 8	0.29 ± 0.11	1.53 ± 0.10	90 ± 131
0.5% NDGA (n=6)	0.31 ± 0.03	26 ± 9	0.22 ± 0.10	1.88 ± 0.28	47 ± 53
EDC-NHS (n=6)	0.17 ± 0.02	126 ± 39	0.53 ± 0.08	2.62 ± 0.23	134 ± 134
DPPA (n=5)	0.20 ± 0.03	70 ± 32	0.53 ± 0.07	2.03 ± 0.40	140 ± 193
4% epoxy compound (n=5)	0.22 ± 0.04	51 ± 26	0.37 ± 0.05	1.76 ± 0.37	271 ± 341
Trans-glutaminase (n=6)	0.16 ± 0.02	173 ± 47	0.48 ± 0.04	3.27 ± 0.17	356 ± 424
Ribose (n=6)	0.15 ± 0.01	145 ± 24	0.34 ± 0.07	2.63 ± 0.18	1002 ± 274
UV (n=6)	0.16 ± 0.01	52 ± 10	0.08 ± 0.02	1.03 ± 0.10	532 ± 360
Photo-oxidation (n=6)	0.18 ± 0.02	106 ± 27	0.45 ± 0.04	2.56 ± 0.14	146 ± 259
DHT (n=6)	0.17 ± 0.01	47 ± 8	0.13 ± 0.02	1.07 ± 0.08	36 ± 17
0.625% Genipin (n=5)	0.24 ± 0.03	40 ± 12	0.55 ± 0.06	1.71 ± 0.09	64 ± 46
1.3% Starch Di-aldehyde (n=7)	0.20 ± 0.03	90 ± 24	0.41 ± 0.09	2.63 ± 0.21	183 ± 174
0.05% Sodium Borohydride (n=6)	0.10 ± 0.01	403 ± 46	0.30 ± 0.02	3.23 ± 0.28	1786 ± 414

As was shown in previous chapters, the diameter of the fibres produced can be affected by several factors (animal species, the collagen concentration / extraction method, co-agent type / concentration, the diameter of the extrusion tube) and as a consequence changes in the mechanical properties of the fibres produced occurred. Similarly, cross-linking appeared to affect the diameter of the fibres produced and as a consequence the tensile stress. Fitting a linear regression model to the Stress at break Vs Diameter for each treatment, correlations (R^2 values) ranging from 0.52 for the myrica fibres to 0.99 for the trans-glutaminase fibres were obtained (Table 8 and Figure 8).

Table 8. Correlation (R^2 values) between tensile strength and fibre diameter for dry cross-linked and control reconstituted collagen fibres.

	R^2
Control	
Made & Dried as before	0.88
DW overnight	0.77
PBS overnight	0.93
100% isopropanol overnight	0.89
Tris-HCl	0.99
Cross-linked	
1% BCS	0.94
1% Myrica	0.52
0.625% Formaldehyde	0.91
1% Formaldehyde	0.90
0.625% GTA	0.96
1% GTA	0.95
5% HMDC	0.97
0.5% NDGA	0.82
EDC-NHS	0.94
DPPA	0.77
4% epoxy compound	0.94
Trans-glutaminase	0.99
Ribose	0.83
UV	0.81
Photo-oxidation	0.94
DHT	0.79
0.625% Genipin	0.98
1.3% Starch Di-aldehyde	0.90
0.05% Sodium Borohydride	0.77

Figure 8. Correlation (R^2 values) between tensile strength and fibre diameter for dry cross-linked reconstituted collagen fibres

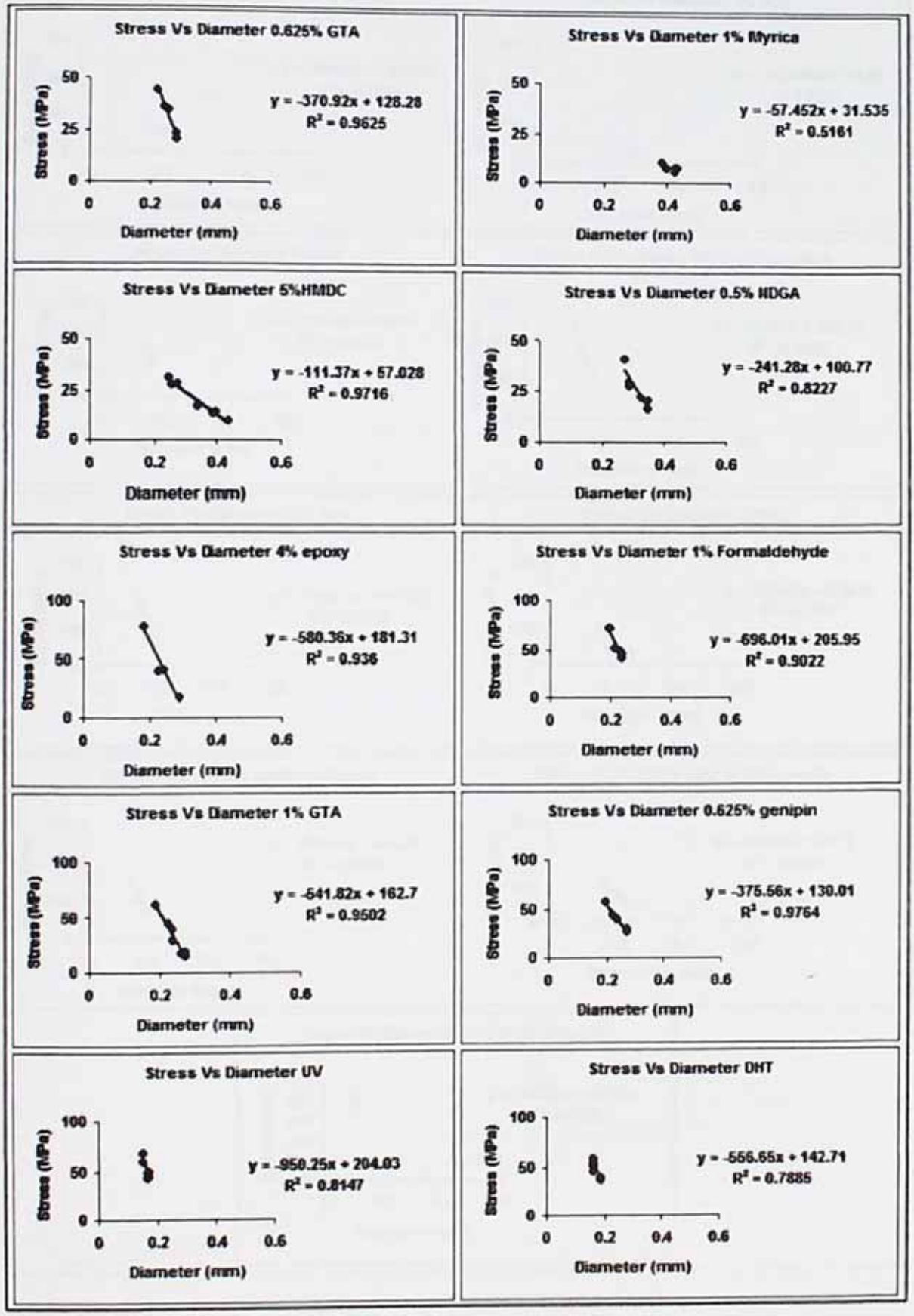
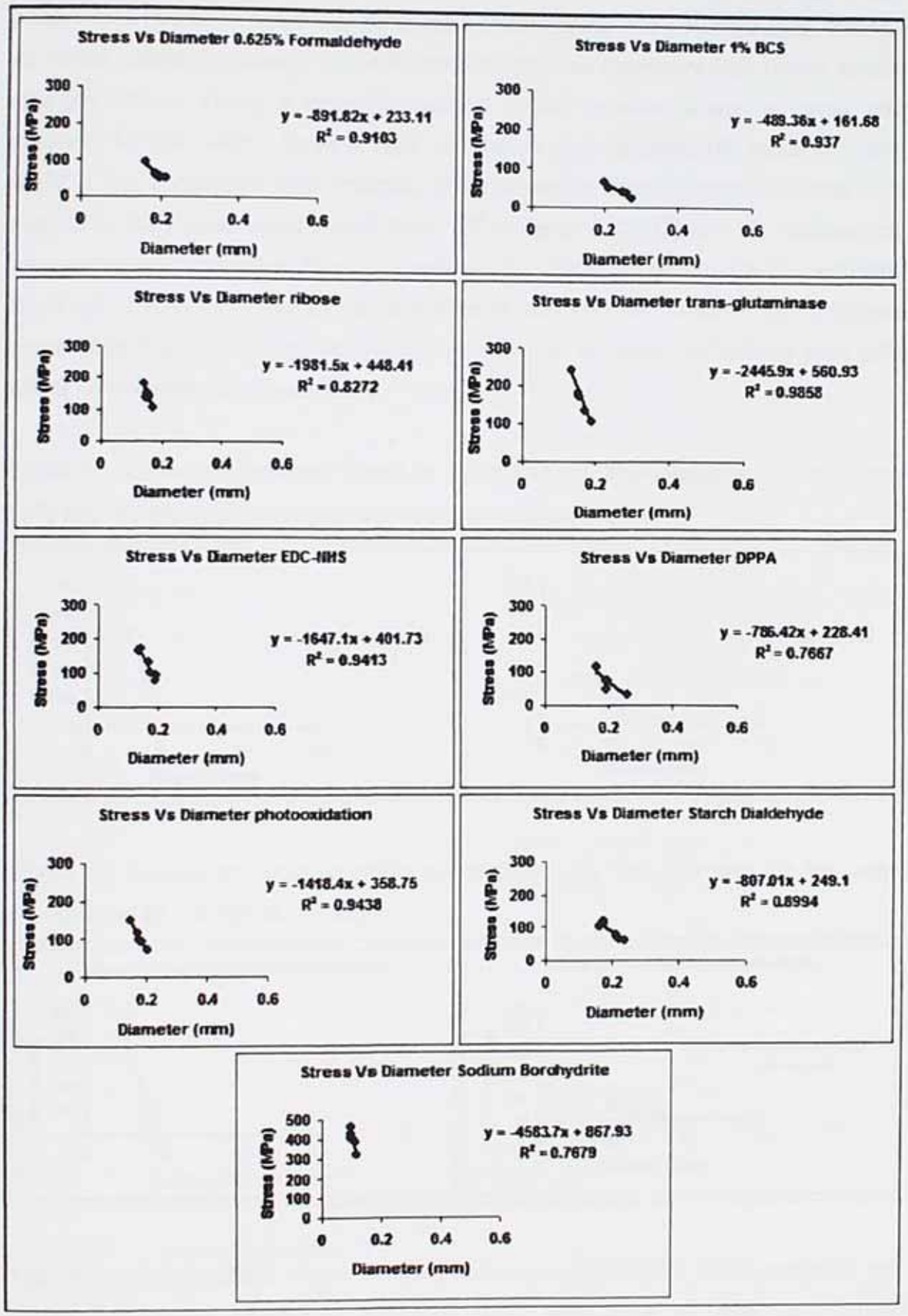


