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## **ABSTRACT**

### **SPINNING OF COLLAGEN FIBERS AND CHARACTERIZING THERMAL, MECHANICAL, TENSILE AND STRUCTURAL PROPERTIES**

**by  
Grace Evangelin Tatagiri**

Collagen is the most important building block in the entire animal world. It plays an important role in regeneration of broken bones and wound healing, also helps grow blood vessels to feed the healing areas. The use of synthetic materials has been extensive, but these materials have their own limits and drawbacks. Poor biocompatibility of these materials is the main issue and often result in inflammations. Hence collagen is being heralded as one of the most appropriate replacement as an implantable material. The reasons being that it is a naturally occurring material, exhibits high biocompatibility low antigenicity and host response. This study involves spinning collagen fibers using dispersion made from bovine tendon, and to modify their properties via cross-linking by treating the fibers with glutaraldehyde. These fibers are characterized for mechanical properties, effect of temperature on dimension changes, temperature dependent heat flow, and temperature dependent weight. The above tests are conducted using TMA, DSC and TGA. The fiber diameters and surface features are studied using SEM. The results of these analyses are compared with cross-linking and without cross-linking for each of the three dispersion percentages. The mechanical behavior of cross linked collagen fibers was enhanced relative to the non cross linked fibers with higher denaturation temperature and lower tensile deformation.

**SPINNING OF COLLAGEN FIBERS AND CHARACTERIZING THERMAL,  
MECHANICAL, TENSILE AND STRUCTURAL PROPERTIES**

**by  
Grace Evangelin Tatagiri**

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**APPROVAL PAGE**

**SPINNING OF COLLAGEN FIBERS AND CHARACTERIZING IN TERMS OF  
THERMAL, MECHANICAL, TENSILE AND STRUCTURAL PROPERTIES**

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Dedicated to:  
My beloved  
Grandparents, Parents, Sisters and Brothers.

*“Thy word is a lamp unto my feet, and a light unto my path” Psalms 119:105*



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# CHAPTER 1

## INTRODUCTION

### 1.1 General Statement of Problem

Collagen is the most important building block in the entire animal world; it is the tie that binds the animal kingdom together. Being nature's most abundant protein polymer, more than a third of the body's protein is collagen making up 75% of the skin. The more Science learns about the body, the more integral we see collagen to be. Collagen acts as scaffolding for bodies, controls cell shape and differentiation. It plays an important role in regeneration of broken bones and wound healing and also helps grow blood vessels to feed the healing areas. Collagen is the fibrous protein constituent of skin, cartilage, bone, and other connective tissue.

The use of synthetic materials, such as polyester fiber or polytetrafluoroethylene as implants designed to replace diseased or damaged body parts has been extensive. These materials have however, enjoyed limited success. This has been due to the poor biocompatibility of many of these materials, which among other problems frequently initiate persistent inflammatory reactions. Additionally, the failure of the body to integrate these materials, because they do not break down and do not lend themselves to remodeling by tissue cells that may come into contact with them, causes further problems (1).

Collagen is being heralded as one of the most useful biomaterials in the realm of drug delivery applications, for surgical purposes (2), and collagen scaffolds for tissue regeneration (3). The reasons are very clear as collagen offers many unique and advantageous properties that make it a versatile material. First and foremost, because

collagen is a naturally occurring material, it exhibits an extremely high biocompatibility and safety, or conversely elicits a low antigenicity and host response. Secondly, collagen is biodegradable and bioreabsorbable. These properties, in turn, are controlled via regulating the degree of cross-linking. Lastly, collagen is easily modifiable and can be combined with several synthetic polymers to produce a variety of medical devices and drug delivery systems.

## **1.2 Objective**

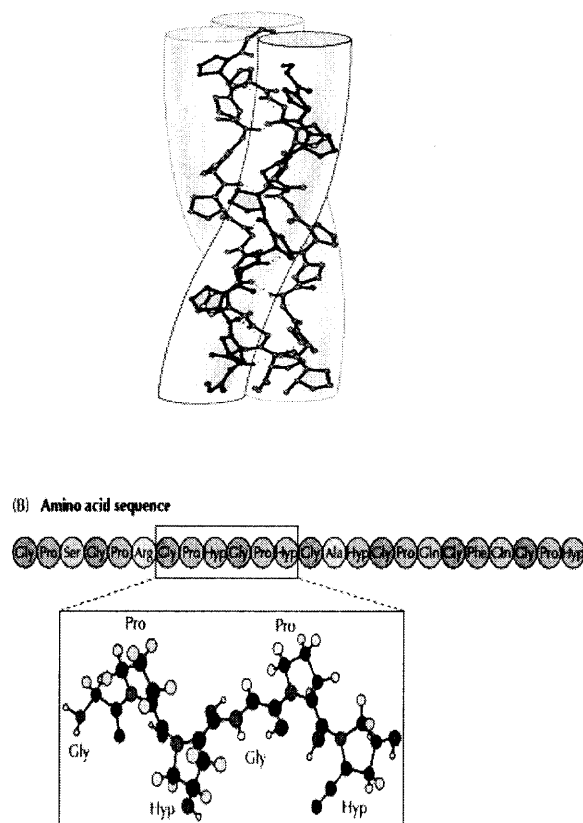
The main objective of this work is to spin collagen fibers using dispersion made from bovine tendon, and to modify their properties via cross-linking by treating the fibers with gluteraldehyde. This work is directed at incorporating the ability to cross link collagen fibers into the set of capabilities of Medical Device and Concept Laboratory. Fibers are spun using a gel, which has various percentages of collagen dispersed in ethanol. The percentages of dispersion under study are 1%, 1.6% and 2%. These fibers are characterized in terms of mechanical behavior, effect of temperature on dimension changes, temperature dependent heat flow, and temperature dependent weight. The above tests are conducted using Thermal Mechanical Analysis (TMA), Differential Scanning Calorimetry (DSC) and Thermal Gravimetric Analysis (TGA) respectively. The fiber diameters and surface features are studied using a Scanning Electron Microscope (SEM). The results of these analyses are compared with cross-linking and without cross-linking for each of the three dispersion percentages.

## CHAPTER 2

### BACKGROUND INFORMATION

#### 2.1 Chemistry of Collagen

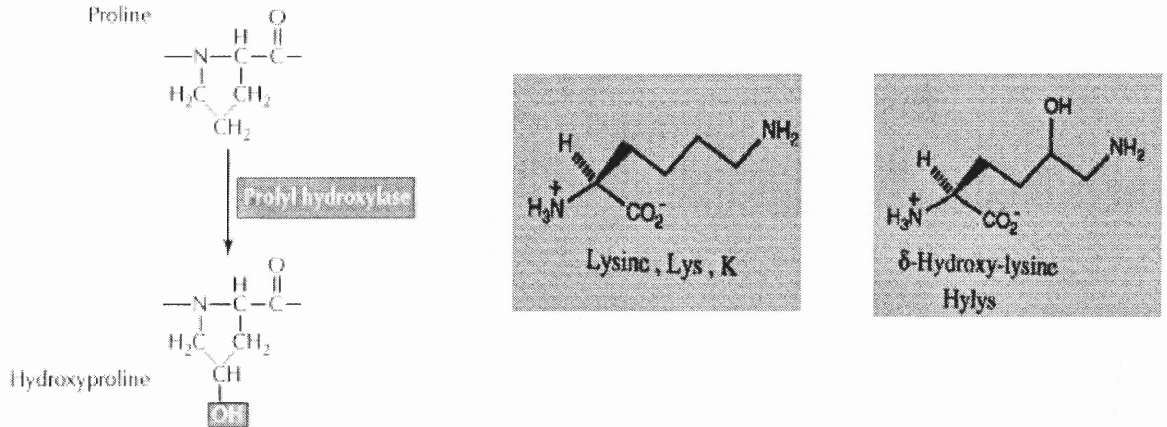
Collagen is characterized by the formation of triple helices in which three polypeptide chains are wound tightly around one another in a ropelike structure. The triple helix domains of the collagens consist of repeats of the amino acid sequence Gly-X-Y. A glycine (the smallest amino acid, with a hydrogen side group) is required in every third position in order for the polypeptide chains to pack together close enough to form the collagen triple helix.



**Figure 2.1** Three-polypeptide chains coil around one another in a characteristic triple helix structure



Proline is frequently found in the X position and hydroxyproline in the Y position, because of their ring structure, these amino acids stabilize the helical conformations of the polypeptide chains (4).



**Figure 2.2** Major Amino Acids of Collagen Proline, Hydroxyproline, Lysine, Hydroxy-lysine.

The primary structure has complete sequence of amino acids along each of three polypeptide chains as well as the location of interchain cross-links in relation to this sequence (5). Approximately one-third of the residues are glycine and another quarter or so are proline or hydroxyproline. The structure of the bifunctional interchain cross-link is the relatively complex condensation product of a reaction involving lysine and hydroxylysine residues; this reaction continues as the organism matures, thereby rendering the collagens of older animals more difficult to extract (6).

The secondary structure is the local configuration of a polypeptide chain that results from satisfaction of stereochemical angles and hydrogen-bonding potential of

peptide residues. In collagen, the abundance of glycine residues plays a key configurational role in the triplet Gly-X-Y, where X and Y are frequently proline or hydroxyproline, respectively, these two amino acids that direct the chain configuration locally by the rigidity of their ring structures. The absence of a side group in glycine permits the close approach of polypeptide chain in the collagen triple helix (7). The tertiary structure refers to the global configuration of the polypeptide chains; it represents the pattern according to which the secondary structures are packed together within the complete macromolecule and it constitutes the structural unit that can exist as a physiochemically stable entity in solution, namely, the triple helical collagen molecule.

The fourth-order quaternary structure denotes the repeating supermolecular unit structure, comprising several molecules packed in a specific lattice, which constitutes the basic element of the solid state (microfibril). Collagen molecules are packed in a quasi-hexagonal lattice at an interchain distance of about 1.3 nm, which shrinks considerably when the microfibril is dehydrated. Higher levels of order, eventually leading to gross anatomical features, which can be seen with the naked eye, have been proposed but there is no agreement on their definition (5, 6, 7).

## **2.2 Types of Collagen**

At this stage 13 kinds of vertebrate collagen are known, plus some smaller molecules looking like collagen parts suspected to be members of the family and each type has evolved to serve a distinct purpose. Collagen varies depending on the anatomical region. From muscle to bone to cartilage to blood vessels to nerves to various parts of the skin, which itself is the largest organ in the body.

The differences among these collagen siblings come at the ends of each collagen molecule. It's as though nature created specified arms able to share their fingertips with the tips of different molecules in appropriate cells. That's how they connect. But all collagen middles are the same. Three strands of repeating amino acids coil themselves, left-handed, into the unique collagen triple helix. Then these coils weave themselves right-handed into a cable, like small steel wires braided into the cables of a suspension bridge. In fact, collagen has a greater tensile strength than steel. Presumably, this complex structure was devised by nature to be invulnerable to the circulating enzymes and other materials in the body. Nature accomplished this purpose superbly, which is why no other enzyme (of the many thousands in the body) but a "collagenase" can break it into its component parts.

When the body needs to build any new cellular structure as in the healing process, for example, collagen and/or collagen fragments play a central role. Although the role of collagen as scaffolding has been known for some time, we now know that collagen controls cell shape and differentiation, migration, and the synthesis of a number of proteins.

They are insoluble, stable and have long biological half-life, high tensile strength and contractibility. They are secreted by connective tissue cells, as well as by a variety of other cell types. As a major component of skin, bone teeth, tendons, ligaments, cartilage, and connective tissue. The characteristic feature of a typical collagen molecule is its long, stiff, triple-stranded helical structure in which three collagen polypeptide chains are wound around one another in a ropelike super helix. Its properties are diverse and remarkable. In tendon it has a tensile strength equal to that of light steel wire. In the

cornea it is as transparent as water. It accounts for the toughness of leather, the tenacity of glue and the viscousness of gelatin. So far about 25 distinct collagen chains have been identified, each encoded by a separate gene. Different combinations of these genes are expressed in different tissues. Although in principle more than 10,000 types of collagen molecules could be assembled from various combinations of the 25 or so chains, only about 15 types of collagen molecules have been found. The main types of collagen found in connective tissues are types I, II, V, XI-type I being the principal collagen of skin and bone and by far the most common. These are the fibrillar collagens and have the ropelike structure. After being secreted into ordered polymers called collagen fibrils, which are thin (10-300nm in diameter) structures, many hundreds of microns long in mature tissues and clearly visible in electron micrographs.

