ABSTRACT

EXTRUSION AND EVALUATION OF DEGRADATION RATE AND POROSITY OF SMALL DIAMETER COLLAGEN TUBES

by
Bipinkumar G. Patel

The limited availability of autografts and failure of small diameter synthetic vascular grafts has stimulated continuing efforts to develop small diameter vascular grafts based on natural materials. The small diameter collagen tubes were extruded using bovine collagen type I. The biodegradation rate was determined and compared for small diameter collagen tubes crosslinked with glutaraldehyde and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) to evaluate physio-chemical properties. The samples were also evaluated for their surface area and porosity using mercury porosimeter. The non-crosslinked tubes almost completely degraded after 2 hours. The tubes crosslinked with EDC/NHS degraded slower than the ones crosslinked with glutaraldehyde. The biodegradation rate seemed to be dependent on concentration of crosslinking agent and collagen suspension. The *in-vitro* model equation for small diameter collagen tubes showed linear behavior. The porosity characterization study showed that EDC/NHS treated tubes are more porous and have more surface area than glutaraldehyde treated tubes. Future work on this area will be to study biodegradation rate for small diameter collagen tubes over longer period of time.

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by
Bipinkumar G. Patel

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Bipinkumar G. Patel

Dr. Michael Jaffe, Thesis Advisor — Date
Professor of Biomedical Engineering, NJIT

Dr. George Collins, Committee Member — Date
Visiting Professor of Biomedical Engineering, NJIT

Dr. Treena Arinzeh, Committee Member — Date
Assistant Professor of Biomedical Engineering, NJIT

BIOGRAPHICAL SKETCH

Author: Bipinkumar G. Patel

Degree: Master of Science

Date: May 2004

Undergraduate and Graduate Education:

- Master of Science in Biomedical Engineering,
  New Jersey Institute of Technology, Newark, NJ, 2004

- Bachelor of Science in Chemical Engineering,
  South Gujarat University, Surat, India, 2001

Major: Biomedical Engineering

To my dad, for always believing in me and encouraging me
To my mom, for always supporting me in every step of life
To my brother, whose wishes came as an inspiration to me

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CHAPTER 1

INTRODUCTION

The collagens are a family of highly characteristic fibrous proteins found in all multicellular animals. It is a biodegradable, biocompatible, and non-nonimmunogenic structural protein, which makes it a suitable compound for a variety of biomedical applications. This family of proteins accounts for 25% or more of total body protein and provides an important structural framework for most body structures, including skeleton, skin and blood vessels. The word collagen is derived from kolla the Greek word for glue. It is present in high amounts in the ECM of numerous tissues. Collagen is regarded as one of the most useful biomaterials. It has found ample usage in the biomedical field due to its low antigenicity, its biodegradability and its good mechanical, haemostatic and cell-binding properties [1, 2].

1.1 Collagen Overview

Collagen is a fibrous protein and constitutes the major protein component of skin, bone, tendon, ligament, cartilage, basement membrane and other forms of connective tissue. It is the most abundant protein in the animal kingdom. Collagen fibers function in biological structures to maintain tissue shape, transmit and absorb loads, prevent premature mechanical failure, partition cells and tissues into functional units, and act as a scaffold that supports tissue architecture [8]. In bone, for example, collagen fibers reinforce the calcium phosphate mineral base. Despite its great strength, bone retains flexibility because of its collagen content.

Collagen has been used extensively in medicine and in surgery. Collagen based devices have been used, as noted above, as nerve regeneration tubes, as sutures, haemostatic fiber and sponges, wound dressings, neurosurgical sponges, injectable implants for soft tissue augmentation, pharmaceutical carriers, ophthalmic aqueous-venous shunts, contact lenses and the like.

The properties of collagen which favor its use as a biomaterial are many. It has a high order of tensile strength and low extensibility. Collagen is biodegradable, and when implanted in the body, is absorbed at a rate that can be controlled by the degree of intra or intermolecular cross-linking imparted to the collagen molecule by chemical or physical treatment. Collagen products can thus be designed such that, on implantation, they will be completely absorbed in a few days or in months. The collagen can also be chemically treated so that it becomes non-absorbable while still retaining its hydrophilic character and its good tissue response. Although native collagen is a very weak antigen, it can be made, for all practical purposes, immunologically inert by means well known to those skilled in the art.

The main sources of collagen for commercial applications are bovine tendons, calf, steer or pig hide. Generally, reconstituted collagen products are prepared by purification of native collagen by enzyme treatment and chemical extraction. The purified collagen is then dispersed or dissolved in solution, filtered and retained as such, or is reconstituted into fiber, film or sponge by extrusion, casting or lyophilization techniques. Although the collagen of skin, tendons, bone, cartilage, blood vessels and basement membrane are similar in structure and composition, they do differ slightly in relative

amino acid content, amino acid sequence and in architecture. The collagen of native skin, tendons, ligaments and bone are primarily Type I collagen [5].

Collagen appears as bundles of individual, nonbranching fibrils, varying greatly in diameter from tissue to tissue. In skin, under an ordinary light microscope, these bundles appear to be woven together at random, but a definite order emerges if larger areas of tissue are examined. In tendon, collagen fibers are arranged in long parallel bundles. In the cornea of the eye, transparency depends upon the orderly arrangement of collagen fibers that probably have a refractive index identical to that of the substance in which they are embedded. In bone, the collagen fibrils are organized much like the struts and girders of a bridge. In cartilage, which coats the inner surface of joints and which must have considerable elasticity and smoothness, the collagen fibers are usually very thin, randomly oriented and embedded in a large volume of extracellular matrix [6].

In skin and basement membrane, it occurs as a reinforced fiber. It can also function as the winding of a pressure vessel, as in nematodes, earthworms and sharks. In tendon and muscle, it is concerned with transmitting tensile stresses and it is in this form, notably rat tail tendon, that it has been most studied. Just as with keratins, the basic microfibril (in this instance, tropocollagen) is assembled into larger and larger units giving a hierarchy of structure which can finally form such components as tendon (Figure 1.1) [7].

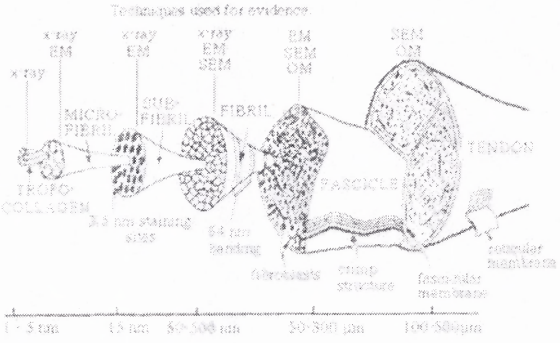

Figure 1.1 Hierarchy of Collagen Structure.

1.2 Structure of Collagen

The common structural feature found in all collagens is a triple helix that consists of three left-handed helixes that are wound into a right-handed triple helix. Individual α-chains, which are the basic units of collagen, contain one or more polypeptide sequences (Gly-X-Y, where X and Y positions are frequently occupied by proline and hydroxyproline) that form the triple helix with one or more nontriple helical modules (Figure 1.2a). The α-chains vary in size from 600 to 3,000 amino acids [4].

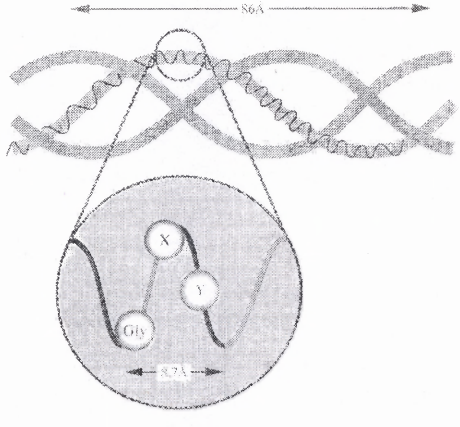

Figure 1.2a Collagen Triple Helix.

First, the amino acid composition of the collagen α-chain will be discussed. Glycine constitutes about one-third of the residues, proline about 13%, and hydroxyproline about 10%. Because only small quantities of hydroxyproline are present in other proteins, assays for hydroxyproline are frequently used to determine collagen content [2]. The α-chains have been classified into fibrillar, nonfibrillar, and novel collagens. The fibrillar collagens include types I, II, III, V, and XI, which form cross-striated fibrils, and all share a triple helical region containing about 1,000 amino acids per chain, which has a length of about 300 nm (Figure 1.2b). Nonfibrillar collagens may associate with fibrillar collagens or form separate networks of microfibrils. These collagens contain triple helical

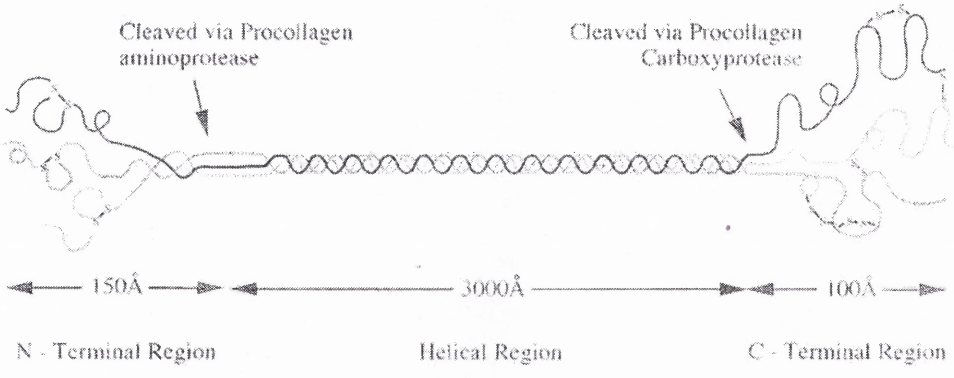

Figure 1.2b Diagram of Procollagen Molecule.

segments of varying lengths interrupted by sequences containing larger segments of noncollagenous sequences. The noncollagenous sequences include modules containing sequences found in von Willebrand factor (binds to and protects factor VIII, which is necessary for blood coagulation), collagen type IV, and fibronectin. The novel collagens are similar to the nonfibril-forming collagens because they consist of triple helical regions separated by nontriple helical regions [4].

The different α-chains are bonded to each other in the following way:

1) By H-bridges. NH... from the glycines and CO... from residues of the other chain. These H...O bonds are perpendicular on the axis of the collagen chains.

2) By H-bridges of hydroxyl-groups of hydroxyprolines.

3) By H-bridges with water molecules.

All these bonds stabilize the triple-helical structure of collagen. However, the repulsion of the prolines already gives the helical structure and turns the H-side chain of the glycines to the inside of the helix. This happens without the help of H- bridges. Inside the triple helix there would be no more space than for the small H- side chains of glycines. All the bulky side chains have to point to the outside of the triple helix of the collagen. In summary: glycines, prolines and hydroxyprolines are mainly responsible for the triple helix structure. The remaining amino acid residues are expected to be responsible for higher order structural regularities.

Two special amino acids occur in collagen almost exclusively:

1) 4- and 3-hydroxyproline,

2) 5-hydroxylysine

The amino acid residues hydroxyproline and hydroxylysine exhibit special functions for the collagen structure. The 4- and 3-hydroxyprolines are important for interchain linking with H-bridges to stabilize the helical structure. The stability of the collagen helix depends strongly on the percentage of prolines and these hydroxyprolines (Table 1.1).

When the temperature rises at T_{melt}, the viscosity drops and altering optical rotation properties can be observed. This is caused by the thermal movements, which

become larger than the cooperative interaction that stabilizes the triple-helix of collagen. In this way T_m is a measure for the stability of the helix structure of a particular collagen.

Table 1.1 Stability of Collagen Helix

Sample	Pro+Hyp	T_m(°C)	Body Temperature(°C)
Cod skin*	155/1000	16	10-14
Frog skin*	174/1000	25	?
Shark skin*	191/1000	29	24-28
Calf skin*	232/1000	39	37
$(Gly\text{-}Pro\text{-}Pro)_n$**	333/1000	24	-
$Gly\text{-}Pro\text{-}Hyp)_n$**	333/1000	58	-

* Data taken from [10]
** Data taken from [11]

From Table 1.1 it might be concluded that the percentage of prolines and especially the contents of hydroxyprolines positively affects the stability.

The 5-hydroxylysines are covalently bonded to oligosaccharides, mostly disaccharides of glucose and galactose. In nascent collagen, before it become helical, the sugars are bonded to the hydroxyl group of hydroxylysines. This occurs with help of the enzymes galactosyl transferase and glucosyl transferase. The number of sugar residues and the kind of sugar residues depends on the tissue, but in general fibrils contain relatively small amounts of sugars while sheets are relatively rich in it. For example, the fibrillar tendon has only six sugar residues per collagen monomer, while the lenscapsule has 110 sugar residues. Hydroxylysines are also enormous important for extensive crosslinking of collagen molecules after secretion of the polypeptides in the extracellular space.

There are 3.3 amino acid residues per turn and 2.9Å per amino acid residue. In a normal α-helix this is 1.5Å per amino acid residue. After self-assemblance of the loose monomers, cross-links generate the tight, covalently bond complex.

The amino acid sequence is not totally unique to collagen. If the collagen sequence is compared with other proteins, homology is found for:

a. C1q subcomponent (chain A, B and C) from the complement system.

b. Acetylcholinesterase.

c. Fibronectin.

d. Osteonectin.