Figure 8. (Continue).



The control fibres appeared to possess lower diameters than the cross-linked ones and as a consequence they exhibited a higher stress at break than the cross-linked ones, which seems to agree with other work (Rajini *et al.*, 2001; Angele *et al.*, 2004). As noted before, in general those fibres that had low diameters had higher tensile strength values. Fitting a linear regression model between stress at break and diameter for the control fibres a high correlation was obtained (R^2 value of 0.94), whilst a low correlation was obtained when a similar linear correlation model was applied to the overall cross-linked fibres (R^2 value of 0.45) (Figure 9). However, by separating the cross-linked fibres according to the diameter; treatments that exhibited fibres with diameter similar to the control ones and another for those that produced higher than the control, high correlation values were observed (R^2 values were 0.86 and 0.74 respectively) (Figure 10).

Figure 9. Correlation between stress at break and dry fibre diameter for the control (left) and the cross-linked (right) reconstituted collagen fibres.

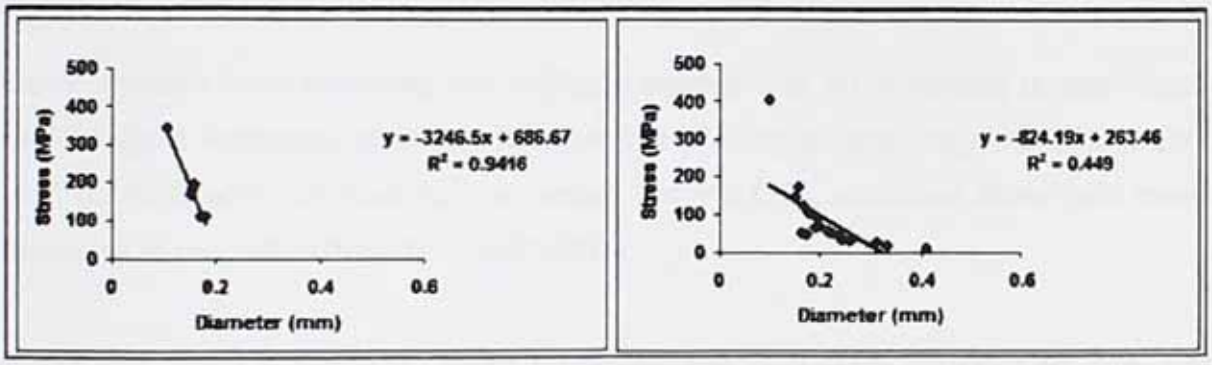
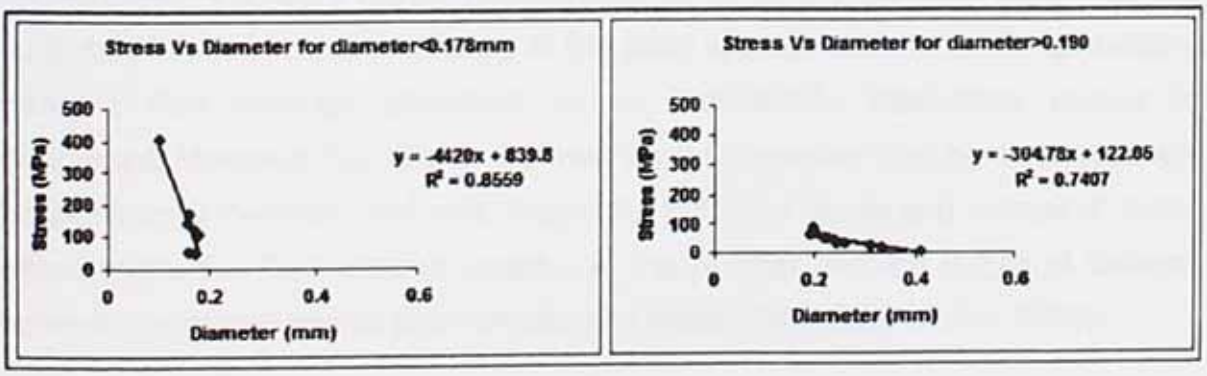


Figure 10. Correlation between stress at break and dry fibre diameter for the cross-linked fibres as a factor of diameter.



The sodium borohydride, ribose, trans-glutaminase, EDC/NHS, photo-oxidation and starch di-aldehyde treatments produced fibres with force at break and tensile strength values similar to the control ones, while the rest of the treatments exhibited lower values than the control. Additionally, these treatments with the exception of the

EDC/NHS had denaturation temperatures similar to the control ones. These observations suggest caution in correlating *in vitro* properties, such as shrinkage temperature and biomechanical strength, with each other or with *in vivo* performance including durability, as was suggested previously (Moore *et al.*, 1996).

As regards the strain at break, UV, DHT, NDGA, HMDC and Myrica fixed fibres gave lower values than the control, while both formaldehyde, BCS, genipin and 1%GTA treated fibres extended more than the control ones. The rest of the treatments produced an extension comparable to the control.

The DPPA, EDC/NHS, DHT, photo-oxidation, 1%formaldehyde, BCS, genipin, both GTA concentrations, NDGA, HMDC and myrica treated fibres exhibited modulus values lower than the control. The production of cross-linked collagen scaffolds with lower modulus values than the control ones has been attributed to the failure of brittle collagen fibres (Angele *et al.*, 2004).

Other workers have observed that collagen treated with an increased concentration of GTA gave increased mechanical properties (Rousseau and Gagnieu, 2002). This is in contrast with our observations, where the 1% GTA produced fibres with lower mechanical properties than the 0.625%GTA.

In general, non cross-linked fibres were stronger and more extendable than the cross-linked ones. Similar results have been reported for UV irradiated rat-tail tendon fibres (Sionkowska and Wess, 2004) and PEG-collagen (Sionkowska, 2006) and PVA-collagen irradiated blends (Sionkowska *et al.*, 2004) and was suggested that such deterioration is a consequence of the chain scission and cross-linking reactions within a fibril although alterations to the inter-fibrillar interactions cannot be discounted. Moreover, the loss of the mechanical properties may be connected with breaking up of the inter- and intra- molecular hydrogen bonds and release of water, which controls H-O-H-collagen bonds, i.e. the number and the length of distance between the protein chains (Sionkowska and Wess, 2004; Sionkowska, 2006).

As was demonstrated, the different cross-linking techniques appeared to produce dry reconstituted collagen fibres with mechanical and physical properties different to the control. Similarly, as can be observed from Table 9, the different cross-linking techniques appeared to affect the properties of the wet reconstituted fibres as well.

Table 9. Properties of wet reconstituted collagen fibres.

