ABSTRACT

EVALUATION OF EFFECT OF EXTRUSION PROCESS VARIABLES ON MECHANICAL PROPERTIES OF EXTRUDED COLLAGEN TUBES AND EFFECT OF POST EXTRUSION TREATMENTS

by
Mayank Patel

This study evaluated the effect of extrusion process variable on mechanical properties of extruded collagen tubes made using a specialized extruder, made by ZOKO Corporation of Czechoslovakia. Three extrusion variables were considered; extrusion speed, linear draw rate and rotation speed. Using Taguchi L_4 matrix, a design of experiment (DOE) was run. Two mechanical properties, wall thickness and estimated elastic modulus, were considered as response of this DOE. Data was analyzed statically to evaluate the effect of extrusion variables on both these properties. Result of this study showed that linear draw rate of the extrusion process has the highest influence on wall thickness and rotation speed has highest influence on elastic modulus.

Additional studies evaluated effects of post-extrusion treatment on properties of the tubes. Collagen is a biodegradable material so it degrades very fast in the body. A proposed use of these tubes is as vascular grafts. To serve this purpose the tubes have to be treated to degrade slowly in the body so that when it degrades newly formed vessel replace it. Studies were performed to compare two different cross-linking methods, coagulation methods and cross-linking time for a glutaraldehyde crosslinking process. Two crosslinking methods, considered in this study, are crosslinking by glutaraldehyde and EDC/NHS. Result of these studies showed that, crosslinking with glutaraldehyde gives higher degree of crosslinking than EDC/NHS; and the coagulation with acetone and

ammonium hydroxide is also more effective than coagulation with just ammonium hydroxide solution. Crosslinking time study for glutaraldehyde showed that increase in degree of crosslinking after 15 minutes is very slow.

EVALUATION OF EFFECT OF EXTRUSION PROCESS VARIABLES ON MECHANICAL PROPERTIES OF EXTRUDED COLLAGEN TUBES AND EFFECT OF POST EXTRUSION TREATMENTS

by
Mayank K. Patel

A Thesis
Submitted to the Faculty of
New Jersey Institute of Technology
In Partial Fulfillment of Requirement for Degree of
Master of Science in Biomedical Engineering

Biomedical Engineering Department

January 2004

APPROVAL PAGE

EVALUATION OF EFFECT OF EXTRUSION PROCESS VARIABLES ON MECHANICAL PROPERTIES OF EXTRUDED COLLAGEN TUBES AND EFFECT OF POST EXTRUSION TREATMENTS

Mayank K. Patel

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

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

Dr. George Collins, Committee Member — Date
Visiting Scientist, NJIT

BIOGRAPHICAL SKECH

Author: Mayank Kashyapbhai Patel

Degree: Master of Science

Date: January 2004

Undergraduate and Graduate Education:

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

- Bachelor of Engineering in Chemical Engineering
  Sardar Vallabhbhai Regional Collage of Engineering and Technology, India, 2001

Major: Biomedical Engineering

To my beloved parents and wife,
who supported me throughout this grand endeavor.

ACKNOWLEDGEMENT

I would like to express my deepest appreciation, to Dr. Michael Jaffe who served as my thesis advisor and research supervisor and provided me with valuable assistance in the completion of this paper. I would also like to give special thanks to Dr. George Collins and Dr. Treena Arinzeh for serving as member of my committee.

I would also like to thank Nels Lauritzen for helping in preparation of collagen suspension and Joseph Pickton for his assistance in the research and testing done for this thesis.

I would also like to thank Bipin Patel for working with me in the lab and helping me in experimental work. Thanks to Varsha Tandra for ordering the chemicals and materials timely.

Finally, I would like to thank my wife, Poonam Patel, for keeping me motivated and on track and supporting me at every step of the way. Last but not the least, my family, for their assistance and constant belief in my abilities and their support over the years.

TABLE OF CONTENTS

Chapter		Page
1	INTRODUCTION	1
	1.1 Collagen Overview	1
	1.2 Collagen Structure	4
	1.3 Collagen Fibril Formation	9
	1.4 Types of Collagen	12
2	APPLICATION OF COLLAGEN TUBES	16
	2.1 Collagen as Biomaterial	16
	2.1.1 Collagen Antigenicity	18
	2.1.2 Collagen Thrombogenicity	19
	2.2 Use of Collagen in Small Diameter Vascular Grafts	19
	2.3 Crosslinking of Collagen	23
3	MATERIAL AND EXPERIMENTAL METHODS	27
	3.1 Collagen Suspension Preparation	27
	3.2 Collagen Tubes Extrusion Protocols	29
	3.2.1 Protocol for Pre-extrusion	30
	3.2.2 Protocol for Extrusion	30
	3.2.3 Protocol for Post- Extrusion	32
	3.3 Glutaraldehyde Crosslinking Protocol	33
	3.4 EDC/NHS Crosslinking Protocol	34
	3.5 Protocol for DOE on Extrusion Variables	35
	3.6 Study of Coagulation Method and Collagen Suspension Concentration	37

TABLE OF CONTENTS
(Continued)

Chapter **Page**

3.7 Study of Crosslinking Methods 38

3.8 Study of Crosslinking Time for Glutaraldehyde 38

3.9 Swelling Test Protocol 39

3.10 Protocol for Wall thickness Measurement 39

3.11 Protocol for DSC, TGA and TMA 40

3.11.1 Thermal Gravimetric Analysis (TGA) 40

3.11.2 Differential Scanning Calorimetry (DSC) 40

3.11.3 Thermal Mechanical Analysis (TMA) 42

4 RESULTS AND DISCUSSION 43

4.1 Results of DOE 44

4.2 Results of Coagulation and Collagen Concentration Study 52

4.3 Results of Study of Crosslinking Method 60

4.4 Results of Crosslinking Time study for Glutaraldehyde 61

5 CONCLUSION 66

APPENDIX 68

REFERENCES 69

LIST OF TABLES

Table		Page
1.1	Stability of Collagen Helix	6
1.2	Classification of Collagens	15
2.1	Natural Polymer Examples and Functions in Host Animal	17
2.2	Properties of Collagen as a Favorable Biomaterial	17
3.1	L_4 Taguchi matrix for Design of Experiment	37
3.2	Matrix for Study of Coagulation Method and Collagen Concentration	38
4.1	Wall Thickness Values for DOE Runs	46
4.2	Main Effects of Extrusion Variables on Wall Thickness of Tube	49
4.3	Results of Elastic Modulus for DOE Runs	52
4.4	Main Effects of Extrusion Variables on Elastic Modulus	55
4.5	Weight % at Different Temperature	56
4.6	Temperature at Highest and Lowest Rate of Weight Loss	58
4.7	Denaturation Temperature for Coagulation Study	59
4.8	TMA Results for Coagulation Study	62
4.9	Swelling Test Results for Coagulation Study	64
4.10	Weight% at Different Temperature	65
4.11	Weight % at Different Temperature for Crosslinking Time Study	67
4.12	Denaturation Temperature for Crosslinking Time Study	67
4.13	TMA Results for Crosslinking Time Study	68
4.14	Results of Swelling Test for crosslinking Time Study	69

LIST OF FIGURES

Figure		Page
1.1a	Model of the α-chain structure of collagen	4
1.1b	Model of the triple helix structure of collagen	4
1.2	Major events in the biosynthesis of fibrous collagens	11
2.1	Mechanism of crosslinking with glutaraldehyde	25
2.2	Mechanism of crosslinking with EDC/NHS	36
4.1	Average effects graph of extrusion variables on wall thickness of tube	47
4.2	Influence of extrusion process variables on wall thickness of tubes	48
4.3	Stress strain curve for DOE samples	51
4.4	Average effects graph of extrusion variables on elastic modulus of tube	53
4.5	Influence of extrusion process variables on elastic modulus of tubes	54
4.6	Graph of TGA test	57
4.7	DSC graph of heat flow vs. temperature	60
4.8	TMA graph of dimension change vs. temperature	63
4.9	Graph of break point temperature vs. crosslinking time	68
4.10	Graph of 1/swelling ratio vs. crosslinking time	69

CHAPTER 1

INTRODUCTION

The word collagen is derived from the Greek word meaning to produce glue. In the past the collagen of bones and tendons was used in industry to produce glue. Also in organisms collagen is a kind of glue. The English adaptation "collagen" was first used around 1865. The Oxford Dictionary (1893) defines collagen as: "that constituent of connective tissue which yields gelatin on boiling." Much of the early work was indeed done on heat-denatured collagen. On the other hand, the presence of fibers in connective tissues had been known since the 19th century from the work of early histologists such as Henle and Ranvier. Only in the 1920s did the pioneering work of Nageotte reveal that acid-solubilized collagen could precipitate into a material, later shown by x-ray diffraction and electron microscopy to be collagen fibers. [1]

1.1 Collagen Overview

The protein collagen is the main substance of connective tissue and is present in all multicellular organisms. In mammals collagen is the most abundant protein making up a quarter of the total weight of proteins. Collagen gives many different organs and tissues substantial, stout and elastic properties. It has been found in many different tissues and organs like bones, tendons, (hyaline) cartilage, blood vessels, teeth, cornea, inter vertebral disks, vitreous bodies, placenta, (fetal) skin, etc. Collagen is a protein that enwraps the organs and parts of it to hold specialized cells together in discrete units. It prevents organs/tissues to tear or loose their functional shape when they are exposed to sudden and wild movements. Besides the structural role in mature tissues, collagen plays

a regulating role in developing tissues as well. Collagen functions as a kind of trigger that influences the proliferation and differentiation of unspecialized cells. It has a key function in the regulation of cell type-specific gene expression and developmental control and diseases like cancer [2].

Besides glue, many new applications have been developed in industry for these proteins. The most popular uses are found in: cosmetics, nutrition, and medical applications for example as surgery stitch cotton [3].

However, collagens attract attention not only for commercial motives. Also from a clinical point of view there is much interest in collagens, because many different diseases are related to disorder in collagen. A better understanding of the spatial structure will give more insight in collagen related disorder diseases. These can be congenital, for example like the Ehler-Danlos syndromes, or the consequence of a deficiency like scurvy.

The collagen proteins belong to the larger family of fibrous proteins, like the fibrins from blood. They form mostly insoluble super strong fibers that have a high tensile strength. A fiber of only 1 mm thick can easily resist a force of 10 kg.

Collagen occurs not only in the shape of fibers. Collagen has been observed as ropes, straps, woven sheets, filtration membranes, supporting skeletal frameworks, bearing materials lubricated with proteoglycans and in all other specialized tissues that must be strong and yet have unusual properties, for example as the light-transmitting cornea and fatigue-resistant heart valves [4]. All these examples concern genetically distinct types of collagen that have evolved into a particular structural function outside the cell. At least 20 types of collagen have been described and characterized so far. They

are distinguished on the basis of their chemical differences. These 20 types differ in the way they associate with one another and the way they interact with other molecules.

The most important types are the fibrillar collagen types I, II, III, IV and V. Type I is with 90% the most abundant collagen type. It forms the largest and strongest fibrillar component that provides tensile strength to bones, skin, tendons and ligaments. Type II collagen is unique to articular cartillage and fibrocartilage, the vitreous body of the eye and certain other organs. Type III collagen is similar in structure to type I but less abundant and is often encountered in areas of rapid new collagen synthesis. Type IV is a major component of all basement membranes and type V is found in some veins and arteries.

Collagens are known to form highly ordered aggregates. The periodicity in these macromolecular structures makes them suitable for investigation by means of X-ray diffraction.

This chapter will describe the spatial organization of collagen structures in particular of the collagen type I. Collagen type I is the most regular one and most structure research has been done for this collagen type.

First, a detailed description will be given of the collagen monomers and the different types. Next it will be shown how they are synthesized (fibrile formation). After that the packing of collagen into collagen fibers will be discussed. The mode of packing will be divided in an axial and an equatorial direction.

1.2 Structure of Collagen

Collagen fibers are analogue to fibrin fibers. Both protein fibers are made from repeating building units. In both cases the building units become active after specific proteolytic cleavages and the active monomers that arise are both self-assembling. Active collagen monomers exist of three different chains. These are called the α-chains.

Figure 1.1a Model of the α-chain structure of collagen.
The collagen model was taken from the Brookhaven PDB. The model is of an artificial collagen with the sequence $(Gly\text{-}Pro\text{-}Pro)_{12}$ [5].

Each α- chain consists of about 1000 amino acid residues. Three of these chains together form a stiff helical cable of 3000Å*15Å, which is one of the largest known proteins.

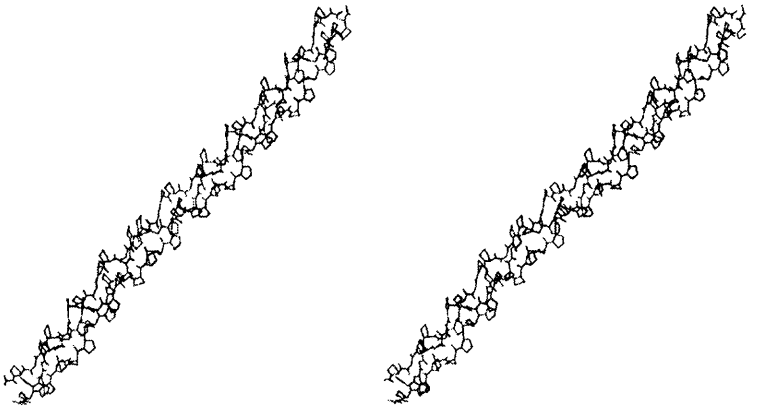

Figure 1.1b Model of the triple helix structure of collagen.

First, the amino acid composition of the collagen α-chain will be discussed. The percentage of prolines in the α-chains is 20%. This is such an extremely high percentage that it easily can be used as a measure for the collagen contents in tissues. Almost a third of the 1000 amino acid residues are glycines (see Appendix)[6]. This is also an enormous high percentage. For example, in hemoglobin this percentage is only 5%. If we take a closer look at the amino acid sequence we will see that every third position is occupied by glycine with an extreme regularity. In many cases glycines are followed by proline or even by two prolines. Roughly, the overall amino acid sequence of the collagen α- chain can be described with $(Gly\text{-}Pro\text{-}Y)_n$.