These proteins contain similar stretches of $(Gly\text{-}X\text{-}Y)_n$ [5]. It is expected that these different proteins somehow interact with collagen to anchor (a and b) or to form tight complexes (c and d). In this way specific cells can use collagen as a kind of anchor field with help of specific antibodies.

In all multicellular organisms, many different kinds of tissue can be identified. Most of these tissues need distinct collagen structures, all with their own specific properties. For this purpose organisms have genetically distinct collagen α-chains. These α-chains have been well defined as distinct gene products in higher animals and they are encoded by many distinct collagen genes [6].

Although from combinatorial point of view, more than a thousand different types of triple helices can be assembled from the various combinations of the α-chains, only a few types of collagen have been described and characterized so far.

To obtain the diversity of collagens needed in all different tissues, the distinct collagen types are modified by some external factors;

a. glycosylation in the endoplasmatic reticulum,

b. post-translational modifications.

These are mechanisms of introducing a greater degree of variability. Owing to these modifications, many tissue- specific collagens can be built from one and the same collagen type [8,14]. The glycosylation takes place intracellular and the post-translational modifications take place extracellular. Both modifications lead to the fine-tuning of the collagen structures.

Adaptability to local environment: It has been shown [25] that specific proteoglycans influence significantly the diameter of collagen fibres. The presence of small proteoglycans results in collagen fibrils that was significantly thinner in width.

The collagen fibrils in various tissues are organized in ways that largely reflect the functions of the tissues (Table 1.2)[14].

Table 1.2 The Arrangement of Collagen Fibrils in Various Tissues

Tissue	Arrangement
Tendon	Parallel bundles
Skin	Sheets of fibrils layered at many angles
Cartilage	No distinct arrangement
Cornea	Planar sheets stacked crossways so as to minimize light scatter

1.3 Collagen Biosynthesis

The individual collagen polypeptide chains are synthesized on membrane-bound ribosomes and injected into the lumen of the endoplasmic reticulum (ER) as larger precursors, called pro- α chains. These precursors not only have the short amino-terminal signal peptide required to direct the nascent polypeptide to the ER, they also have additional amino acids, called propeptides, at both their amino- and carboxyl-terminal ends. In the lumen of the ER, selected proline and lysine residues are hydroxylated to form hydroxyproline and hydroxylysine, respectively, and some of the hydroxylysine

residues are glycosylated. Each pro- α chain then combines with two others to form a hydrogen-bonded, triple-stranded helical molecule known as procollagen.

Hydroxylysines and hydroxyprolines (Figure 1.3a) are infrequently found in other animal proteins, although hydroxyproline is abundant in some proteins found in the plant

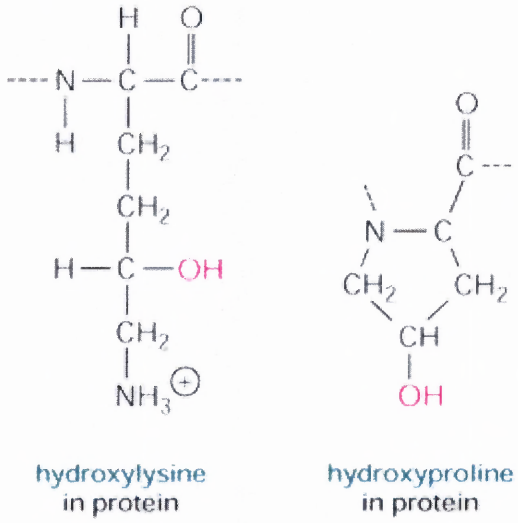

Figure 1.3a Hydroxylysine and Hydroxyproline.
These modified amino acids are common in collagen; they are formed by enzymes that act after the lysine and proline are incorporated into procollagen molecules.

cell wall. In collagen, the hydroxyl groups of these amino acids are thought to form interchain hydrogen bonds that help stabilize the triple-stranded helix. Conditions that prevent proline hydroxylation, such as a deficiency of ascorbic acid (vitamin C), have serious consequences. In scurvy, the disease caused by a dietary deficiency of vitamin C that was common in sailors until the nineteenth century, the defective pro- α chains that are synthesized fail to form a stable triple helix and is immediately degraded within the cell. Consequently, with the gradual loss of the preexisting normal collagen in the matrix, blood vessels become extremely fragile and teeth become loose in their sockets. This implies that in these particular tissues degradation and replacement of collagen is relatively rapid. In many other adult tissues, however, the turnover of collagen (and other

extracellular matrix macromolecules) is thought to be very slow: in bone, to take an extreme example, collagen molecules persist for about 10 years before they are degraded and replaced. By contrast, most cellular proteins have half-lives of hours or days.

After secretion, the propeptides of the fibrillar procollagen molecules are removed by specific proteolytic enzymes outside the cell. This converts the procollagen molecules to collagen molecules, which assemble in the extracellular space to form much larger collagen fibrils. The propeptides have at least two functions: (1) they guide the intracellular formation of the triple-stranded collagen molecules, and (2) because they are removed only after secretion, they prevent the intracellular formation of large collagen fibrils, which could be catastrophic for the cell.

The process of fibril formation is driven, in part, by the tendency of the collagen molecules, which are more than thousandfold less soluble than procollagen molecules, to self-assemble. The fibrils begin to form close to the cell surface, often in deep infoldings of the plasma membrane formed by the tandem fusion of secretory vesicles with the cell surface. The underlying cortical cytoskeleton can therefore influence the sites, rates, and orientation of fibril assembly.

When viewed in an electron microscope, collagen fibrils have characteristic cross-striations every 67 nm, reflecting the regularly staggered packing of the individual collagen molecules in the fibril. After the fibrils form in the extracellular space, they are greatly strengthened by the formation of covalent cross-links between lysine residues of the constituent collagen molecules (Figure 1.3b). The types of covalent bonds involved are found only in collagen and elastin. If cross-linking is inhibited, the tensile strength of

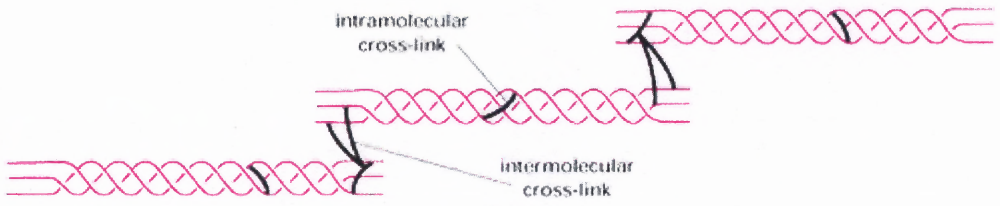

Figure 1.3b Cross-links Formed Between Modified Lysine Side Chains Within a Collagen Fibril.
The cross-links are formed in several steps. First, certain lysines and hydroxylysines are deaminated by the extracellular enzyme lysyl oxidase to yield highly reactive aldehyde groups. The aldehydes then react spontaneously to form covalent bonds with each other or with other lysine or hydroxylysine residues. Most of the cross-links form between the short nonhelical segments at each end of the collagen molecules.

the fibrils is drastically reduced; collagenous tissues become fragile, and structures such as skin, tendons, and blood vessels tend to tear. The extent and type of cross-linking varies from tissue to tissue: collagen is especially highly cross-linked in the Achilles tendon, for example, where tensile strength is crucial.

Figure 1.3c summarizes the various steps in the synthesis and assembly of collagen fibrils. Given the large number of enzymatic steps involved in forming a collagen fibril, it is not surprising that there are many human genetic diseases that affect fibril formation. Mutations affecting type I collagen cause osteogenesis imperfecta, characterized by weak bones that easily fracture. Mutations affecting type II collagen cause chondrodysplasias, characterized by abnormal cartilage, which leads to bone and joint deformities. Mutations affecting type III collagen cause Ehlers-Danlos syndrome, characterized by fragile skin and blood vessels and hypermobile joints.

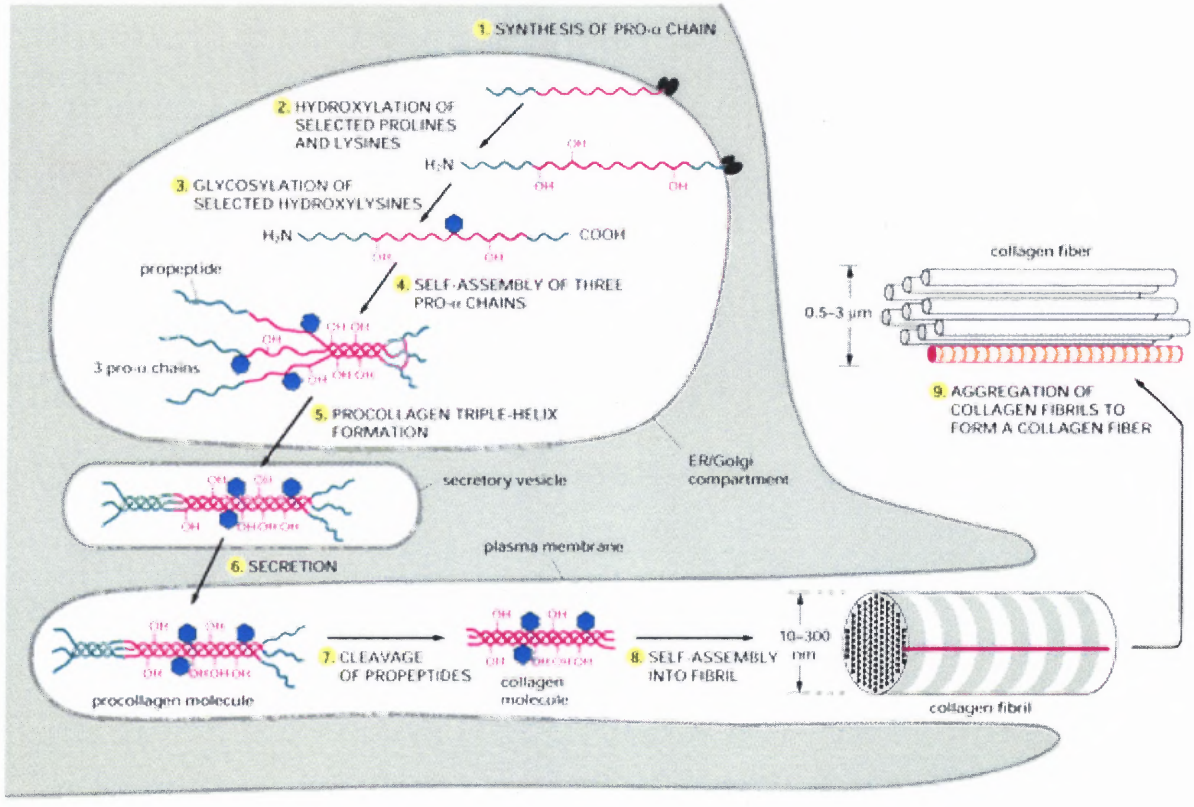

Figure 1.3c The Intracellular and Extracellular Events Involved in the Formation of a Collagen Fibril.

Note that collagen fibrils are shown assembling in the extracellular space contained within a large infolding in the plasma membrane. As one example of how the collagen fibrils can form ordered arrays in the extracellular space, they are shown further assembling into large collagen fibers, which are visible in the light microscope. The covalent cross-links that stabilize the extracellular assemblies are not shown.

In contrast to GAGs, which resist compressive forces, collagen fibrils form structures that resist tensile forces. The fibrils have various diameters and are organized in different ways in different tissues. In mammalian skin, for example, they are woven in a wickerwork pattern so that they resist tensile stress in multiple directions. In tendons they are organized in parallel bundles aligned along the major axis of tension. In mature bone and in the cornea, they are arranged in orderly plywoodlike layers, with the fibrils in each layer lying parallel to one another but nearly at right angles to the fibrils in the layers on either side. The same arrangement occurs in tadpole skin (Figure 1.3d).

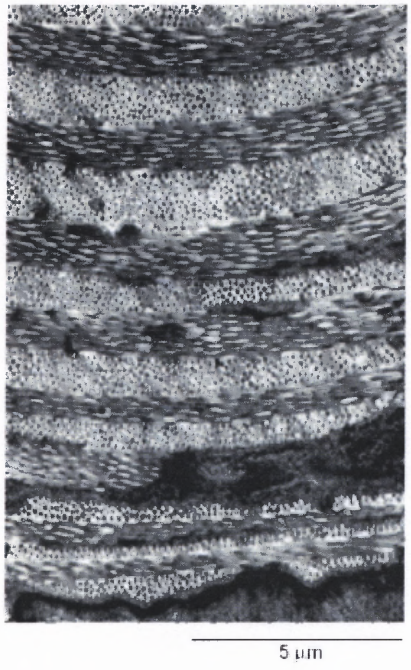

Figure 1.3d Collagen Fibrils in the Tadpole Skin.
This electron micrograph shows the plywood like arrangement of the fibrils. Successive layers of fibrils are laid down nearly at right angles to each other. This organization is also found in mature bone and in the cornea. (Courtesy of Jerome Gross.).