	Diameter (mm)	Stress at Break (MPa)	Strain at Break	Force at Break (N)	Modulus at 2.2% Strain (MPa)
Control					
Made & Dried as before (n=5)	0.30 ± 0.02	2.97 ± 0.87	0.33 ± 0.07	0.20 ± 0.04	3.78 ± 1.10
DW overnight (n=5)					
PBS overnight (n=5)	0.28 ± 0.02	3.67 ± 1.44	0.22 ± 0.05	0.23 ± 0.06	10.48 ± 8.56
100% isopropanol overnight (n=5)	0.27 ± 0.01	3.07 ± 0.88	0.23 ± 0.02	0.17 ± 0.03	7.84 ± 4.61
Tris-HCl (n=3)	0.65 ± 0.06	0.09 ± 0.02	0.47 ± 0.11	0.03 ± 0.01	0.10 ± 0.04
Cross-linked					
1% BCS (n=3)	0.31 ± 0.04	16.19 ± 5.90	0.57 ± 0.16	1.19 ± 0.14	10.43 ± 5.96
0.625% Formaldehyde (n=5)	0.31 ± 0.04	12.00 ± 4.18	0.57 ± 0.08	0.82 ± 0.15	6.72 ± 4.57
1% Formaldehyde (n=4)	0.34 ± 0.02	6.23 ± 2.30	0.68 ± 0.03	0.57 ± 0.16	2.87 ± 1.35
0.625% GTA (n=5)	0.31 ± 0.03	10.85 ± 2.85	0.43 ± 0.04	0.77 ± 0.04	6.90 ± 3.29
5% HMDC (n=5)	0.34 ± 0.03	13.25 ± 7.02	0.45 ± 0.15	1.11 ± 0.41	6.39 ± 6.55
0.5% NDGA (n=5)	0.45 ± 0.06	6.35 ± 2.57	0.58 ± 0.10	0.92 ± 0.09	4.86 ± 3.18
EDC-NHS (n=4)	0.37 ± 0.05	3.16 ± 0.63	0.54 ± 0.11	0.34 ± 0.03	1.76 ± 0.33
DPPA (n=3)	0.31 ± 0.04	5.11 ± 1.32	0.44 ± 0.13	0.37 ± 0.02	2.95 ± 1.33
4% epoxy compound (n=5)	0.25 ± 0.03	32.21 ± 8.01	0.29 ± 0.04	1.55 ± 0.28	54.42 ± 57.93
Trans-glutaminase (n=3)	0.62 ± 0.05	0.10 ± 0.05	0.61 ± 0.14	0.03 ± 0.02	0.13 ± 0.09
Ribose (n=4)	0.23 ± 0.02	5.38 ± 1.47	0.25 ± 0.09	0.22 ± 0.02	22.12 ± 21.57
UV (n=5)	0.25 ± 0.03	2.91 ± 1.92	0.21 ± 0.13	0.17 ± 0.13	6.00 ± 2.33
Photo-oxidation (n=4)	0.39 ± 0.02	1.71 ± 0.49	0.47 ± 0.11	0.20 ± 0.05	0.84 ± 0.14
DHT (n=4)	0.26 ± 0.02	2.46 ± 0.64	0.26 ± 0.08	0.14 ± 0.06	4.41 ± 1.58
0.625% Genipin (n=5)	0.34 ± 0.05	6.89 ± 2.52	0.40 ± 0.03	0.59 ± 0.09	5.54 ± 3.95
1.3% Starch Di-aldehyde (n=5)	0.31 ± 0.03	2.44 ± 0.73	0.28 ± 0.07	0.18 ± 0.02	1.55 ± 0.61
0.05% Sodium Borohydride (n=5)	0.31 ± 0.04	2.03 ± 0.70	0.26 ± 0.05	0.15 ± 0.03	4.01 ± 2.91

The largest structural unit in the anterior cruciate ligament is the fascicle, which is 20-400μm in diameter. The fascicles are composed of sub-fascicles or fibre bundles 1-20μm in diameter, and collagen fibrils 25-150nm in diameter. Due to the high collagen content and fibre alignment, the anterior cruciate ligament can withstand high mechanical loads and has a tensile strength of about 38MPa in humans (Dunn *et al.*, 1993). The collagen fibres evaluated in this study had wet diameters and wet tensile strengths comparable to that of the anterior cruciate ligament.

It has been mentioned that an increased amount of suture material causes thickening of the tendon at the repair site, which, in some cases, restricts the gliding of the tendon in the narrow tendon sheath. Development of suture materials with minimal cross-sectional area and sufficient strength to withstand the range of forces generated in the early rehabilitation protocol might provide a solution to the problem (Kujala *et al.*, 2004). As was shown in previous sections, by utilising different collagen extraction methods to different species, by controlling the concentration of the collagen solution, by using different co-agents or cross-linking methods the dry fibre diameter can be controlled and a competitive material for tissue repair can be manufactured. Similarly, cross-linking appeared to affect the wet diameter of the fibres produced and as a consequence the tensile stress. Fitting a linear regression model for the Stress at break Vs Diameter for each treatment (Table 10 and Figures 11 and 12), correlations (R^2 values) ranging from 0.55 (for the epoxy treated fibres) to 0.99 (for the 0.625% formaldehyde fixed fibres) were found. Furthermore, for the DHT, UV and trans-glutaminase treatments an increase was observed in tensile strength as the fibre diameter was increased.

Table 10. Correlation values (R^2) between tensile strength and diameter for wet cross-linked and control reconstituted collagen fibres.

	R^2
Control	
Made & Dried as before	0.91
PBS overnight	0.94
100% Isopropanol overnight	0.84
Tris-HCl	0.36
Cross-linked	
1% BCS	0.91
0.625% Formaldehyde	0.99
1% Formaldehyde	0.79
0.625% GTA	0.94
5% HMDC	0.79
0.5% NDGA	0.90
EDC-NHS	0.95
DPPA	0.89
4% epoxy compound	0.55
Trans-glutaminase	0.92
Ribose	0.95
UV	0.81
Photo-oxidation	0.87
DHT	0.94
0.625% Genipin	0.85
1.3% Starch Di-aldehyde	0.77
0.05% Sodium Borohydride	0.78

Figure 11. Correlation values (R^2) between tensile strength and diameter for wet control reconstituted collagen fibres.

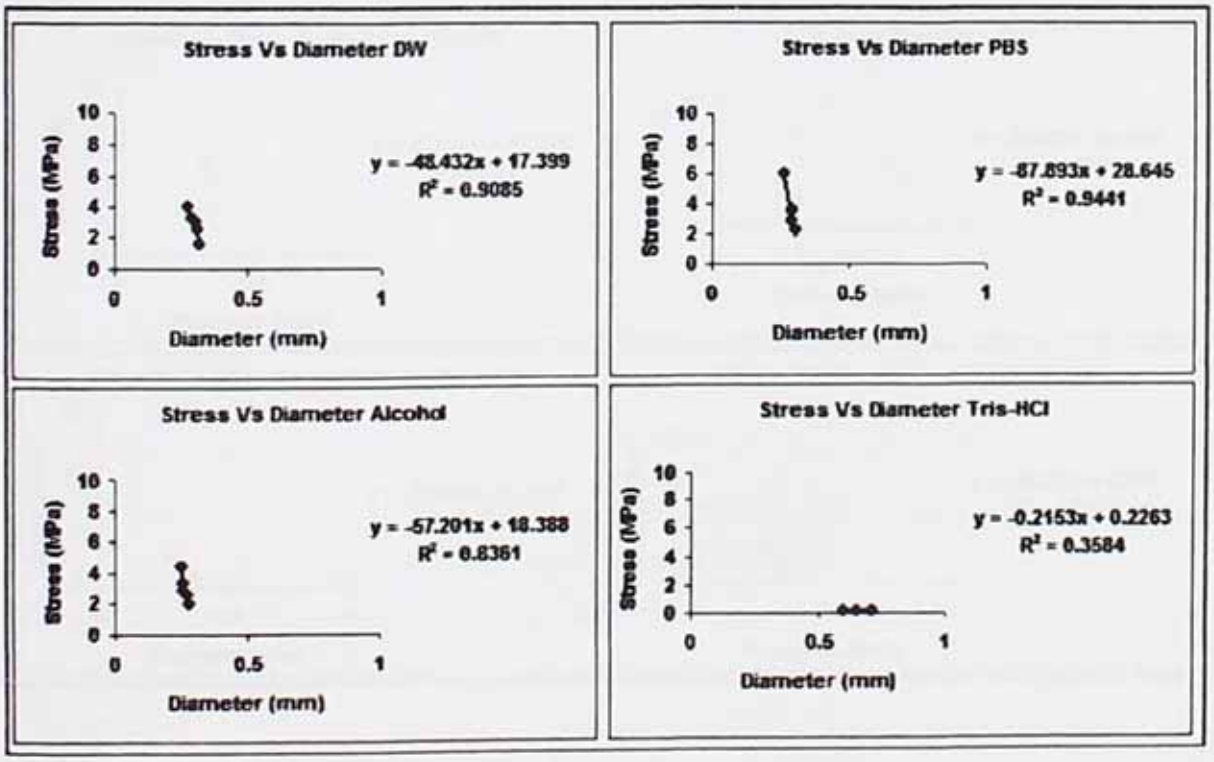


Figure 12. Correlation values (R^2) between tensile strength and diameter for wet cross-linked reconstituted collagen fibres.