The collagen fibrils often aggregate into larger, cable like bundles, which can be seen in the light microscope as collagen fibers several micrometers in diameter. Types IX and XII are called fibril-associated collagens as they decorate the surface of collagen fibrils. They are thought to link these fibrils to one another and to other components in the extracellular matrix. Types IV and VII are network-forming collagens. Type IV molecules assemble into a felt like sheet or meshwork that constitutes a major part of mature laminae while type VII molecules form dimmers that assemble into specialized structures called anchoring fibrils, which help attach the basal lamina multilayered epithelia to the underlying connective tissue and therefore are especially abundant in the skin.

Type I. Major collagen found in skin, tendon, bone, dentin; a fetal form also exists: Mutations in the cleaving of the procollagen I have been shown to be responsible for Ehlers-Danlos syndrome type VII, a disorder characterized by extreme joint hypermobility and congenital bilateral hip dislocations. Other mutations in collagen I produce osteogenesis imperfecta, a highly variable condition (depending on the place of the mutation) characterized by deformed bones, short stature, and abnormalities of teeth.

Type II. Specific for cartilage and vitreous humor.

Mutations in collagen II can cause abnormalities of skeletal development and eye structure.

Type III. Frequently found with type I in skin, muscles, and blood vessels.

Mutations in collagen III cause Ehlers-Danlos syndrome.

This is a life threatening and debilitating disease characterized by extreme arterial and capillary fragility. Death is often due to hemorrhage.

Type V. Fetal tissues, placenta, interstitial tissues

Appears to be necessary for extracellular matrix assembly in connective tissues.

Knockouts of this gene in mice have spinal deformities and poor skin and eye development.

Type XI. Cartilage. Mutations in collagen XI are responsible for the Stickler Syndrome, characterized by cartilage and joint problems, cleft palate, as well as poor retinal development.

### 2.3 Background of Collagen Producing Process

Furukawa et al, (8) describes a process for producing regenerated collagen fiber with excellent water resistance and undergoes substantially no waving on contact with water. This is suitable as a substitute for human hair, animal hair, or as catgut. The starting material used in this invention is a solution of solubilized collagen for which the source was collagen from the raw hide of animals, such as cattle or pigs, fresh or salted, with an alkali or an enzyme and preparing an acidic aqueous solution. This collagen was accomplished by treating soluble collagen having metallic salt aqueous solution. At the end they prepare an aqueous solution with an acid for spinning fiber from it. This acidic collagen solution is spun through a spinneret of an inorganic salt, such as sodium sulfate, sodium chloride, to obtain fibrous solubilized collagen.

To get excellent water resistance, researchers cross link these fibers with a water soluble organic cross linking agents include monoaldehydes, e.g., formaldehyde, acetaldehyde, methylglyoxal, and acrolein; dialdehydes, e.g., glyoxal, malondialdehyde, succindialdehyde, glutaraldehyde, phthalaldehyde, dialdehyde, and starch; epoxy compounds, e.g., glycol glycidyl ether or a polyol glycidyl ether, and a glycidyl ester of a monocarboxylic acid, dicarboxylic acid or polycarboxylic acid, chromic acid; N-methylol compounds, e.g., urea, melamine, acrylamide, methacrylamide, and N-methylol compounds derived from polymers of these compounds; water-soluble polyurethane obtained by introducing an isocyanate group into a polyol or a polycarboxylic acid and adding sodium hydrogen sulfite; chlorotriazine derivatives, e.g., monochlorotriazine and dichlorotriazine; sulfuric ester of oxyethylsulfone or vinylsulfone derivatives, tanning, and synthetic tannin. These water-soluble organic crosslinking agents may be used either

individually or in combination of two or more of them. Among these agents, formaldehyde and glutaraldehyde are preferred because they are generally used in the lather industry and are therefore easily available.

The solution is usually adjusted to a pH of 7 to 13 with for example, boric acid, sodium acetate or sodium hydroxide. If the pH of the solution is less than 7, the cross linking reaction is retarded and if it exceeds 13, the peptide linkage of the solubilized collagen is susceptible to hydrolysis. The temperature of the solution is preferably 40 degree C or less.

#### **2.4 Background of Spinning Process**

The apparatus relates to a method for forming a collagen fiber. The method includes directing liquid collagen solution or dispersion into a coagulation bath to form a continuous collagen fiber. This dehydrated continuous collagen fiber is dried up as it comes out of the coagulation bath using cold air at room temperature.

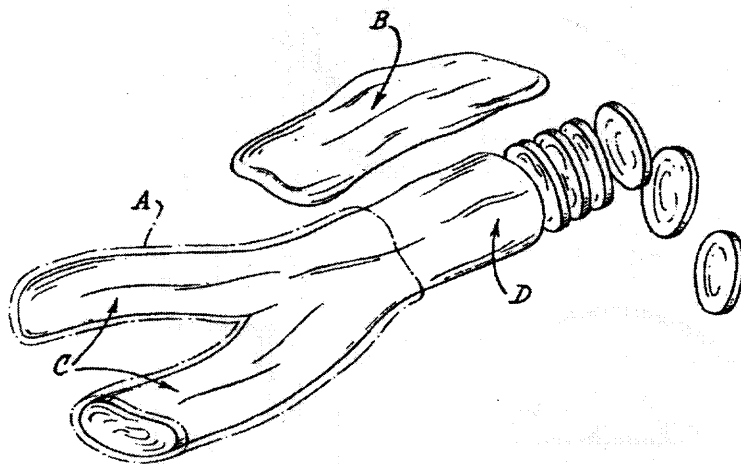
The spinneret of this design is particularly adapted to the extrusion of a dispersion of swollen collagen fibril into an acetone and ammonia-dehydrating bath to form a collagen monofilament. In the production of collagen monofilament, it is the general practice to force an aqueous dispersion of collagen through a spinneret, having single or multiple orifices, into a dehydrating medium, which converts the material into a filament. As the filament-forming collagen emerges from the orifice the dehydrating medium will cause the collagen to set, by removing the solvent from it.

## CHAPTER 3

### EXPERIMENTAL DESIGN

#### 3.1 Making of Collagen Dispersion

The process to extract collagen used in this project is developed with the help of Mr. Nels Lauritzen and Dr. J. Nichols of Prodex Science Inc., located in Princeton, New Jersey, USA. The process starts with complete Bovine Superficial flexor and deep flexor tendons as obtained from cattle.



**Figure 3.1** The various sections of bovine tendon.

The various sections of bovine tendon are illustrated in Figure 3.1. In this figure, the letters “A” through “D” have arbitrarily designated certain sections of the tendon. The “A” portions consist of sheaths, which surround the two “C” sections. The “A” portions are also connected directly to the “B” tendon. The “C” material consists of two small dense shanks which branch off the larger “D” section. These “C” portions contain a large



percentage of reticulin. That section of the single shank identified by the letter “D” in Figure 3.1 is the preferred portion of the tendon for preparing the collagen dispersion. Clean the “D” portion of the tendon and freeze tendon in water. The clean tendons are then sliced to a thickness of about 12-15 millimeters, using the NBI Nantsune deli slicer. Thicker slices swell slowly in aqueous acid solution and are difficult to disperse. Thinner slices disperse readily but the dispersion when extruded has poor tensile strength. It is observed that the tendon sections are sliced across the major axis, as lengthwise slicing seems to result in a slower swelling.

The weight of sliced tendon was 947.1gm. Using electric meat grinder, grind the sliced tendon; transfer the ground tendon into a beaker.

- Weight of beaker + tendon = 1489.0 gm
- Weight of beaker = 625.0 gm
- Weight of ground tendon = 864.0 gm

An adequate sample of the sliced tendon is analyzed at this time for total solids, as the moisture contained in the tendon received from various suppliers at different times is not constant. Take out and weight  $2.0 \pm 0.5$  gm of wet ground tendon in to three different aluminum dry dish. Close the beaker tightly with aluminum foil, so that tendon does not leave moisture.

**Table 3.1** Weights after Grinding the Tendon

		#1	#2	#3
a	Aluminum dish (gm)	1.0046	1.0048	1.0105
b	Aluminum dish + wet tendon (gm)	2.1472	2.1110	2.4448

Place the aluminum dishes with the wet tendon into the oven at 100°C for a minimum of 4 hours, later for 3 days in  $100 \pm 20^\circ\text{C}$ . Take out the aluminum dishes from the oven and weigh them.

**Table 3.2** Weight after Placing in the Oven

		#1	#2	#3
c	Aluminum dish + Dry Tendon	1.5379	1.5200	1.6312

Calculation for the wet weight, dry weight and percent weight.

**Table 3.3** Resultant Weights of the Collagen

d	Wet Weight (b-a) g.	1.1426	1.1062	1.4343
e	Dry Weight (c-a) g.	0.5333	0.5160	0.6207
f	Percent wet solids (e/d x 100) g	46.67	46.64	43.27

Initial dry weight of ground tendon available for purification.

(Wet Weight of ground tendon) x (percent solid) = Dry Weight of tendon to be purified.

The ground tendon is next treated with a buffer solution. Preparation of buffer solution is, 8.4 liter of  $\text{KH}_2\text{PO}_4$  (Potassium phosphate monobasic) solution by adding 41.25 gm of  $\text{KH}_2\text{PO}_4$  to 8.4 liter of distilled or demineralized water. Then 1.5gm of sodium hydroxide is added to the solution to get the pH around 6.

- Recorded pH-6
- Water = 8.4liter,  $\text{KH}_2\text{PO}_4$  = 41.25gm

Add the ground tendon to the above solution. Warm up to 37°C and add 10gm of ficin enzyme derived from plant. Dissolve this ground tendon in 300ml of solution taken from previously prepared 8.4 liter buffer batch. Immediately, add 300 ml ficin premix uniformly to the buffer solution. The buffer enzyme treatment is needed to dissolve the elastin, which encircles and ties together the collagen fibers. By this treatment substantially all of the elastin is dissolved and can be removed. The tendon-enzyme mixture is incubated at a temperature of about 37°C for 15 to 20 hours.

- Wet weight of ground tendon added is 864.0gm.
- Temperature of solution 36°C (ficin added 10.0gm)

Stir intermittently and keep the solution at 37°C  $\pm$ 2 for 1 hour. Now to deactivate the enzyme and to wash the soluble proteins and lipids, prepare a solution of NH<sub>4</sub>NO<sub>3</sub> (Ammonium Nitrate) and NaClO<sub>2</sub> (Sodium Chlorite) in 8.4 liters of distilled water.

- Water = 8.4 liters
- NH<sub>4</sub>NO<sub>3</sub> = 90.2 gm
- pH of the solution = 6
- NaClO<sub>2</sub> = 10 gm

Add NaClO<sub>2</sub> to solution during last 5 minute of preparation. Wash three (3) times, 15 minutes for each time (wash) with 3 liters of distilled water per wash and also squeeze out excess water. Monitor the pH at each wash.

**Table 3.4** pH reading of the Washes

	Start	Finish	pH
Wash 1	5:45 PM	6:00 PM	6.02
Wash 2	6:15 PM	6:30 PM	5.87
Wash 3	6:45 PM	7:00 PM	5.41

Store the squeezed dry collagen in cool and dry place. To prepare the collagen dispersion, 500 ml of distilled water and 500ml of methanol is taken, mix them together, and then 2 gm of cyano acetic acid is added. This mixture is then poured into the beaker with collagen. This is allowed to sit for couple of minutes.

- Weight of collagen = 21.98gm
- Weight of cyano acetic acid = 2.0gm

This mixture is then poured into a blender for three to five continuous cycles. 10 seconds at low speed, 10 seconds at medium speed, and 10 second at high speed. These three speeds count as one cycle. These cycles can be repeated if needed. A 5-minute pause between each cycle is taken to prevent heat due to friction. Centrifuging the mixture at 4000 rpm for 5 minutes then follows this. A very clean, colorless material is obtained. This is now ready to be spun.