The polypeptide-chains form left-handed coils. Three of this helical α-chains assemble to form the collagen monomer. They are twisted around each other, forming a right-handed, triple-helical structure. The different α-chains are bonded to each other in the following way:

a. 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.

b. By H-bridges of hydroxyl-groups of hydroxy prolines.

c. By H-bridges with water molecules [2].

All these bonds stabilize the triple-helical structure of collagen. However, the repulsion of the prolines (pyrrolidon rings, see Figure 1.1a) already gives the helical structure and turns the H-side chain of the glycins 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:

a. 4- and 3-hydroxyproline,

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

* Data taken from [7].

** Data taken from [8].

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.

In a normal α-helix distance per amino acid residue is 1.5Å. 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 $(Gly\text{-}X\text{-}Y)_n$ [9]. 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 [10].

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 (idem).

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 [11] [12]. 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 [13] that specific proteoglycans influence significantly the diameter of collagen fibers. The presence of small proteoglycans results in collagen fibrils that was significantly thinner in width.

1.3 Collagen Fibril Formation

Collagen is most abundant in animal tissues as very long fibrils with a characteristic axial periodic structure. The fibrils provide the major biomechanical scaffold for cell attachment and anchorage of macromolecules, allowing the shape and form of tissues to be defined and maintained. How are the fibrils formed from their monomeric precursors? Collagen fibril formation is basically a self-assembly process (i.e. one which is to a large extent determined by the intrinsic properties of the collagen molecules themselves) but it is also sensitive to cell-mediated regulation, particularly in young or healing tissues. Recent attention has been focused on 'early fibrils' or 'fibril segments' of ~10 μm in length which appear to be intermediates in the formation of mature fibrils that can grow to be hundreds of micrometers in length.

The assembly of collagen molecules into fibrils is an entropy-driven process, similar to that occurring in other protein self-assembly systems, such as microtubules, actin filaments and flagella (for a review, see [6]). These processes are driven by the loss of solvent molecules from the surface of protein molecules and result in assemblies with a circular cross-section, which minimizes the surface area/volume ratio of the final assembly. Although the broad principles of collagen fibril self-assembly are generally accepted, less is known about the molecular mechanisms of the assembly process.

Collagen biosynthesis and assembly follows the normal pathway for a secreted protein. The collagen chains are synthesized as longer precursors called procollagens; the growing peptide chains are co-translationally transported into the lumen of the rough endoplasmic reticulum (ER). In the ER, the procollagen chain undergoes a series of processing reactions. First, as with other secreted proteins, glycosylation of procollagen occurs in the rough ER and Golgi complex. Galactose and glucose residues are added to hydroxylysine residues, and long oligosaccharides are added to certain asparagine residues in the C-terminal propeptide, a segment at the C-terminus of a procollagen molecule that is absent from mature collagen. (The N-terminal end also has a propeptide.) In addition, specific proline and lysine residues in the middle of the chains are hydroxylated by membrane-bound hydroxylases. Lastly, intrachain disulfide bonds between the N- and C-terminal propeptide sequences align the three chains before the triple helix forms in the ER. The central portions of the chains zipper from C- to N-terminus to form the triple helix.

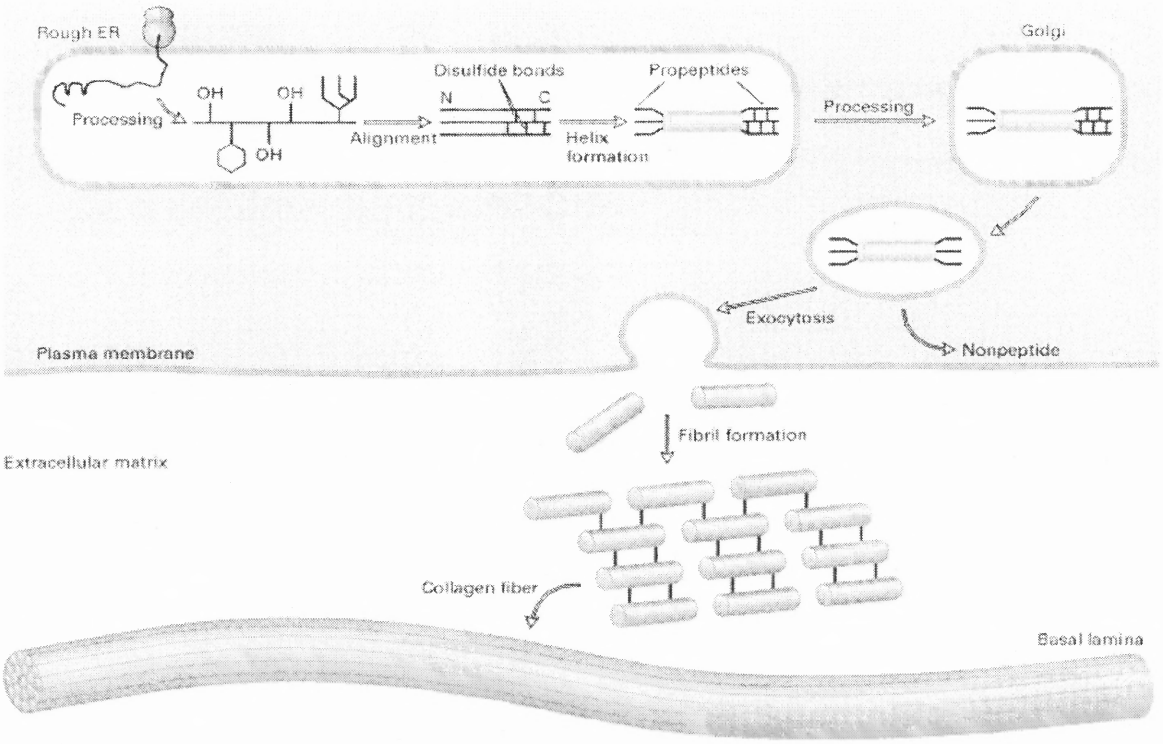

Figure 1.2 Major events in the biosynthesis of fibrous collagens.
Modifications of the procollagen polypeptide in the endoplasmic reticulum include hydroxylation, glycosylation, and disulfide-bond formation. Interchain disulfide bonds between the C-terminal propeptides of three procollagens align the chains in register and initiate formation of the triple helix. The process continues, zipperlike, toward the N-terminus. All modifications occur in a precise sequence in the rough ER, Golgi complex, and the extracellular space, and allow lateral alignment and formation of the covalent cross-linkers that enable helices to pack into 50-nm-diameter fibrils. [After M. E. Nimni, 1993, in M. Zern and L. Reid, eds., Extracellular Matrix, Marcel Dekker, pp. 121 –148.]

After processing and assembly of type I procollagen is completed, it is secreted into the extracellular space. During or following exocytosis, extracellular enzymes, the procollagen peptidases, remove the N-terminal and C-terminal propeptides. The resulting protein, often called tropocollagen (or simply collagen), consists almost entirely of a triple-stranded helix. Excision of both propeptides allows the collagen molecules to polymerize into normal fibrils in the extracellular space. The potentially catastrophic assembly of fibrils within the cell does not occur both because the propeptides inhibit

fibril formation and because lysyl oxidase, which catalyzes formation of reactive aldehydes, is an extracellular enzyme. As noted above, these aldehydes spontaneously form specific covalent cross-links between two triple-helical molecules, which stabilizes the staggered array characteristic of collagen molecules and contributes to fibril strength [18].

1.4 Types of Collagen

There are at least 20 types of collagen, but 80 – 90 percent of the collagen in the body consists of types I, II, and III. These collagen molecules pack together to form long thin fibrils of similar structure. Type IV, in contrast, forms a two-dimensional reticulum; several other types associate with fibril-type collagens, linking them to each other or to other matrix components. At one time it was thought that all collagens were secreted by fibroblasts in connective tissue, but we now know that numerous epithelial cells make certain types of collagens. The various collagens and the structures they form all serve the same purpose, to help tissues withstand stretching [1].

The existence of a family of collagenous proteins in the connective tissues of vertebrates was first identified when cartilage collagen (type II) was found to be genetically distinct from the type I collagen of skin, bone, and tendon. A third collagen (designated type III) was detected in skin. More than 30 different collagenous polypeptides have been found in the extracellular matrix in the form of at least 20 different collagen types. The collagen numbering system (with Roman numerals for each collagen type and Arabic numerals for individual α-chains) to some extent reflects the

relative abundance of the various collagens, in that generally the more abundant collagens were identified earliest [17].

In addition to these collagens, there exist a number of secreted proteins that contain collagenous amino acid sequences and short triple-helical conformations, such as the complement component, C1q, acetylcholine esterase, lung surfactant protein, conglutinin, serum mannose-binding protein, scavenger receptors (AR-I and AR-II), and MARCO. The collagenous sequences in these proteins contribute to their distinctive structures and functions. Since they have no known structural role in the extracellular matrix, however, they are not classified as collagens.

From the data derived from amino acid and gene sequencing, collagen molecules can be grouped into the groups shown in Table 1.2. Fibrillar collagen molecules are characterized by an uninterrupted helical domain of approximately 300 nm. They are synthesized as procollagens comprised of three pro a chains that undergo processing to a chains and subsequently assemble into collagen fibrils and fibers [15]. Fibrillar collagen molecules (i.e. types I, II, III, V, and XI) exhibit several common structural features that reflect the highly conserved exon-intron structure of the genes. Polygonal meshwork-forming collagens (type IV collagen polypeptides) have large triple- helical domains (> 160 kDa) with a length of >350 nm. Their primary structures are characterized by imperfections in the Gly-X-Y- triplet sequence. These interruptions are a particular feature of type IV collagen, in which the helical domain contains more than 20 short stretches of non-helix-forming amino acids [16].

Short triple-helical collagen molecules (types VIII and X) contain interruptions in the helical domains (as in types IX, XII, XIV, and XIX). Collagen types VIII and X show remarkable homology and might, have similar roles in tissues. Type XII and type XIV collagens have similarities to type IX collagen in the helical domain structures. A portion of these triple-helical domains have the potential to interact with fibrillar collagen. Thus, these three types of collagen, plus type XVI and XIX, comprise a group of fibril-associated collagens with interrupted triple helices (FACIT collagens). An alternative approach for classifying the collagens depends on supramolecular structures that might be related to their physiological function. Individual collagen types may themselves represent a family or group of related collagenous structures in the extracellular matrix. Type IV collagen is a family of six homologous α chains (α1, α2, α3, α4, α5, and α6), and type V/XI is a family of six α chains: α1(V), α2(V), α3(V), α1(XI), α2(XI) and α3(XI) [17].

Table 1.2 Classification of Collagens

Type	Known α-chains	Known or putative chain compositions of molecules at present	Length of the domain	Major distribution	Aggregate structure of purified protein*
		Fibrillar Collagen			
I	α1(I) α2(I)	$[\alpha1(I)]_2\alpha2(I)$	300 nm	Almost all connective tissues except hyaline cartilage	Fibril
II	α1(II)	$[\alpha1(II)]_3$	300 nm	Cartilage	Fibril
III	α1(III)	$[\alpha1(III)]_3$	300 nm	Almost similar to that of type I	Fibril
V	α1(V) α2(V) α3(V)	$[\alpha1(V)]_2\alpha2(V)$ α1(V)α2(V)α3(V)	300 nm	Almost all connective tissues	Fibril
XI	α1(XI)α2(XI) α3(XI)	α1(XI)α2(XI)α3(XI)	300 nm	Cartilage	Fibril
V/XI	α1(XI)α2(V)	$[\alpha1(XI)]_2\alpha2(V)$	300 nm		Fibril
		FACIT Collagen (Fibril-associated collagen with interrupted triple-helices)			
IX	α1(IX)α2(IX) α3(IX)	α1(IX)α2(IX)α3(IX)		Surface of the cartilage fibril	Periodically on the cartilage collagen fibrils
XII	α1(XII)	$[\alpha1(XII)]_3$?		Surface of the collagen fibril	Periodically on the cartilage collagen fibrils?
XIV	α1(XIV)	$[\alpha1(XIV)]_3$?		Surface of the collagen fibril	Periodically on the cartilage collagen fibrils?
XVI	α1(XVI)	$[\alpha1(XVI)]_3$?			
XIX	α1(XIX)	$[\alpha1(XIX)]_3$?			
		Short chain collagen			
VIII	α1(VIII) α2(VIII)	$[\alpha1(VIII)]_3$ $[\alpha2(VIII)]_3$		Basement membrane of endothelial cell	Hexagonal array
X	α1(X)	$[\alpha1(X)]_3$?	150 nm	Hypertrophic cartilage	Hexagonal array
		Multiplexins (Protein with multiple triple-helix domains and interruptions)			
XV	α1(XV)	$[\alpha1(XV)]_3$?	150 nm		
XVIII	α1(XVIII)	$[\alpha1(XVIII)]_3$?			
		MACIT (Membrane associated collagen with interrupted triple-helices)			
XIII	α1(XIII)	$[\alpha1(XIII)]_3$			
XVII	α1(XVII)	$[\alpha1(XVII)]_3$?			
		Others			
VI	α1(VI)α2(VI) α3(VI)	α1(VI)α2(VI)α3(VI) $[\alpha1(VI)]_2\alpha2(VI)$?	100 nm	Almost all connective tissues	Beaded microfibril
VII	α1(VII)	$[\alpha1(VII)]_3$?	420 nm	Anchoring fibril	Short dimer
IV	α1(IV)α2(IV) α3(IV)α4(IV) α5(IV)α6(IV)	$[\alpha1(IV)]_2\alpha2(IV)$ α3(IV)α4(IV)α5(IV) $[\alpha5(IV)]_2\alpha6(IV)$	350 nm	Basement membrane sinusoid	Polygonal meshwork

CHAPTER 2

APPLICATION OF COLLAGEN TUBES

2.1 Collagen as Biomaterial

An important aspect of biomedical applications is to make the intended implant or device as biocompatible as possible. Biocompatibility is defined as the "ability of a material to perform with an appropriate host response in a specific application" [19]. In order for an implant to be as biocompatible as possible, certain materials are often chosen because they do not illicit a harmful response from the body. 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 illicit a harmful response during its time of operation or after it has degraded into byproducts. Many materials are carcinogenic and others often cause clotting or encapsulation.