The connective tissue cells themselves must determine the size and arrangement of the collagen fibrils. The cells can express one or more of the genes for the different types of fibrillar procollagen molecules. But even fibrils composed of the same mixture of fibrillar collagen molecules have different arrangements in different tissues. How is this achieved? Part of the answer is that cells can regulate the disposition of the collagen molecules after secretion by guiding collagen fibril formation in close association with the plasma membrane (see Figure 1.3b). In addition, as the spatial organization of collagen fibrils at least partly reflects their interactions with other molecules in the matrix, cells can influence this organization by secreting, along with their fibrillar collagens, different kinds and amounts of other matrix macromolecules.

The fibril-associated collagens, such as type IX and XII collagen molecules are thought to be especially important in this regard. They differ from the fibrillar collagens in several ways.

(1) Their triple-stranded helical structure is interrupted by one or two short nonhelical domains, which makes the molecules more flexible than fibrillar collagen molecules. (2) They are not cleaved after secretion and therefore retain their propeptides. (3) They do not aggregate with one another to form fibrils in the extracellular space. Instead, they bind in a periodic manner to the surface of fibrils formed by the fibrillar collagens: type IX molecules bind to type-II-collagen-containing fibrils in cartilage, the cornea, and the vitreous of the eye, whereas type XII molecules bind to type-I-collagen-containing fibers in tendons and various other tissues.

Fibril-associated collagens are thought to mediate interactions of collagen fibrils with one another and with other matrix macromolecules. In this way they play a part in determining the organization of the fibrils in the matrix [9].

1.4 Types of Collagen

For simplicity, the superfamily of collagens can be divided into several classes on the basis of the polymeric structures they form or related structural features: (1) collagens that form fibrils (types I, II, III, V, and XI), (b) collagens that form network-like structures (the type IV family, and types VIII and X), (c) collagens that are found on the surface of collagen fibrils and are known as fibril-associated collagens with interrupted triple helices (FACITs that include types IX, XII, XIV, XVI, and XIX), (d) the collagen that forms beaded filaments (type VI), (e) the collagen that forms anchoring fibrils for

basement membranes (type VII), (f) collagens with a transmembrane domain (types XIII and XVII), and (g) the newly discovered types XV and XVIII collagens that have been only partially characterized.

(a) Fibril-Forming Collagens: All these collagens (types I-III, V, & XI) are similar in size and in that they contain large triple-helical domains with about 1000 amino acids or 330 -Gly-X-Y- repeats per chain. In addition, they are also first synthesized as large precursors, and the precursors need to be processed to collagens by cleavage of N-propeptides and C-propetides by specific proteinases. Finally they are similar in that they all assemble into cross-striated fibrils in which each molecule is displaced about one-quarter of its length relative to its nearest neighbor along the axis of the fibril. Type I is the most abundant collagen and is found in variety of tissues. Many of the other fibril-forming collagens have a more selective tissue distribution (Table 1.3)

(b) Network-Forming Collagens: These collagens include the family of type IV collagens found in basement membranes and type VIII and X collagens. The collagenous domain of a type IV collagen molecule is longer than in the fibril-forming collagens and consists of about 1400 amino acids in –Gly-X-Y- repeats that are frequently interrupted by short noncollagenous sequences. Collagen types VIII and X are very different in structure from type IV but similar to each other. The α1(VIII), α2VIII) and α1(X) chains all contain a collagenous sequence of almost the same size.

(c) FACIT Collagens: These collagens (types IX, XII, XIV, XVI, and XIX) do not form fibrils themselves but are found attached to the surfaces of preexisting fibrils of the fibril-forming collagens. All these collagens are characterized by short triple-helical domains interrupted by short noncollagenous sequences. The type IX collagen molecule consists

Table 1.3 Collagen Types and the Location of Their Genes on Human Chromosomes

Type	Gene	Chromosome	Expression
I	COL1A1	17q21.3-q22	Most connective tissues
	COL1A2	7q21.3-q22	
II	COL2A1	12q13-q14	Cartilage, vitreous humor
III	COL3A1	2q24.3-q31	Extensible connective tissues, e.g. skin, lung, vascular system
IV	COL4A1	13q34	Basement membranes
	COL4A2	13q34	
	COL4A3	2q35-q37	
	COL4A4	2q35-q37	
	COL4A5	Xq22	
	COL4A6	Xq22	
V	COL5A1	9q34.2-q34.3	Tissues containing collagen I, quantitatively minor component
	COL5A2	2q24.3-q31	
	COL5A3		
VI	COL6A1	21q22.3	Most connective tissues
	COL6A2	21q22.3	
	COL6A3	2q37	
VII	COL7A1	3p21	Anchoring fibrils
VIII	COL8A1	3q12-q13.1	Many tissues, especially endothelium
	COL8A2	1p32.3-p34.3	
IX	COL9A1	6q12-q14	Tissues containing collagen II
	COL9A2	1p32	
	COL9A3		
X	COL10A1	6q21-q22	Hypertrophic cartilage
XI	COL11A1	1p21	Tissues containing collagen II
	COL11A2	6p21.2	
	COL2A1*	12q13-q14	
XII	COL12A1	6	Tissues containing collagen I
XIII	COL13A1	10q22	Many tissues
XIV	COL14A1		Tissues containing collagen I
XV	COL15A1	9q21-22	Many tissues
XVI	COL16A1	1p34-35	Many tissues
XVII	COL17A1	10q24.3	Skin hermidesmosomes
XVIII	COL18A1	21q22.3	Many tissues, especially liver and kidney
XIX	COL19A1	6q12-q14	Rhabdomyosarcoma cells

* The α3(XI) chain of type XI collagen is encoded by the same gene as the α1(II) chain of type II.

of three triple-helical domains and four noncollagenous domains. Type XII and XIV collagens show several structural similarities to type IX collagen, particularly in the C-terminal collagenous domains.

(d) Beaded Filament-Forming Collagens: The only collagen known to form beaded filament is type VI. Each of the three different chains of the protein contains a very short triple-helical domain and the remainder consists of large N-terminal and C-terminal globular domains.

(e) Collagen of Anchoring Fibrils: Type VII collagen forms anchoring fibrils that link basement membranes to anchoring plaques of type IV collagen and laminin in the underlying extracellular matrix. The triple-helical domain of type VII collagen, which is longer than the triple helix of any other collagen, contains 1530 amino acids in –Gly-X-Y- repeats that are interrupted at 19 separate sites.

(f) Collagens with a Transmembrane Domain: This family of collagens, Type XIII and Type XVII, contain a transmembrane domain. They are not homologous in structure, but they both contain a single transmembrane N-terminal domain that is apparently cytoplasmic.

CHAPTER 2

APPLICATION OF COLLAGEN TUBES

2.1 Collagen as a Biomaterial

Biomaterials is a term used to indicate materials that constitute parts of medical implants, extracorporeal devices, and disposables that have been utilized in medicine, surgery, dentistry, and veterinary medicine as well as in every aspect of patient health care. The National Institutes of Health Consensus Development Conference defined a biomaterial as " any substance (other than a drug) or combination of substances, synthetic or natural in origin, which can be used for any period of time, as a whole or as a part of a system which treats, augments, or replaces any tissue, organ, or function of the body" (Boretos and Eden, 1984) [2]. A complementary definition essential for understanding the goal of biomaterials science, is that of "biocompatibility." Biocompatibility is defined as the "ability of a material to perform with an appropriate host response in a specific application" (Williams, 1987). The definition applies for the lifetime of the implant, therefore if an implant is to be placed permanently into the body, it must never illicit a harmful response. Likewise, a degradable implant must not elicit a harmful response during its time of operation or after it has degraded into byproducts.

Natural polymers offer the advantage of being very similar, often identical, to macromolecular substances which the biological environment is prepared to recognize. The problems of toxicity and stimulation of a chronic inflammatory reaction, which are frequently provoked by many synthetic polymers, may thereby be suppressed. Furthermore, the similarity to naturally occurring substances introduces the interesting

capability of designing biomaterials which function biologically at the molecular, rather than the macroscopic, level. On the other hand, natural polymers are quite immunogenic. Furthermore, because they are structurally much more complex than most synthetic polymers, their technological manipulation is much more elaborate. On balance, these opposing factors have conspired to lead to a substantial number of biomaterials applications in which naturally occurring polymers, or their chemically modified versions, have provided unprecedented solutions. Table 2.1 shows some natural polymers and their general properties [13].

Table 2.1 Natural Polymer Examples and Functions in Host Animal

Polymer	**Incidence**	**Physiological Function**
Silk	Synthesized by arthropods	Protective cocoon
Keratin	Hair	Thermal insulation
Collagen	Connective tissue	Mechanical support
Gelatin	Partly amorphous collagen	(Industrial Product)
Fibrinogen	Blood	Blood clotting
Elastin	Neck ligament	Mechanical support
Actin	Muscle	Contraction, Motility
Myosin	Muscle	Contraction, Motility

The properties of collagen making it suitable for fabrication into medical products are intimately dependent on characteristics of amino acid composition and sequence which determine the three-dimensional structure and interaction among other macromolecules in the environment. As the chief structural protein of the body, collagen is uniquely designed to transmit tensile and compressive forces of great magnitude [12]. Collagen substrates are known to influence the growth characteristics of cells and also to modulate various aspects of cell behavior like cell-adhesion, proliferation and differentiation [15,16,17]. Furthermore, its presence as a constituent of basement membranes in extracellular spaces suggests that it also contributes in the body and may

be of some importance in diffusion. The filtration properties of collagen membranes can have important applications in medicine. The properties of collagen which makes it such a promising substance for use in the biomedical field are summarized in Table 2.2 [12].

Table 2.2 Factors Favoring the Use of Collagen as Biomaterial

1. Physical-mechanical	High tensile strength
	Low extensibility
	Orientation of fibers
2. Physical-chemical	Controllable cross-linking by tanning agents; affects solubility, swelling, resorption
	Ion-exchanger function
	Semipermeability of membranes
3. Biological	Low antigenicity
	Effect on wound healing
	Effect on blood coagulation

Certain application of collagen-based biomaterials are shown in Table 2.3

Table 2.3 Certain Applications of Collagen-based Biomaterials

Application	**Physical state**
Sutures	Extruded tape (Schmitt, 1985)
Hemostatic agents	Powder, sponge, fleece (stengel et al., 1974; Chavapil, 1979)
Blood vessels	Extruded collagen tube, processed human or animal blood vessel (Nimni, 1988)
Heart valves	Processed porcine heart valve (Nimni, 1988)
Tendon, ligaments	Processed tendon (Piez, 1985)
Burn treatment (Dermal regeneration)	Porous collagen-glycosaminoglycan (GAG) polymer (Yannas et al., 1981, 1982 and 1989)
Peripheral nerve regeneration	Porous collagen-GAG copolymer (Chang and Yannas, 1992)
Meniscus regeneration	Porous collagen-GAG copolymer (Stone et al., 1989)
Intradermal augmentation	Injectable suspension of collagen particles (Piez, 1985)
Gynecological applications	Sponges (Chvapil, 1979)
Drug-delivery systems	Various forms (Stenzel et al., 1974; Chvapil, 1979)

2.1.1 Collagen Thrombogenicity

Collagen is highly thrombogenic, as is demonstrated from its use as a hemostatic powder or sponge [18]. Collagen induces platelet adhesion and aggregation as well as activation of the intrinsic coagulation cascade. Fibrillar collagen preparations are used as aggregation agent in diagnostic platelet function tests. Where contact of blood with an injured vessel wall result in extrinsic blood coagulation, contact of blood with purified collagen induces activation of the intrinsic pathway, initiated by adsorption of (activated) factor XII [19]. It has been postulated that crosslinking of collagen may decrease its thromobogenicity [20]. Crosslinking can be done by using different crosslinking agents. Two procedures of crosslinking will be discussed later in this chapter.

2.1.2 Collagen Antigenicity

It has been suggested that the presence of aromatic amino acids, particularly tyrosine, in proteins is responsible for a major portion of antigenicity (Sela and Arnon, 1960). Collagen has a low content of aromatic amino acids, and has only approximately three residues of tyrosine per α-chain. Thus one might expect it to be a weak antigen. It is well known that the N-terminal regions of the collagenous polypeptide chains which contain the tyrosine residues are split off by treating tropocollagen with various proteases (pronase); thus the antigenicity of the collagen sample is reduced (Davidson et al., 1967) [12].

2.2 Application of Collagen as Vascular Grafts

Replacing narrowed or occluded portions of larger arteries is becoming a fairly common medical procedure. Currently, the best vascular graft performance is given by autografts (tissues taken form one site on a patient and transplanted to another site on the same patient). Many patient, however, especially those with pre-existing vascular disease or patients that have already had autograft procedures, do not have blood vessels that are healthy enough to adequately serve as replacements. The main mechanisms of vascular autograft failure are thrombosis, emboli production, poor long term patency and intimal hyperplasia [2]. Failure of the autograft is usually due to some form of occlusion that results from lumenal narrowing. Damage of the vessel during removal and reimplementation may cause the recruitment of factors or cells that adhere to the autograft wall and decrease the diameter or the lumen. The restricted flow then increases the thrombogenicity, making full occlusion ever more likely. Other problems are preparation and preservation of the autograft, procedures that can result in vessel damage or diminished in vivo performance. Finally, due to increased and/or different mechanical forces, endothelial cells can shrink, diminishing barrier performance and degrade, also resulting in increased thrombogenicity [21].