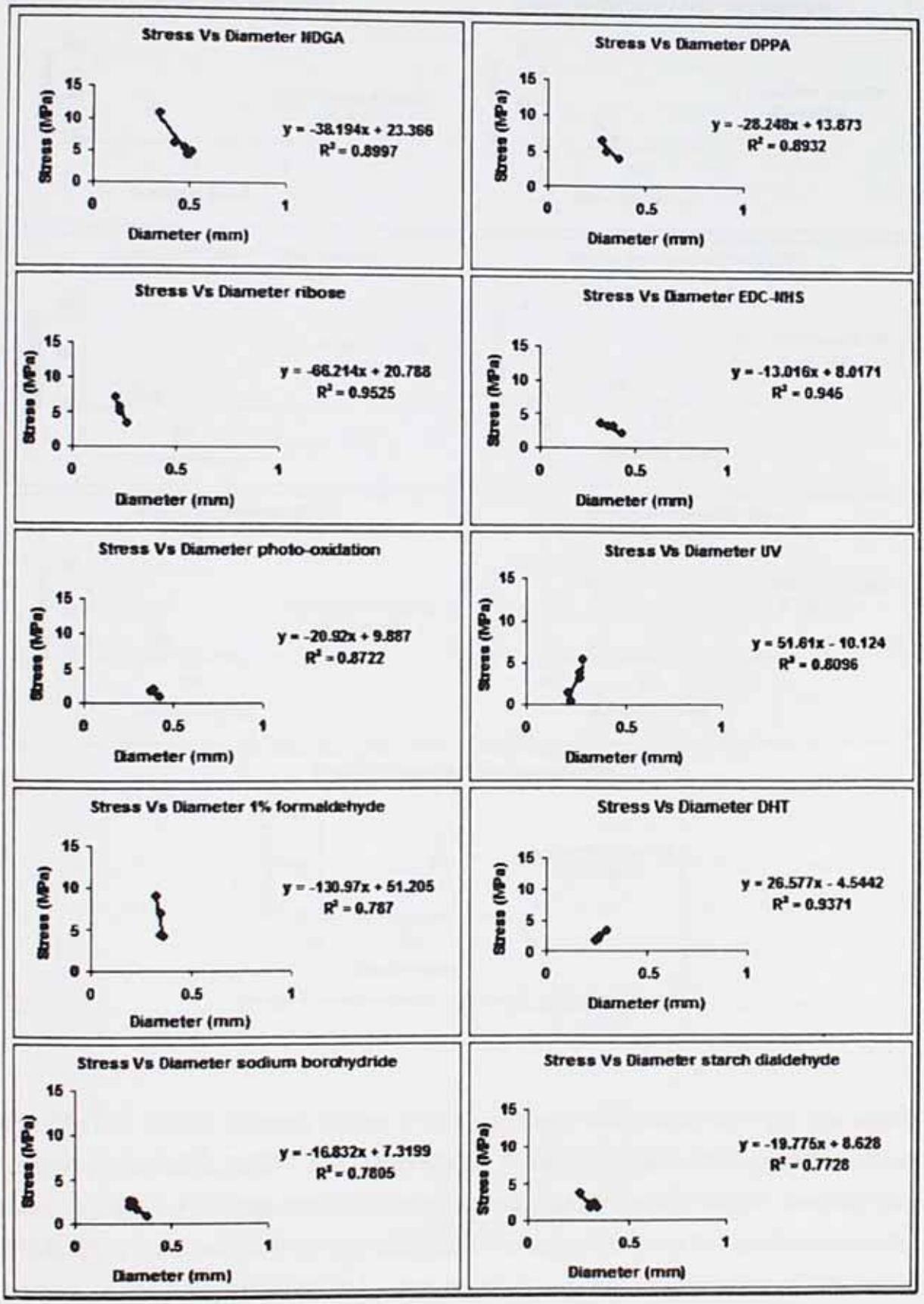
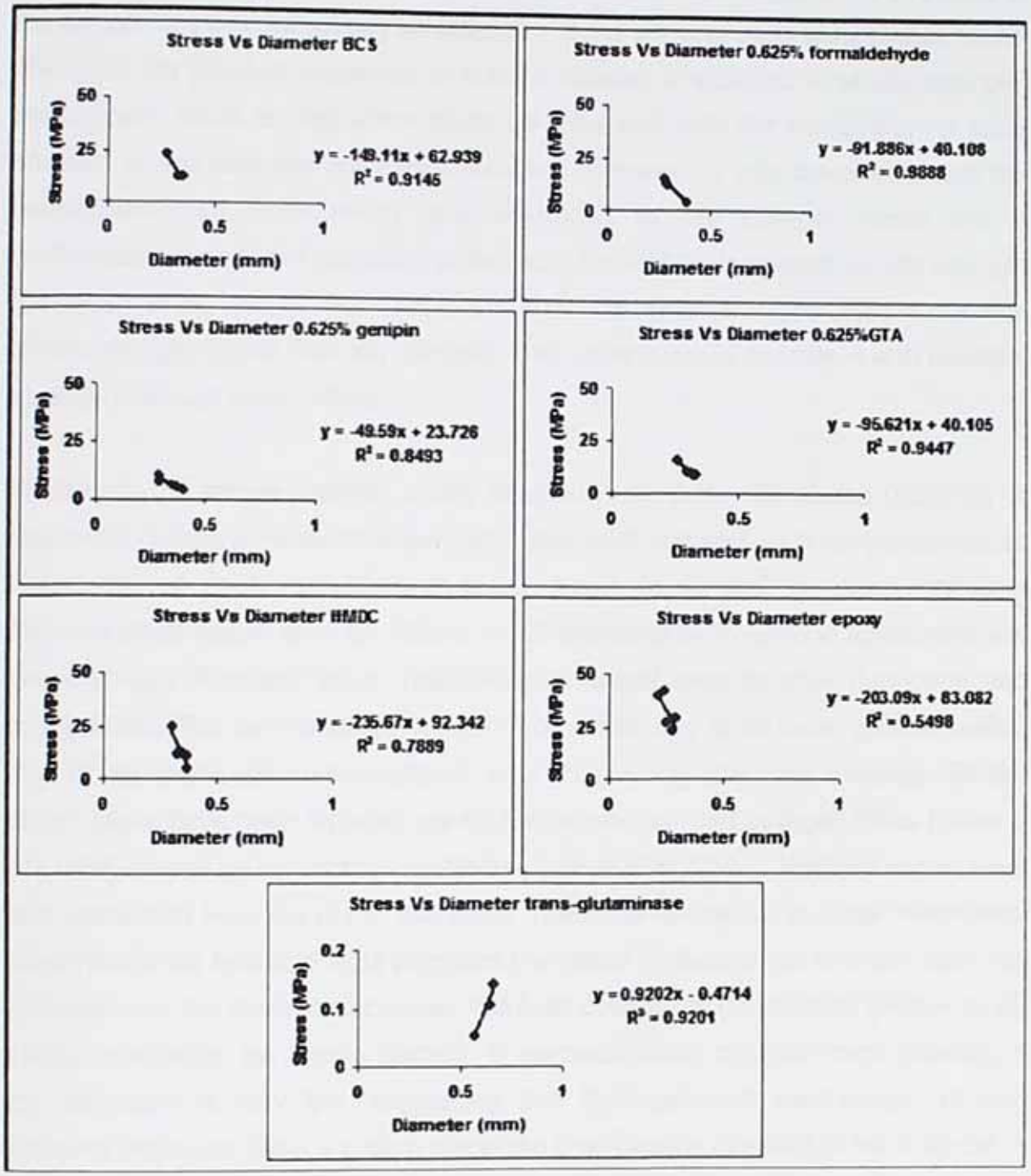


Figure 12. (Continue).



The highest tensile strength values in the wet state were obtained from the epoxy treatment with BCS, HMDC and 0.625%GTA. These treatments also gave the lowest swelling values. Previous studies have demonstrated that a decrease in swelling ratio is related to an increase in tensile strength of collagen fibre. As the equilibrium water content of the fibre is modified by the cross-linking 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 (Wang *et al.*, 1994). Photo-oxidation, sodium borohydride, starch di-aldehyde, DHT, UV, trans-

glutaminase and EDC/NHS treated fibres exhibited tensile strength lower or similar to the control fibres. A preliminary investigation of the effect of trans-glutaminase cross-linking on the physical properties of leather showed a reduction in tensile strength, the opposite effect to that which might be expected from the introduction of more covalent bonds between collagen molecules. However, it was speculated that the trans-glutaminase cross-linking may covalently fix the collagen fibres into a conformation that is not necessarily the most optimal for increased tensile strength (Collighan *et al.*, 2004). The rest of the chemical treatments produced fibres with tensile strength higher than the controls. This observation is consistent with previous studies (Kato and Silver, 1990).