Natural polymers can be more effective than synthetic ones because they are very similar and often identical to the items that are being replaced or augmented. The body therefore may not deem them foreign and an undesirable host response may be avoided. This can avoid the most common problems of toxicity or the inflammatory reaction. A second advantage of a natural material is that it can perform the same tasks on the molecular level as the original as well as the intended function. There is always a chance or immunogenecity of any natural polymer, however, which increases the chance of antibiotic attack on the implant. The immunological response of the body is directed at target sites on the implant proteins. The body can produce antibodies or lymphocytes, which attach to the surface of the implant leading to its degradation. In order to counter

this, these antigenic determinant sites on the protein can be modified chemically. Table 2.1 shows some natural polymers and their general properties [19].

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

Source: Rattner, Buddy et al. Biomaterials Science, Academic Press, N.Y., 84-92, 287-288, 1996.

Collagen is a structural protein which properties make it suitable for various biomedical applications. Collagen has good mechanical properties, a good biocompatibility (depending on e.g. the type of crosslinking, will be discussed later in this chapter), and as a matrix protein it can be used as a scaffold for cell seeding or for regeneration of host tissue [20]. Table 2.2 shows some of the advantages of using collagen as a biomaterial.

Table 2.2 Properties of Collagen as a Favorable Biomaterial

Type of Property	**Property**
Physical- Mechanical	High Tensile Strength Low Extensibility Orientation of Fibers
Physical-Chemical	Controllable Cross-linking (Affects solubility, Swelling, Resorption) Ion Exchanger Function Semipermeability of Membrane
Biological	Low Antigenicity Effect on wound healing/blood clotting

Source: Whyne,C., M.S. Thesis: Evaluation of Crosslinking Methods and Characterization of Surface Features of a Collagen-Based Dermal Equivalent. Biomed. Eng., Rutgers, The State University, N.J., 1-16, 1984.

An added advantage that makes collagen an excellent choice for an implantable biomaterial is the relative similarity among species. Due to collagen's relative standard Gly-X-Y arrangement, species differences among mammals are small [21]. The triple helix configuration does not allow for major substitutes of amino acids between the species as other proteins do, making collagen a relatively constant structure [19]. As a result, the collagen from other species, mainly bovine type I collagen, can be harvested for use in biomedical application. The relatively high amounts of bovine collagen available make it an excellent source of collagen for medical applications after chemical treatment.

2.1.1 Collagen Antigenicity

The collagens are generally considered weak immunogens in comparison to most proteins, which may be due to a lake of tyrosine residues [22]. According to some research, tyrosine had a 1.2% contribution per residue to the antigenicity of a protein. This data suggests that most of the antigenic determinants are of the steric conformation in which tyrosine is frequently involved as immunodominant amino acids [23]. The use of bovine collagen in humans may lead to an antigenic response, however, due to the minor differences in the collagen amino acid sequences and the nonhelical telopeptide region [19]. When purified, collagen is only weakly antigenic [24]. Antigenicity of collagen can be further reduced by (enzymatic) removal of the non-helical telopeptide regions of the molecule [25] or by crosslinking [26]. As a result of these treatment antigenicity is reduced and will allow xenografts to be more accepted in humans without serous immunological response.

2.1.2. Collagen Thrombogenicity

Collagen is highly thrombogenic, as is demonstrated from its use as a hemostatic powder or sponge [20]. 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 [27]. It has been postulated that crosslinking of collagen may decrease its thromobogenicity [28]. Crosslinking can be done by using different crosslinking agents. Two procedures of crosslinking will be discussed later in this chapter.

2.2 Use of Collagen in Small Diameter Vascular Graft

As the incidence and frequency of vascular procedures increases from year to year, researchers are increasingly looking to modified natural materials as a compromise between autografts – replacement vessel taken from the patient's own arteries and veins – and purely synthetic grafts utilize material like ePTFE (extended polytetrafluoroethylene) and Dacron (poly[ethylene terephthalate]). More then 450,000 vascular grafts were used in coronary bypass surgeries in 1999. Other uses for vascular grafts include treatments for blood vessel aneurysms and fistulas, as well as replacement for diseased arteries in other locations in the body. When possible, the best choice for a replacement vessel is an autograft, where sections of the patient's healthy blood vessels (usually veins) are harvested and implanted in the required location. Many patients, 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. There are several other drawbacks to autografts. One problem is the relatively poor long-term patency. 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 thromobogenicity, 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 [40].

Synthetic vascular grafts are usually made of ePTFE, Dacron, or polyester. These materials work well for large diameter vascular grafts (>5-6 mm) but have low long-term patency for small diameter grafts (4 mm). These synthetic graft materials often cause a severe inflammatory reaction. 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 [41].

By incorporating biological materials into a synthetic vascular graft, the host response can be modulated to help insure that the graft will not fail. To reduce the chance of thrombus formation anti-thrombogenic substances can be incorporated into the synthetic graft material system [42]. Incorporating biodegradable materials into the graft material system could further modulate the host response. However, these types of techniques have been able to significantly increase long-term patency.

In these cases, the most common form of treatment has been the use of synthetic polymeric materials, like Dacron, to form either permanent or resorbable replacements for the damaged vessels. In cases where the graft can be of a large diameter (greater than 5-6 mm), the synthetic material has been effective. However, in situations where a smaller vessel diameter is required, the synthetic material 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.

The use of collagen as a material for a synthetic vascular graft is quite promising because it is biodegradable and has good mechanical properties. 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.

According to the discussion in previous section, collagen is antigenic and thromobogenic. It is also biodegradable so if it is not treated it degrades very quickly in body. Collagen must be treated to get desired properties as vascular grafts.

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 at 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 [43].

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 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.

2.3 Cross-linking of Collagen

Cross-linking of collagen biomaterials is often applied to control or reduce the in vivo resorption rate or to improve mechanical properties of materials [29][30]. 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.

The more prevalent method of cross-linking is through chemical treatment. 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 [32].

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 [30].

Crosslinking of collagen in commercial collagen-coated synthetic vascular grafts is commonly carried out using glutaraldehyde or formaldehyde [33]. 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[33]. Figure 2.1 shows the mechanism of crosslinking with glutaraldehyde.

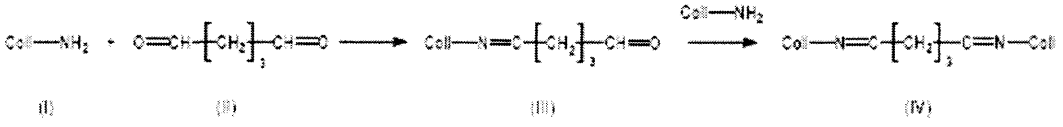

Figure 2.1 Mechanism of crosslinking with glutaraldehyde.

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 [34]. It has been shown that residual glutaraldehyde completely inhibits in vitro fibroblast proliferation at concentrations as low as 3 ppm [35]. 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 [36].

Cross-linking 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, [37] 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.

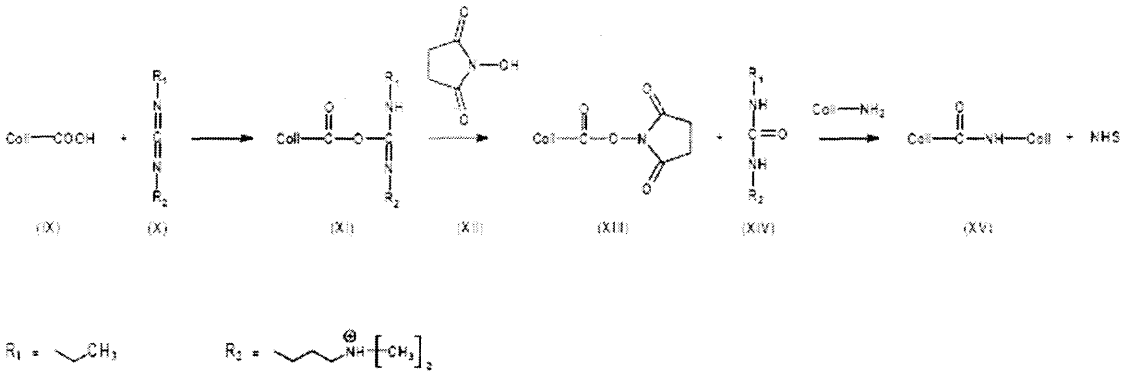

Figure 2.2 Mechanism of crosslinking with EDC/NHS.

EDC/NHS crosslinked collagen is reported to be no-cytotoxic in vitro, and biocompatibility was demonstrated in animal models [38]. When subcutaneously implanted in rates, calcification of EDC/NHS crosslinked collagen proved to be very low compared to glutaraldehyde crosslinked collagen [39].

CHAPTER 3

MATERIAL AND EXPERIMENTAL METHODS

3.1 Collagen Suspension Preparation

The collagen suspension used to prepare the tubes for these experiments was prepared by the process, 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 gms of frozen bovine tendon was sliced using the NBI Nantsune deli slicer. The slices of tendon were ground using electric meat grinder (w/4.5 mm grinder plate). To determine the % solid weight in the ground tendon small sample the wet tendon was weighed and then dried it in an 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.

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 the enzyme activity was noted. 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 fibers were strained and hand squeezed to remove excess water and placed into the enzyme deactivation solution. 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 three 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 treatment, fibers are 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. 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 the fibers in the water. The fibers were allowed to soak in this condition for 10-15 minutes. This mixture was than blended for 30 second in laboratory blender followed by allowing it to sit for 15 minutes and dissipate the heat it received by blending and not letting the temperature go up. Blend the mixture 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 were become thick paste. The paste then transferred in bottles and stored in refrigerator. Collagen suspension was centrifuged for enough time to remove air bubbles if necessary before using them to extrude tubes.

3.2 Collagen Tubes Extrusion Protocol

The collagen tubes were extruded using a special collagen extrusion machine developed by ZOKO spol. S r.o. of Czechloslovakia. 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 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 are then cut in half lengthwise to provide adequate length and ease of access.

3.2.1 Protocol for Pre-extrusion

Two types of rods were used as guide rod in extrusion process, 36" long Teflon rods and 36" Stainless Steel rod. Teflon rod didn't need any treatment except just cleaning up. But stainless steel rods needed pre-extrusion treatment. For that 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 providing a coating to the rods. Then the guide rods were hung for 1-2 minute to drain off excess glycerin to the bath.

Before starting up for new study or new batch with different collagen concentration, all the parts of the extrusion head were disassembled and cleaned to prevent any contamination of the previous batch traces.

3.2.2 Protocol for Extrusion

After turning the machine on the allowing to 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. Collagen was then hand packed tightly into the cylinder until it was approximately ¾ full. The desired extrusion head and nozzle were attached to the

machine after ensuring the guide rod would fit through the nozzle without any interference. The nozzle used was 3/16 in. diameter and extrusion head was 3/8 in. diameter. The cylinder was then sealed and the tube from the cylinder head was run into the extrusion head manifold. The swinging arm was closed so that it sat directly over the extrusion head. A coated stainless steel rod was taken from the hanging rack and inserted through the bottom of the nozzle up through the extrusion head and then clamped into the swinging arm. The machine settings were then entered for the desired extrusion rate, rotation speed, and linear pull speed. In order to ensure no stoppage of the tightly packed collagen, the extrusion was started and collagen filled the tube from the cylinder to the extrusion head manifold.

Once the collagen began filling the manifold, the rotation motor was started. 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 detect for gaps in the tubes. Once the guide rod had completely passed through 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 clear 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 of the tubes with only minor changes to the settings when necessary. After four tubes, however, the cylinder was refilled with collagen to prevent gaps from forming in the tubes. After each trial was finished, the rods were taken for post-extrusion treatment and the machine was cleaned.

3.2.3 Post-extrusion Tube Treatment

The post-extrusion process was primarily concerned with chemically treating the collagen and drying the tubes for removal from the guide rods. Chemical treatment can be divided into two part, coagulation and cross-linking. The tubes prepared from the above protocols were treated differently according to the protocols for different studies. The protocols for different studies will be discussed later in this chapter. Regardless of subsequent treatments, the tubes were extruded according to the protocols written in Section 3.2.1 and 3.2.2 and dried according to procedure described as follows unless mentioned specifically.

After allowing stainless steel rods to sit for 18 to 24 hours under normal atmospheric conditions, the tubes deemed dry. The tubes were immersed completely in the water baths in order to rehydrate the tubes. Tubes were kept in water baths for approximately 1 ½ hour to allow enough water to absorb through the entire tube. At this point, the tubes were cut to the desired length and slipped off of 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 off smoothly without damaging the tube or causing it to lose 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 of the stainless steel rods.

With 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 cardboard boxes for drying. A pair of holes were drilled on the

opposite side wall of specially prepared cardboard boxes. Screws of approximately diameter same as the 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.

3.3 Glutaraldehyde Crosslinking Protocol

Ammonium Hydroxide used in this protocol was received from Fisher Scientific as 5% v/v solution. It was then diluted using distilled water to 1%. Glutaraldehyde, used for this treatment, was also received from Fisher Scientific as 25% v/v solution. It was then diluted using distilled water to 5%. In order to ensure adequate chemical concentration for all the tubes, these chemicals were refilled after every four tubes with the reminder of the chemical being removed for disposal.

Using baths, 1 liter of ammonium hydroxide and 1 liter of glutaraldehyde were prepared for treatment of the tubes. The rod with the extruded collagen tube on it was immersed completely in the ammonium hydroxide solution. The tube soaked for 10 minutes with occasional agitation and rotation of the rod to ensure good chemical absorbance. After the 10 minutes had passed, 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 rod was immersed completely in the glutaraldehyde solution bath and allowed to soak for 5 minutes with occasional agitation and rotation. After 5 minute treatment, the rod was removed from the glutaraldehyde and washed 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.