Synthetic vascular grafts are made from expanded polytetrafluoroethylene (ePTFE), Knitted polyethylene terephthalate (Dacron) or polyester. Although grafts made from synthetic biomaterials perform well when used to replace larger blood vessels, they are inadequate for replacing small diameter (<6 mm) vessels [3]. In large diameter synthetic vascular grafts the accumulation of fibrous and cellular material is not great enough to cause the graft to shrink in diameter significantly. On the other hand, in small

diameter synthetic vascular grafts the accumulation of material is often significant enough to cause the blood vessel to become occluded. In order to reduce thrombus formation anticoagulation drugs are necessary. The use of these drugs often results in undesirable systemic side effects and can be very problematic. Therefore synthetic materials are poor choices for materials for small diameter vascular grafts [21].

In cases where the graft can be of a large diameter (> 5-6 mm), the synthetic material has been effective. However, in situations where a smaller vessel diameter is required, the synthetic materials cannot be used due to high rates of stenosis and thrombus formation. One possible solution is to use natural materials like collagen, either modified or combined with a synthetic material, to form a graft that more closely mimics the body's natural function and has low thrombogenicity and low incidence of stenosis [22].

The use of collagen as a material for a synthetic vascular graft is quite promising because it is biodegradable, antigenic and has good mechanical, haemostatic and cell-binding properties [1,2]. Since collagen is biodegradable, as the device degrades tissue can grow into the device. This is advantageous because ideally as the collagen implant degrades the newly formed tissue will replace it, which results in a gradual transfer of stress from the implanted device to the newly formed tissue. Collagen can be crosslinked to form a polymer with sufficient mechanical strength to resist the collapse of the blood vessel.

Heparin is a negatively charged anti-thrombogenic protein that can be incorporated into collagen, which is inherently thrombogenic. The incorporation of heparin significantly reduces the thrombogenic properties of collagen and allows it to be

used as a material for vascular implants. If a collagen vascular implant material were seeded with endothelial cells so that they coat the lumen, the surface would theoretically be more biocompatible. Recently, endothelial cells have been cultured onto the collagen small diameter vascular grafts. The growth of endothelial cells into the collagen vascular graft can be increased by incorporating growth factors into the vascular graft material. Basic fibroblast growth factor (bFGF) can be immobilized by the heparin that is already incorporated into the collagen that is used for a vascular implant. bFGF caused more tissue to grow into the implant material and helped form a coating of endothelial cells on top of the implant material. Therefore by incorporating bFGF into the collagen vascular implant material endothelial cells can be seeded onto the top of the material to create a lumenal surface that is comprised of endothelial cells to more closely mimic the natural biological environment [23].

To reduce the antigenicity, as discussed before, collagen should be crosslinked with different method. Crosslinking of collagen will also enhance mechanical strength and time needed for degradation of collagen tubes. Many crosslinking methods can be used i.e. chemical cross-linkage using formaldehyde, hexamethylene diisocyanate, glutaraldehyde (GA) or N-(3-dimethyaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS), physical cross-linking using irradiation, ultraviolet(UV) irradiation or heat treatments.

Depending on the intended application, the ability for natural polymers to be broken down by enzymes allows for the complete degradation of an implanted material. This can be an advantage if the implant is only meant for short-term usage and eventual replacement by the body's own materials. For collagen, the cells can synthesize new

collagen, which normally forms a new architectural arrangement, such as scar tissue, in wounds. This is one step in the wound healing process that the body begins as a result of damage caused by implantation.

This degradation can also be a disadvantage if these enzymes destroy the implant before it completes its intended task. Collagen is susceptible to attack by collagenases in a physiological setting. These collagenases are present in healing wounds and are a primary reason for the degradation of collagen implants. Luckily, through various physical or chemical methods, the degradation rate of collagen implants can be controlled [24].

2.3 Physical Modification of Collagen

The porosity of collagenous implants normally makes an indispensable contribution to its performance. A porous structure provides an implant with two critical functions. First, pore channels are ports of entry for cells migrating from adjacent tissues into the bulk of the implant or for the capillary section of blood from a hemorrhaging blood vessel nearby. Second, pores endow a solid with a frequently enormous specific surface which is made available either for specific interactions with invading cells or for interaction with coagulation factors in blood flowing into the device (e.g. hemostatic sponges) [13].

Porosity is significant because it controls the tendency to hemorrhage during and after implantation and controls the ingrowth of tissue into the wall of the graft. It is desirable that the vascular graft substrate be sufficiently blood-tight to prevent the loss of blood during implant, yet the structure must be sufficiently porous to permit ingrowth of fibroblast and smooth muscle cells in order to attach the graft to the host tissue. Grafts

which are impermeable to blood after the time of implantation do not permit the subsequent ingrowth of cells which is necessary for uniform and satisfactory bonding of the internal lining of the prosthesis. The general procedure for implantation includes the step of pre-clotting, wherein the graft is immersed in the blood of the patient and allowed to stand for a period of time sufficient for clotting to insue. After pre-clotting, hemorrhaging does not occur when the graft is implanted and growth of tissue is not impeded [26].

Cross-linking of collagen biomaterials is often applied to control or reduce the in vivo resorption rate or to improve mechanical properties of materials [27,28]. In general, there are two methods for crosslinking of collagen: physical and chemical.

The most common physical method is dehydrothermal treatment. By heating in an oven, collagen can be severely dehydrated which creates interchain amide links. By exposure to temperature in excess of 105° C with atmospheric pressure for a few hours, the collagen can produce cross-links which help to prevent degradation of the helix. The ultimate tensile strength may be improved by preventing interfibrillar slippage and also removing the water molecules which swell the matrix and prevent hydrogen and other forms of electrostatic bonding between the collagen. Dehydrothermal treatment of collagen requires a careful balance between the amount of time exposed to heat to provide adequate cross-links and the amount of time which could lead to the denaturing or degrading of the tissue.

Another less widely used method for cross-linking collagen is through exposure to short wave ultraviolet irradiation of gamma radiation. An issue with this however, is in determining how deep the radiation will penetrate and subsequently how many cross-

links it will produce. Although these methods do not introduce potentially harmful chemical to the collagen, they are not as effective as chemical treatments which have been used commercially for years and, as previously stated, can help lower antigenicity while increasing the tensile strength of the collagen.

2.4 Chemical Modification of Collagen

Collagen-based implants are normally degraded by collagenases, naturally occurring enzymes which attack the triple helical molecule at a specific location. An effective method for reducing the degradation rate of collagen by naturally occurring enzymes is chemical cross-linking.

Reagents like glutaraldehyde, formaldehyde and diisocyanates introduce crosslinks between two ε-amino groups of lysine and/or hydroxylysine residues of reconstituted collagen. Secondly, crosslinking can be carried out by introducing amide bonds between carboxylic acid groups from aspartic or glutamic acid residues and ε-amino groups. Examples are cyanamide crosslinking, acyl crosslinking and the use of carbodiimide [29].

Several studies have been directed towards the use of novel epoxy compounds for cross-linking of collagen. It has been demonstrated that, depending on pH, bis-epoxy compounds introduce crosslinks between either carboxylic acid groups, or primary amino groups of collagen [28].

Crosslinking of collagen in commercial collagen-coated synthetic vascular grafts is commonly carried out using glutaraldehyde or formaldehyde. The mechanism of crosslinking by these reagents is complex and poorly understood. Using glutaraldehyde it

is assumed that Schiff base intermediates are formed by reaction of aldehyde groups of glutaraldehyde with amino groups of collagen (I). Subsequent reactions of these intermediates results in the formation of large variety of possible crosslink-moieties [30]. Figure 2.1 shows the mechanism of crosslinking with glutaraldehyde.

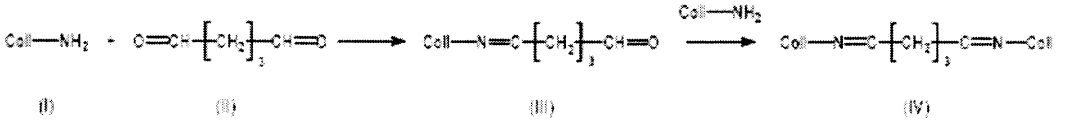

Figure 2.1 Mechanism of Crosslinking with Glutaraldehyde.

The use of glutaraldehyde has an important drawback as it is incorporated in collagen material. Glutaraldehyde is known to induce local cytotoxicity by the release of (unreacted) crosslink-agents or derivatives thereof, during both in vitro and in vivo application of collagen biomaterials [31]. It has been shown that residual glutaraldehyde completely inhibits in vitro fibroblast proliferation at concentrations as low as 3 ppm [32]. It can be concluded that upon implantation, cytotoxic reactions hamper endothelialization of currently available collagen coated synthetic vascular graft materials. In addition, glutaraldehyde crosslinking of collagen- based biomaterials is associated with enhanced calcification of the implant, which has an adverse effect on the mechanical properties of material [33].

Crosslinking of collagen using N-(3-dimethyaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) is based on a well known method in peptide synthesis. The reaction of EDC with carboxylic acid groups of collagen results in the formation of O-acylisourea. When O-acylisourea reacts with NHS reactive NHS-esters are formed [34], and release of water-soluble 1-ethyl-3(3-aminopropyl)urea (EDU)

occurs. Subsequently, reaction of NHS-esters with free ε-amino groups of (hydroxy)lysine residues results in the formation of peptide crosslinks and liberated NHS. Direct reaction of O-acylisourea with free ε-amino groups also results in formation of peptide crosslinks. NHS, however, prevents side reactions of O-acylisourea groups like hydrolysis and N-acyl shift to the stable N-acylisourea. Figure 2.2 shows the mechanism of crosslinking with EDC/NHS.

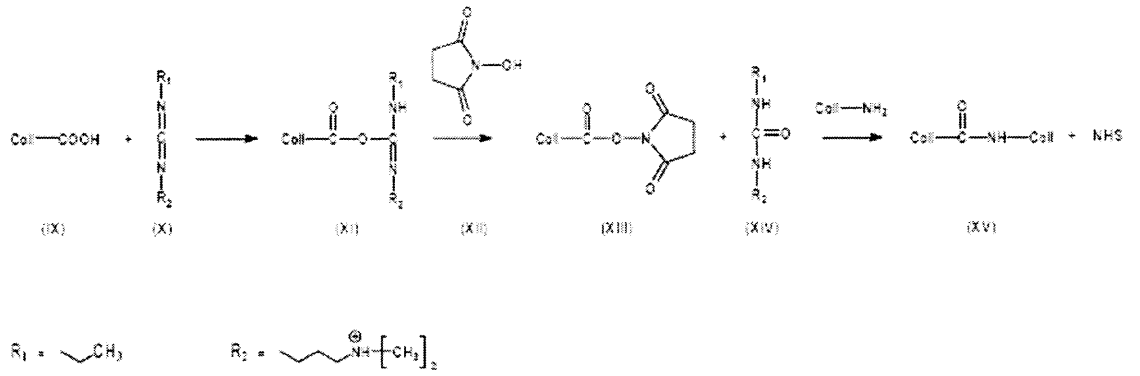

Figure 2.2 Mechanism of Crosslinking with EDC/NHS.

The EDC/NHS crosslinked collagen is reported to be non-cytotoxic in vitro, and biocompatibility was demonstrated in animal models [35]. When subcuteneously implanted in rates, calcification of EDC/NHS crosslinked collagen proved to be very low compared to glutaraldehyde crosslinked collagen [36].

CHAPTER 3

MATERIALS AND EXPERIMENTAL METHODS

3.1 Collagen Extraction Process

The process to extract collagen from bovine tendon is developed with the help of Mr. Nels Lauritzen and Dr. Joseph Nichols of Prodex Science Inc., located in Princeton, New Jersey, USA. The process uses bovine superficial flexor and deep flexor tendons as collagen source.

Approximately 1000 grams of frozen bovine tendon was sliced using the NBI Nantsune deli slicer. The sliced tendon was ground using electric meat grinder (w/4.5 mm grinder plate). To determine the % solid weight in the ground tendon, small sample of the wet tendon was weighed and then dried it in a oven for 4 hr to overnight at 100 °C to determine the dry weight. The % solid was calculated by using the value of wet weight and dry weight.

The 8.4 liters of KH_2PO_4 (Potassium Phosphate Monobasic) solution was prepared by adding 41.25 grams of KH_2PO_4 to 8.4 liters of distilled or demineralized water. Then 1.77 grams of NaOH was added to solution to get the pH of 6.15 ± 0.15. The weighed out ground tendon was added to above solution. The mix was then warmed up to 37 °C using hot plate. Then 300 ml of previously prepared buffer solution was taken and 10 grams of Ficin was dissolved in it. Immediately 300 ml of ficin premix was added in the buffer solution and noted the enzyme activity. The solution was stirred intermittently and kept it at 37 ° C ± 2 for 1 hour.

Enzyme deactivation solution was prepared by adding 84 gms of NH_4NO_3 and 10 grams of $NaClO_2$ in 8.4 liters of distilled water. Wearing latex gloves, the enzyme treated fiber was strained and hand squeezed to remove excess water and placed into the enzyme deactivation solution. A perforated funnel (colander) was used to obtain last bits of fibers. The fibers were kept in this solution for 1 hour with intermittent stirring. After that wearing latex gloves, once again fibers were strained and hand squeezed to prepare for washing. Then fibers were washed 3 times for 15 minutes using 3 liters of distilled water.