### 3.2 The Experimental Setup for Spinning

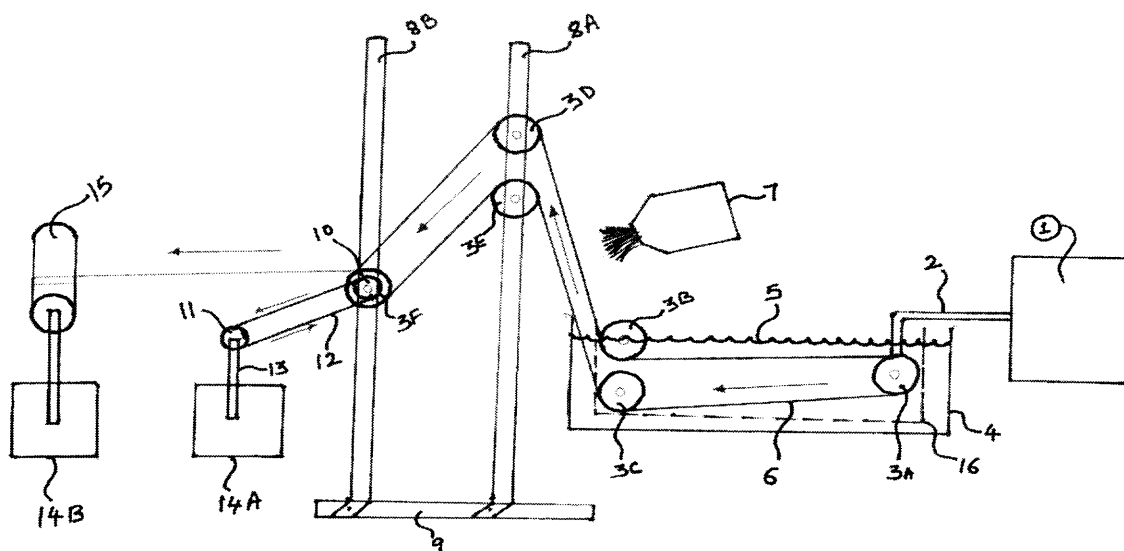
Shown in Figure 3.2 in a schematic view, is an apparatus for spinning a collagen fiber monofilament. The term “monofilament” as used here, means a single thread of oriented collagen fibril as extruded through a single orifice spinneret. Item 1 is syringe pump

made by Harvard Systems Model number 901 on which syringes of 10 cc, 20 cc, 30 cc, or 50 cc filled with collagen dispersion can be fixed. Item **2** is the needle attached to syringe, made up of stainless steel with diameter ranges from 16, 18, 20, 22 gauges. The needle is bent at 90° angle so that its orifice can face right above the conveyer belt item **6**. Needles made of stainless steel are preferable because they have the property to resist corrosive environment of acetone and ammonia. Items **3A, B, C, D, E, F** are nylon rollers which are 1 inch in diameter. They have bore in the center of about 0.30 inch which make them look like wheels and their function is to rotate the conveyer belts. In order to resist the corrosive environment of coagulation bath, the rollers used are made up of nylon. These nylon rollers are attached to a stainless steel frame, item **16**, inside the coagulation bath item **4**. Coagulation solution item **5** which may contain 2 liters of solution originally made up by adding 1.5 milligrams of  $\text{NH}_3\text{OH}$  (Ammonium Hydroxide) and 60 grams of water to 2 liters of fresh HPCL grade acetone. The original water content of this HPCL grade acetone is 4 grams per liter. This will give pH between 9-10 in the coagulation bath.

If less ammonia is present in the spin bath, the extruded filament is too soft when formed at the needle opening and if too much ammonia is present in the bath, the filament is brittle and cannot be stretched to obtain the desired orientation. In the coagulation bath, acetone takes out the water from collagen fiber and ammonia neutralizes the acid to give strength to the monofilament.

The water present in the spin bath has the opposite effect, in that too much water will result in an excessively soft filament, and too little water will give a brittle filament that cannot be stretched. Thus, the ammonia present in the acetone bath will compensate

to some extent for the water present and vice versa. The composition of the spin bath may be maintained relatively constant by adding ammonia, acetone and water at every 15 minutes and constantly checking pH by using hand held pH meter.



**Figure 3.2** Schematic diagram of spinning of collagen fiber.

**Item 6** is the conveyer belt made up of polyethylene and has thickness of 0.15 cm, width of 3.5 cm and 48 inches in length. Polyethylene is resistant to corrosive environment of acetone and ammonia, it also does not like collagen, that means after drying the collagen fiber does not stick to the surface of the conveyer belt, so the fiber readily comes off the conveyer belt. **7** is the cold air blower, very much similar to hair dryer, which is aimed on the conveyer belt right near the end of coagulation bath to increase the amount of time required for drying. **Item 8A and B** is the clamp made up of

aluminum onto which rollers are attached. Item **9** is the rail made up of iron on which these clamps are mounted. Items **10** and **11** are two pulleys. Item **10** is attached to roller Item **3F** with 0.25 inch bore size, where as Item **11** is attached to electric motor's Item **14A** shaft with 0.5 inch bore size. Item **12** is the timing belt made up of rubber of 4 inches in length, which connect two pulleys. Item **13** is the shaft of electric motor of size 0.5 inches. Items **14A**, **14B** are two low speed DC motors with speed controllers. The lowest speed that can be achieved is 2 inches/min, and the fastest speed that can be achieved is 10 inches/min. Item **15** is the take up roller coated with Teflon or Polyethylene film. From the trial and error experiment it is observed that to get uniformity in fiber the identical speed of motor is 3 inch/min and speed of syringe pump is 2 inches/min.

Using this apparatus we make collagen monofilament from three different dispersion concentrations; i.e. 1%, 1.6%, and 2%. It is observed that with 1% dispersion, it gives us a film like monofilament which has the tape or film like structure instead of round fiber because the viscosity of 1% dispersion is low and when the fiber comes out from needle orifice it sets on the conveyer belt throughout the coagulation bath. Since the viscosity is very low it gets flat to form a tape or film like structure.

With 1.6% dispersion, which has more collagen in it and has comparatively more viscosity than 1%, it almost looks like gel and produces round fiber. When 1.6% dispersion comes out of the needle orifice it does not get flat because of the higher viscosity. This also happens with 2% dispersion. Fibers with concentration higher than 2% cannot be spun because 2% in itself is very viscous and is found to be difficult to get through needle with small orifice.

There are two drawbacks of this apparatus. One is it does not have any mechanism to remove air bubbles from the dispersion. Due to this problem, at times leads to the breakage of the continuous fiber that is spun using the syringe pump. This problem can be overcome by centrifuging the dispersion at more than 5000 rpm. The other solution of this problem is filling the syringe with desired concentration of dispersion and let it sit for one or two days. This will remove the air bubble automatically, if not all, at least most of them. The second drawback is it does not have any cross-linking bath. This problem here in this project is overcome by using a separate bath to which cross linking agent is added. This bath is used in the place of coagulation bath in above design. The fiber is then passed through this cross-linking bath for cross-linking the fibers.

### **3.3 Chemical Modification of Collagen to Cross Link the Fibers**

The primary structure of collagen is made up of long sequences of some 20 different amino acids. Since each amino acid has its own chemical identity, there are 20 types of pendant side groups, each with its own chemical reactivity, attached to the polypeptide chain backbone. As examples, there are carboxylic side groups (from glutamic acid and aspartic acid residues), and primary amino groups (lysine, hydroxylysine, and arginine residues), and hydroxlic groups (tyrosine and hydroxylysine). The collagen molecule is therefore subject to modification by a large variety of chemical reagents. Because of the chemical versatility there are many ways for the collagen to be chemically modified. In addition it is possible to react with only a specific type of amino acid rather than all amino acid residue types carrying the same functional group, also requires chemical analysis (9).



Historically, the chemical modification of collagen has been practiced in the leather industry (since about 50 % of the protein content of cowhide is collagen) and in the photographic gelatin industry. Today, the increasing use of collagen in biomaterials applications has provided renewed incentive for novel chemical modification, primarily in two areas. First, implanted collagen is subject to degradable attack by collagenases, and chemical cross-linking is a well-known means of decelerating the degradation rate.

Second, collagen extracted from an animal source elicits production of antibodies (immunogenicity). Although it is widely accepted that collagen elicits synthesis of a far smaller concentration of antibodies than other proteins e.g., albumin, treatment with specific reagents, including enzymatic treatment is occasionally used to reduce the immunogenicity of collagen (10).

Collagen-based implants are normally degraded by collagenases, naturally occurring enzymes, which attack the triple helical molecule at a specific location. Two characteristic products result, namely, the N-terminal fragment which amounts to about two thirds of the molecule, and the one-quarter C-terminal fragment. Both of these fragments become spontaneously transformed (denatured) to gelatin at physiological temperatures via the helix-coil transition and the gelatinized fragments are then cleaved to oligopeptides by naturally occurring enzymes, which degrade several other tissue proteins (non specific proteases) (11).

Collagenases are naturally present in healing wounds and are credited with a major role in the degradation of collagen fibers at the site of trauma. At about the same time the degradation of collagen and of the extra cellular matrix components proceeds in the wound bed, these components are being synthesized by cells in the wound bed (12).

Eventually, new architectural arrangements, such as scar tissue form a stable between adjacent organs, which allows the healed organ to continue functioning at a nearly physiological level. The combined process of collagen degradation and scar synthesis is often referred to as remodeling. One of the frequent challenges in the design of collagen implants is to modify collagen chemically in a way, which either accelerates or slows down the rate of its degradation at the implantation site to a desired level (12).

An effective method for reducing the degradation rate of collagen by naturally occurring enzymes is chemical cross-linking (13). A very simple self-cross-linking procedure, dehydrative cross-linking, is based on the fact that removal of water below about 1% by weight insolubilizes collagen as well as gelatin by inducing formation of interchain peptide bonds. The nature of the cross-links formed can be inferred from the results of studies using chemically modified gelatins. Gelatin which had been modified either by esterification of the carboxylic groups of aspartyl-gluamyl residues or by acetylation of the  $\Sigma$ -amino groups of lysyl residues remained soluble in aqueous solvents after exposure of the solid protein to high temperature, while unmodified gelatins lost their solubility. Insolubilization of collagen and gelatin following severe dehydration has been, accordingly, interpreted as the result of drastic removal of the aqueous product of a condensation reaction, which led to the formation of interchain amide links. The proposed mechanism is consistent with results, obtained by titration; showing that the number of free carboxylic groups and free amino groups in collagen are both significantly decreased following high temperature treatment (14).

Removal of water to the extent necessary to achieve a density of cross-links in excess of  $10^{-5}$  moles of cross-links/g dry gelatin, which corresponds to an average

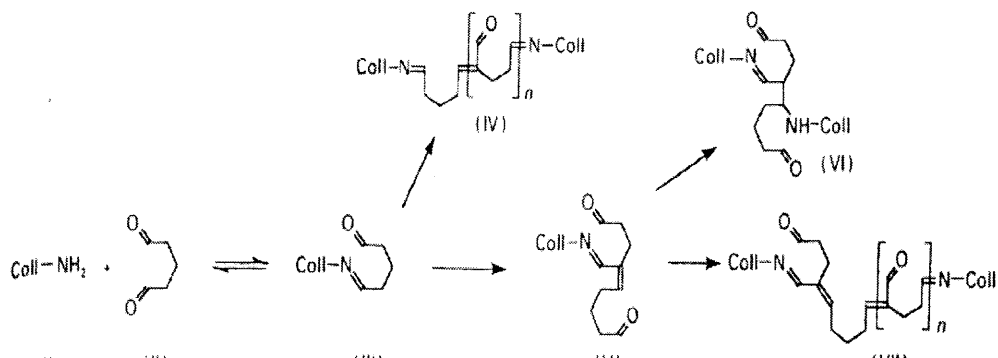
molecular weight between cross-links,  $M_c$ , of about 70 kDa, can be achieved within hours by exposure to temperatures in excess of 105°C under atmospheric pressure. The possibility that the cross-linking achieved under these conditions is caused by a pyrolytic reaction has been ruled out (12). Furthermore, chromatographic data have shown that the amino acid composition of collagen remains intact after exposure to 105°C for several days. In fact, it has been observed that gelatin can be cross-linked by exposure to temperatures as low as 25°C provided that a sufficiently high vacuum is present to achieve the drastic moisture removal, which appears to drive the cross-linking reaction (15).