The procedure was repeated for the remaining samples. Stainless steel rods with the collagen tubes were left to dry under atmospheric conditions for 18 to 24 hours.

3.4 EDC/NHS Cross-linking Protocol

Collagen tubes were crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS). In order to minimize hydrolysis of EDC, crosslinking was carried out in a buffer of 2-morpholinoethane sulfonic acid (MES buffer, 0.05 M, pH adjusted using 10 M NaOH). Extruded collagen tubes were coagulated by 1% ammonium hydroxide for 10 minutes and then washed 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 crosslinking 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 tube were crosslinked so 3.655 gms of EDC and 2.075 gms of NHS was dissolved in 500 ml of MES solution After 1 hour the crosslinking reaction was stopped by washing the collagen film with 0.1 M Na_2HPO_4 solution for 2 hours.

3.5 Protocol for DOE on Extrusion Variable

Primarily three variable effected the extrusion process; extrusion rate, linear pull rate of swinging arm and rotation speed of extrusion head. To evaluate the effect of all the variables on extrusion process and mechanical properties of extruded tubes, a design of experiment (DOE) was run. As previously mentioned, three variables were considered as input parameters in this design of experiment. The mechanical tensile strength and wall thickness of extruded tubes were considered as response. To make the DOE simpler no noise factor were considered. Otherwise concentration of collagen suspension and diameter of extruded tube can be considered as noise factor in this DOE. Before setting up the levels in this DOE, a range study was performed using waste collagen approximately of same concentration of the collagen would be used in this DOE. The machine was run at different setting and the extruded tubes were visually checked for wall thickness. As a result of this study all the parameter were bracketed for two levels, which can give significant effect in response parameter. To minimize the number of runs, only two levels of the parameters were considered and L_4 Taguchi matrix was used for determining runs. To check the effect very precisely L_8 Taguchi matrix of full factorial matrix can be used with two level DOE or more than two level can also be used. From the experiments, the two levels were decided as given on next page:

Extrusion rate (E): Low (E_1) – 25 cm^2/min

High (E_2) – 40 cm^2/min

Linear Pull rate (L): Low (L_1) – 300 mm/min

High (L_2) – 450 mm/min

Rotation Speed (R): Low (R_1) – 30 rpm

High (R_2) – 80 rpm

Using statistic software Minitab, L_4 matrix was prepared for two levels of three variables. Factorial design and Taguchi design can be used for this study; however, Taguchi matrix was selected because it requires fewer numbers of runs for study. According to matrix, four runs were carried out and two tubes were extruded for each run. 1.6% collagen suspension was used to prepare all the samples for this study. Coagulation of tubes was done by 1% ammonium hydroxide for 10 minutes and cross-linking of the samples was done by 5% glutaraldehyde for 5 minutes. Detailed protocols for coagulation and cross-linking are given in Section 3.3.

Table 3.1 L_4 Taguchi matrix for Design of Experiment

Run Number	Extrusion Rate (E) cm^2/min	Linear Draw Rate (L) mm/min	Rotation Speed (R) rpm
1	E_1 (25)	L_1 (300)	R_1 (30)
2	E_1 (25)	L_2 (450)	R_2 (80)
3	E_2 (40)	L_2 (450)	R_1 (30)
4	E_2 (40)	L_1 (300)	R_2 (80)

Samples prepared for each run were labeled by the DOE - run number followed by sample number; i.e. 2nd sample of 1st run was labeled as DOE-1-2. Five samples were prepared from each run for testing. Two types of responses were evaluated for this study; estimated elastic modulus and wall thickness. Data collected from these tests were analyzed statistically to prepare graphs of effect of all the input parameters on responses and bar chart of effects using demo version of design and analysis of Taguchi experiments software Qualitek-4 from Nutek Inc.

3.6 Study of Coagulation Methods and Collagen Suspension Concentration

Coagulation of tubes was tested for three different methods. 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. Three different concentration of collagen suspension were used for this study 1.6%, 2.0% and 2.5%. Three tubes of all three different concentrations were extruded. Then one tubes of each concentration were reconstituted by using 1% ammonium hydroxide for 10 minutes. Another three tubes was reconstituted using 1% ammonium hydroxide for 5 minutes and then 50-50% of acetone and 1% ammonium hydroxide for 10 minutes. The last three tubes were reconstituted using 50-50 % of acetone and 1% ammonium hydroxide for 15 minutes. Table 3.2 shows the matrix and how the samples were numbered.

Table 3.2: Matrix for Study of Coagulation Method and Collagen Concentration

	1% NH_4OH 10 min.	1% NH_4OH 5min. 1:1 (NH_4OH: Acetone) 10min	1:1 (NH_4OH: Acetone) 15min
1.6% Collagen	1-1	1-2	1-3
2.0% Collagen	2-1	2-2	2-3
2.5% Collagen	3-1	3-2	3-3

The tube that was coagulated using just 1% ammonium hydroxide was washed with water for 5-7 minute in water bath and then cross-linked by 5% glutaraldehyde for 5 minutes. After cross-linking, it was given another water wash for 5-7 minute and then hung on drying rack for 18-24 hours. The other two tubes were directly transferred into

5% glutaraldehyde bath for cross-linking without washing them with water. The samples were tested for DSC, TGA, TMA, swelling test.

3.7 Study of Different Cross-linking Method

Samples for this study were prepared using 2% collagen suspension. The extrusion parameters were set at extrusion speed 25 cm^2/min, linear draw rate 300 mm/min, rotation speed 60 rpm. Then tubes were cross-linked using two different cross-linking methods. Two tubes were prepared for each method. Two tubes were extruded and cross-linked using the protocol documented in Section 3.3 for glutaraldehyde cross-linking. The other method used for cross-linking is described in Section 3.4 with using EDC/NHS. Samples prepared for this study was tested for DSC, TGA, TMA, Swelling ratio.

3.8 Study of Cross-linking Time for Glutaraldehyde

Samples for this study were prepared using 2% collagen suspension. The extrusion parameters were set at extrusion speed 25 cm^2/min, linear draw rate 300 mm/min, rotation speed 60 rpm. Tubes were coagulated with 1% ammonium hydroxide for 10 minutes and then washed in water bath for 5 minutes. Coagulated tubes were then cross-linked with 5% glutaraldehyde for 5 min., 15 min., 25 min. and 35 min. After cross-linking, tubes were washed in water bath for 5 minutes and than hung to dry for 18-24 hours. Dried tubes were removed from the guide rods as described in Section 3.2.3 and analyzed for DSC, TGA, TMA, swelling ratio test.

3.9 Swelling Test Protocol

Approximately 1 inch long sample was cut from the dried collagen tubes. Dry weight of the samples was found using a Cahn C-30 electric microbalance calibrated for milligrams. After the dry weight was determined, the samples were placed into phosphate buffer solution (PBS) solution of pH 7.4 for 1 hour at room temperature. Separate aluminum pan was used for each sample to soak. After 1 hour, the samples were removed from the PBS solution and excess solution accumulated inside the tube was removed vie paper towel as well as any excess surfaced water. The wet weight of the sample was determined. The following formula was used to determine the percentage swelling of the samples.

Swelling Ratio = (Wet weight/ Dry weight) × 100 %

3.10 Protocol for Wall Thickness Measurement

Dried collagen tubes were moistened by immersing them into distilled water for 2-3 minutes. Then very thin cross-section of the tube wall was cut with the razor blade and placed on a standard microscope slide. The cross-section of tube wall was dried for 5-10 minutes at atmospheric condition to loss the moisture it gained while moistening it to cut. It was dried to get exact wall thickness as with the moisture trapped inside the wall will swell a little and exact thickness can't be measured. After drying the cross-section was examined under the Zeiss microscope to measure wall thickness. Wall thickness was measure using 6.3x magnification.

3.11 Protocol for DSC, TGA and TMA Tests

3.11.1 Thermal Gravimeteric Analysis (TGA)

Thermal gravimetric analysis was carried out on Q50 Thermogravimetric Analyzer. Non-isothermal experiments were performed in the temperature ranges of 30 to 250 °C at heating rate of 10 degree C per min on each sample. The average sample size was 8 mg and the nitrogen flow-rate was 50 cm^3 per min.

The thermogravimetric data was acquired by a computer connected to Q50 Thermogravimetric Analyzer and analyzed using the associated TGA-Q50 software. The loss of mass in terms of percentage loss in weight from the dependence of the heating rate was the apparent results of this test.

The collagen tubes were cut with a razor blade into tiny bits of pieces and weighed. The scan rate was 10 degree C/min over a range of room temperature to 250 degrees. These parameters were selected based on the previous studies conducted on rat-tail tendon and collagen of pericardium.

3.11.2 Differential Scanning Calorimetry (DSC)

Thermal analysis of collagen tubes was used as a diagnostic tool to evaluate the effect of temperature and stress conditions. The effect of temperature on the collagen fibers, however, has not been adequately studied. This method while easy to apply does not provide any insight into structural changes occurring in the fiber. This study is based on the understanding of collagen structure. Collagen chains consist of three helical polypeptide chains held together by hydrogen bonds. The thermoreversible transformation of collagen to gel is interpreted as the disintegration of these helical structures into random coils. Upon cooling, random coils undergo a conformational coil

to helix transition during which they attempt to reform the original collagen structure. Depending on the temperature and time at which the random coils are allowed to cool, they form less organized gel. In this sense gel is prepared by complete thermal denaturation of collagen [23] followed by partial renaturation through nucleation and growth of crystalline links [24]. The resulting three-dimensional network is responsible for the strength and integrity of the gel. Only a fraction of the macromolecules comprises the crystalline network. The space between the fibrils is composed of disordered amorphous polypeptide chains [25], plasticizers and water providing the elasticity to the collagen fiber.

The objective of this study was to gain an understanding of the structural changes of collagen fiber when exposed to higher temperature on heating and lower temperature on cooling by observing the relative heat flow using ramp and heat-cool-heat using thermal analysis.

Thermal behavior and denaturation temperatures were determined with Q100 differential scanning calorimetry, TA Instruments New Castle, DE. Samples for thermal analysis were prepared as follows. The collagen tubes was cut with a razor blade into tiny bits of pieces and weighed. The weight was 5-9 mg then hermetically sealed into aluminum DSC pans and crimped. An empty pan with a cover was also crimped and placed as a reference sample. The equipment was first operated on Heat-Cool-Heat mode. The scan rate was 20 degree C/min over a range of -50 to 225 °C for first cycle and –50 to 300 °C for second cycle. For heat cool heat operation mode, the scan rate was kept at 10 degree C/min for cooling. These parameters were selected based on the previous studies conducted on rat-tail tendon and collagen of pericardium. The same procedure

were used for all the samples. The heat flow at different temperature was measured and the data was acquired by a computer connected with the DSC instrument. Denaturation temperatures for all the samples were determined by analyzing the data of heat flow vs. temperature.

3.11.3 Thermal Mechanical Analysis (TMA)

This test was conducted using the TMA 2940 Thermo mechanical Analyzer made by TA Instruments. This test was used to evaluate the effect of temperature on the change in dimensions. The results were analyzed using the software. It is expected that as the firmness of the collagen tubes increases, the temperature at which it breaks, also increases.

Method for determining break point

Collagen tubes were cut, using a razor blade, to 3 mm wide films and placed between the clamps. Before the sample was mounted onto the thermocouple tube, the probe was zeroed. The sample was then mounted onto the thermocouple tube. The initial length of the film was measured automatically by the instrument. The sample was then subjected to the non-isothermal temperature ranging from room temperature to 325° C. The same procedure was used for all the samples. Heating rate was kept at 20 °C/min for all the testing.

Method for the stress strain curve

This type of TMA test was performed to measure the estimated elastic modulus of DOE samples. Tubes were moistened with distilled water to make them flexible. Then the tubes were flattened carefully and placed between the clamps. After zeroing the probe, the sample was placed on the thermocouple tube. The initial length of the film

was measured automatically by the instrument. The sample was then subjected to isothermal temperature. The temperature is kept constant at body temperature i.e. 37 degree C. After equilibrating at this temperature for 5 minutes, the sample was subjected to force ranging from 0 to 1 N at a rate of 0.1 N/min. The same procedure was used for all the samples of DOE.

CHAPTER 4

RESULTS AND DISCUSSION

According to extrusion protocols, tubes were successfully extruded. Two different types of guide rods were used for extrusion; stainless steel rod and Teflon rod. Handling of Teflon rod was easy, as it didn't need to be glycerin coated before using it in the extrusion equipment. However, Teflon rod can not remain straight and hence, when the guide rod comes out from the extrusion head it can not remain in center and because of this, the wall thickness varies in longitudinal cross-section of the tube. Because of this problem with Teflon rod, the stainless steel rods were used for all the studies. A solution for this problem could be to use Teflon coated metal rod. Teflon coated metal rods can remain straight and coating of Teflon can serve the advantage of Teflon surface.

As part of different studies, tubes were extruded at different settings and treated according to the study protocols. The wall thickness varied by changing the extrusion process variables. The tubes, which were crosslinked with glutaraldehyde, became yellowish after treatment. The thicker the tube the darker it is in color. After taking it out from the guide rod and drying it in the specially prepared drying box, it was observed that the thicker tubes shrinks more. After final drying, the tubes with thicker wall had small inner diameters. From this phenomenon it can be derived that if specific inner diameter is required for dried tube it can not just be obtain by using the same diameter guide rod but the wall thickness and the shrinkage of tube has to be considered as well.