For alkalai treatment, a solution was prepared by adding 1400 gms of anhydrous sodium sulfate and 350 gms of NaOH to 6.8 liters of distilled water. The temperature was stabilized at 25 ± 1 °C. Fibers were kept in this solution for 42 hours at 25 ± 1 °C. After 42 hours the fibers were washed 3 times with 3 liter sodium sulfate solutions for 15 minutes. Following these washes fibers were once again washed 3 times with 3 liters of distilled water, adjusted to pH of 4.6 by using dilute H_2SO_4, for 15 minutes.

For isopropanol (IPA) treatment, fibers were placed into 2-3 liters of 100% isopropanol at 60 °C slowly and carefully, avoiding the hot spots and potential degradation. The blend was kept stirring to avoid hot spots. Fibers were kept in this condition for 2 hours and then prepared for second wash with isopropanol. The second wash proceeded with harvesting the fibers from the first wash by hand squeezing the fibers and colander staining to remove excess IPA. The fibers of second wash were allowed to remain in IPA at 60 ° C for 1 day. After that fibers were teased and placed into Pyrex dish to dry it in to oven at 45 °C overnight. Dried collagen fibers were stored in refrigerator.

3.2 Collagen Suspension Preparation

To prepare the collagen suspension of C %, 10 × C grams of collagen fibers were soaked in 1000 ml of distilled water. C ml of lactic acid was also added to break down the fibers in the water. The fibers were allowed to soak in this condition for 10-15 minutes. Then this mixture was blended for 30 second in laboratory blender followed by allowing it to set for 15 minutes and loose the heat it received by blending and not letting the temperature go up. The mixture was blended for 30 seconds followed by 15 minutes break and last cycle of 30 second blending. At the end of third cycle of blending the mixture became thick paste. The paste then transferred in bottles and stored in refrigerator. The collagen suspension was centrifuged for enough time to remove air bubbles if necessary before using them to extrude tubes.

3.3 Collagen Tubes Extrusion Protocol

The collagen tubes were extruded using a special collagen extrusion machine developed by ZOKO spol. S r.o. of Czechoslovakia. The extrusion machine used a piston to force the collagen gel through a rotating mandrel where it was deposited on a guide rod. The rod itself was lifted away at uniform speed from the mandrel to form an even coating. The collagen was deposited onto the rod after passing through a rotating head which oriented the get to the left or the right according to the its rotation in left or right direction. The rotation head had speeds ranging from 22- 260 rpm in either direction. In order to adjust the thickness of the tube, the pulling device also had variable speeds ranging from 152 – 1522 mm/min in the upward or downward direction. The rate of extrusion was also adjustable by controlling the upward motion of the piston. The

extrusion rate had the range from 2 – 50 cm^2/ min. The extrusion machine had a cylinder with a volume of approximately 1925.625 cc. The maximum pressure allowed in the cylinder head was 3 MPa and was regulated by an electronic meter attached to an oil reservoir which shut off the extrusion if the pressure exceeded the maximum value. To allow for chemical treatment of the tubes, homemade baths were made from PVC piping 40 in. long and sealed on both ends with caps adding an additional 1 in. length. These baths were then cut in half lengthwise to provide adequate length and ease of access.

3.3.1 Protocol for Pre-Extrusion

Two types of rods were used as guide rods for extrusion process, stainless steel and teflon both 36" in length. Because of good surface properties of teflon, teflon rods didn't need any pre-extrusion treatment. To facilitate isolation of tube from stainless steel rod, one of the PVC baths was filled with 99% pure glycerin from Fisher Scientific company of Somerville, New Jersey. The stainless steel rods were placed into the glycerin bath and allowed to soak for about 10 minutes to provide a uniform coating to the rods. Then the guide rods were hung for 1-2 minute to drain off excess glycerin into the bath.

In order to prevent contamination, parts of the extrusion machine that comes in direct with collagen were disassembled and washed before the start up of new experiment or batch.

3.3.2 Protocol for Extrusion

After switching on the machine, letting it warm up, the piston was lowered to its lowest point and the piston head was manually pushed into place at the bottom of the cylinder. Cylinder then was filled tightly up to $3/4^{th}$ of its height with collagen dispersion. The desired extrusion head and nozzle were attached to the machine to ensure that the guide rod would pass through the nozzle without any interference. The cylinder top was then covered and a 1½ ft. PVC tube was connected from the cylinder head to the extrusion head manifold. The swinging arm was lowered close to the extrusion head to clamp the guide rod. A glycerin coated stainless steel rod was inserted through the bottom of the nozzle up through the extrusion head and then clamped into the swinging arm. To ensure continuous extrusion of collagen, the PVC tube was allowed to purge. The machine was set for the desired extrusion rate, rotation speed, and linear pull speed.

The rotation motor was started as soon as collagen began filling the manifold. The collagen was continuously filling the manifold and finally became visible around the rod through the top of the extrusion head. At this point, the linear drawing arm was then activated and moved at the desired speed. The collagen was now being extruded onto the rod and a visual check was made to ensure uniform coating of collagen slurry around the tubes. Once the guide rod had completely entered the manifold, the extrusion and rotation was stopped to prevent wasting of collagen. The swinging arm was allowed to rise until the guide rod was completely out of the manifold at which point the arm was opened and lowered. The rod was removed and hung on the drying racks.

This process was repeated for each tube of various collagen concentration. After each trial, the rods were prepared for post-extrusion treatment and the machine was cleaned.

3.3.3 Post-extrusion Tube Treatment

The main goal of this treatment is to chemically treat the collagen and dry the tubes to facilitate the easy removal from guide rods. The chemical treatment is composed of two parts, namely coagulation or reconstitution and cross-linking. The tubes prepared from the above protocols were treated differently according to the protocols for different studies. The tubes were extruded according to the protocols written in section 3.3.1 and 3.3.2 and dried according to procedure described as follows.

After drying stainless steel rods for 18 to 24 hours under ambient conditions, the tubes were deemed dry. The tubes were allowed to soak in the water bath to rehydrate the tubes. They were kept in the water bath for approximately 1 ½ hour to allow enough water to absorb through the entire tube. Then the tubes were immediately cut to the desired length and slipped off from the stainless steel rods. The rehydration made the collagen tubes more flexible and with slight twists at small intervals along the tube, the bond with the rod was loosened enough to pull the cut tubes smoothly without damaging the tube or causing it to loose its shape. Some of the rods were not completely rehydrated under the standard time, particularly the thick tubes, so they were returned to the water bath for an additional 30 minutes until they were easily slipped off from the stainless steel rods.

Now the tubes no longer supported by the guide rod, curling would occur with the final drying without intervention. To prevent this undesirable shape change, the collagen tubes were placed in drying cardboard boxes. A pair of holes were drilled on the opposite side wall of specially prepared cardboard boxes. Screws with diameter approximately same as guide rods were placed in the holes. Two ends of cut tube were then fixed on the two opposite screws on the box. Tubes were actually cut of the length, so that it can fit on the screws with slight tension. The tubes were allowed to dry for 18 to 24 hours in this condition and then removed from the rack and stored in boxes to prevent any damage.

3.4 Glutarldehyde Cross-linking Protocol

Ammonium hydroxide (NH_4OH) and glutaraldehyde, the crosslinking agent, used in this protocol were received from Fisher Scientific as 5% v/v and 25% v/v solution respectively. NH_4OH was diluted to 1% and glutaraldehyde was diluted to 0.6% & 5 % using distilled water. In order to ensure adequate chemical concentration for all the tubes, these chemicals were refilled after treating every two tubes with the reminder of the chemical being removed for disposal.

Three baths each with 1 liter of ammonium hydroxide, 1 liter of glutaraldehyde and 1 liter of water in it were prepared for treatment of extruded tubes. The extruded collagen tubes were incubated in ammonium hydroxide solution. The tube was soaked for 10 minutes with occasional agitation and rotation of the rod to ensure good chemical absorbance. The rod was removed from the bath and washed for 5-7 minutes in a water bath to remove excess chemicals from the surface.

After washing, the rods were incubated in glutaraldehyde solution bath and allowed to soak for 5 minutes with occasional agitation and rotation followed by washing in a water bath for 5-7 minutes. The rod was subsequently hung on the vertical hanging rack to allow excess moisture to drip off and the tube dried.

3.5 EDC/NHS Cross-linking Protocol

The cross-linking agents used for this treatment were N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). Cross-linking was carried out in a buffer of 2-morpholinoethane sulfonic acid (MES buffer, 0.05 M, pH=7.4 was adjusted using 10 M NaOH) to minimize hydrolysis of EDC. These chemicals were ordered from Fischer Scientific. Extruded collagen tubes were coagulated by 1% ammonium hydroxide for 10 minutes followed by washing in water bath for 5 minutes. Washed tubes were hung on drying rack for 15-18 hours to dry. Dried collagen tubes were incubated in MES buffer for 30 minutes. Subsequently, the tubes were immersed in a solution of EDC and NHS in MES buffer, and cross-linking was carried out under gentle shaking.

Typically, crosslinking was carried out using 0.731 g EDC and 0.415 g NHS in 500 ml MES- buffer per gram of collagen (1.29 mmol carboxylic acid groups, Coll-COOH, per gram of collagen, resulting in a molar ratio of EDC : NHS : Coll-COOH of 7.0 : 2.8 : 1). Approximately 2.5 feet long tubes were crosslinked so 3.655 gms (0.019M) of EDC and 2.075 gms (0.018 M) of NHS was dissolved in 500 ml of MES solution. After 1 hour, crosslinking was stopped by washing the collagen film with 0.1 M sodium

phosphate dibasic (Na_2HPO_4) solution for 2 hours. Then the rod was hung on vertical hanging rack to dry for 15-18 hours under ambient conditions.

3.6 Degradation Study Protocol

The collagenase, Tris-HCl, $CaCl_2$ and EDTA used for this study were ordered from Sigma Aldrich Co., St. Louis, MO. The solution of 0.05 M $CaCl_2$ and 0.25 M EDTA were prepared using distilled water. For this study collagen tubes were extruded using setting of extrusion speed 25 cm^2/min, linear draw rate 300 mm/min, rotation rate 60 rpm. Two different concentration of collagen suspension were used for this study 2.0% and 2.5%. Two tubes of each concentration were extruded. All the tubes were reconstituted by using 1% ammonium hydroxide for 10 minutes followed by washing in water bath for 5 minutes. One tube of each concentration was cross-linked by using 0.6% glutaraldehyde for 5 minutes while other two tubes were cross-linked by using EDC/NHS according to the protocol described in section 3.5. After cross-linking all the tubes were given water wash for 5-7 minutes and then hung on drying rack for 18-24 hours. After drying all the tubes were separated from guide rod as explained in section 3.3.3.

Seven samples of each tube, each weighing approximately 25 mg, were prepared for this study. Initial dry weight of the samples was recorded before the start of study. Glutaraldehyde and EDC/NHS crosslinked samples were tested for 2 hr, 4 hr, 6 hr, 8 hr, 10 hr, 12 hr and 14 hr.

First the samples were incubated for 1 hr in 5 ml 0.1 M Tris-HCl (pH 7.4), containing 10 ml 0.05 M $CaCl_2$ at 37 °C. Separate aluminium pans were used for each sample and covered with aluminium foil to avoid possible evaporation of solution.

Subsequently 30 mg 291 U bacterial collagenase (Clostridium histolyticum, EC 3.4.24.3, Sigma Chemical Co.) was added to above solution. After specified time for each sample, the reaction was stopped by the addition of 1 ml 0.25 M EDTA and cooling the mixture on ice. Then samples were dried under atmospheric conditions for 18-24 hours. The final dry weight of the samples was recorded. The extent of degradation was determined from the final weight of samples and expressed as percentage weight loss after the collagenase treatment.

3.7 Porosity Characterization

The Mercury Porosimeter is a versatile and accurate instrument used to determine properties such as pore size distribution, total pore volume, surface area, and bulk and absolute densities of solid and powder samples. The Mercury Porosimeter fills the penetrometer and sample chamber with mercury under vacuum and takes a volume reading. The sample, however, is not initially intruded with mercury because of the high surface tension. Gradually, increasing amounts of pressure are applied. For each incremental increase in pressure, the change in intrusion volume is equal to the volume of the pores whose diameters fall within an interval that corresponds to the particular pressure.

The theory of all mercury porosimeters is based on the physical principle that a non-reactive, non-wetting liquid will not penetrate pores until sufficient pressure is applied to force its entrance. The relationship between the applied pressure and the pore

size into which mercury will intrude is given by the Washburn equation:

$$PD = -4\gamma \cos \theta$$

where P is the applied pressure, D is the diameter, γ is the surface tension of mercury (480 dyne cm^{-1}) and θ is the contact angle between mercury and the pore wall, usually near 140°. As pressure increases, the instrument senses the intrusion volume of mercury by the change in capacitance between the mercury column and a metal sheath surrounding the stem of the sample cell. As the mercury column shortens, the pressure and volume data are continuously acquired and displayed by an attached personal computer.