Similarly to the tensile strength, epoxy treatment with BCS, HMDC and 0.625%GTA required the highest forces to failure, while the DHT and sodium borohydride treated fibres required the lowest forces to break. Again, in general, the chemically fixed fibres required higher force for failure, which seems to be in general agreement with previous work (Kato and Silver, 1990). Wet fibres had lower breaking load compared to dry fibres. The tensile stress values were diminished to an even greater extent, due to an increased cross-sectional area caused by the fibre swelling. Similar observations have been reported previously for reconstituted collagen fibres (Dunn *et al.*, 1993) as well as for collagen scaffolds (Angele *et al.*, 2004). Modulus values were also decreased from the dry to wet state. The large decreases in these mechanical properties in the hydrated state suggests that water molecules act to break down the hydrogen and the electrostatic bonds that hold collagen fibrils together (Pieper *et al.*, 1999). In addition, the tensile strength of un-cross-linked collagen fibres (control) in the wet state is very low, suggesting that hydrogen and electrostatic bonding between molecules plays a critical role in the load-bearing capacity of this material. A prime role of cross-linking appears to be that of maintaining the distances between neighbouring molecules, preventing the incorporation of excess water that would disrupt 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 between per collagen α-chain), 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 inter-molecularly. Furthermore, it has been suggested 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 inter-molecularly to stiffen the collagen triple helix and prevent slippage and translation to occur between neighbouring molecules (Wang *et al.*, 1994).

As regards to the strain at break, 1% formaldehyde fixed fibres gave an exceptional 68% elongation whilst trans-glutaminase fixed fibres were slightly less extendable at 61%. In general, all treatments produced more extendable fibres than the controls. This increase of the strain values has been observed previously and has been attributed to the two distinct effects that cross-linking has on collagen. The first is the planar shrinkage that occurs as the wavy collagen fibres become more tightly crimped during cross-linking. If gauge length is defined under a small initial load, the cross-linked tissue will appear to have higher extensibility. The second effect of cross-linking is the production of inter-fibrillar cross-links, which reduce the ability of collagen fibres to slip past each other, particularly in response to high loads. As noted earlier, the strain at fracture in fresh tissue is the sum of un-crimping of tissue, extension of collagen fibres under tension and finally slippage of fibres. After cross-linking the degree of extension can be associated with the un-crimping mechanism of deformation which is enhanced and the slippage mechanism which is reduced. However, the net result is an increase in strain at fracture (Chachra *et al.*, 1996). It has been suggested that cross-linking enhances the tensile strength of collagenous matrices, whilst it reduces the strain at break (Charulatha and Rajaram, 2001). However, this observation does not appear to be in agreement with our results, where no correlation was observed between tensile stress and strain at break.

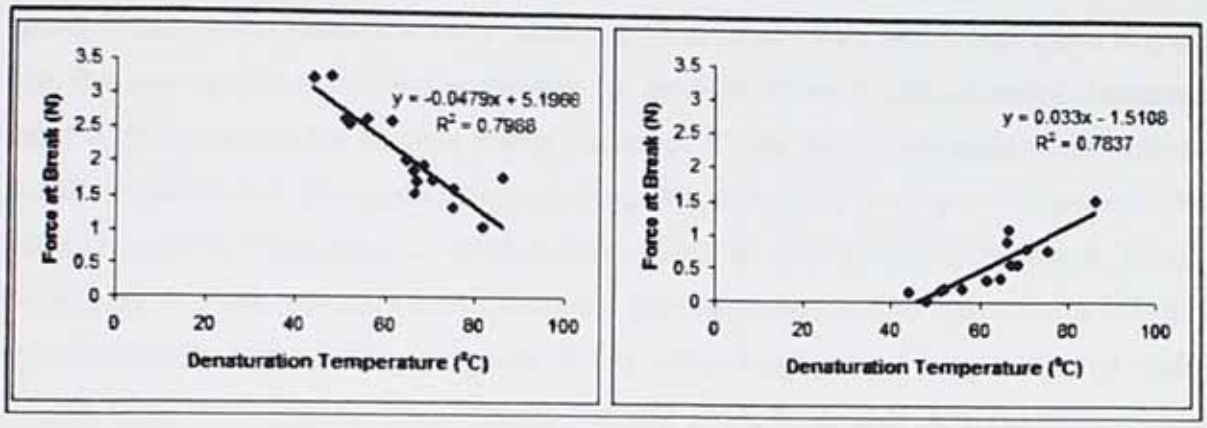
The highest modulus values were obtained from the epoxy fixed fibres with ribose to follow, while the trans-glutaminase and the photo-oxidation treated fibres were found to give the lowest modulus values. It has been reported that the incorporation of cross-linking agents in collagen results in brittle materials (Chachra *et al.*, 1996; Osborne *et al.*, 1998). Furthermore, it is known that the increase in fibre stiffness is the result of an increase in cross-link density (Wang *et al.*, 1994; Lavagnino *et al.*, 2005) indicating that epoxy and ribose induced fibres had higher cross-linking densities. Our results indicate that the *in vitro* glycation influenced the matrix stability and are in accord with recent work, where it was demonstrated that the glucose mediated glycation modulated specifically the biomechanical stability of soft tissue (tendons), but had no effect on bone biomechanics (Reddy, 2003). Additionally, *in*

vitro glycation resulted in an increase of tensile strength and stiffness as has been reported previously (Reddy *et al.*, 2002). Furthermore, it was demonstrated that genipin treatment increased both the stiffness and the modulus of the fibres, which seems to agree with previous work on sheep tendons, suggesting that genipin has a potential utility in soft tissue repair and tissue-engineering applications (van Kleunen and Elliott, 2003).

Other workers have reported that in connective tissues increases in mechanical strength due to increase of the cross-linking density (Vogel, 1977; Charulatha and Rajaram, 2001). Such changes occur during maturation as well as after treatments with corticosteroids and cross-linking methods (Vogel, 1977). Cross-linking of porcine pericardium with different agents resulted in increased mechanical strength in the order of epoxy < GTA < genipin (Chen *et al.*, 1997; Sung *et al.*, 1998). However, these results are in disagreement with our observations. We support that the epoxides resulted in higher cross-linking density than the aldehyde or iridoid fixation as can be seen from the denaturation temperature and the swelling ratio. In collagen-chondocytes matrices the observed cross-linking order was found to be DHT < UV < GTA (Lee *et al.*, 2001), which appears to be in agreement with our results. As expected, the chemical treatments can induce higher cross-linking densities than the physical methods; results that can be confirmed from the denaturation and swelling studies.

As has been seen, the mechanical and thermal properties of collagen fibres depend on the degree of covalent cross-linking, but tensile strength and shrinkage temperature were found to be affected in different ways since no correlation was observed between these two parameters. Similar observations have been reported previously (Cavallaro *et al.*, 1994). However, fitting a linear regression model for Denaturation Temperature Vs Force to break for the chemical cross-linking methods (except for the BCS where denaturation temperature was a lot higher than the other treatments) a high correlation was observed (R^2 values of 0.80 and 0.78 for dry and wet state respectively). It was found that in the dry state a decrease of force was observed as denaturation temperature increased, while in the wet state, a decrease in force was observed with decreasing denaturation temperature (Figure 13).

Figure 13. Correlation between denaturation temperature and force at break for the dry (left) and wet (right) cross-linked reconstituted collagen fibres.



Furthermore, it was noted that fibres may have similar shrinkage temperatures (HMDC / NDGA) and yet have different wet material properties, suggesting that different mechanisms are responsible for each (Cavallaro *et al.*, 1994). Similarly, it was shown for HMDC fixation, that the mechanical characteristics can be maximised by increasing the concentration and the reaction time (Vizarova *et al.*, 1994). However, by increasing the formaldehyde concentration in the wet state, a decrease in tensile strength was observed. These observations imply that different chemical cross-linking agents have higher cross-linking efficiency than others. Thus the degrees of cross-linking produced by GTA and genipin were greater than those produced by epoxy in fixation of pericardium (Chen *et al.*, 1997; Sung *et al.*, 1998). For the reconstituted collagen fibres of this study, the degrees of cross-linking were greater for epoxy, followed by GTA and genipin. The physical methods (DHT, UV) were found to be less efficient than the chemical methods (GTA) for reconstituted collagen fibres. These observations could indicate that other factors than the cross-linking method alone can affect the efficiency of the method utilised.