4.1 Results of DOE

According to the protocol of DOE, tubes were extruded using different settings and treated with ammonium hydroxide and glutaraldehyde. Samples for all the runs were tested according to the protocols of wall thickness measurement and elastic modulus. Both wall thickness and estimated elastic modulus were determined successfully. Wall thickness values for all the DOE runs are given in table 4.1

Table 4.1: Wall Thickness Values for DOE Runs

Run Number	Extrusion Rate (E) cm^2/min	Linear Draw Rate (L) mm/min	Rotation Speed (R) rpm	Avg. Wall Thickness in μm
1	E_1 (25)	L_1 (300)	R_1 (30)	116.3
2	E_1 (25)	L_2 (450)	R_2 (80)	81.4
3	E_2 (40)	L_2 (450)	R_1 (30)	100
4	E_2 (40)	L_1 (300)	R_2 (80)	400

The determined values were statistically analyzed using the demo version of Taguchi design and analysis software Qualitek-4. Analysis of the results gave the effect of extrusion variable.

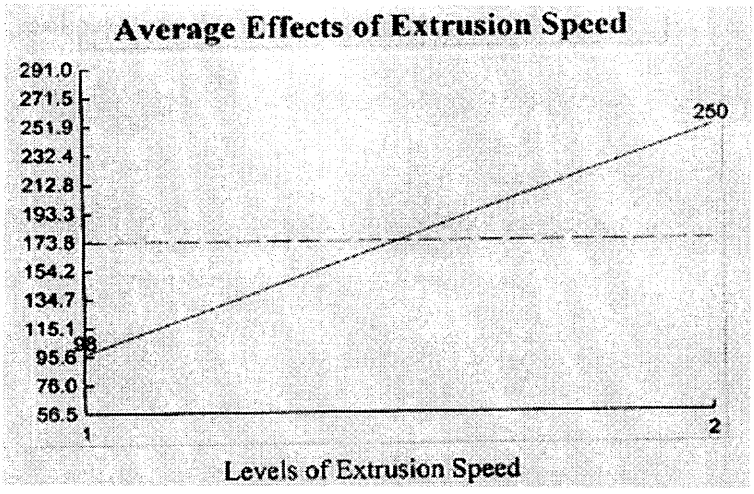

Figure 4.1a Average effects graph of extrusion speed on wall thickness of tube.

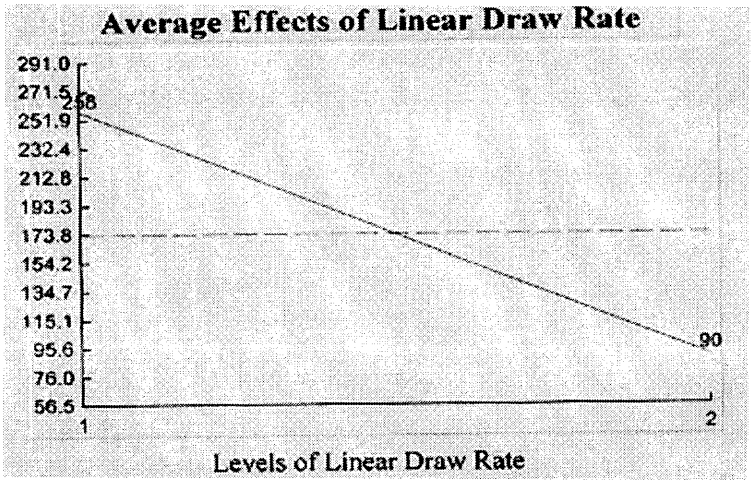

Figure 4.1b Average effects graph of linear draw rate on wall thickness of tube.

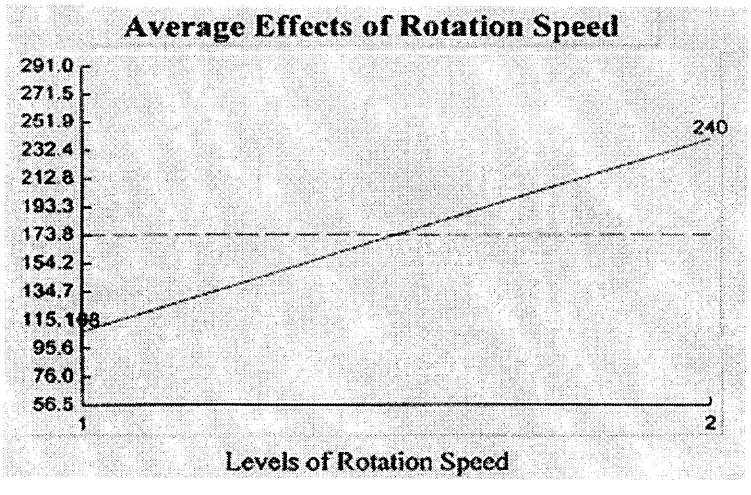

Figure 4.1c Average effects graph of rotation on wall thickness of tube.

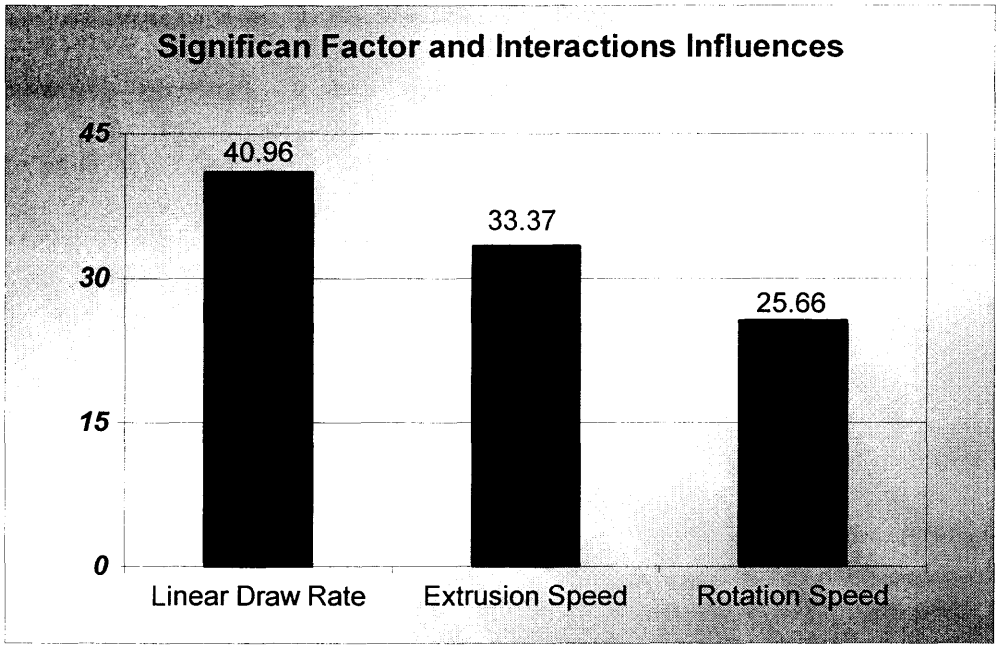

Figure 4.2 Influence of extrusion process variables on wall thickness of extruded tubes.

From figure 4.2 it is concluded that linear draw rate has maximum effect on wall thickness of extruded tubes and rotation speed has the minimum influence on wall thickness. Therefore, linear draw rate of guide rod is the significant factor to control the wall thickness of tubes. Figure 4.1a, 4.1b and 4.1c shows the effect of two levels variables on the wall thickness. Figure 4.1a, graph of average effect of extrusion speed shows that, if the extrusion speed is increased with keeping the other two factors constant, the wall thickness will increase. Graph of average effect of linear draw rate in Figure 4.1b has negative slope. From this it can be derived that linear draw rate has negative effect on wall thickness of tube. Therefore, when linear draw rate is increased and the other two variables are kept constant, the wall thickness of tube will decrease. Graph of average effect of rotation speed has positive slope hence it shows that when the rotation speed is increase and the other two variables are kept constant wall thickness will increase. However, Figure 4.2 shows that the influence of rotation speed is lower than

extrusion speed and linear draw rate so change in rotation speed doesn't cause as much difference in wall thickness as linear draw rate and extrusion speed do.

Table 4.2 Main Effects of Extrusion Variables on Wall Thickness of Tube

Factors	Wall thickness in mm at level 1 (L_1)	Wall thickness in mm at level 2 (L_2)	$L_2 - L_1$
Extrusion Speed	97.5	250	152.5
Linear Draw Rate	257.5	90	-167.5
Rotation Speed	107.5	240	132.5

Table 4.2 highlights the numbers graphed in figure 4.1 and 4.2. Second and third column of the table give the value of wall thickness, which particular variable will give at level 1 and 2.Fourth column of the table gives the slope of the all three graph given Figure 4.1. The values of the slope give the measure of influence of that variable on the process and the negative sign indicates that that variable has negative effect.

To determine the estimated elastic modulus of tubes, graph of TMA stress-strain test was used. From the TMA stress-strain test data, graph of force vs. dimension change was prepared and slope of this graph was calculated. Then cross-sectional area of the tube was calculated by considering the inner diameter of tube as 4.762mm and wall thickness, calculated in previous section.

Cross-sectional area of tube (A) = $\pi((\text{ID of tube} + \text{wall thickness})^2 - (\text{ID of tube})^2)/4$

The length of all the samples (l_0) was kept 12.6 mm. From this data the estimated elastic modulus of the tube was caluculated as follows:

Estimated elastic modulus $\approx$ (slope of graph) $\times$ (l_0/A)

Figure 4.3 shows the graph of force vs. dimension change for DOE run 1. Similar graphs were prepared for all four runs and estimated elastic modulus was calculated for all the runs. Table 4.3 gives the value of estimated elastic modulus for all the runs.

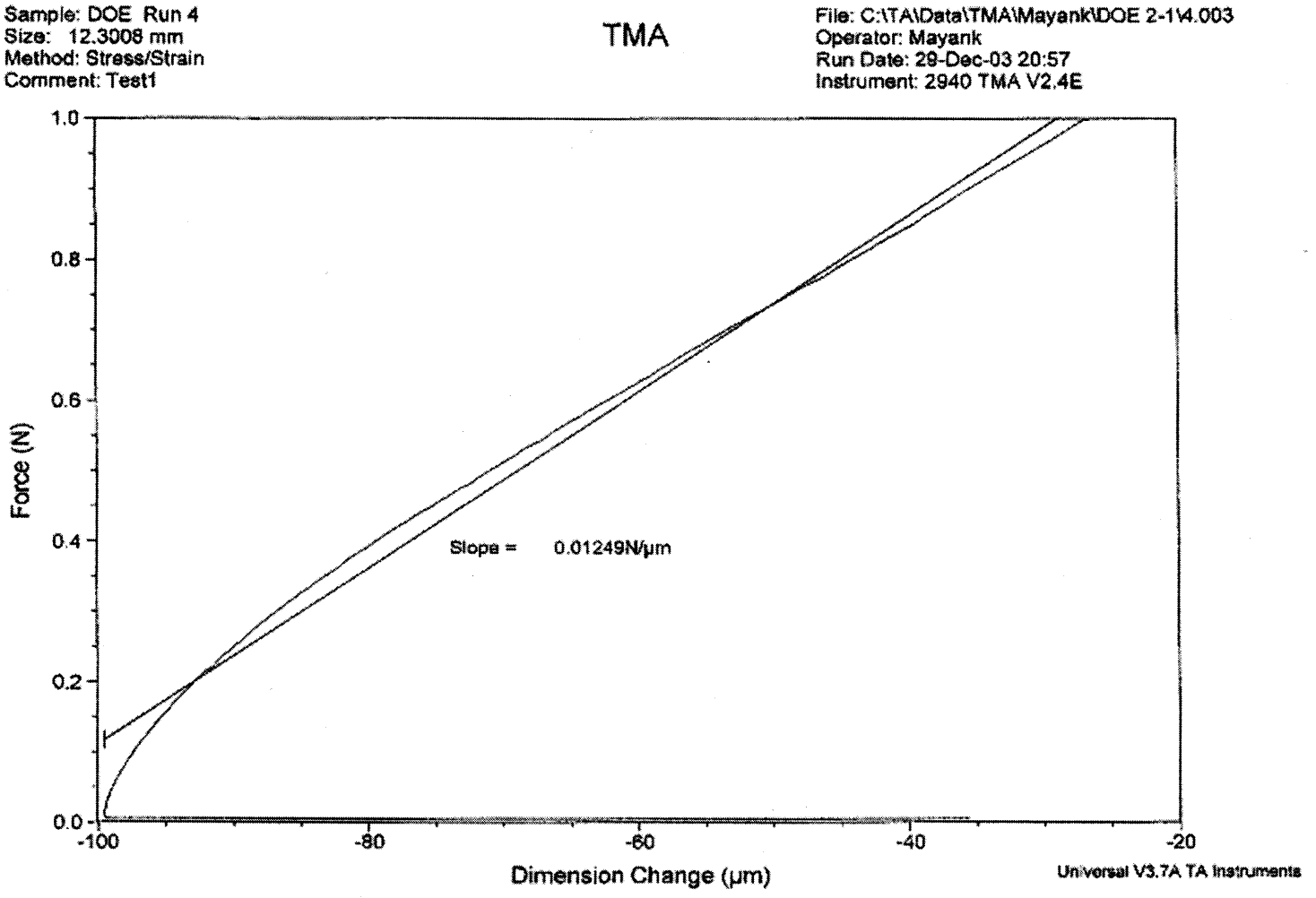

Figure 4.3 Stress strain curve for DOE samples.

Table 4.3 Results of Elastic Modulus for DOE Runs

Run Number	Extrusion Rate (E) cm^2/min	Linear Draw Rate (L) mm/min	Rotation Speed (R) rpm	Slope of Force-Dimes. Change plot N/m^2
1	E_1 (25)	L_1 (300)	R_1 (30)	1.117×10^9
2	E_1 (25)	L_2 (450)	R_2 (80)	1.024×10^9
3	E_2 (40)	L_2 (450)	R_1 (30)	1.226×10^9
4	E_2 (40)	L_1 (300)	R_2 (80)	0.509×10^9

The determined values were statistically analyzed using the demo version of Taguchi design and analysis software Qualitek-4. Analysis of the results gave the effect of extrusion variable. The analysis was performed considering the bigger the better option with averaging the data.