The concentration of glutaraldehyde used for this study was 5%. The samples were cut in to 2.7 cm H x 2 cm D dimensions and were prepared for pore volume, pore size distribution, surface area and porosity characterization. Mercury contact angle (θ) was taken as 140 degrees. The samples were identified as follows:

Table 3.1 Sample Identification for Porosity Characterization

Sample No.	**Description**
1	2% Collagen, NH_4OH coagulation, Glutaraldehyde cross-linked
2	2% Collagen, NH_4OH coagulation, EDC/NHS cross-linked
3	2.5% Collagen, NH_4OH coagulation Glutaraldehyde cross-linked
4	2.5% Collagen, NH_4OH coagulation, EDC/NHS cross-linked

CHAPTER 4

RESULTS AND DISCUSSION

The collagen tubes were successfully extruded using collagen extrusion machine. Stainless steel rods were used for extrusion. Thanks to glycerin coating on guide rods, tubes were easily isolated from guide rods. Drying was carried out under normal atmospheric conditions. Extrusion variables had a great effect on wall thickness. Glutaraldehyde treated tubes turned reddish yellow after drying, while there was no change in color for EDC/NHS treated tubes. For porosity characterization and degradation study, different glutaraldehyde concentration (5% & 0.6% respectively) was used because collagenase had no significant effect on samples crosslinked with 5% glutaraldehyde for up to 72 hours. The inner diameter of the tubes reduced after drying due to shrinkage of tubes.

4.1 Results of Degradation Study

Collagen crosslinking using EDC involves the activation of carboxylic groups of glutamic and aspartic acid residues, and the formation of amine bonds in the presence of lysine or hydroxylysine residues. The addition of NHS increases the rate and degree of crosslinking.

The enzymatic degradation of extruded collagen tubes was performed using collagenase (Clostridium histolyticum, EC 3.4.24.3, Sigma Chemical Co.). The samples crosslinked with glutaraldehyde and EDC/NHS were evaluated for their enzymatic stability against collagenase digestion. Non-crosslinked samples almost completely

degraded after 2 hours. The dry weight before and after the collagenase treatment was used to calculate the percentage weight loss after the treatment as shown in Table 4.1.

Table 4.1 Results of Degradation Test

Time(hr)	% Weight loss			
	Sample #1 (2% Coll., GTA Crosslinked)	Sample #2 (2% Coll., EDC/NHS Crosslinked)	Sample #3 (2.5% Coll., GTA Crosslinked)	Sample #4 (2.5% Coll., EDC/NHS Crosslinked)
2	1.58	0.32	1.84	0.42
4	2.68	0.75	3.09	1.12
6	3.87	0.88	4.1	1.59
8	5.69	1.22	6.86	1.49
10	7.8	1.48	8.78	1.76
12	9.49	1.95	9.96	2.26
14	10.35	2.91	12.49	3.35

The data in Table 4.1 was used to plot percentage weight loss as a function of time (Figure 4.1 and Figure 4.2).

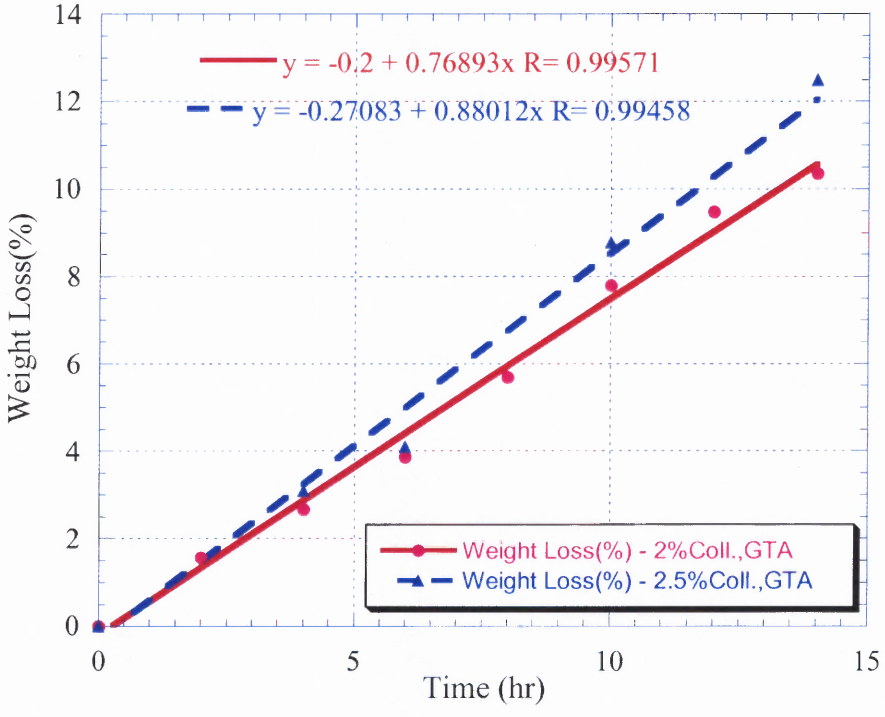

Figure 4.1 Plot Showing Percentage Weight Losses as a Function of Time for Glutaraldehyde Treated Tubes.

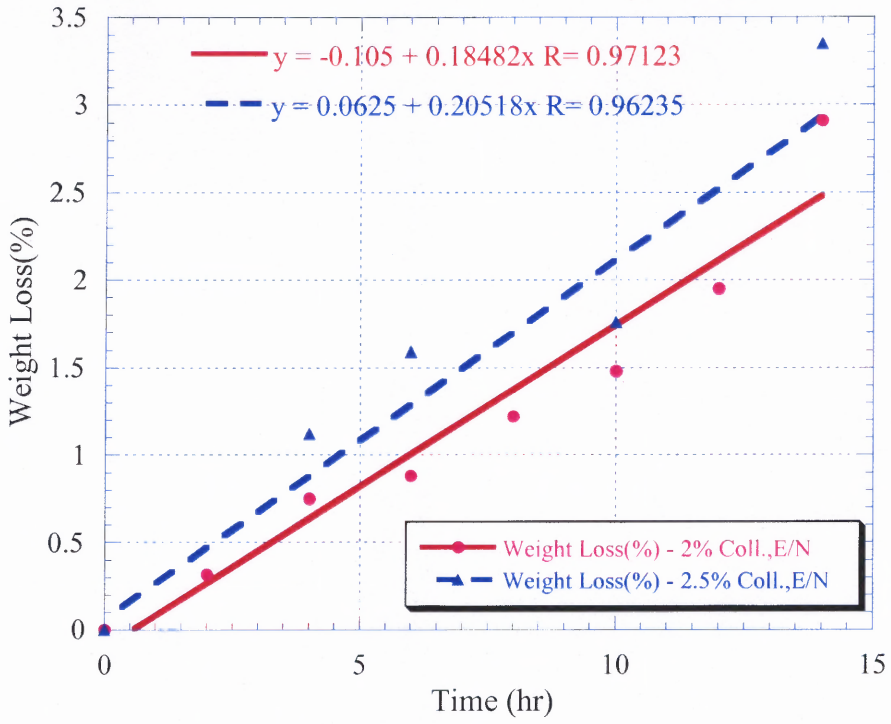

Figure 4.2 Plot Showing Percentage Weight Losses as a Function of Time for EDC/NHS Treated Tubes.

Figures 4.1 and 4.2 show the effect of collagenase on glutaraldehyde and EDC/NHS crosslinked samples as well as collagen concentration. As can be seen from these plots, the slopes of curves for sample number 1 and 3 are much steeper than those for sample number 2 and 4. This suggests that percentage weight loss is much higher for samples crosslinked with glutaraldehyde than the ones crosslinked with EDC/NHS. That means that EDC/NHS treated tubes degraded slower than glutaraldehyde treated tubes. Collagenase cleaves a unique site of peptide bonds in triple-helical collagen, i.e. the primary sequence of the amino acids leucine and glycine [37]. The structural integrity after chemical crosslinking caused enzymes to have difficulty in reaching attack sites in collagen, possible due to many intermolecular cross-links [38].

Furthermore, as can be seen from the plots, it can be concluded that 2.5% collagen samples showed less resistance to degradation compared to 2% collagen samples because of the difference in amount of fibers they contain.

Considering simple best linear fit for the curves, predictive equations for the curves of weight loss vs. time are derived. These predictive equations shows the degradation rate of collagen tubes inside body and can further be used to design an in vitro model of these tubes as small diameter vascular grafts.

These predictive equations are:

1. $y = 0.76893(x) - 0.2$; $R = 0.99571$
2. $y = 0.18482(x) - 0.105$; $R = 0.97123$
3. $y = 0.88012(x) - 0.27083$; $R = 0.99458$
4. $y = 0.20518(x) + 0.0625$; $R = 0.96235$

Where, y = % weight loss

x = time (hr)

R = correlation coefficient

Since the degradation rate is zero at the start of experiment, there is no weight loss at time t = 0. Also the point (0, 0) is included in each data set for samples. So the y intercepts in the above equations can be neglected theoretically. Hence, these equations take the form,

$$\Delta W = kt$$

Where ΔW is percentage weight, t is time in hour and constant k can be taken as 0.82 for glutaraldehyde treated tubes and 0.19 for EDC/NHS treated tubes. The above equation is the model equation for degradation rate of collagen tubes which shows the relationship between the mass degraded and time.

4.2 Results of Porosity Characterization

The collagen tubes samples were characterized for porosity using mercury porosimeter. The data recorded were pressure (psia), pore diameter (microns) and cumulative pore volume (cc/g). Some of the nomenclatures used in the data are explained below:

1. Cumulative pore volume = Pore volume belonging to the pores of diameter > D
2. % Total pore volume = % of total cumulative pore volume belonging to the pores of diameter > D
3. Average pressure = square root of P(I) * P(I-1)
4. Pore size distribution function = $\Delta V / \Delta \log D$

Table 4.2 Summary of Porosity Characterization Results

	#1	#2	#3	#4
Sample weight (g)	0.2000	0.0700	0.1800	0.1700
Total intrusion volume (cc/g)	0.2179	0.6559	0.2585	0.4434
Total surface area (m^2/g)	80.6494	216.1894	92.9195	110.100
Median pore dia. (Based on Vol.) (μ)	0.0153	0.0259	0.0165	0.062
Median pore dia. (Based on Surf. area)(μ)	0.0052	0.0053	0.0052	0.0055
Standard deviation (Based on Vol.) (μ)	0.0054	0.006	0.0056	0.0078
Standard deviation (Based on Surf. area) (μ)	0.0019	0.002	0.0019	0.0023
Average pore dia. (4V/S) (μ)	0.0108	0.0121	0.0111	0.0161
Dia. of min. value of dV/dlogD	0.00383	0.003683	0.003620	0.00372

Table 4.2 shows some properties of collagen tubes obtained by porosity characterization.

From the intrusion volume data in Table 4.2, it can be concluded that EDC/NHS treated samples are more porous than glutaraldehyde treated samples. Also from Table 4.2 and Figure 4.7, EDC/NHS treated samples have more surface area than glutaraldehyde treated samples.

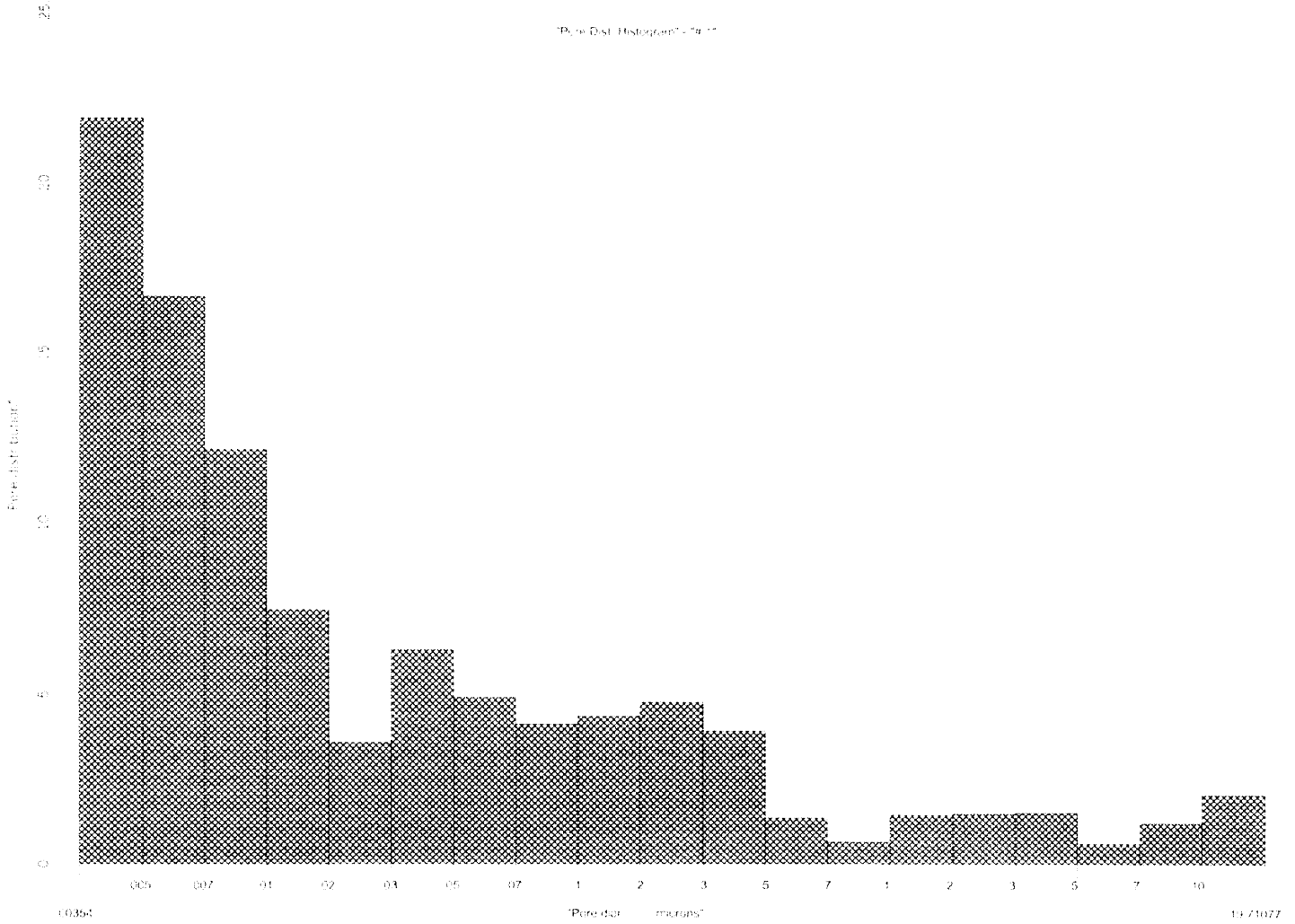

Figure 4.3 Pore Distribution Histogram for 2% Collagen, GTA Crosslinked Samples.