Exposure of highly hydrated collagen to temperatures in excess of about 37°C is known to cause reversible melting of the triple helical structure, as described earlier. The melting point of the triple helix increases with the collagen-diluent ratio from 37°C, the helix-coil transition of the infinitely dilute solution, to about 120°C for collagen swollen with as little as 20% wt. diluent and up to about 210°C, the melting point of anhydrous collagen. Accordingly, it is possible to cross-link collagen using the drastic dehydration procedure described earlier without loss of the triple helical structure. It is sufficient to adjust the moisture content of collagen to a low enough level prior to exposure to the high temperature levels required for rapid dehydration (16).

Dialdehydes have been long known in the leather industry as effective tanning agents and in histological laboratories as useful fixatives. Both of these applications are based on the reaction between the dialdehyde and the  $\Sigma$ - amino group of lysyl residues in the protein, which induces formation of interchain cross-links. Glutaraldehyde cross-linking is a relatively widely used procedure. The nature of the cross-link formed has

been the subject of controversy, primarily because of the complex, apparently polymeric, character of this reagent. Considerable evidence supports the proposed anabilysine structure, which is derived from two lysine side chains and two molecules of glutaraldehyde (13, 16).

Evidence for other mechanisms has been presented. Compared with other aldehydes, glutaraldehyde has shown itself to be a particularly effective cross-linking agent, as judged, for example, by its ability to increase the cross-link density. The  $M_c$  values provide the experimenter with a series of collagens in which the enzymatic degradation rate can be studied over a wide range, thereby affording implants, which effectively disappear from tissue between a few days and several weeks following implantation. Although the mechanism of the reaction between glutaraldehyde and collagen at neutral pH is understood in part, the reaction in acidic media has not been studied extensively (13,16). Evidence that covalent cross-linking is involved comes from measurements of the equilibrium tensile modulus of films that have been treated to induce cross-linking and have subsequently been gelatinized by treatment in 1 M NaCl at 70°C. Under such conditions, only films that have been converted into a three-dimensional network support an equilibrium tensile force; by contrast, uncross-linked specimens dissolve readily in the hot medium (15).



**Figure 3.3** Collagen cross-linked with glutaraldehyde (27).

The Figure 3.3 shows how glutaraldehyde reacts with the amino acids of collagen to form cross-links. There is reaction of amine with aldehydes through condensation. The immunogenicity of the collagen used in implants is significant and has been studied assiduously using laboratory preparations. However, the clinical significance of such immunogenicity has been shown to be very low, and it is often considered to be negligible. This immense simplification of the immunological problem of using collagen as a biomaterial was recognized a long time ago by manufacturers of collagen-based sutures. The apparent reason for the low antigenicity of type I collagen stems from the small species difference among type I collagens (e.g., cows vs. human). Such similarity is, in turn, probably understandable in terms of the inability of the triple helical configuration to incorporate the substantial amino acid substitutions, which characterize species differences with other proteins. The relative constancy of the structure of the triple helix among the various species is, in fact, the reason why collagen is sometimes referred to as a “successful” protein in terms of its evolution or, rather, the lack of it (17, 18,10).

In order to modify the immunogenicity of collagen, it is useful to consider the location of its antigenic determinants, i.e., the specific chemical groups that are recognized as foreign by the immunological system of the host animal. The configurationally (or conformational) determinants of collagen depend on the presence of the intact triple helix and, consequently, are abolished when collagen is denatured into gelatin; the latter event occurs spontaneously after the triple helix is cleaved by a collagenase (10,11). Gelatinization exposes the sequential determinant of collagen over the short period during which gelatin retains its macromolecule character, before it is cleared away following attack by one of several non-specific proteases. Controlling the stability of the triple helix during processing of collagen, therefore, prevents the display of the sequential determinants (19, 4).

Sequential determinants also exist in the nonhelical end (telopeptide region) of the collagen molecule and this region has been associated with most of the immunogenicity of collagen-based implants. Several enzymatic treatments have been devised to cleave the telopeptide region without destroying the triple helix. Treating collagen with glutaraldehyde not only reduces its degradation rate by collagenase but also appears to reduce its antigenicity as well. The mechanism of this effect is not well understood.

### **3.4 Characterization of Collagen Fibers**

The purpose of this work is to determine whether the physical behaviour change of collagen fiber change as a function of chemical treatment. In all the cases there is a need to know whether an inflammatory response results in the presentation to the implanted

implanted tissue the cellular components that lead to calcification, or whether a change in the protein structure causes the initiation of the calcification process.

The tissue evaluated here is the bovine tendon collagen. Several different fiber treatment processes were evaluated to see if changes in protein structure could be identified like treatment with glutaraldehyde. The analytical methods used for the initial studies were developed to study physical changes in polymers. They include differential scanning calorimetry (DSC), thermal mechanical analysis (TMA), thermal gravimetric analysis (TGA). The diameters and surface features are analyzed using SEM and Universal Research Microscope.

### **Materials**

1. Collagen gel with 1% viscosity treated with ethanol and spun into fibers dehydrated using a acetone bath (pH 9-10).
2. Collagen gel with 1% viscosity treated with ethanol and spun into fibers. These fibers are treated with glutaraldehyde during the dehydration process in acetone. This results in producing fibers with cross-links.
3. Collagen gel with 1.687% viscosity treated with ethanol and spun into fibers dehydrated using a acetone bath (pH 9-10).
4. Collagen gel with 1.687% viscosity treated with ethanol and spun into fibers. These fibers are treated with glutaraldehyde during the dehydration process in acetone. This results in producing fibers with cross-links.
5. Collagen gel with 2% viscosity treated with ethanol and spun into fibers dehydrated using a acetone bath (pH 9-10).
6. Collagen gel with 2% viscosity treated with ethanol and spun into fibers. These fibers are treated with glutaraldehyde during the dehydration process in acetone. This results in producing fibers with cross-links.

### **3.5 Thermal Gravimetric Analysis**

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

The thermogravimetric data was analysed 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 fiber was cut with a scissors into tiny bits of pieces and weighed. The pan with the collagen fibers was covered with a porous metal screen to keep the light fibers from blowing out of the pan. 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.

### **3.6 DSC- Differential Scanning Calorimetry**

Thermal analysis of collagen fibers is used as a diagnostic tool to evaluate the effect of temperature and stress conditions. The effect of temperature on the collagen fibers has 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 (21). The thermoreversible transformation of collagen to gel is interpreted as the disintegration of these helical structures into random coils. Upon cooling, random coils (22) undergo a conformational



coil to helix transition during which they attempt to reform the original collagen structure(23). 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 helical structure. The space between the fibrils is composed of disordered amorphous polypeptide chains (25) providing the elasticity to the collagen fiber.

The purpose of this DSC experiments is to gain an understanding of the structural changes of collagen fiber when exposed to heating and cooling by observing the relative heat flow using ramp and heat-cool-heat using thermal analysis.

Thermal behavior and melting temperatures are determined with Q100 differential scanning calorimetry, TA Instruments New Castle, DE. Samples for thermal analysis are prepared as follows. The collagen fiber was cut with a scissors into tiny bits of pieces and weighed. The weight was 2-5 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 Ramp mode and then followed by Heat-Cool-Heat mode. The scan rate was 10 degree C/min over a range of -50 to 250 degrees. For heat cool heat operation mode the scan rate for the cooling cycle was kept at 5 degree C/min in order to accurately study the thermal events. These parameters were selected based on the previous studies conducted on rat-tail tendon and collagen of pericardium.

### 3.7 Thermal Mechanical Analysis

This test is conducted using the TMA 2940 Thermo mechanical Analyzer made by TA Instruments. This test is used to evaluate the effect of temperature on the change in dimensions. The results are analyzed using the software. It is expected that as the strength of the collagen fiber increase, the temperature at which it break also increase.

Collagen fiber is cut using a scissors and placed between the clamps used to mount the sample. The sample is then mounted onto the sample fixture. The initial length of the fiber is measured automatically by the instrument. It is then subjected to the non-isothermal temperature ranging from  $-50$  to  $250$  degrees C. It is heated at the rate of  $10$  degree C per min. Since an internal cooling system was not available, it was cooled below room temperature using liquid nitrogen in the furnace.

### 3.8 Tensile Modulus

This test is conducted using the TMA 2940 Thermo mechanical Analyzer made by TA Instruments to find the Young's modulus of the collagen fiber at isothermal temperature. The process of cross-linking after treating the collagen gel with gluteraldehyde is realized and the slope is listed. It shows that there is cross-linking of terminal amino groups with treatment of gluteraldehyde. This is demonstrated by the fact that the slope of the plot of tensile deformation versus applied force decreases when the fiber is cross-linked. The equipment software plots the changes in the length of the fiber with respect to increase in force applied. The maximum amount of force applied here  $1$  Newton.

Collagen fiber is cut using a scissors and placed between the clamps used to mount the sample. The sample is then mounted onto the sample fixture. The initial length

Collagen fiber is cut using a scissors and placed between the clamps used to mount the sample. The sample is then mounted onto the sample fixture. The initial length of the fiber is measured automatically by the instrument. In this method, the test is conducted at isothermal temperature. The temperature is kept constant equal to body temperature i.e 37 degree C.

### **3.9 Scanning Electron Microscope**

The size and surface features of the collagen fibers produced by the different amount of dispersion and cross-linked and non-cross linked were studied by scanning electron microscope. Collagen samples about 2-3mm in length were mounted on aluminum stubs. The instrument used was Leo 1530 VP.

Collagen fibers are placed on the aluminum stubs. Then a double-sided tape, a piece large enough to place on top of the aluminum stub is cut. The paper on the tape is peeled off and placed on the stub. Tweezers are used to place the sample onto the tape. Drop the aluminum stubs into the numbered sample holder and turn screws until tight. The inner sample holders are placed into the outer sample holder and again the screws are turned to tightness. The samples were then introduced into the specimen chamber of a scanning electron microscope and examined for surface structure and diameter.

### **3.10 Universal Research Microscope**

This study was performed on the collagen fibers using a universal research microscope for obtaining polarized and non-polarized images. About 3-4 mm of the collagen fiber in length was cut with scissors and mounted on a glass slide. This was then placed URM.

## **CHAPTER 4**

### **RESULTS AND DISCUSSION**

The table below shows the different fibers spun in this study to characterize their thermal stability, mechanical, tensile and structural properties.

**Table 4.1** Different Types of Collagen Fibers Spun for the Study.

Percentage of collagen dispersion	PH of the coagulation bath	Speed of syringe pump*** (inches/min)	Time inside the bath (min)	Speed of extrusion (inches/min)	Diameter of the fiber(micrometer)	Observation
1 Non crosslinked	9-10	2	3	3	Film*	Flat film, transparent, uniform thickness
1.6 Non cross linked	9-10	2	3	3	20.4**	uniform diameter, cylindrical fiber
2 Non cross linked	9-10	2	3	3	18.3**	Uniform diameter, cylindrical fiber
1 Cross linked	9-10	2	3	3	Film*	flat fiber, uniform thickness
1.6 Cross linked	9-10	2	3	3	14.5**	uniform diameter, cylindrical, firm
2 Cross linked	9-10	2	3	3	20.1**	uniform diameter, cylindrical, firm

\* spun using 18 guage needle (0.033" inside diameter)

\*\* spun using 20 guage needle (0.023" inside diameter)

\*\*\* Volume per minute

#### 4.1 Thermal Gravimetric Analysis

The thermogravimetric curves for three dispersions with and without cross-linking are shown below in Figures 4.1a to 4.1f. All the samples showed similar behavior with the main stage of mass loss. This stage showed at about 250 degree C the loss of adsorbed water and bound water. These graphs show the weight loss as the temperature increases. The water content in these fibers is completely evaporated at an average temperature of 245.93, it is seen that there is maximum loss in weight for all the fibers or no further weight loss irrespective of the amount of percentage dispersion of collagen and cross-linking at this temperature. It is also observed that there is rapid loss between room temperature and 100 degree C followed by linear weight loss until 246 degree, which shows that there is some loosely bound water and some adsorbed water. Figures below show the plots and the rest in the Appendix B.