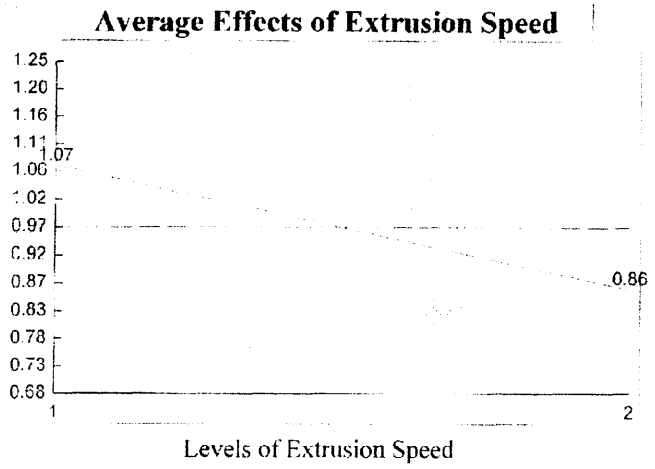

Figure 4.4a Effect of extrusion speed on estimated elastic modulus.

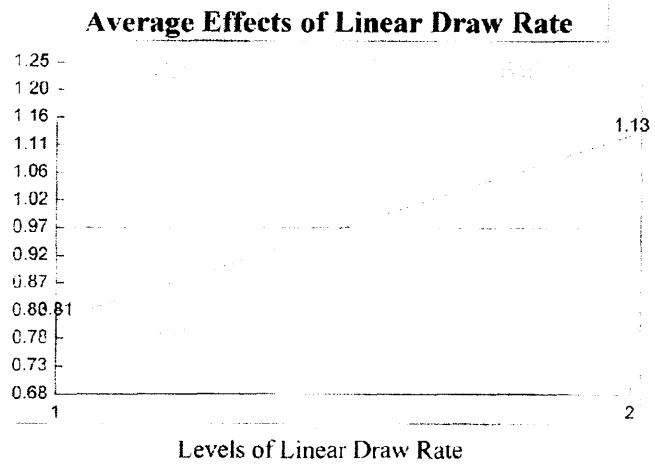

Figure 4.4b Effect of linear draw rate on estimated elastic modulus.

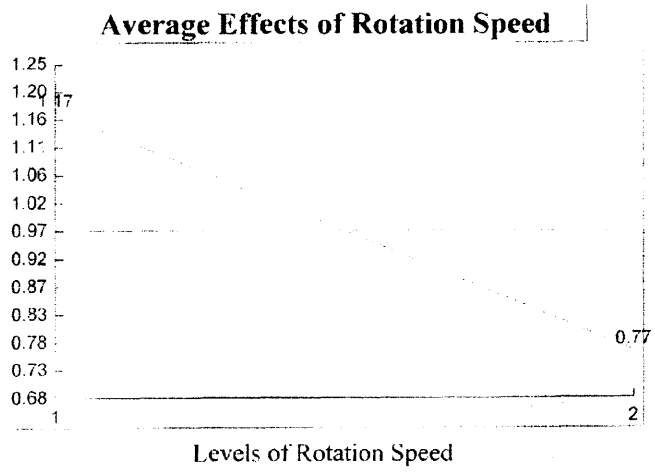

Figure 4.4c Effect of rotation speed on estimated elastic modulus.

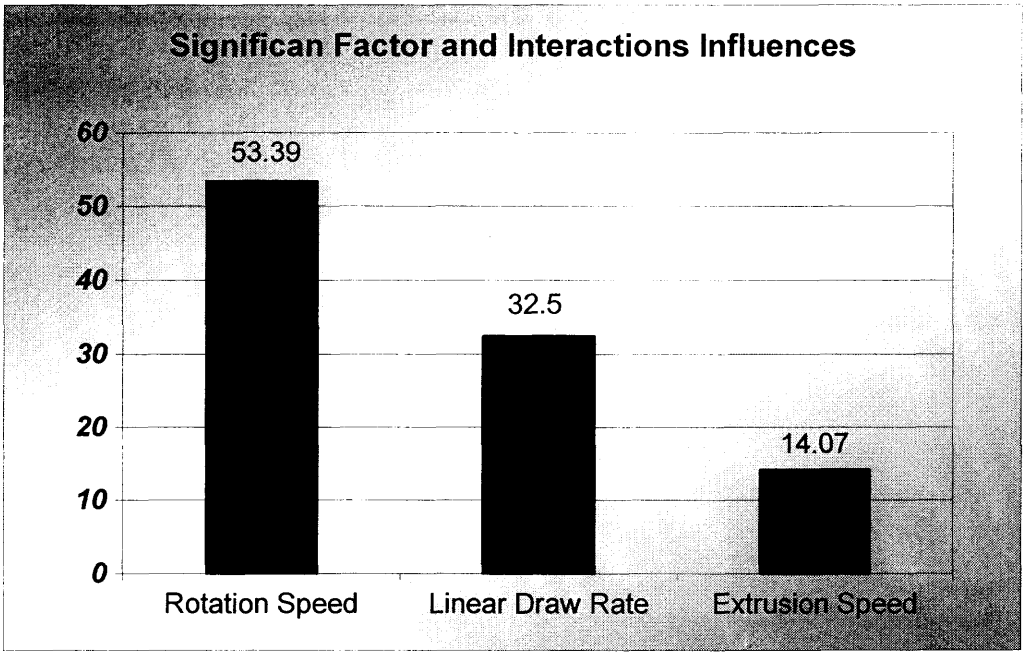

Figure 4.5 Influence of extrusion process variables on estimated elastic modulus of extruded tubes.

Figure 4.5 shows the influence of all three variables on elastic modulus of extruded tubes. The numbers of the columns gives the percentage of influence on estimated elastic modulus. This results shows that rotation speed has highest influence on elastic modulus and extrusion speed has minimum influence. From the graphs of effect of each variable in Figure 4.4a, 4.4b and 4.4c, it is observed that rotation speed and extrusion speed has negative effect on elastic modulus. That means, by increasing the rotation speed and extrusion speed within the specified range of both the variables, elastic modulus decreases therefore tube becomes more compliance. Linear draw rate has positive effect on elastic modulus therefore by increasing the linear speed within the specified range of linear draw rate, elastic modulus will increase and tube becomes less compliance.

Table 4.4 Main Effects of Extrusion Variables on Elastic Modulus

Factors	Elastic Modulus in N/m^2 at Level 1 (L_1)	Elastic Modulus in N/m^2 at Level 2 (L_2)	$L_2 - L_1$
Extrusion Speed	1.07×10^9	0.86×10^9	-0.21×10^9
Linear Draw Rate	0.81×10^9	1.13×10^9	0.32×10^9
Rotation Speed	1.17×10^9	0.77×10^9	-0.40×10^9

Table 4.4 gives the highlights of Figure 4.3 and 4.4. Second and third column of the table give the value of estimated elastic modulus, which particular variable will give at level 1 and 2. The fourth column of the table gives the slope of the all three graph given Figure 4.3a, 4.3b and 4.3c. The values of the slope give the measure of influence of that variable on the process and the negative sign indicates that that variable has negative effect.

4.2 Results of Coagulation Method and Collagen Concentration Study

To study the effect of collagen concentration and coagulation method this study was performed according to the protocol. The tubes were extruded and treated according to the protocol successfully. Results of TGA test are given in Table 4.

Table 4.5 Weight % at Different Temperature

Run #	Weight % at 100°C	Weight % at 200°C	Weight % at 250°C
1-1	95.1	90.2	88.6
1-2	94.7	90.0	88.4
1-3	95.4	90.4	88.8
2-1	96.5	91.2	88.8
2-2	96.4	90.5	88.8
2-3	96.5	90.7	88.9
3-1	95.6	89.8	88.4
3-2	95.6	89.6	88.2
3-3	95.5	90.0	88.6

Figure 4.6 gives a graph of weight% vs. temperature and rate of weight loss vs. temperature. Weight% at different temperature is determined as given in Figure 4.6.

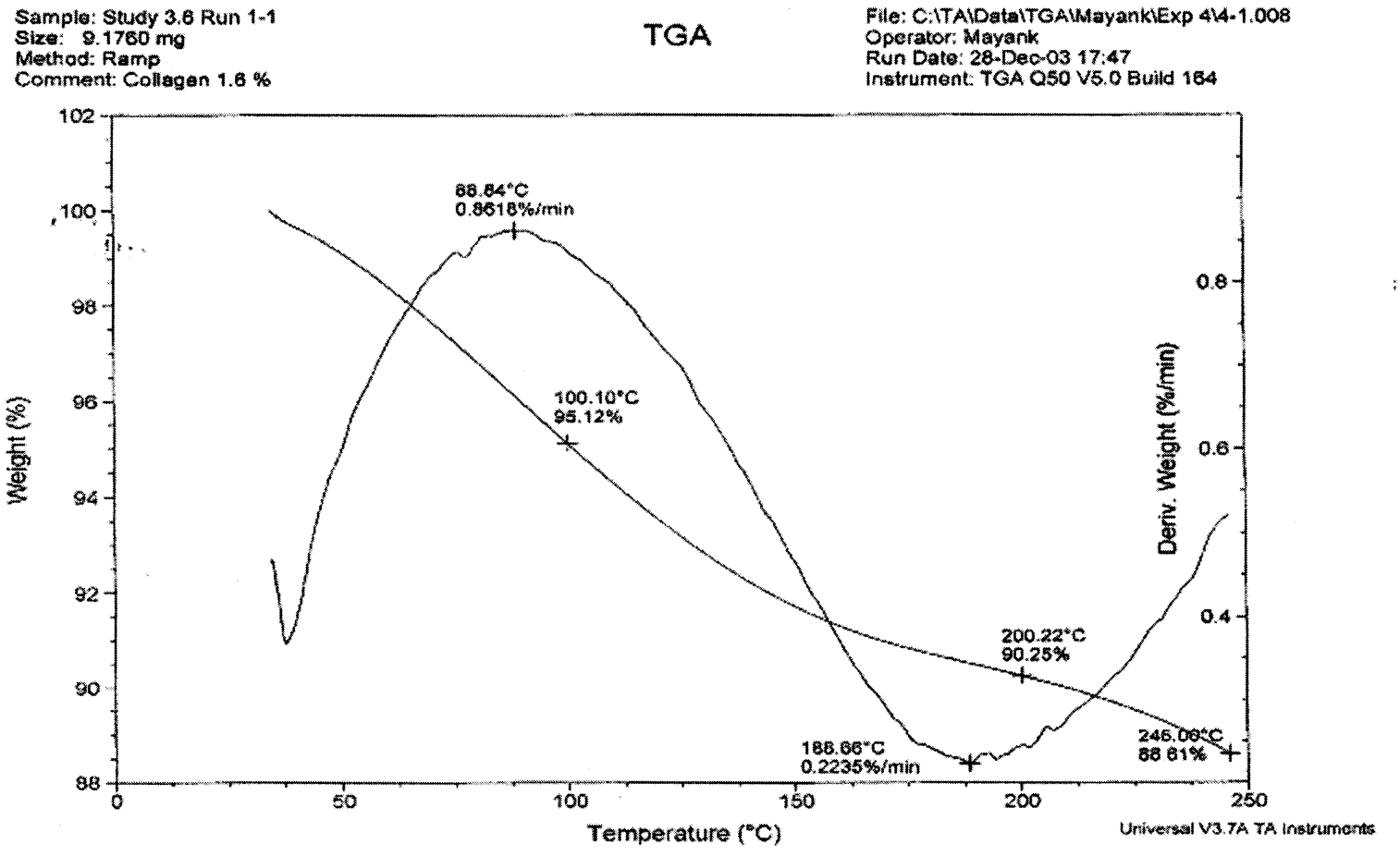

Figure 4.6 Graph of TGA test.

From the data of weight % at different temperature given in Table 4.5 it is observed that weight% at different temperature for all the runs is almost the same. From this it is concluded that there is no effect of collagen concentration or coagulation method on the weight loss of the tubes. But the rate of weight loss at particular temperature varies for different concentration. Temperature at which highest and lowest rate of weight loss was observed is given in Table 4.6.

Table 4.6 Temperature at Highest and Lowest Rate of Weight Loss

Run #	Highest rate of weight loss at Temp °C	Lowest rate of weight loss at Temp °C
1-1	88.38	188.67
1-2	84.10	185.11
1-3	94.65	194.12
2-1	110.39	213.79
2-2	113.45	215.10
2-3	102.09	201.47
3-1	109.71	216.19
3-2	119.91	216.46
3-3	105.46	203.11

To provide the rationale for this behavior the wall thickness of all three different concentration tubes was measured. The wall thickness of lower collagen concentration tube was observed less as when tubes were extruded at same extrusion variable setting wall thickness of the tube at the time of extrusion is same but because of the more water content in lower collagen concentration tubes the physically bound water evaporates at

the time of drying and leaves the tube with smaller thickness. So tubes extruded with 1.6% collagen concentration has the smallest the wall thickness and tubes extruded with 2.5% collagen concentration has the largest wall thickness. The highest and lowest rate of weight loss was observed at lower temperature for lower collagen concentration tube. This could be because the water from the large wall thickness tubes needs higher temperature to evaporate so 1.6% collagen concentration tubes with smaller wall thickness has higher and lower rate of weight loss at lower temperature compared to 2.5% collagen concentration tubes.

Differential Scanning Calorimetry was performed on all the samples of this study according the protocol for DSC. Table 4.7 gives the data of denaturation temperature for all the runs.