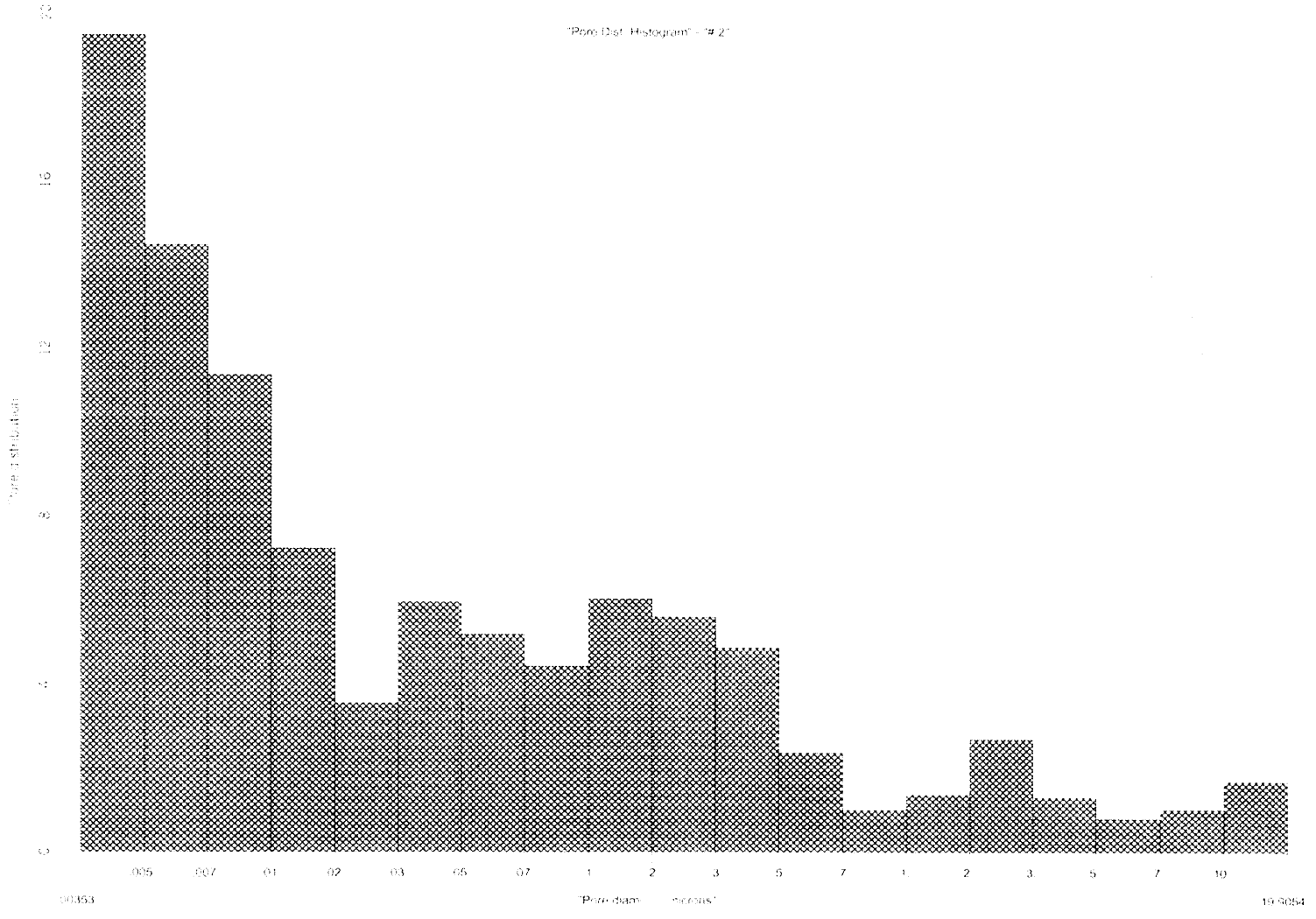

Figure 4.4 Pore Distribution Histogram for 2% Collagen, EDC/NHS Crosslinked Samples.

For the application of endothelial cells on vascular grafts, higher surface area is advantageous, but it might be disadvantageous from the point of view of thrombus formation. Figures from 4.3 to 4.6 represent pore distribution for each kind of sample.

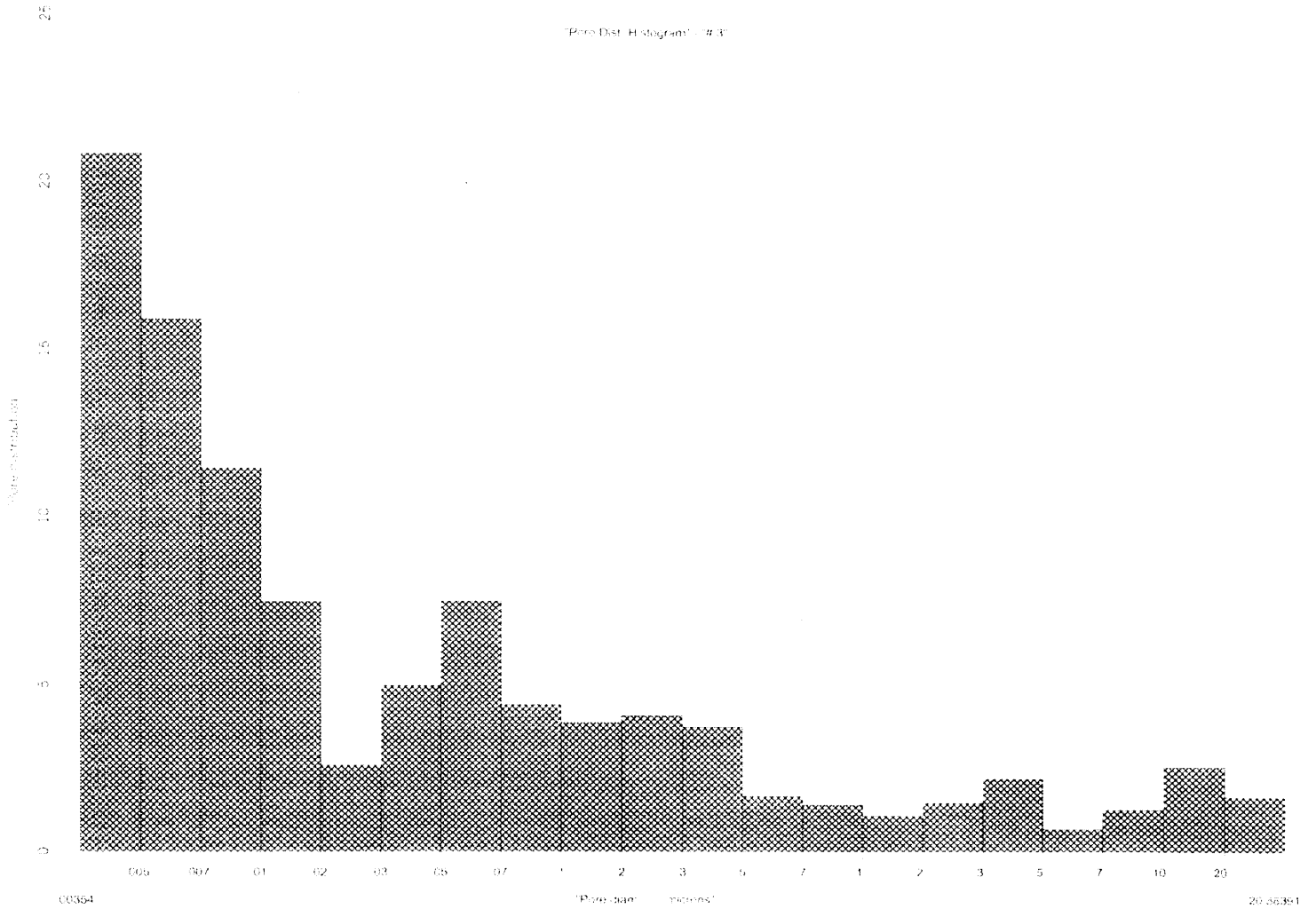

Figure 4.5 Pore Distribution Histogram for 2.5% Collagen, GTA Crosslinked Samples.

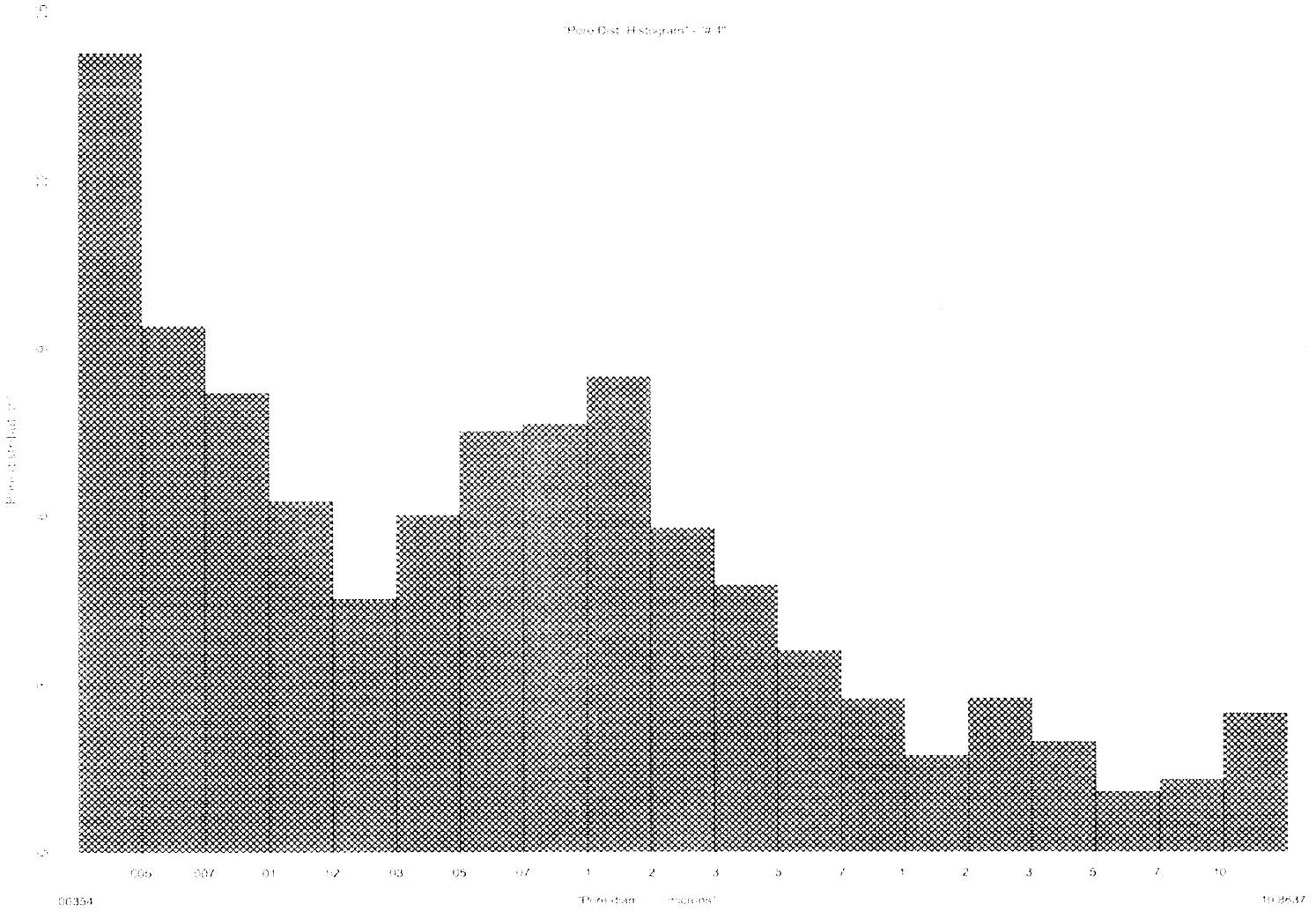

Figure 4.6 Pore Distribution Histogram for 2.5% Collagen, EDC/NHS Crosslinked Samples