Since the molecules of GTA can self-polymerise the cross-linking of collagenous fibrils by GTA may be in the form of a three-dimensional network with long-range cross-links spanning larger gaps, which can affect collagen fibres properties by fibre stiffening and strengthening and prolonged biodegradation (Kato *et al.*, 1989; Kato and Silver, 1990). In contrast, because the epoxy ether is a bi-functional agent, only linear cross-linking within the collagenous tissues is formed. As a result, it would have been expected that GTA would produce fixed tissues with greater thermal stability and mechanical strength than its epoxy counterpart, and indeed this has

been found previously for pericardium tissue (Chen *et al.*, 1997). However, in this study the epoxy fixed fibres were found to be superior to those fixed with GTA as judged by stress at break and thermal stability values. It was also found that the epoxy fibres had a lower diameter than the GTA fixed ones and it has been shown that the strength of the fibres increases for smaller cross-sectional areas, because there is less chance for defects being included. It has also been suggested that as the fibre diameter is decreased, cross-linking efficiency may be improved, because of the increased surface area to volume ratio (Dunn *et al.*, 1993). Furthermore, it may be argued that the adjacent fibrils and fibril bundles were too far apart to be bridged by small length cross-linking reagents (GTA), while longer chain length (epoxy) could enable cross-linking of reactive groups that are spatially distant, and hence increase the cross-linking density. Similar observations have been reported with dermal sheep collagen after fixation with GTA / HMDC / EDC/NHS / epoxy (Damink *et al.*, 1995; Chachra *et al.*, 1996; Damink *et al.*, 1996a; Osborne *et al.*, 1998).

In the wet state, similar tensile strength values were obtained for the GTA and the HMDC treatments, and a decrease in modulus was observed in comparison to the non-cross-linked samples. Similar results have been reported for dermal sheep collagen (Damink *et al.*, 1995). Cross-linking with EDC/NHS resulted in fibres (in both the wet and dry state) with decreased modulus and tensile strength values in comparison with the GTA. Similar results have been reported for tendon prosthesis (Goldstein *et al.*, 1989) and for anterior cruciate ligament reconstruction using collagenous prosthesis (Dunn *et al.*, 1992). Lysyl residues on reconstituted collagen react with GTA, resulting in increased strength. EDC/NHS treatment of collagen catalyses the formation of synthetic peptide bonds without introducing cytotoxic aldehydes, however the number of cross-links is decreased relative to aldehyde cross-linked collagen (Dunn *et al.*, 1992). The decrease in tensile strength has been attributed to the production of local stress concentrations due to the early failure of brittle collagen fibres formed after extensive cross-linking (Damink *et al.*, 1996a).

It has been shown that the material properties of reformed collagen fibres cross-linked with NDGA equal the tensile strength, stiffness and strain at failure of native tendon (Koob *et al.*, 2001a; Koob *et al.*, 2001b), however in later studies NDGA polymerised fibres appeared to have nearly twice the tensile strength of native bovine tendon, while the stiffness appeared to be 80% greater than the stiffness of bovine tendon. Furthermore, the superiority of NDGA fixed fibres in comparison to GTA fixed

in regards to their mechanical properties has been demonstrated (Koob and Hernandez, 2002). UV light could affect the cross-linked polymer, altering the solubility of polymer adducts or changing the polymer chemistry. Furthermore, it was shown that UV treatment appeared not to liberate toxic by-products. The tensile strength of UV-treated cross-linked fibres appeared to be lower, whilst the modulus was higher than the counterpart NDGA-fixed fibres (Koob *et al.*, 2001b). GTA treated fibres had a higher tensile strength, lower elongation to break and higher stiffness than the NDGA ones. Both NDGA and GTA cross-linked fibres had mechanical properties higher than the UV treated fibres as well as the un-cross-linked controls.

It can be concluded that depending on the application, tailored made biomaterials can be produced by the selection of the appropriate cross-linking agent / technique. For example when a strong material is required, anything with similar properties to those of GTA would be ideal, as long as it is more biocompatible. It is known that wound dressings need to be flexible and adhere to and protect the wound from infection and fluid loss. Therefore a flexible material would be an ideal replacement (Charulatha and Rajaram, 2003).

The mechanical characteristics of collagenous tissue have been a topic of great interest. This has been very useful in obtaining a clear idea of the mechanical integrity, as well as strength of these connective tissues and correlating them with the animal age, specimen length and weight, deformation rate, collagen content, presence of other extra-cellular components (proteoglycans), chemical modification, and sampling position were found to play important roles in the study of the collagenous tissue (Vogel, 1978; Haut, 1986; Danielsen and Andreassen, 1988; Arumugam *et al.*, 1992; Lavagnino *et al.*, 2005). Furthermore, it has been shown in this study that several factors can affect the mechanical properties of reconstituted collagen fibres (species, tissue, extraction method, collagen types / concentration, co-agent type / concentration and cross-linking method). Although all the above factors play an important role on the properties of the reconstituted collagen materials nevertheless reference values will be provided for comparison reasons (Table 10 and 11) and it can be observed that the mechanical properties of the fibres of this study are comparable with other studies. Any deviations could be attributed to the reasons described above. However, it should be pointed out that compatible biomaterials were produced.

Table 10. Mechanical properties of dry reconstituted cross-linked collagen fibres.

	Diameter (mm)	Stress at Break (MPa)	Strain at Break (%)	Force at Break (N)	Modulus (MPa)	Reference
GTA Vapour (Bovine) Cyan. Vapour / DHT RTT		139-180 168-184 363-366	11.8-15.3 19.5-22.2 15.60-13.80		3070-4860 3600-4320 2130-2690	(Kato *et al.*, 1989)
AS BAT GTA EDC		224 175 197				(Cavallaro *et al.*, 1994)
Bovine Corium GTA Vapour Cyan. Vapour / DHT	0.024-0.088 0.022-0.078 0.023-0.080	75-160 77-205 76-210		0.08-0.46 0.07-0.43 0.06-0.40		(Dunn *et al.*, 1993)
DHT RTT		194-605	15.65-26.74		396-2688	(Wang *et al.*, 1994)
RTT Formaldehyde GTA BCS		120.02 82.34 108.43 105.66	31 40 42 47			(Rajini *et al.*, 2001)
RTT BAT-EDC 0.05mm BAT EDC 1.02mm BAT EDC 1.27mm	0.271 0.059 0.125 0.158	115 50 36 25			1175 485 360 270	(Gentleman *et al.*, 2003)

Table 11. Mechanical properties of wet reconstituted cross-linked collagen fibres.

	Diameter (mm)	Stress at Break (MPa)	Strain at Break (%)	Force at Break (N)	Modulus (MPa)	Reference
Bovine corium GTA Vapour Cyanamide Vapour	0.125 0.070 0.080	2.4 44.1 17.4	24 14.2 22.2	0.26 1.75 0.91	13.2 340.6 131	(Kato and Silver, 1990)
RTT DHT Decorin Native RTT	0.309 0.111 0.296 0.250	0.772 38.44 1.359 53.5	47.8 19.03 54.8 13.9	0.53 3.53 0.82 26.86	2.32 252 4.50 498	(Pins *et al.*, 1997b)
RTT DHT	0.327 0.094	0.908 46.81	67.97 15.59	7.55 31.73	1.819 383	(Pins *et al.*, 1997a)
Bovine Tendon NDGA GTA Native Bovine Native Rabbit		0.7 28.8-91.2 61.1 46.8 42.8	46-40 12-11 8 19 15		1-4 244-696 489 327 366	(Koob and Hernandez, 2002)
Native BAT GTA Vapour Cyan. Vapour / DHT		36.7 31.4 11.6				(Kato *et al.*, 1991)
GTA Vapour (Bovine) Cyan. Vapour / DHT RTT		50-66.2 23.9-31.3 32.6-39	13.6-16.10 14.70-17.70 6.70-8.10		384-503 170-198 478-570	(Kato *et al.*, 1989)
AS BAT GTA EDC		1.2 23.9 27.7				(Cavallaro *et al.*, 1994)
Native RTT Insoluble corium AS RTT	0.250 0.085 0.103	53.5 26.6 37.2	13.9 17.9 11.86	2.63 0.16 0.28	498.8 204 378	(Pins and Silver, 1995)
Bovine Corium GTA Vapour Cyan. Vapour / DHT	0.050-0.180 0.025-0.099 0.025-0.100	10-15 25-110 35-75		0.025-0.16 0.055-0.248 0.045-0.26		(Dunn *et al.*, 1993)
DHT RTT		7.9-91.8	7.4-20.57		58.3-895.8	(Wang *et al.*, 1994)
Auto-genous graft Bov. Corium GTA Bov. Corium Cyan.		24 32 13	38 24 36		154 190 44	(Goldstein *et al.*, 1989)

4.4. Conclusions

Repair and regeneration of tissues is dependent on the use of biodegradable polymer scaffolds, which support, reinforce and organise the regenerating tissue. Reconstituted collagen fibres have been shown to be a competitive biomaterial for soft tissue repair and regeneration. In this chapter for first time an overall comparison between the available cross-linking techniques on one tissue has been presented. It was found that reconstituted collagen fibres could be prepared with a wide range of thermal stabilities and mechanical properties depending on the cross-linking method utilised. Therefore, depending on the application, tailor made biomaterials can be produced. A direct relation between the tensile strength and the fibre diameter was observed, while correlation between the shrinkage temperature and the degree of swelling was not observed. However, the shape of the stress-strain curves and the mechanical properties of the fibres were dependent on the degree of swelling and the denaturation temperature was found to be strongly correlated with the force at break. Finally, competitive biomaterials for tissue engineering applications were produced.