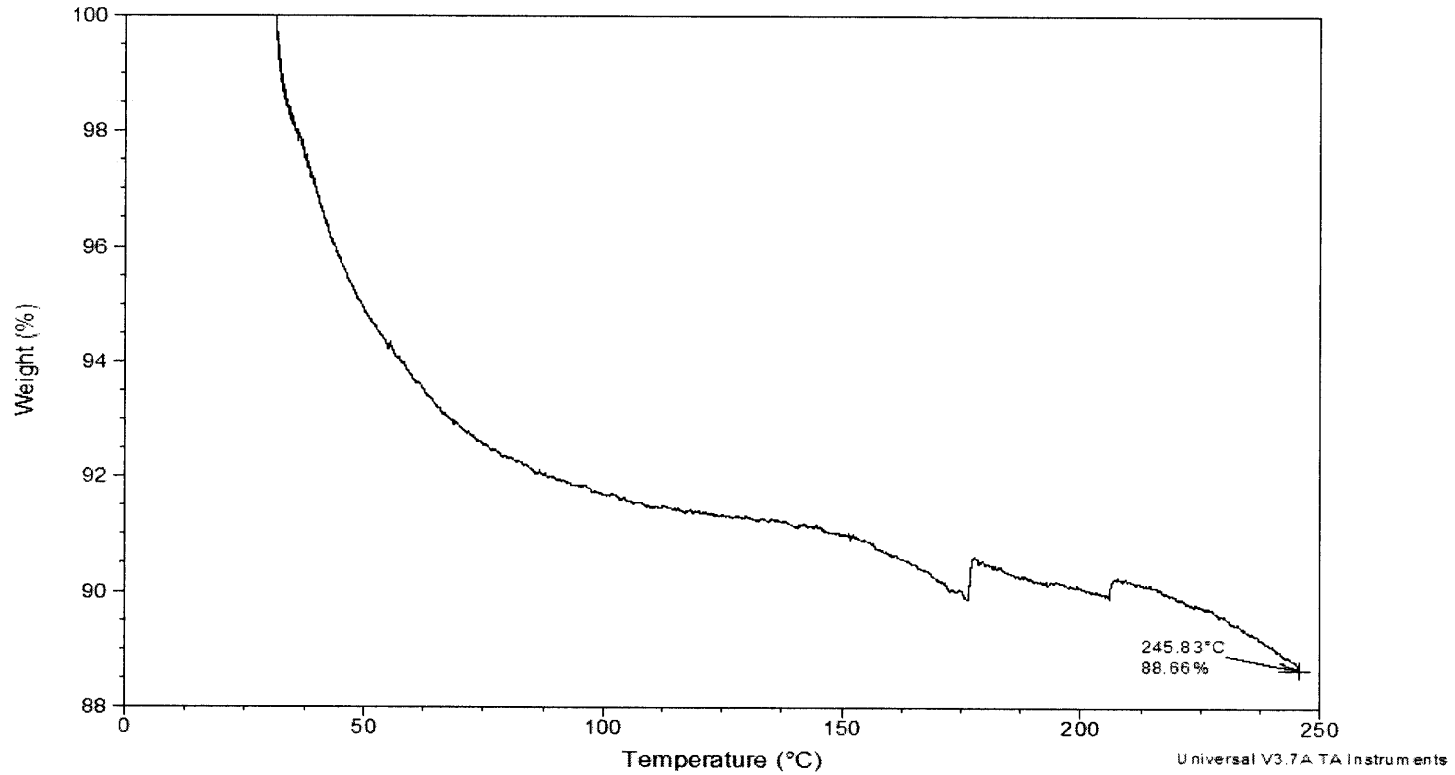
**Table 4.2** Temperatures and the Corresponding Weight Loss of Different Percentage Dispersions of Collagen and also with Cross-linked and Non Cross-linked Fibers.

Percentage dispersion of collagen	Temperature in degree C	Weight retain(%)
1 - Non-Cross linked	245.83	88.66
1.6 - Non-Cross linked	246.02	86.50
2 - Non Cross linked	246.12	85.36
1 - Cross linked	245.94	87.23
1.6 - Cross linked	245.90	86.67
2 - Cross linked	246.06	85.74

Sample: Collagen Film 1%  
Size: 0.2840 mg  
Method: Ramp  
Comment: with screen

### TGA

File: C:\...tga\Grace\_TGA\CF1%.002  
Operator: Grace  
Run Date: 18-Nov-03 12:52  
Instrument: TGA Q50 V5.0 Build 164

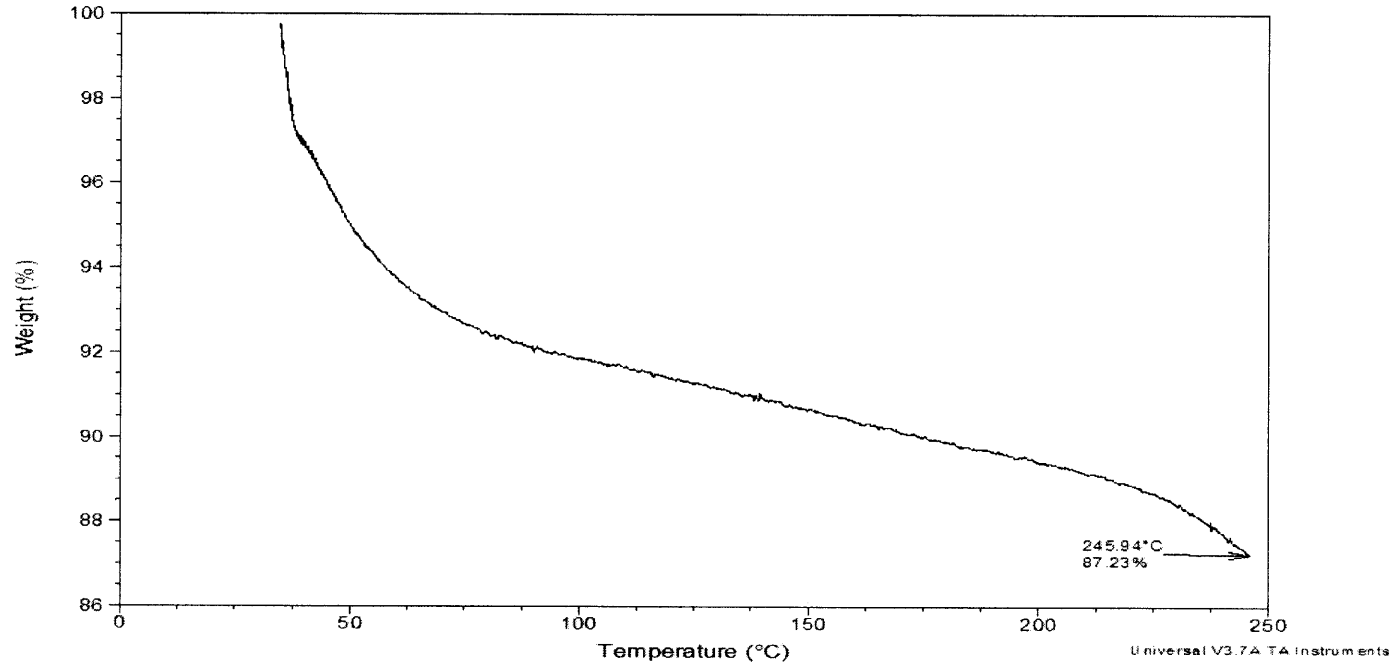


**Figure 4.1** Thermogravimetric analysis (TGA) plot of collagen with 1% dispersion and non cross-linked.

Sample: Collagen Film X 1%  
Size: 0.3200 mg  
Method: Ramp  
Comment: with screen

### TGA

File: C:\Atga\Grace\_TGA\CFX1%.001  
Operator: Grace  
Run Date: 18-Nov-03 12:03  
Instrument: TGA Q50 V5.0 Build 164



**Figure 4.2** Thermogravimetric analysis (TGA) plots of collagen with 1% dispersion and cross-linked.



## 4.2 Differential Scanning Calorimetry

During the ramp test, the broad endotherm given by the total heat flow corresponds to the loss of water content as shown by TGA of the collagen fiber. It is important to remember that hydrogen bonds that maintain the secondary and tertiary structure of collagen are disrupted with an increase of temperature inducing the uncoiling of the triple helix in  $\alpha$  chains of random conformation, individually or covalently linked depending on the degree of heating. The denaturation phenomenon, distinct from degradation implies that the rupture of peptide bonds leads to the formation of an amorphous polymer, namely gelatin. It can be used as a test to study the thermal stability of collagen.

This is shown in the graphs at the end of the ramp cycle. Previous calorimetric measurements on different collagens have shown that the denaturation of the dry protein occurs in the 180-250° C temperature range as an endothermic peak. This endothermic peak, which can be assigned, as a first order transition is not reversible on successive heating scans in the dry state. In this case a peak is observed, which is the denaturation temperature. On subsequent heat/cool/heat operation the glass transition temperature at an average temperature of 195degree C is observed.

From the table below we also observe that the denaturation for cross linked fibers occurs at a higher temperature compared to the collagen fibers without the cross links. Figures below show the plots and the rest followed in the Appendix B.

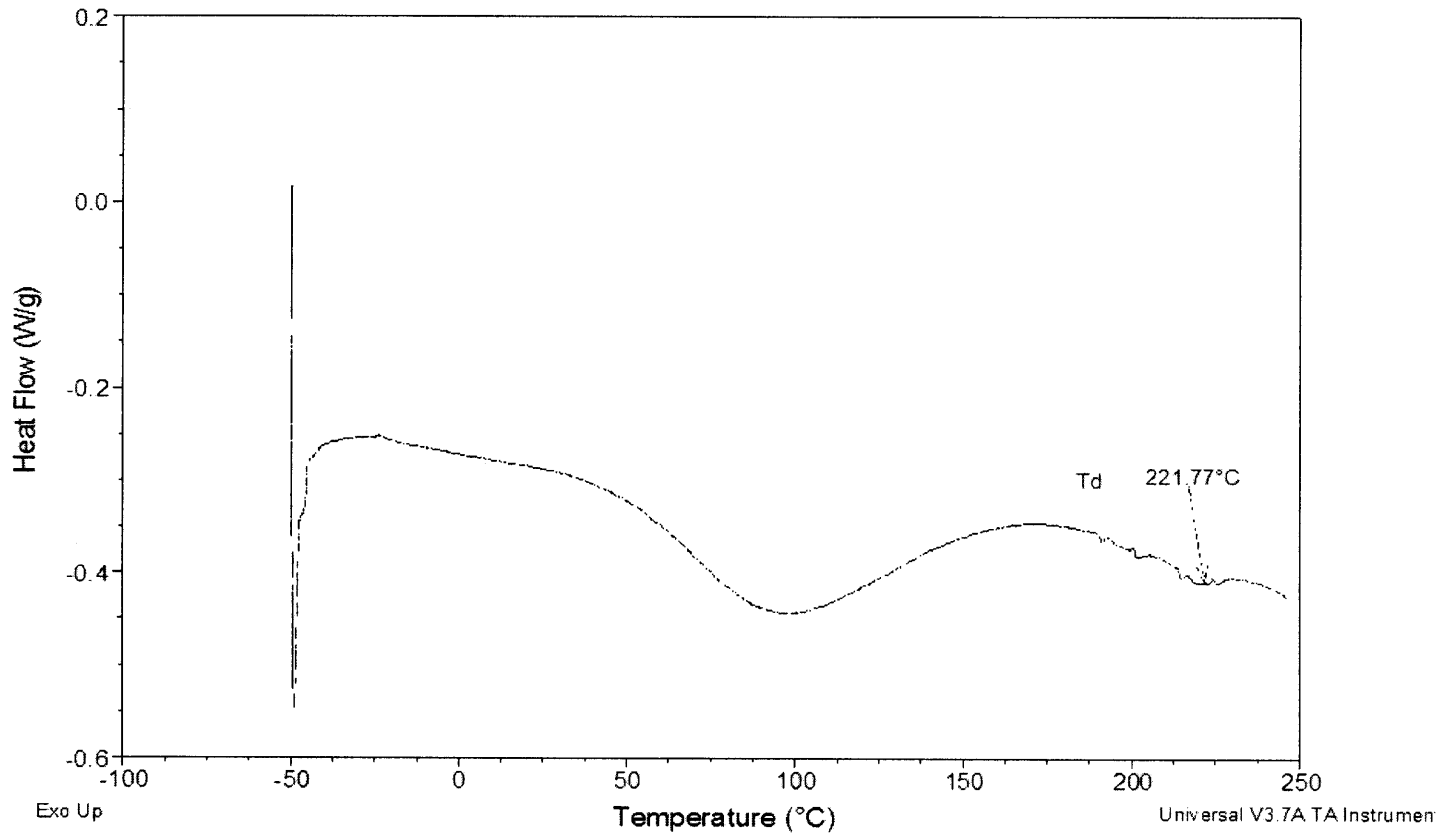
**Table 4.3** Shows Different Fibers with their Corresponding Td and Tg.