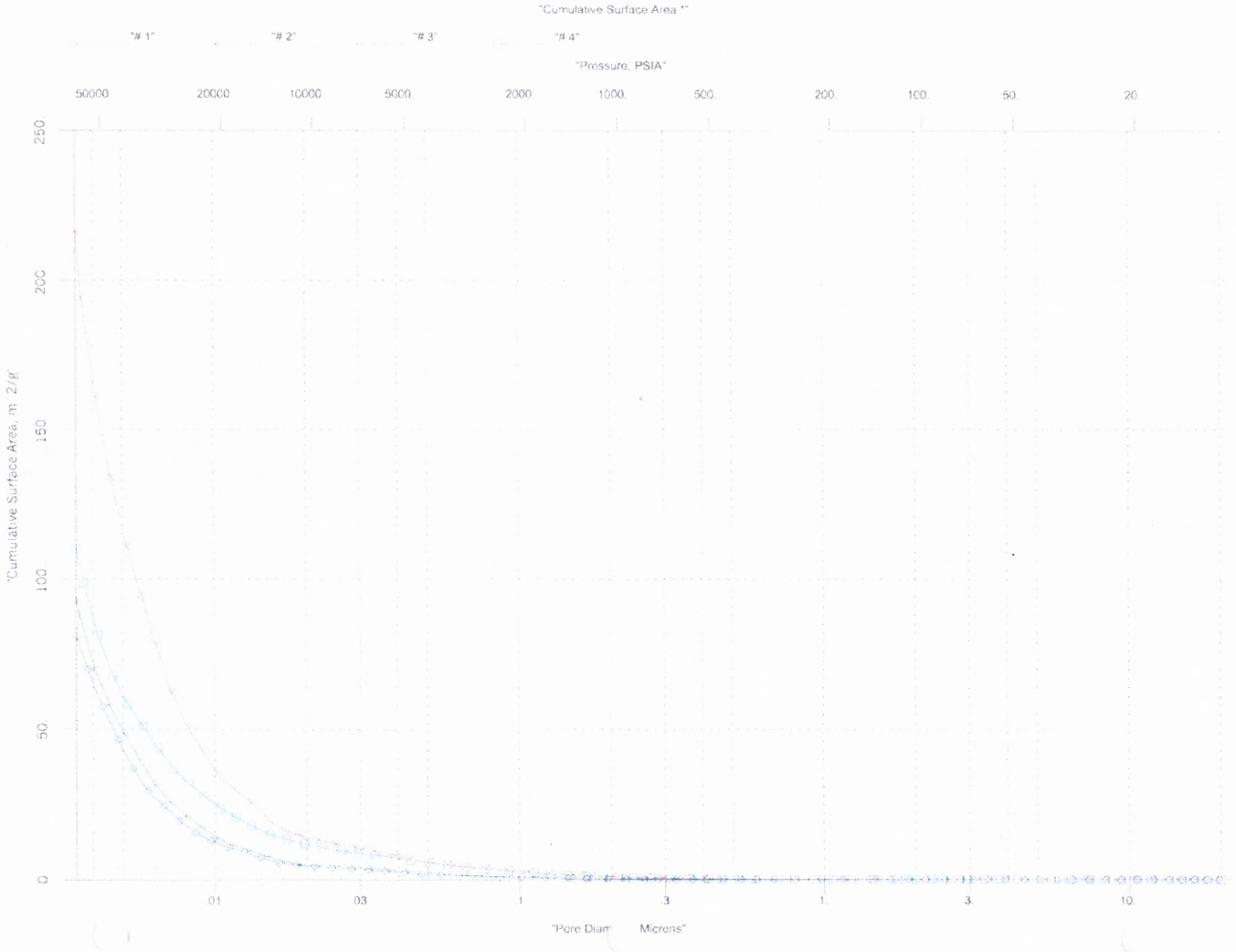

Figure 4.7 Cumulative Surface Area of Samples.

CHAPTER 5

CONCLUSION

One effect of crosslinking is increased resistance to enzymatic degradation by bacterial collagenase. In the present study, enzymatic degradation of non-crosslinked tubes resulted in a removal of non-fibrillar collagenous substance. Tubes crosslinked with 5% glutaraldehyde had developed higher resistance against enzymatic degradation. From degradation study results, it can be inferred that the EDC/NHS treated tubes showed greater resistance to enzymatic degradation than glutaraldehyde treated tubes. It can also be concluded that the higher the collagen concentration, the higher the degradation rate. The predictive equations derived from the data of degradation study can be used to design an in vitro model for small diameter collagen tubes. The *in-vitro* model equation for the degradation rate of small diameter collagen tubes showed linear behavior.

From porosity characterization study, it can be concluded that EDC/NHS treated samples are more porous than glutaraldehyde treated samples. The EDC/NHS treated samples have more surface area than glutaraldehyde treated samples. This is advantageous in cases where endothelial cell seeding is desired.

Also the tubes are not porous enough and do not have optimum surface area to mimic the natural blood vessel. The amount of thrombus deposition is proportional to the surface area of the exposed device and may be increased by deposition of fibrinogen into cracks on the surface [4]. Future work will be to study biodegradation rate for small diameter collagen tubes over longer period of time. The possible research could be in the direction to reduce the thrombogenicity and optimize the porosity of EDC/NHS treated

tubes and to develop a graft with porosity such that it prevents the loss of blood during implant but permits ingrowth of fibroblast and smooth muscle cells in order to attach the graft to the host tissue. The reduction in thrombogenicity can be accomplished by coating the tubes surface with endothelial cells.

REFERENCES

1. M. Chvapil, L. Kronenthal, W. van Winkle. Medical and surgical applications of collagen. Int Rev Connect Tissue Res 1973; 6:1–61.

2. J. M. Pachence. Collagen-based devices for soft tissue repair. J Biomed Mater Res 1996; 33:35–40.

3. K. C. Dee, D. A. Puleo, R. Bizios. An Introduction to Tissue-biomaterial Interactions. John Wiley and Sons, c2003.

4. F. H. Silver, D. L. Christiansen. Biomaterials Science and Biocompatibility. Springer, c1999.

5. R. Smith. The molecular genetics of collagen disorders. Clinical Science, 1986; 71:129-135.

6. K. S. E. Cheah. Collagen genes and inherited connective tissue disease. Biochemical Journal, 1985; 229:287-303.

7. Vincent, F. V. Julian. Structural Biomaterials. Princeton University Press, c1990.

8. E. J. Miller. The Structure of Collagen. Monographs in Pathology, 1983; 24:4-15.

9. B. Alberts et al. Molecular Biology of the Cell. Garland Publishing, c2002.

10. P. L. Privalov. Stability of proteins; Proteins which do not present a single cooperative system. Advances in Protein Chemistry, 1982; 35:1-104.

11. D. A. D. Parry and A.S. Craig. The molecular structure of collagen. Developments in Biochemistry, 1981; 22:63-67.

12. R. Fleischmajer, B. R. Olsen, and K. Kuhn. Biology, Chemistry, and Pathology of Collagen. New York Academy of Sciences, 1985.

13. B. Ratner et al. Biomaterials Science, Academic Press, N.Y., 84-92,287-288, 1996.

14. M. L. Tanzer. Collagen cross-linking. NATO ASI Ser., Ser. A, 1985; 93:65-72.

15. B. Kinner, J. Zaleskas, M. Spector. Contractile behavior of human connective tissue and mesenchymal stem cells in collagen-GAG matrices for tissue engineering. Tissue Eng 2001; 7:617.

16. S. Nehrer, H. A. Breinan, A. Ramappa, G. Young, S. Shortkroff, L. K. Louie, C. B. Sledge, I. V. Yannas, M. Spector. Matrix collagen type and pore size influence behavior of seeded canine chondrocytes. Biomaterials 1997; 18:769–76.

17. S. Nehrer, H. A. Breinan, A. Ramappa, H. P. Hsu, T. Minas, S. Shortkroff, C. B. Sledge, I. V. Yannas, M. Spector. Chondrocyte seeded collagen matrices implanted in a chondral defect in a canine model. Biomaterials 1998; 19:2313–28.

18. J. S. Bennett, Platelet aggregation. in: Hematology, W. J. Williams, et al. (Eds.), McGraw-Hill, New York, 1990, pp. 1778-1781.

19. H. Kambic, S. Murabayashi, R. Yozu, T. Morimoto. Small vessel replacement with elastomeric protein composite materials: Preliminary studies. ASAIO Trans., 30: pp. 406-410 (1984).

20. W. J. Quinones-Baldrich, W. S. Moore, S. Ziomek, M. Chapvil 1986: Development of a leakproof knitted dacron vascular prosthesis. J Vasc Surg 3: 895-903.

21. T. Huynh, G. Abraham, J. Murra , et. al., "Remodeling of an acellular collagen graft into physiologically responsive neovessel", Nature Biotechnology, Vol 17, Nov 1999, 1083-1086.

22. M. J. B. Wissink, R. Beernink, A. A. Poot, et. al., "Improved endothelialization of vascular grafts by local release of growth factor for heparinized collagen matrices", Journal of Controlled Release 2000; 64:103-114.

23. D. Kallmes, L. Horng-Ban, N. Fujiwara, et. al. "Comparison of small-diameter type I collagen stent-grafts and PTFE stent-grafts in a canine model", J Vasc Interv Radiol,Vol 12, Number 10, 2001, 1127-1133.

24. H. Colft, D. Kallmes, L. Horng-Ban, et. al., "Bovine type I collagen as endovascular stent graft material: biocompatibility study in rabbits", Radiology 2000; 214:557-562.

25. K. G. Vogel and J. A. Trotter. The effect of proteoglycans on the morphology of collagen fibrils formed in vitro. Collagen Rel. Res., 1987; 7:105-114.

26. Hoffman, Jr., H. Schankereli, Kemal, Chvapil, Milos. Drug delivery collagen-impregnated synthetic vascular graft. US Pat Pending.

27. M. Chvapil, D. P. Speer, H. Holubec, T. A. Chvapil, D. H. King, Collagen fibers as a temporary scaffold for replacement of ACL in goats. J.Biomed.Mat.Res., 27: pp. 313-325 (1993).

28. R. Zeeman, Cross-linking of collagen-based materials. Ph.D. thesis, University of Twente, Enschede, 1998.

29. E. Khor, Methods for the treatment of collagenous tissues for bioprostheses. Biomaterials, 18: pp. 95- 105 (1997).

30. L. H. H. Olde Damink, P. J. Dijkstra, M. J. A. van Luyn, P. B. van Wachem, P. Nieuwenhuis, J. Feijen, Glutaraldehyde as a crosslink agent for collagen-based biomaterials. J.Mat.Sci.: Mat. Med., 6: pp. 460- 472 (1995).

31. L. L. H. Huang-Lee, D. T. Cheung, M. E. Nimni, Biochemical changes and cytotoxicity associated with the degradation of polymeric glutaraldehyde derived crosslinks. J.Biomed.Mat.Res., 24: pp. 1185-1201 (1990).

32. D. P. Speer, M. Chvapil, C. D. Eskelson, J. Ulreich, Biological effects of residual glutaraldehyde in glutaraldehyde-tanned collagen biomaterials. J.Biomed.Mat.Res., 14: pp. 753-764 (1980).

33. F. J. Schoen, H. Harasaki, K. M. Kim, H. C. Anderson, R. J. Levy, Biomaterial-associated calcification: Pathology mechanisms and strategies for prevention. J.Biomed.Mat.Res., 22: pp. 11-36 (1988).

34. L. H. H. Olde Damink, P. J. Dijkstra, M. J. A. van Luyn, P. B. van Wachem, P. Nieuwenhuis, J. Feijen, Cross-linking of dermal sheep collagen using a water-soluble carbodiimide. Biomaterials, 17: pp. 765- 773 (1996).

35. M. J. A. van Luyn, P. B. van Wachem, L. H. H. Olde Damink, Relations between in vitro cytotoxicity and crosslinked dermal sheep collagens. J.Biomed.Mat.Res., 26: pp. 1091-1110 (1992).

36. P. B. van Wachem, M. J. A. van Luyn, L. H. H. Olde Damink, P. J. Dijkstra, P. Nieuwenhuis, Biocompatibility and tissue regenerating capacity of crosslinked dermal sheep collagen. J.Biomed.Mat.Res., 28: pp. 353-363 (1994).

37. M. E. Nimni, R. D. Harkness Molecular structure of collagen, vol.1. in: M. E. Nimni, editor. Boca Raton, FL: CRC Press. 1988. pp. 1-77 [Chapter 1].

38. K. Vizárová, D. Bakos, M. Rehákova, M. Petríkova, E. Panáková and J. Koller. Modification of layered atelocollagen: enzymatic degradation and cytotoxicity evaluation. Biomaterials 1995; 16:1217-21.