Chapter 5. Conclusions

It was demonstrated throughout this work that there is an inverse relationship between the dry and wet diameter of reconstituted collagen fibres and their tensile strength at break; the smaller the fibre diameter, the greater the tensile strength. This finding would be of high importance for tissue engineering applications and tissue reconstruction. For example, by measuring the tissue to be replaced fibre diameter, we can manufacture a reconstituted implant with similar diameter and tensile strength.

In spite of previous uncertainty it can be concluded that fibres can be routinely prepared from pepsin extracted collagen. Such fibres have structures and mechanical properties which are similar to those produced from acid extracted collagen. This is a highly significant finding since pepsin solubilisation will give greater yields of collagen from a wider variety of sources and produce a more biocompatible biomaterial.

The preparation of reconstituted collagen fibres from porcine tissue is a highly significant result, bearing in mind the high correlation, physiology speaking, between human and porcine species. These results, not only add a new tissue in the quest of the ideal raw material for collagen extraction and consequently scaffold preparation, but also contributes to minimising biological waste, promoting that way recycling in all aspects of life.

The importance of a co-agent present in the fibre formation bath was demonstrated. Based on this finding, for first time, a number of different co-agents were utilised, providing not only an important insight of the mechanical properties of the reconstituted collagen fibres, but also a wide range of alternative options depending on the application in mind.

Furthermore, for first time, the cross-linking methods currently available were examined on the same tissue, providing an overall picture of the techniques and their influence on the reconstituted collagen properties. The significance of this systematic work lays on that now we are able to choose the ideal cross-linker depending on the aim of the work in mind.

It can be concluded that reconstituted collagen fibres can be tailor made to suit a diversity of surgical needs with properties similar or even superior to native tissues or other synthetic or natural materials that are already in practice. These results confirm the position of collagen, Nature's favourite biomaterial, in the leading position as a scaffold for tissue engineering applications.

Chapter 6. Future Work

Over the last few decades extensive research has been carried out on biomaterials derived from natural resources. At the same time, medical-grade collagen became easier to obtain, the processing technology improved and new collagen products were successfully placed on the market. As a result, the expansion of tissue engineering technology found an ideal substrate to utilise as a scaffold. Despite the various applications of collagen as a biomaterial, still several draw backs exist: the high cost of preparation of pure type I collagen; variability of isolated collagen (cross-linking density, fibre size, impurities); hydro-philicity which leads to swelling and more rapid release compared with synthetic polymers; side effects such as possible transmitted diseases (BSE) and calcification. However, the advantages of using collagen for tissue engineering applications (good biocompatibility and low anti-genicity, high mechanical properties, availability in high quantities, haemostatic properties, easily formulated in various forms) are clear. Thus, the interest of establishing common aims between different research-groups, further work as indicated below is suggested which will underpin future developments and uses of collagen.

Overall, the work reported in this thesis clearly showed that many factors need to be taken into account in order to optimise the properties of reconstituted collagen fibres and to produce a competitive biomaterial of high calibre. Although several factors were addressed during this research work, there are still many that remain of interest. After this systematic study, more than ever before, it is of importance to assess:

- alternatives sources of collagen (e.g. pig skin, fish skin)
- reconstitution of different types of collagen present
- different co-agents / cross-linking agents
- the elasticity of the fibres using load extension cycle tests
- the fibril orientation using X-ray diffraction
- the mineralization of the reconstituted collagen fibres
- the internal structure of the reconstituted fibres with transmission electron microscopy
- the differences between discrete and continuous reconstituted collagen fibres
- braiding the fibres into scaffolds and evaluating the properties of the resulting material
- the *in vitro* biocompatibility and bioactivity of the material
- the cross-linking stability and durability of the fixed tissue as a function of storage and chronic implantation

To conclude, engineering the material and evaluating the wound healing response would seem to be an appropriate goal. Recombinant technology and knowledge of collagen and tissue repair can be combined to generate biologically enhanced collagen-based biomaterials with specifically designed characteristics to allow interaction and manipulation of the intended tissue environment. Finally, it is worth pointing out that the full potential of this versatile material has yet to be realised.

Chapter 7. References

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Chapter 8. Appendix

8.1. Hydroxyproline Assay

Reagents

Citrate buffer (1l): 34.38g of sodium acetate anhydrous, 37.50g of tri-sodium citrate.$2H_2O$, 5.50g of citric acid, 400ml of distilled water and 400ml of isopropanol. Dissolve solids in the distilled water, add the isopropanol and make up the volume with distilled water.

Chloramine T reagent (45-90 determinations): 24g di-methyl-amino-benzaldehyde, 36g of perchloric acid and 200ml of isopropanol. The solids were dissolved in the perchloric acid and the isopropanol was added immediately prior to use.

Diluent: isopropanol: distilled water; 2 to 1 ratio

Hydroxyproline standards: 0.1g/l

Procedure

5ml of the collagen solution was placed in a digestion tube. Then 5ml of concentrated HCl was added to the tube and it was immediately closed with a lid containing PTFE insert.

The tubes were put in oven at 100^0C for 16 hours.

After that, the tubes were left to cool down and then transferred into volumetric flasks (100ml) containing approximately 40ml distilled water. The tubes were further rinsed with distilled water and the washings were added to the volumetric flask up to the mark and mixing was carried out.

5, 10 and 20 ml of the solution were further diluted in 100ml volumetric flasks and mixing was carried out.

Standard hydroxyproline solutions of 2.5, 5, 10, 15 and 20 mg/ml were prepared.

Blank, using distilled water instead of the sample or standard, was prepared.

0.55ml of standard or sample or blank, 1.27ml of diluent and 0.88ml Chloramine T reagent were added in test tubes.

Thorough mixing was carried out and then the samples were left to settle for 5 minutes at room temperature.

Then 2.30ml of Ehrlichs reagent was added followed by mixing.

The sample tubes were then immersed in a water bath at 70^0C for 10 minutes.

Finally, they were left to cool down, mixed well and the absorbance was measured in a spectrophotometer at 555nm.

Standards and samples were measured in duplicate.

8.2. SDS-PAGE Assay

Appropriate volumes of sample (25-100µg) were loaded onto a vertical acryl-amide gel (4% stacking gel, 5.5 / 7.5% resolving gel) and electrophoresed for approximately 30min (50mA per gel) using a modified Laemmli system. Electrophoresis was then interrupted and 5µl of 2-mercaptoethanol (30% v/v in sample buffer) added to each well. Gels were stained overnight with Coomassie Brilliant Blue and then de-stained prior to densio-metric analysis. Known weights of collagen standards were also analysed in a similar manner to provide a calibration curve and to determine the lower limits of sensitivity. Electrophoresis was carried out using the Bio-Rad Protean II system, 160mm inner plate and 1.0mm spacers.

Stock reagents:

30% acryl-amide (29:1)

1.0M Tris-HCl pH 6.8 & 8.8

10% SDS (lauryl sulfate)

1.5% APS (ammonium persulfate)

N, N, N, N-tetra-methyl-ethylene-di-amine (TEMED)

30% 2-mercaptomethanol (v/v in sample buffer)

0.01% Bromophenol blue

Resolving gel (ml/10ml of gel)

	5.5%	7.5%
30% acryl-amide	1.83	2.50
1.0M Tris-HCl pH 8.8	3.75	3.75
10% SDS	0.10	0.10
Distilled Water	4.00	3.33
1.5% APS	0.30	0.30
TEMED	0.112	0.112

Stacking gel 4% (ml/10ml of gel)

	4%
30% acryl-amide	1.33
1.0M Tris-HCl pH 6.8	1.30
10% SDS	0.10
Distilled Water	6.74
1.5% APS	0.30
TEMED	0.02

Electrophoresis Buffer approximate pH 8.6

	g/L
Tris	3.0
Glycine	14.4
SDS	1.0

Sample Buffer approximate pH 6.8

	g/100ml
Tris	1.51
SDS	2.0
Glycerol	10.0
Bromophenol blue	1.0ml of a 1% stock

Dissolve in 80ml of distilled water, adjust to pH 6.8 using 1M HCl and make up to 100ml.

Stain

	g/L
Coomassie Brilliant Blue	2.0
Methanol	(400ml)
Acetic Acid	(170ml)

De-stain

	ml/L
Methanol	100
Acetic Acid (conc.)	70
Distilled Water	830

8.3. Materials

All materials, chemicals and consumables were persuaded from Sigma-Aldrich, UK, except those detailed in sections 8.3.1. and 8.3.2.