Percentage of collagen dispersion	Denaturation Temperature Td in degree C	Glass Transition Temperature Tg in degree C
1 Non Cross linked	221.77	195.65
1 Cross linked	229.65	189.01
1.6 Non cross linked	215.97	182.79
1.6 Cross linked	230.48	203.53
2 Non Cross linked	220.47	181.13
2 Cross linked	233.98	198.55

Sample: Collage film 1%  
Size: 3.2000 mg  
Method: Ramp

DSC

File: C:\...My Documents\CF1%.001  
Operator: Grace  
Run Date: 29-Dec-03 12:03  
Instrument: DSC Q100 V7.0 Build 244

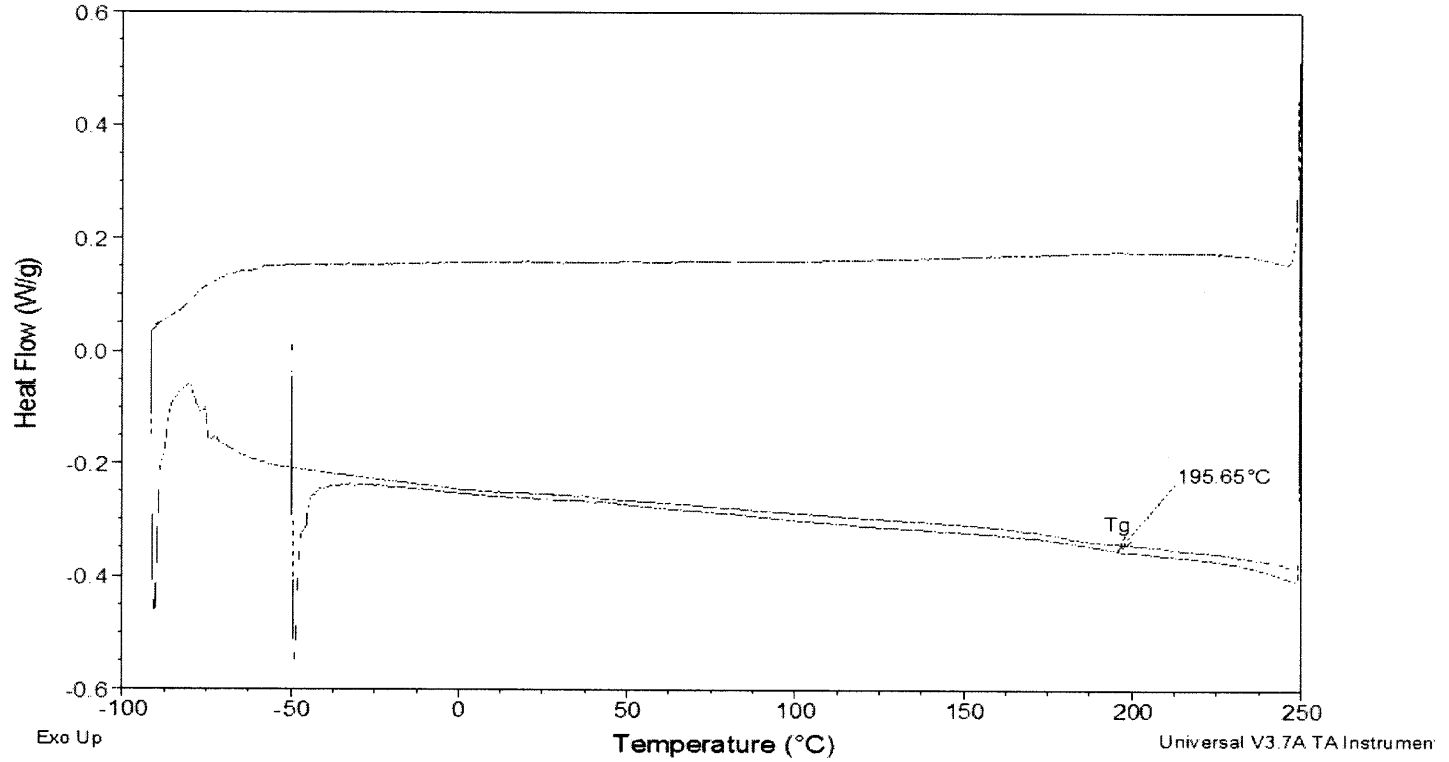


**Figure 4.3** DSC plot above shows the heating cycle for 1% dispersed collagen fiber.

Sample: Collage film 1%  
Size: 3.2000 mg  
Method: Heat/Cool/Heat

DSC

File: C:\My Documents\CF1%.002f  
Operator: Grace  
Run Date: 29-Dec-03 12:55  
Instrument: DSC Q100 V7.0 Build 244



**Figure 4.4** DSC plot above shows the heat/cool/heat cycle for 1% collagen fiber.

### 4.3 Thermal Mechanical Analysis

The influence of cross-linking the fibers is studied using thermal mechanical analysis. It is observed that the and temperature at which a cross-linked fiber breaks is greater than the fiber without cross-linking. It is also observed that a fiber with higher percentage of collagen dispersion also breaks at a greater temperature. Table 4.3a shows the different percentages of collagen and also the temperature at which they break down. As the heating increases there is contraction in the helical structure. At some point it denatures and loses its mechanical integrity. The contraction is caused by the rotation of the structural units in the protein macromolecule, which are oriented. Figures below show the plots and the rest are given in Appendix B.

**Table 4.4** Shows the Different Fibers and their Corresponding Temperature at which they Contract and Break Down.

Percentage of dispersion of collagen	Temperature in degree C at which it breaks
1 Non Cross linked	222.45
1.6 Non Cross linked	227.23
2 Non cross linked	233.39
1 Cross linked	237.18
1.6 Cross linked	240.85
2 Cross linked	241.20

Sample: Collagen fiber -1%  
Size: 12.8832 mm  
Method: Ramp

TMA

File: C:\...Desktop\TMA Files\Grace.002  
Operator: Grace  
Run Date: 15-Oct-03 12:04  
Instrument: 2940 TMA V2.4E

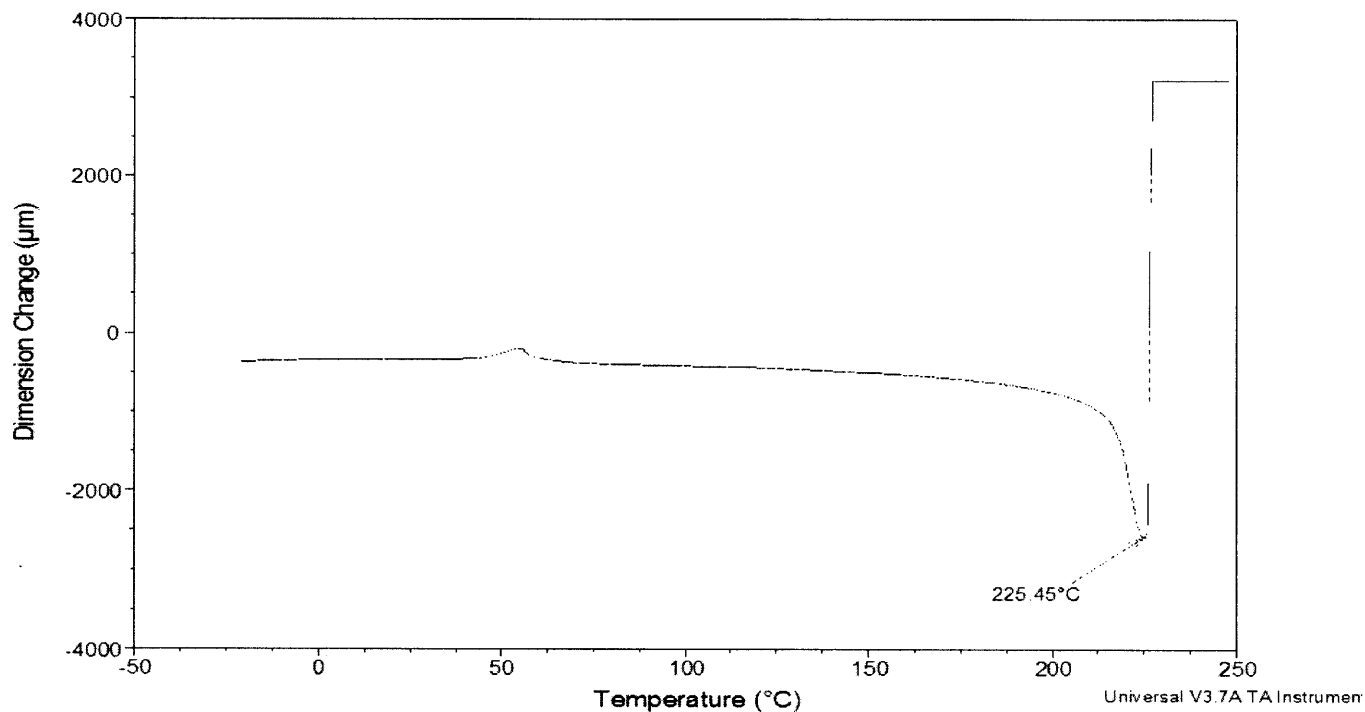
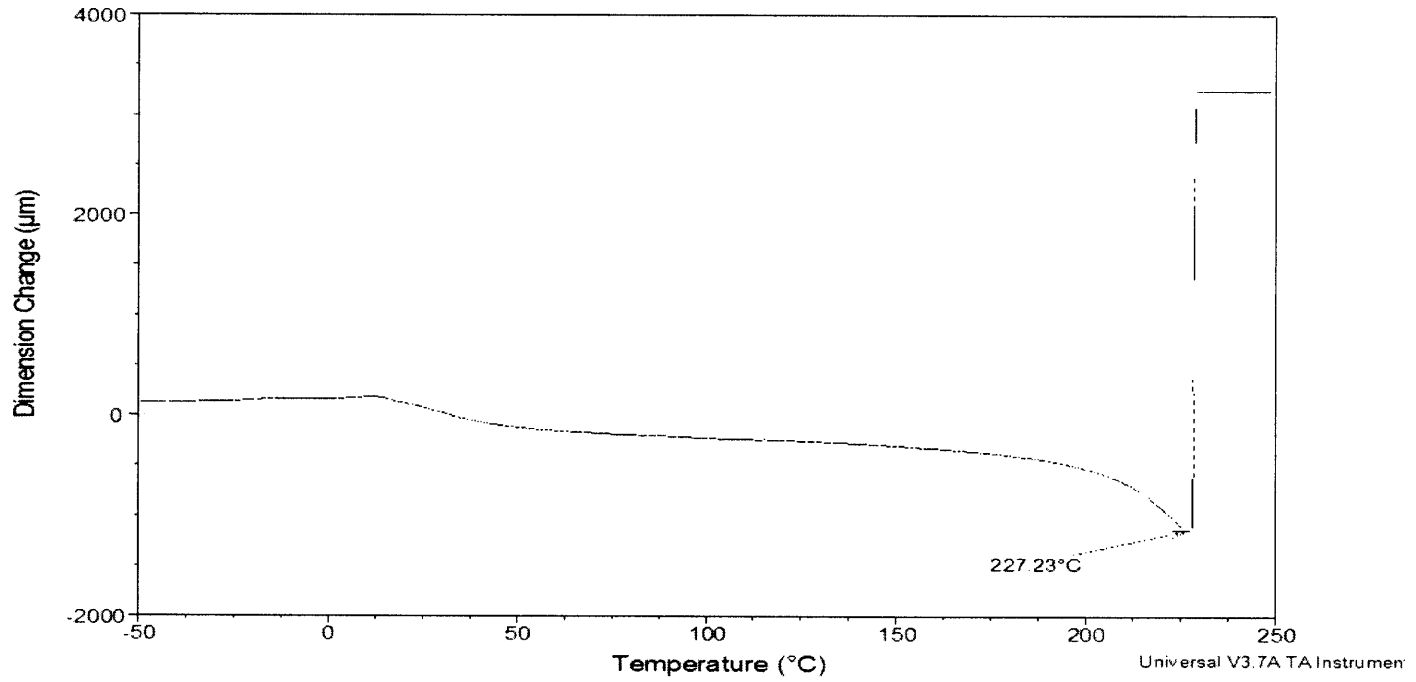


Figure 4.5 TMA plot above shows the contraction and break point of collagen fiber with 1% dispersion.