Table 4.7 Denaturation Temperatures for Coagulation Study

Run #	Denaturation temp. °C
1-1	218.69
1-2	237.35
1-3	240.26
2-1	239.83
2-2	236.52
2-3	237.09
3-1	241.44
3-2	241.50
3-3	239.43

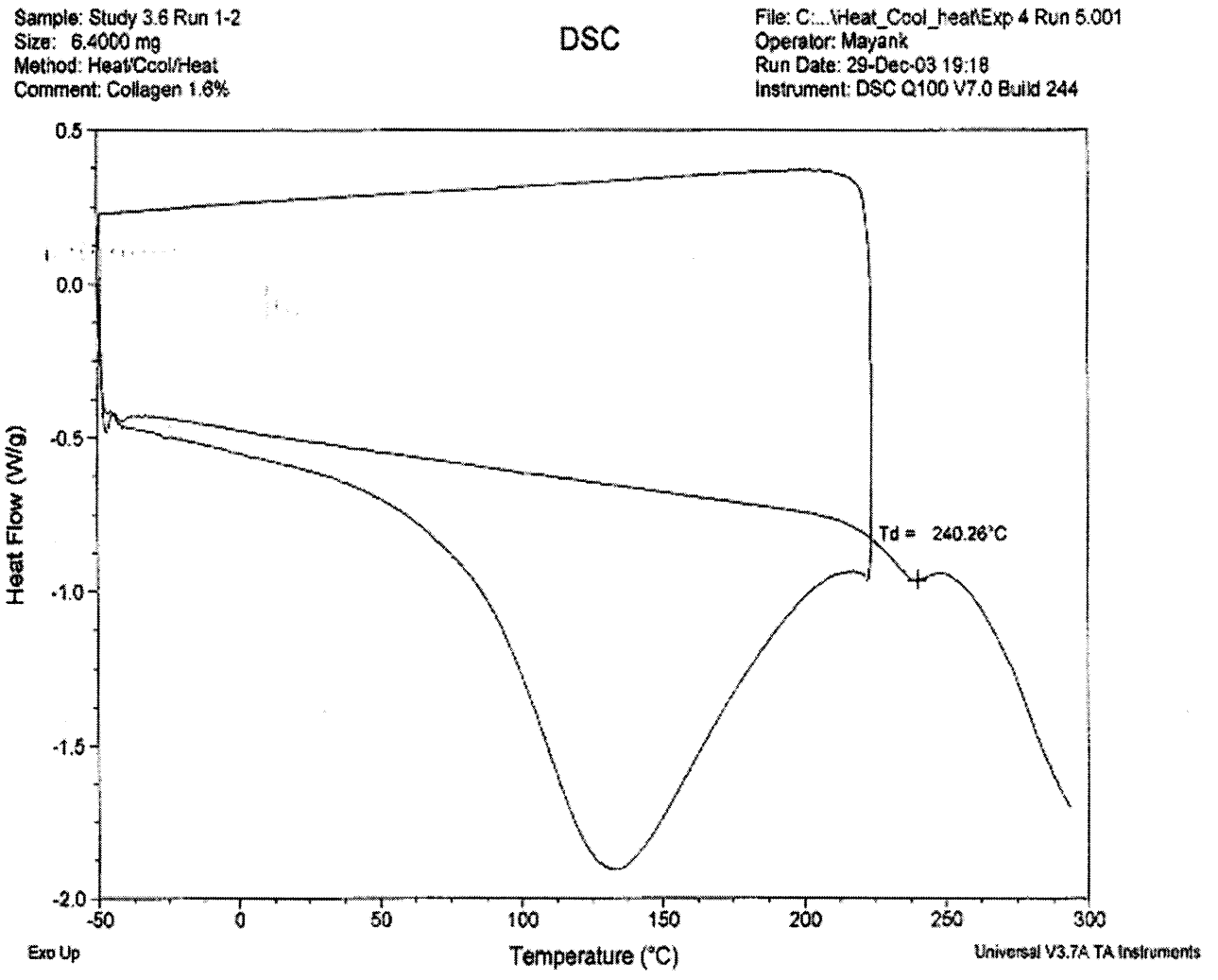

Figure 4.7 DSC graph of heat flow vs. temperature

Figure 4.7 shows a DSC test graph of heat flow vs. temperature and how the denaturation temperature was determined. It is observed from the data on the Table 4.7 that denaturation temperature for all the samples is the same except one of 1.6 % collagen concentration, which was coagulated with just ammonium hydroxide. The reason could be that the 1.6% collagen concentration tube contains more water and when it was removed by acetone the degree of crosslinking can be achieved higher in collagen matrix. That's why 1.6% sample, which is coagulated with ammonium hydroxide and acetone have higher denaturation temperature.

Results of TMA studies and swelling test are documented in Table 4.8 and 4.9 respectively. Figure 4.8 gives the graph of dimension change vs. temperature during the TMA study. Break point temperature was determined from where the graph of dimension change vs. temperature suddenly jumps up. The TMA results show that the break point is highest for coagulation method third samples for all the concentration. The break point increases with increase in degree of crosslinking. However, the results of TMA test are not reliable because of the limitations of the instrument. In Figure 4.8, it is observed that the graph of dimension change vs. temperature becomes straight line parallel to x-axis for some times before it breaks. This happened with all the samples as instrument cannot measure dimension change beyond that point and the graph shows no dimension change after that point. Therefore measurement of break point is also not exact. However, the samples were tested for swelling test, which is the most reliable test to measure the degree of crosslinking.

Table 4.8 TMA Results for Coagulation Study

Run #	Break point °C
1-1	293.53
1-2	268.34
1-3	300.97
2-1	300.27
2-2	289.01
2-3	303.11
3-1	295.71
3-2	277.82
3-3	303.11

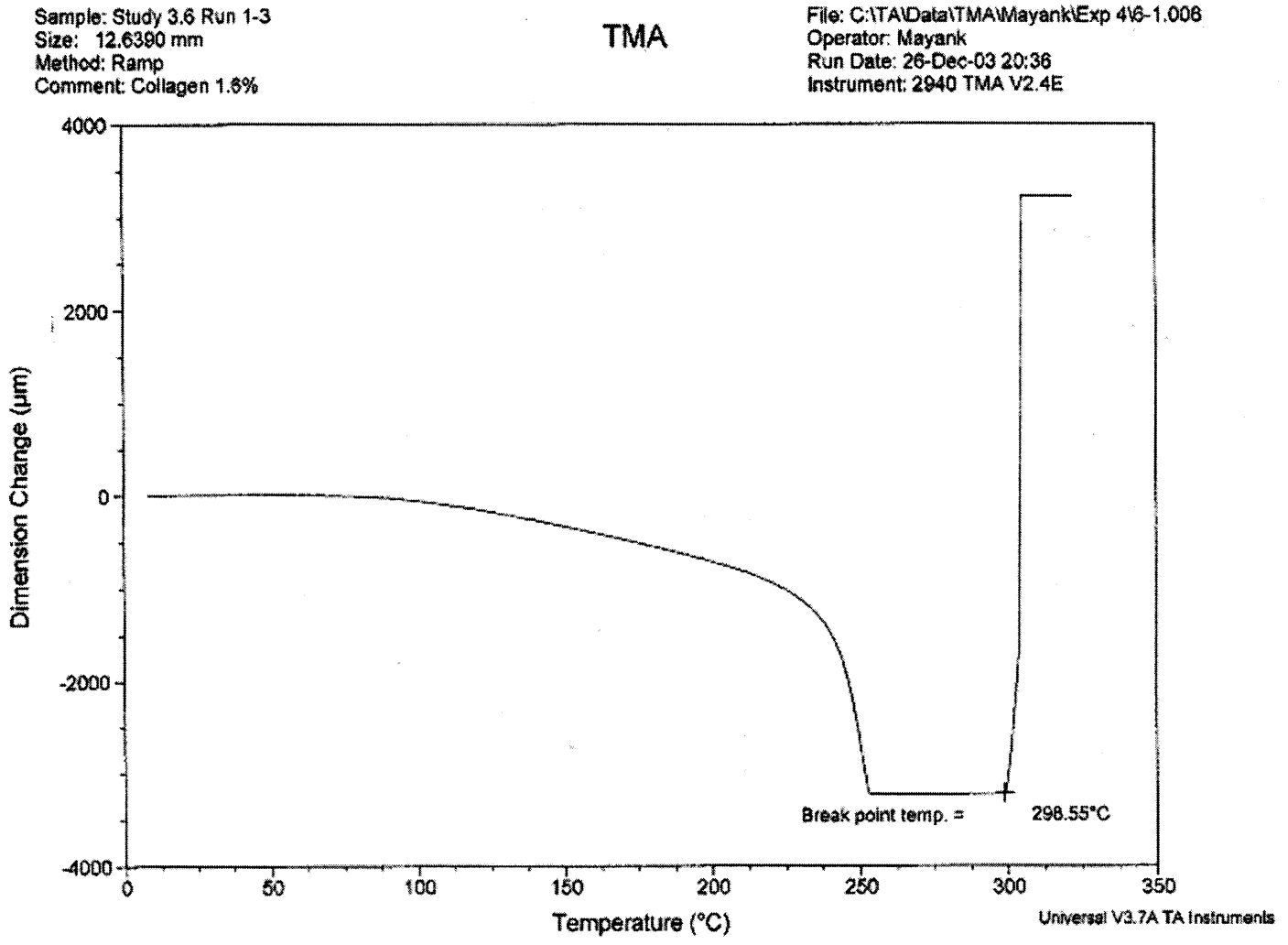

Figure 4.8 TMA graph of dimension change vs. temperature

Results of swelling test also show the swelling ratio is lowest for samples coagulated by third method of the protocol. Swelling ratio decreases when degree of crosslinking increases, as the number of sites available for water to bind is less. Therefore, these results show that the tubes coagulated with third method of protocol, 15 minutes treatment in 1:1 mixture of acetone and 1% ammonium hydroxide, gives highest degree of cross-linking with glutaraldehyde. From this, it can be derived that tubes coagulated by acetone/ammonium hydroxide mixture gives highest degree of crosslinking with 5 % glutaraldehyde. The rationale for this could be that acetone removes the water molecules, which swell the matrix and prevent hydrogen and other forms of electrostatic bonding between the collagen.

Table 4.9 Swelling Test Results for Coagulation Study

Run #	Swelling Ratio
1-1	231.2896406
1-2	224.6062992
1-3	209.1139241
2-1	240.2967523
2-2	230.9872611
2-3	224.0117132
3-1	263.6828645
3-2	249.6296296
3-3	216.5275459

4.3 Study of Crosslinking Methods

Study for crosslinking was performed according to protocols. Tubes were extruded at the same extrusion variable and with same collagen suspension. After the coagulation with 1% ammonium hydroxide tubes were crosslinked with two different methods. Both the samples were removed from the guide rod by swelling the collagen by immersing it in water for one and half hour. It was observed that the tubes crosslinked with glutaraldehyde swelled more and easy to remove from the guide rod while the tubes, crosslinked with EDAC/NHS swelled less and was a little harder to remove from the guide rod. Tubes crosslinked with EDAC/NHS were clear with white shades while tubes crosslinked with glutaraldehyde were yellowish. Besides this, there is no major difference observed between the two samples. Samples were tested for TGA, DSC, TMA, swelling test and universal microscope analysis.

Weight% at different temperature for both the samples is given in table 4.10. From the TGA results it is concluded that there is no much difference in weight% through out the range of 30°C to 250°C.

Table 4.10 Weight% at Different Temperature

Cross-linking method	Weight % at 100°C	Weight % at 200°C	Weight % at 250°C
Glutaraldehyde	96.5	91.5	90.1
EDAC/NHS	96.57	90.94	89.72

Results of DSC test gave the denaturation temperature for both the samples. Denaturation temperature of sample crosslinked with glutaraldehyde is 239.82 ° C and for EDC/NHS sample denaturation temperature is 238.18. There is not big difference

observed in denaturation temperature of both the samples. From the DSC data it cannot be concluded that which crosslinking method gives higher degree of crosslinking.

TMA was performed on both the samples. Break point temperature for sample crosslinked with glutaraldehyde is 300.27 °C and for EDC/NHS crosslinked sample is 273.32 °C. From the results of TMA it can be observed that breakpoint temperature of sample crosslinked with EDC/NHS is less that means the degree of crosslinking is less compared to crosslinking with glutaraldehyde.

Swelling ratio for sample crosslinked with EDC/NHS is 266.25% and for sample crosslinked with glutaraldehyde is 240.29%. As the swelling ratio increases with decrease in degree of crosslinking, this result suggests that the degree of crosslinking achieved by glutaraldehyde is more than can be achieved by EDC/NHS. This is also consistent with the results of the TMA test.

4.4 Crosslinking Time Study Results

To evaluate the effect of time on crosslinking process with 5% glutaraldehyde this study was performed. Tubes were extruded at mentioned in protocol with 2.0% collagen suspension. After coagulation, tubes were treated for crosslinking for different time period. One tube was not crosslinked at all to set the base line. The results for TGA test are given in table 4.11. This results shows that there is no effect of crosslinking time on weight loss by increase in temperature.

Table 4.11 Weight % at Different Temperature for Crosslinking Time Study

Crosslinking time	Weight % at 100°C	Weight % at 200°C	Weight % at 250°C
No crosslinking	93.4	86.9	83.5
5 min	96.5	91.2	88.8
15 min	96.4	90.7	88.9
25 min	96.2	90.2	88.3
35 min	96.5	90.7	88.7

Denaturation temperature was determined using the DSC data. Denaturtion temperatures for all the runs are given in table 4.12. From this data it is seen that denaturation temperature increases with crosslinking as sample without crosslinking has danaturation temperature much lower than crosslinked tubes. However, denaturation temperature for all the tubes crosslinked for different time is almost same. From this data we cannot say any difference in degree of crosslinking by increasing time of crosslinking.

Table 4.12 Denaturation Temperature for Crosslinking Time Study

Crosslinking time	Denaturation temp °C
No crosslinking	209.98
5 min	240.26
15 min	241.50
25 min	241.92
35 min	237.35

Results of TMA test are documented in Table 4.13. These results show the increase in break point temperature with increase in cross-linking time. Graph of break point temperature vs. crosslinking time is given in figure 4.9. The increase in break point temperature is little after 15 minutes time so it can be concluded that the at 15 minutes almost the highest degree of crosslinking with 5% glutaraldehyde is achieved. After 15 minutes also it continues to crosslink but the process is almost complete by the time and very little further crosslinking can be done.