8.3.1. Materials / Chemicals / Consumables

Bovine Achilles tendon: Kindly provided from the British Leather Centre, Northampton, UK

Rat tail tendon: Kindly provided from the Department of Biomedical Sciences, Sheffield University, Sheffield, UK

Pig tail tendon: Kindly provided from the National Pig Association, Warwickshire, UK

Pepsin: Porcine gastric mucosa pepsin (2500 U/mg), Roche Diagnostics, UK

Trans-glutaminase: Microbial trans-glutaminase (10% Tg and 90% maltodextrin), Forum Products Ltd., Redhill, UK

Genipin: Challenge Bioproducts, Co, Ltd, Taiwan

Chromium Salt: Elementis Chromium, Eaglescliffe, UK

Myrica: Kindly provided from Prof A. D. Covington, British School of Leather Technology, University of Northampton, UK

SDS-PAGE chemicals: Bio-Rad Laboratories Ltd, Hertfordshire, UK

Syringes: Terumo 5ml plastic disposable syringes, Terumo Medical Corporation UK Ltd, Merseyside, UK

Laboratory Tubing: Samco Silicone Products, Ltd., Warwickshire, UK

Separation: Filter paper and units, Whatman Plc, Middlesex, UK

Centrifuge tubes: Nalgene Polycarbonate tubes and Nalgene Polypropylene Screw Closure, Nalge Ltd, Hereford, UK

8.3.2. Equipment

Syringe Pump System: KD-Scientific 200, KD-Scientific Inc., Massachusetts, USA
SDS-PAGE: Bio-Rad Laboratories Ltd, Hertfordshire, UK
Centrifuge: Gr20.22 Jouan refrigerated centrifuge, Thermo Electron Corporation, Bath, UK
Electron Microscopy: S-3000N Hitachi Variable Pressure Scanning Electronic Microscope, Hitachi, Berkshire, UK
Optical Microscope: Nikon Eclipse E600, Nikon Instruments, Surrey, UK
Stereo Microscope: Olympus Ltd, Middlesex, UK
Scanning Calorimetry: 822e Mettler-Toledo DSC, Mettler-Toledo International Inc., Leicester, UK
Viscometer: DV-III Brookfield Viscometer, Brookfield Viscometers Ltd, Essex, UK
Material Testing: 1122 Instron Universal Testing Machine, Instron Ltd, Buckinghamshire, UK
Mincer: OMTS12 Rowlett Meat Mincer, O'Gormans Ltd., Oxfordshire, UK
Spectrophotometer: Spectronic 501; Milton Roy Company, Berkshire, UK
Water bath: SS40-3, Grant Instruments Ltd, Cambridge, UK
Controlled Environment: Air Conditioning & Humidity Unit, Model U8 x 8H #A3987; Denco Limited, Cheshire, UK

Chapter 9. Scientific Contribution up to Present

9.1. Healthcare and Medical Textiles '03, International Conference and Exhibition, Bolton, UK

Title: Reformed Collagen Fibres

Authors: D. Zeugolis, R. G. Paul, G. E. Attenburrow

Abstract:

Collagen is the primary structural component of connective tissue and because of its inherent biocompatibility is an ideal candidate for biomedical applications such as scaffolds for tissue regeneration. One route to create a collagenous biomaterial which can be produced in a variety of forms is to render the collagen soluble and then reconstitute it. The research reported here is concerned with reconstituted collagen fibres and specifically compares different solubilisation processes (acid soluble collagen, ASC Vs pepsin soluble collagen, PSC), and the influence of polyethylene glycol (PEG) solution on fibre formation. The structure and mechanical properties of reformed fibres were examined. It was found that PEG was essential for the formation of fibres from PSC but its use caused a decrease in the strength of the fibres from ASC. The yield of fibres from PSC was much greater than that from ASC. Some dried reformed fibres produced from both acid soluble and pepsin soluble collagen had a tensile strength equal to that of dried native collagen fibres.

Paper published at: Medical Textiles and Biomaterials for Healthcare (2004), Editors: Anand, S.C., Miraftab, M., Rajendran, S., Kennedy, J.F. and King, M. W., Woodhead Publishing Limited, England, ISBN 1 85573 683 7

9.2. XIX Federation of European Connective Tissue Societies Conference, 2004, Giardini Naxos, Sicily, Italy

Title: Reconstitution of collagen fibres in the presence of a co-agent

Authors: Dimitrios Zeugolis, Gordon Paul, Geoff Attenburrow

Abstract:

Introduction: Collagen is the primary structural component of the extra-cellular matrix and it is of interest to consider the factors, which influence the self-assembly of collagen molecules to form fibres. Previous studies have demonstrated that the in vitro self-assembly of collagen fibrils with a D-periodic banding similar to that of native fibrils may be achieved by extruding a collagen solution into a series of neutral buffers (Pins and Silver, 1995; Cavallaro et al., 1994). For the self-assembly of Pepsin Soluble Collagen derived from Bovine Achilles Tendon, the use of co-agents [e.g. polyethylene glycol (PEG)] has been shown to be essential (Zeugolis et al., 2003).

Methods: This study investigates the effect of the co-agent (PEG Mw 8000) as well as the collagen solution concentration, and the diameter of the tube through which the extrusion of the fibre takes place.

Results and Discussion: The mechanical properties of the fibres were evaluated and correlated to the microscopic structure of the fibres as revealed by microscopic techniques.

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Cavallaro J. F., Kemp P. D., Kraus K. H., (1994), Biotechnology and Bioengineering, 43: 781-791

Pins G. D., Silver F. H. (1995), Materials Science and Engineering, 3: 101-107

Zeugolis D., Paul G., Attenburrow G., (2003), Medical Textiles and Biomaterials for Healthcare (2004), Editors: Anand, S.C., Miraftab, M., Rajendran, S., Kennedy, J.F. and King, M. W., Woodhead Publishing Limited, England, ISBN 1 85573 683 7

9.3. Cell Based Therapies, British Society for Matrix Biology, Autumn Meeting 2004, Bristol, UK

Title: Reconstitution of Collagen Fibres in the Presence of a Strong Electrolyte

Authors: Dimitrios Zeugolis, Gordon Paul, Geoff Attenburrow

Abstract:

Collagen is the major component of the extra-cellular matrix (ECM). It can be found in most animal phyla and it is the main structural protein of skin, bone and tendon. Fibrous, natural proteins such as collagen and silk exhibit useful mechanical properties in addition to their biocompatibility and low antigenicity, in contrast to the catalytic and molecular recognition functions displayed by globular proteins and as a result they provide important materials in the field of controlled drug release, biomaterials and scaffolds for tissue engineering. In vitro, under appropriate conditions (temperature, pH, ionic strength, composition of fibre formation medium), collagen molecules spontaneously form fibres (fibrillogenesis) that are similar to native fibres. Since the natural cross-linking pathway does not occur in vitro and there are no mechanisms currently known for catalysing lysine-derived cross-link formation in the reconstituted fibrils, reformed collagen fibres tend to be relatively weak in tension. However by controlling the composition of the aqueous medium in which fibres form from pepsin soluble collagen it has been found that the strength and uniformity of fibres made can be much improved.

This study is concerned with the properties of reformed collagen fibres prepared by extrusion of pepsin soluble collagen into various buffered aqueous media. In particular the effects of (a) the concentration of Sodium Chloride in the fibre formation medium and (b) the collagen concentration of the extrudate are investigated. The mechanical properties of the fibres were evaluated and are discussed in relation to their microscopic structure.

Abstract Selected and Published at:

International Journal of Experimental Pathology, 2005, 86, A1–A56

9.4. Collagens – From Genes to Fibrils, British Society for Matrix Biology, Spring Meeting 2005, Liverpool, UK

Title: Mechanical and Structural Properties of Reconstituted Cross-linked Collagen Fibres

Authors: Dimitrios Zeugolis, Gordon Paul, Geoff Attenburrow

Abstract:

Currently available different surgical suture materials, including absorbable and non-absorbable types, have provided satisfactory performance for many years in surgical practice. Suture serves an important function in wound repair, namely to hold tissues in apposition as the wound heals. In the selection of an appropriate suture for a given situation the main characteristics that should be considered are tensile strength, ease of handling, knot security and tissue reactivity. The use of collagen as a biomaterial has been advocated based on several factors such as its favourable role in cellular development, wound healing and blood coagulation. Fibrous, natural proteins such as collagen and silk exhibit useful mechanical properties in addition to their biocompatibility and low antigenicity, and as a result they provide important materials in the field of controlled drug release, biomaterials and scaffolds for tissue engineering. In vitro, under appropriate conditions (temperature, pH, ionic strength, composition of fibre formation medium), collagen molecules spontaneously form fibres (fibrillogenesis) that are similar to native fibres. Since the natural cross-linking pathway does not occur in vitro, reconstituted forms of collagen can lack sufficient strength and may disintegrate upon handling or collapse under the pressure from surrounding tissue in vivo. Thus, it is often necessary to introduce exogenous cross-links (chemical or physical) into the molecular structure, in order to control mechanical properties, the resistance time in the body and to some extent the immunogenicity of the device. This study is concerned with the major cross-linking techniques, which were applied to extruded pepsin soluble bovine Achilles tendon derived collagen fibres. The cross-linking efficiency of the different treatments was estimated by analysing the thermal transitions of the collagenous materials. The mechanical properties of the fibres were evaluated and discussed in relation to their microscopic structure.

Abstract Selected and Published at:
International Journal of Experimental Pathology, 2005, 86, A57–A94