Sample: Collagen Fiber (X) 1 %  
Size: 12.7850 mm  
Method: Ramp

TMA

File: C:\Desktop\TMA Files\Grace.011  
Operator: Grace  
Run Date: 18-Nov-03 11:34  
Instrument: 2940 TMA V2.4E



**Figure 4.6** TMA plot above shows the contraction and break point of collagen fiber with 1% dispersion and cross linked.

#### 4.4 Tensile Modulus

It is a well-known fact that the mechanical properties of tendons and ligaments change with age. With growth the collagen fibril length and diameter increase which is associated with increased ultimate tensile strength and moduli and decreased failure strain. At maturity the tensile strength as well as failure strain appear to decrease which is associated with the conversion of cross-links to non cross-links. In this study it is determined that the dependence of tensile extension on force applied to the collagen fiber up to a maximum force of 1 Newton. The inverse slope of plots made in the manner is related to the Young's modulus. It is observed in the table 4.4 that the slope decreases for the cross-linked fibers. Using the cross sectional area the estimated modulus is calculated taking the initial length as 12.6 mm. Figures below show the plots and the rest are followed in Appendix B.

**Table 4.5** Shows the Fibers and their Corresponding Slope of their Tensile Modulus.

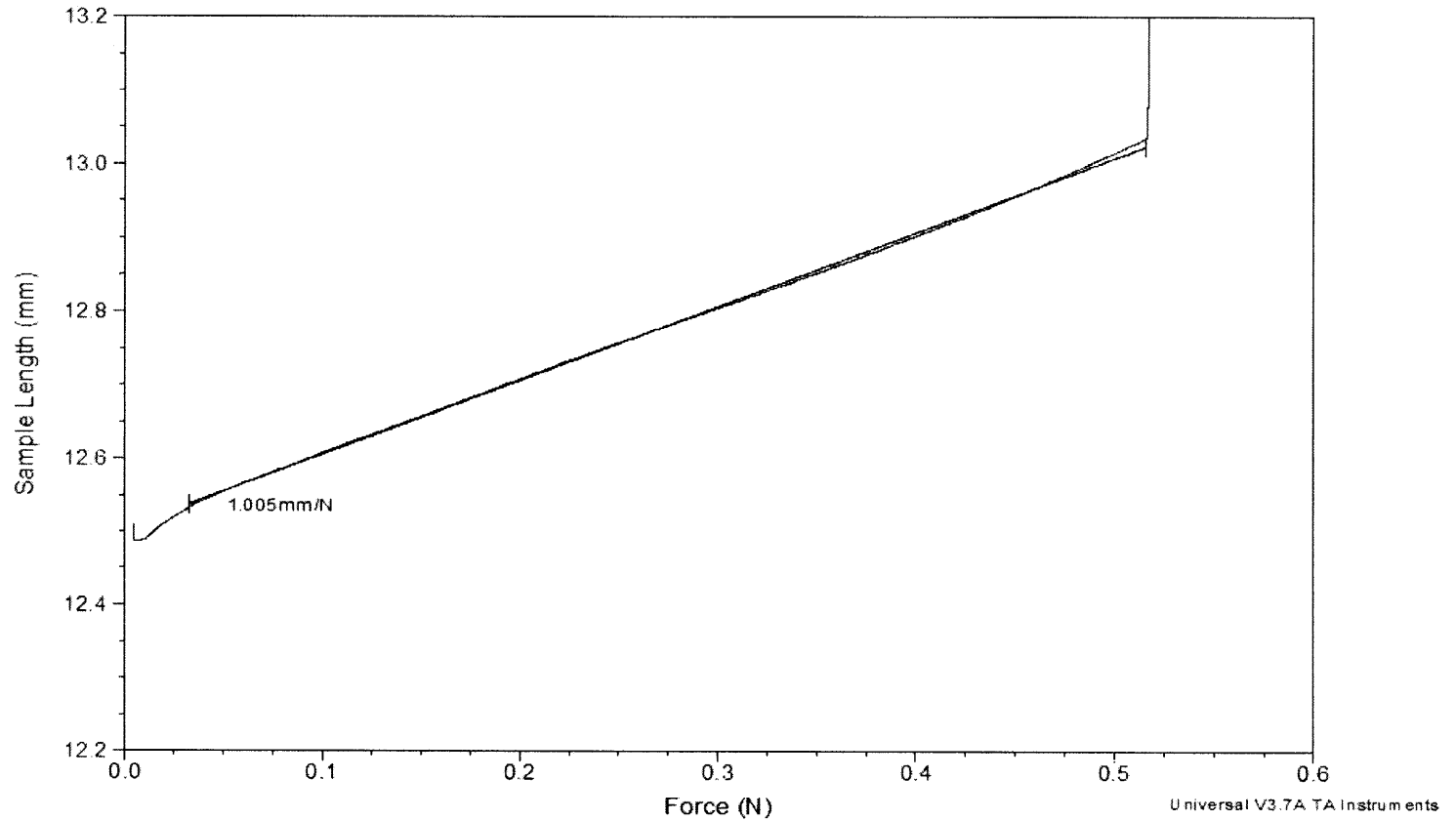
Percentage of dispersion of collagen	Slope mm/Newton	Estimated Modulus Newton/Meter <sup>2</sup>
1 Non Cross linked	1.005	-
1 Cross linked	0.9050	-
1.6 Non Cross linked	0.2816	$0.1 * 10^{12}$
1.6 Cross linked	0.1675	$0.4 * 10^{12}$
2 Non Cross linked	0.3067	$0.13 * 10^{12}$
2 Cross linked	0.1954	$0.20 * 10^{12}$



Sample: Collagen Fiber 1%  
Size: 12.5261 mm  
Method: Stress/Strain  
Comment: Test1

TMA

File: C:\...Desktop\ts\CF1.001f  
Operator: Grace  
Run Date: 23-Dec-03 11:30  
Instrument: 2940 TMA V2.4E

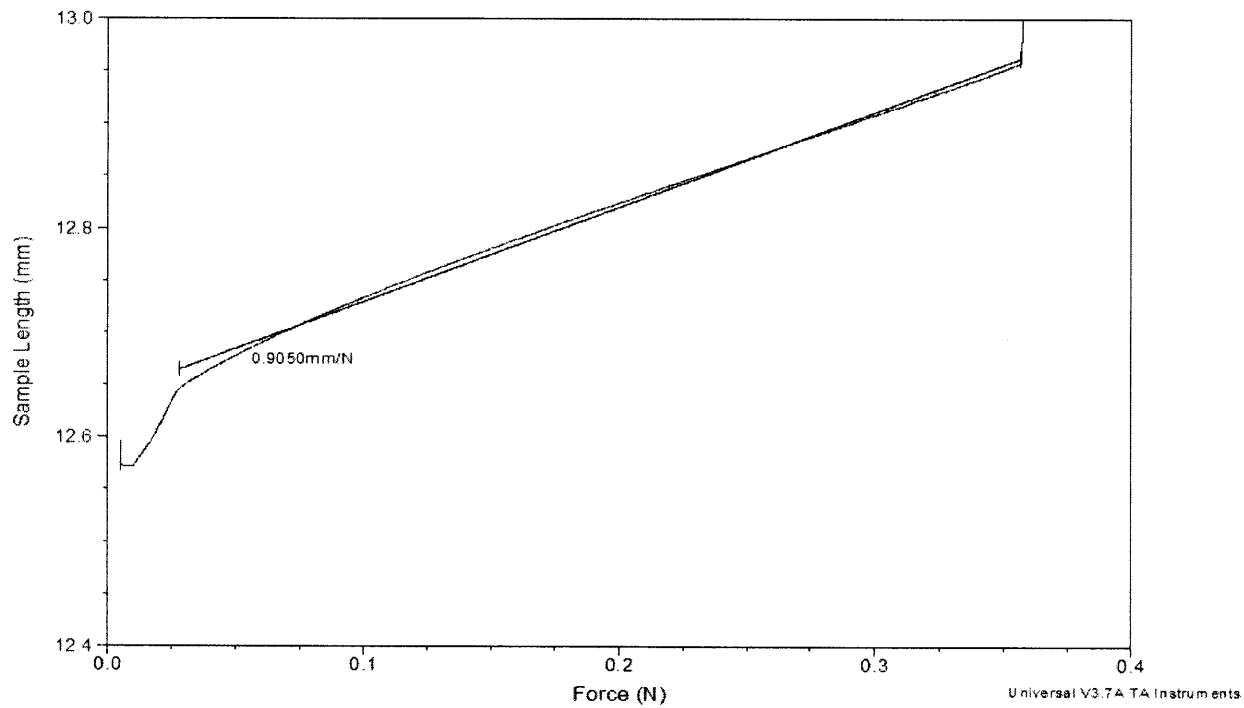


**Figure 4.7** This plot above shows the slope of the stress strain curve of collagen film with 1% dispersion.

Sample: Collagen Fiber 1 (X)  
Size: 12.6127 mm  
Method: Stress/Strain  
Comment: Test1

TMA

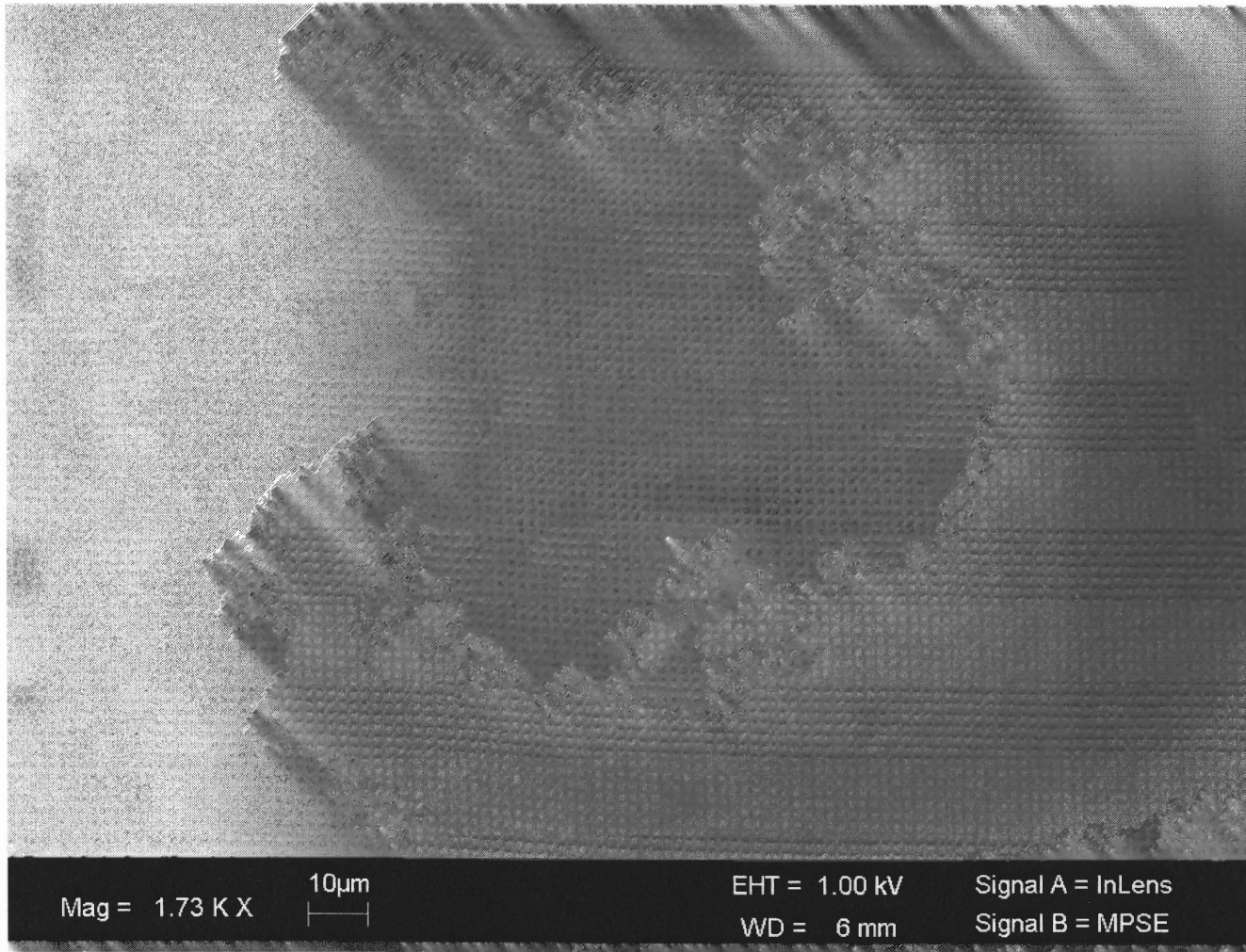
File: C:\Desktop\ts\CF1X.001f  
Operator: Grace  
Run Date: 23-Dec-03 13:23  
Instrument: 2940 TMA V2.4E



**Figure 4.8** This plot above shows the slope of the stress strain curve of collagen film with 1% dispersion and cross-linked.