Table 4.13 TMA Results for Crosslinking Time Study

Run #	Break point °C
1-0	202.75
1-1	300.27
1-2	306.84
1-3	307.59
1-4	308.92

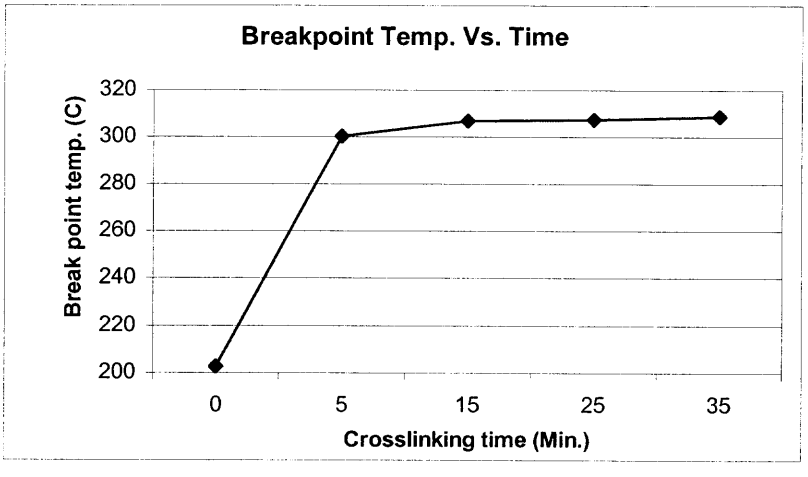

Figure 4.9 Graph of break point temperature vs. crosslinking time.

Swelling test results are given in table 4.14. Swelling ratio decreases with increasing the time of crosslinking. And as mentioned, swelling ratio is inversely proportional to degree of crosslinking. So lower the swelling ratio higher the degree of crosslinking. The results of swelling test show that the increase in time of crosslinking increases the degree of crosslinking. Figure 4.10 gives the graph of 1/swelling ratio vs. crosslinking time. The graph shows that the decrease in swelling ratio after 15 minutes is slow. This supports the results of TMA, which shows that the increase in degree of crosslinking after 15 minutes is slower.

Table 4.14 Results of Swelling Test for crosslinking Time Study

Run #	Swelling Ratio
1	240.296752
2	225.8673
3	220.3007519
4	217.6165803

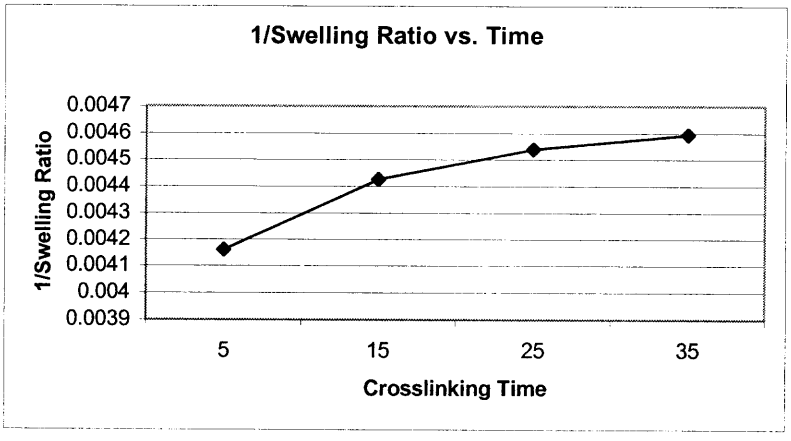

Figure 4.10 Graph of 1/swelling ratio vs. crosslinking time.

CHAPTER 5

CONCLUSIONS

A design of experiment was run and evaluated the effect of extrusion variable on extruded tubes by ZOKO extruder. Graphs of effect of all the variables on wall thickness and elastic modulus of tubes were obtained as a result of DOE. With these graphs the wall thickness and elastic modulus can be controlled. In other words, with the use of these graphs, desirable wall thickness and elastic modulus can be obtained depending on the requirement of its application. From the results of DOE it can be concluded that to reduce the wall thickness, linear draw rate should be increased or extrusion speed and rotation speed should be decreased and vise versa. To reduce the elastic modulus, rotation speed and extrusion speed should be increased and linear draw rate should be decreased.

From the post extrusion studies it was concluded that coagulation with acetone and ammonium hydroxide mixture is more effective than just with ammonium hydroxide. The results of this study showed that samples coagulated with acetone and NH_4OH achieves higher degree of crosslinking. This method is more effective particularly in tubes extruded with lower collagen concentration suspension as acetone removes the water from collagen matrix and thus crosslinking becomes more effective.

Crosslinking study showed that crosslinking with glutaraldehyde is more effective that crosslinking with EDC/NHS. Both TMA and swelling test results showed higher degree of crosslinking with glutaraldehyde. Study of crosslinking time for glutaraldehyde showed that degree of crosslinking increases with time. After 15 minutes of crosslinking increase in degree of crosslinking is slower than in the initial 15 minutes.

From the results of all the post extrusion studies it is concluded that optimized and strongest tubes can be prepared by treating the tubes 15 minutes each with acetone and NH_4OH mixture for coagulation and glutaraldehyde for crosslinking.

Future work on this would be surface characterization to reduce the thrombogenicity of collagen. This may include incorporation with heparin, which is anti-throbogenic. This study showed that glutaraldehyde is more effective way for crosslinking but there is some drawback with glutaraldehyde such as cytotoxicity by release of unreacted glutaraldehyde and its derivative. Possible research could be in direction of reducing the cytotoxic effect of glutaraldehyde in vivo or development of other crosslinking method more effective that glutaraldehyde crosslinking.

From the biological point of view, the future work also includes testing of these tubes in vivo. Develop a relation between the degree of crosslinking and degradation in vivo will help a lot to control the degradation of this tube as graft in vivo. Other possible work includes characterizing the tube biologically to mimic the function of blood vessel.

APPENDIX

HUMAN COLLAGEN CHAIN α(1)

Table A.1 gives amino acid sequence of human collagen chain α(1) including the propeptides.

Table A.1 The Sequence of Human collagen α1(I)

	5	10	15	20	25	30
1	MFSFV	DLRLL	LLLAA	TALLT	HGQEE	GQVEG
31	QDEDI	PPITC	VQNGL	RYHDR	DVWKP	E=-\tPCQI
61	CVCDN	GKVLC	DDVIC	DETKN	CPGAE	VPEGE
91	CCPVC	PDGSE	SPTDQ	ETTGV	EGPKG	DTGPR
121	GPRGP	AGPPG	RDGIP	GQPGL	PGPPG	PPGPP
151	GPPGL	GGNFA	PQLSY	GYDEK	STGGI	SVPGP
181	M(GPSG	PRGLP	GPPGA	PGPZG	FZGPP	GZPGZ
211	PGASG	P)M(GPR	GPPGP	PGKBG	BBGZA	GKPGR
241	PGZRG	PPGPZ	GARGL	PGTAG	LP)GMK	GHR(GF
271	SGLBG	AKGBA	GPAGP	KGZPG	SPGZB	GAPGZ)
301	M/GPPG	PKGNS	GEPGA	PGSKG	DTGAK	GEPGP
331	VGVQG	PPGPA	GEEGK	RGARG	EPGPT	GLPGP
361	PGERG	GPGSR	GFPGA	DGVAG	PKGPA	GERGS
391	PGPAG	PKGSP	GEAGR	PGEAG	LPGAK	GLTGS
421	PGSPG	PDGKT	GPPGP	AGQDG	RPGPP	GPPGA
451	RGQAG	VMGFP	GPKGA	AGEPG	KAGER	GVPGP
481	PGAVG	PAGKD	GEAGA	QGPPG	PAGPA	GERGE
511	QGPAG	SPGFQ	GLPGP	AGPPG	EAGKP	GEQGV
541	PGDLG	APGPS	GARGE	RGFPG	ERGVQ	GPPGP
571	AGPRG	ANGAP	GNDGA	KGDAG	APGAP	GSQGA
601	PGLQG	MPGER	GAAGL	PGPKG	DRGDA	GPKGA
631	DGSPG	KDGVR	GLTGP	IGPPG	PAGAP	GDKGE
661	SGPSG	PAGPT	GARGA	PGDRG	EPGPP	GPAGF
691	AGPPG	ADGQP	GAKGE	PGDAG	AKGDA	GPPGP
721	AGPAG	PPGPI	GNVGA	PGAKG	ARGSA	GPPGA
751	TGFPG	AAGRV	GPPGP	SGNAG	PPGPP	GPAGK
781	EGGKG	PRGET	GPAGR	PGEVG	PPGPP	GPAGE
811	KGSPG	ADGPA	GAPGT	PGPQG	IAGQR	GVVGL
841	PGQRG	ERGFP	GLPGP	SGEPG	KQGPS	GASGE
871	RGPPG	PMGPP	GLAGP	PGESG	REGAP	GAEGS
901	PGRDG	SPGAK	GDRGE	TGPAG	PPGAX	GAXGA
931	PGPVG	PAGKS	GDRGE	TGPAG	PAGPV	GPAGA
961	RGPAG	PQGPR	GDKGE	TGEQG	DRGIK	GHRGF
991	SGLQG	PPGPP	GSPGE	QGPSG	ASGPA	GPRGP
1021	PGSAG	APGKD	GLNGL	PGPIG	PPGPR	GRTGD
1051	AGPVG	PPGPP	GPPGP	PGPPS	AGFDF	SFLPQ

1081	PPQEK	AHDGG	RYYRA	DDANV	VRDRD	LEVDT
1111	TLKSL	SQQIE	NIRSP	EGXRK	NPART	CRDLK
1141	MCHSD	WKSGE	YWIDP	NQGCN	LDAIK	VFCNM
1171	ETGET	CVYPT	QPSVA	QKNWY	ISKNP	KDKRH
1201	VWFGE	SMTDG	FQFEY	GGQGS	DPADV	AIQLT
1231	FLRLM	STEAS	QNITY	HCKNS	VAYMD	QQTGN
1261	LKKAL	LLXGS	NEIEI	RAEGN	SRFTY	SVTVD
1291	GCTSH	TGAWG	KTVIE	YKTTK	SSRLP	IIDVA
1321	PLDVG	APDQE	FGFDV	GPVCF	L	

REFERENCES

1. B. R. Olsen, Y. Ninomiya 1999 Collagens in Guidebook to the extracellular matrix, anchor, and adhesion proteins (2nd ed.) eds by T Kreis & R Vale, pp. 380-408. Oxford University Press Inc., New York, USA.

2. D. R. Eyre. Collagen: Molecular Diversity in the Body's Protein Scaffold. Science, 1980, 207, 1315-1322.

3. M. Chvapil. Industrial uses of Collagen. Fibrous Proteins: Scientific, Industrial and Medical Aspects, 1979, 1, 247-269.

4. G. R. Martin, R. Timpl, P.K. Mu"ller and K. Ku"hn. The genetically distinct collagens. Trends in Biochemical Science, 1985, 10, 285-287.

5. J. M. Chen, C.E. Kung, S.H. Feairheller and E.M. Brown. An Energetic Evaluation of a "Smith" Collagen Microfibril Model. Journal of Protein Chemistry, 1991, 10(5), 535-552.

6. M. L. Chu, W. d. Wet, M. Bernard, J.F. Ding, M. Morabito, J. Myers, C. Williams and F. Ramirez. Human proalpha1(I) collagen gene structure reveals evolutionary conservation of a pattern of introns and exons. Nature, 1980, 310, 337-340.

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

8. D. A.D. Parry and A.S. Craig. The Molecular Structure of Collagen. Developments in Biochemistry, 1981, 22, 63-67.

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

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

11. E. J. Miller. The structure of Collagen. Monographs in Pathology, 1983, 24, 4-15.

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

13. 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.

14. B. Brodsky, and N. K. Shah 1995 The triple helix motif in proteins FASEB J. 9, 1537-1546.

15. K. E. Kadler 1995 Extracellular Matrix 1: Fibril-forming collagens. Protein Profile 2:491-619.

16. Kadler K. E., Holmes D. F., Trotter J. A., Chapman JA Collagen fibril formation. Biochem J 316:1-11, 1996.

17. J. F. Bateman, S. R. Lamandé, and J. A. M. Ramshaw (1996) Collagen Superfamily in Extracellular Matrix Vol. 2 W. D. Comper ed. harwood academic publishers.

18. Lodish, Berk, Zipursky, Matsudaira (4th edition) Molecular Cell Biology, pp. 979-985. W. H. Freeman and Company.

19. Rattner, Buddy et al. Biomaterials Science, Academic Press, N.Y.,84-92,287-288, 1996.

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

21. J. Kroschwitz, Collagen in Encyclopedia of Polymer Science and Engineering, Volume 3, Wiley Interscience Publishers, N.Y., 699-725, 1985.

22. G. N. Ramachandran,. and A. H. Reddi, Biochemistry of Collagen, Plenum Press, N.Y., Chapter 1, 1976.

23. M. Sasaki, S. Takeda, T. Kato, K. Matsuba, Antigenicities of stem bromelain. Contribution of three-dimensional structure and individual amino acid residues, Journal of Biochemistry (Tokyo, 87(3), 817-824, 1980.

24. H. Furthmayr, R. Timpl, Immunochemistry of collagens and procollagens. Int.Rev.Connect.Tissue Res., 7: pp. 61-99, 1976.

25. A. Arem, Collagen modifications. Clin.Plast.Surg., 12: pp. 209-220, 1985.

26. J. Hardin-Young, R.M. Carr, G.J. Downing, K.D. Condon, P.L. Termin, Modification of native collagen reduces antigenicity but preserves cell compatibility. Biotechnol.Bioeng., 49: pp. 675-682 1996.

27. 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.

28. 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.

29. 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).

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

31. T. Koide, M. Daito, Effects of various collagen crosslink techniques on mechanical properties of collagen film. Dental Mat.J., 16: pp. 1-9 (1997).

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

33. 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).

34. 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).

35. 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).

36. 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).

37. 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).

38. 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).

39. 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).

40. 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

41. 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 64, 2000, 103-114.

42. 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.

43. 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.