#### **4.5 Scanning Electron Microscope**

Observation of the appearance of surface and the diameter of these collagen fibers were carried out using scanning electron microscope. The surface of the collagen is smooth without pores. The collagen film made with 1% dispersion appears more like a film in both cross linked and non cross linked fiber. Other fibers made of 1.6% and 2% appeared cylindrical with diameters ranging from 10-24micrometers. Figures below show the SEM pictures and the rest are followed in the Appendix B.



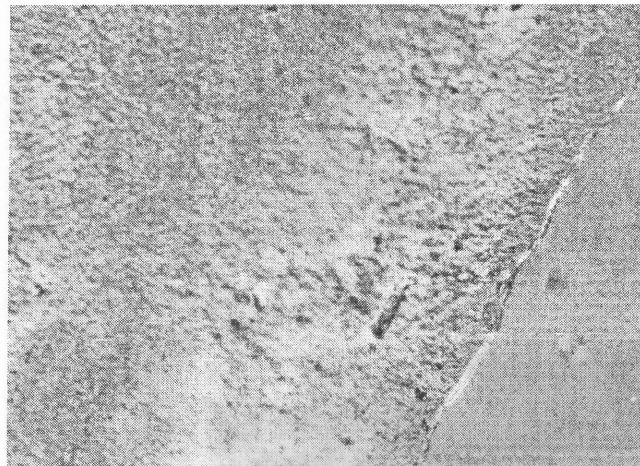
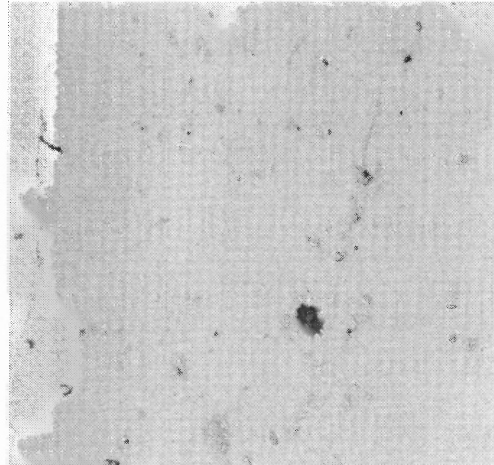
**Figure 4.9** The surface of the collagen film with 1% dispersion non cross-linked.



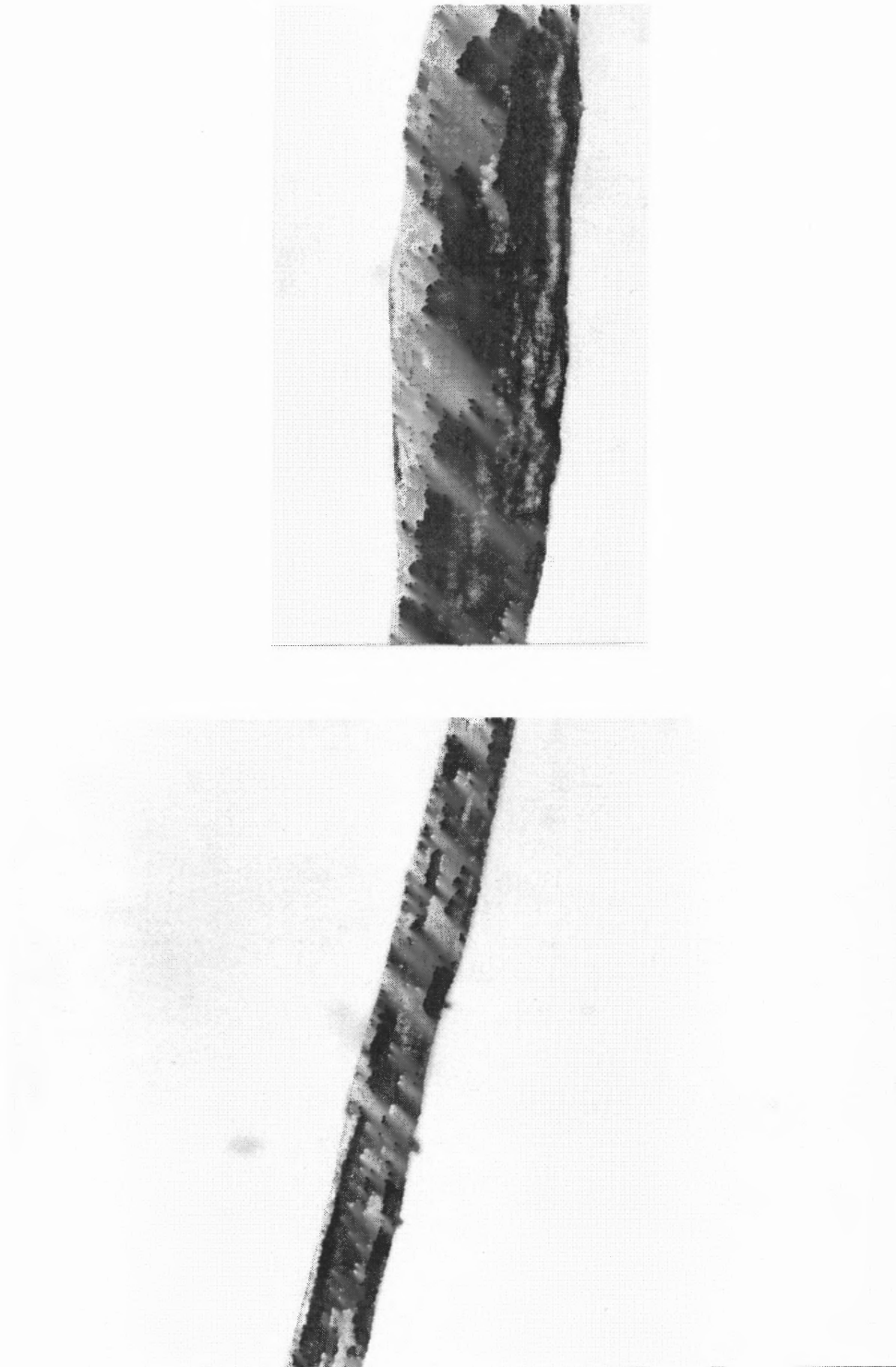
**Figure 4.10** The surface of the collagen film with 1% dispersion cross-linked ,

#### 4.6 Universal Research Microscope

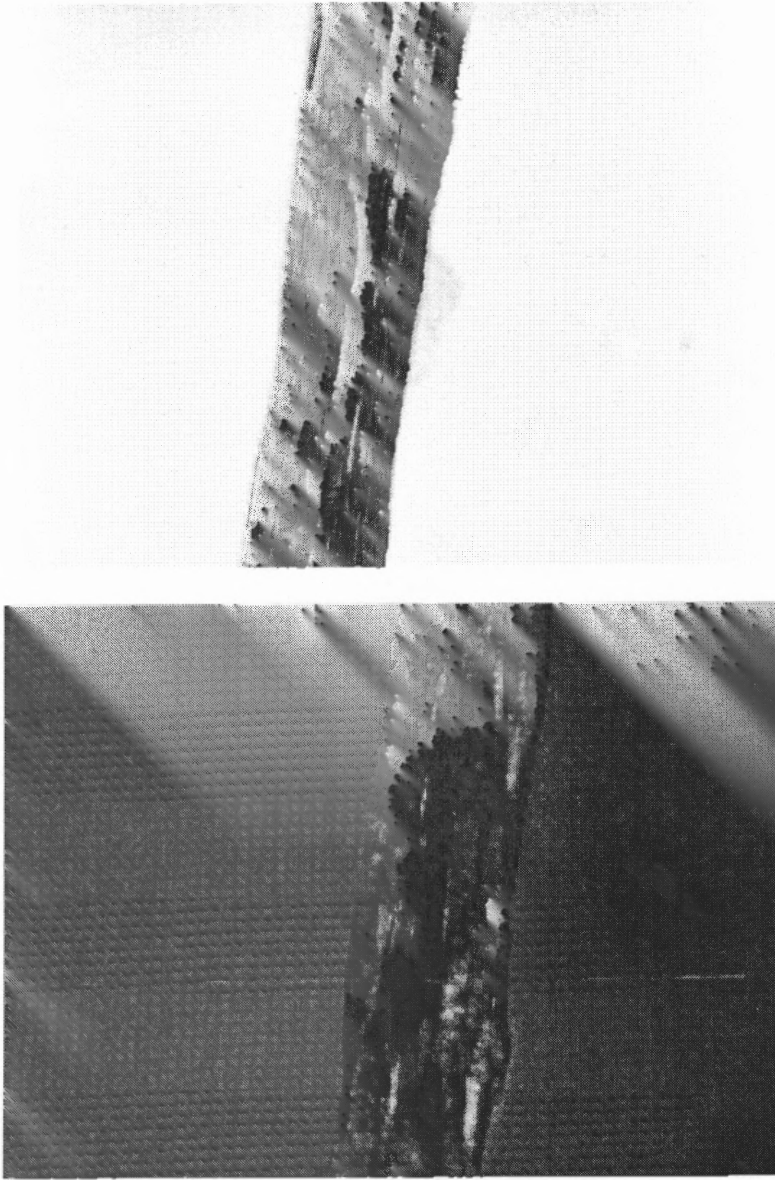
The following figures show the polarized and non polarized images of collagen fibers with different collagen dispersion percentages and also with cross-linked and non cross-linked fibers. They exhibit the property of birefringence showing that the fibers are oriented.



**Figure 4.11** Collagen film with 1% dispersion non-polarized (top) and polarized (bottom).



**Figure 4.12** Collagen film with 1.6% dispersion non-polarized (top) and polarized (bottom).



**Figure 4.13** Collagen film with 2% dispersion non-polarized (top) and polarized (bottom).



## **CHAPTER 5**

### **CONCLUSION**

In this study procedures for spinning bovine tendon collagen at three different dispersion levels (1%, 1.6% and 2%) were developed. Further techniques for cross-linking these fibers using gluteraldehyde were demonstrated.

Thermal analysis techniques were used to evaluate the physical behavior of cross-linked and non cross-linked collagen fiber. Based on the results from the thermo analysis data the mechanical behavior of cross-linked collagen fibers was enhanced relative to the non cross-linked collagen fibers. This enhancement was evident in higher denaturation temperature of cross linked collagen fiber compared to non cross linked collagen fibers as well as a lower tensile deformation of the cross linked fiber per unit force applied compared to non cross linked fibers.

Based on these results the ability to cross-link wet spun collagen fiber has been clearly demonstrated. These fibers with further processing and modification could be used to make scaffolds for tissue engineering and other surgical purposes. With the ability to tailor physical behavior via cross-linking these fibers can be engineered for specific applications.

#### **5.1 Recommendations**

The major drawback to spin fibers of longer lengths should be overcome by finding better techniques to remove the air bubbles that usually block the continuous flowing collagen gel. Using different concentrations of gluteraldehyde the degree of cross-linking can be regulated and enhanced to make stronger fibers.

## **APPENDIX A**

### **SAFETY PROCEDURES**

The safety procedures of spinning are summarized in this appendix.

- Always wear eye protection, hand protection and protective clothing.
- Handle chemicals used during experimental with care and store in a proper place
- Spinning should always be done inside the hood to avoid the fumes that are highly inflammable
- Clean syringes, needles, beakers should be used.
- Used solutions should not be disposed in the drains.
- Close the lid of ammonium hydroxide bottle immediately after use.
- Electrical Safety during Spinning:
- Proper care should be taken while connecting the wires
- Keep the wires from crossing the acetone bath

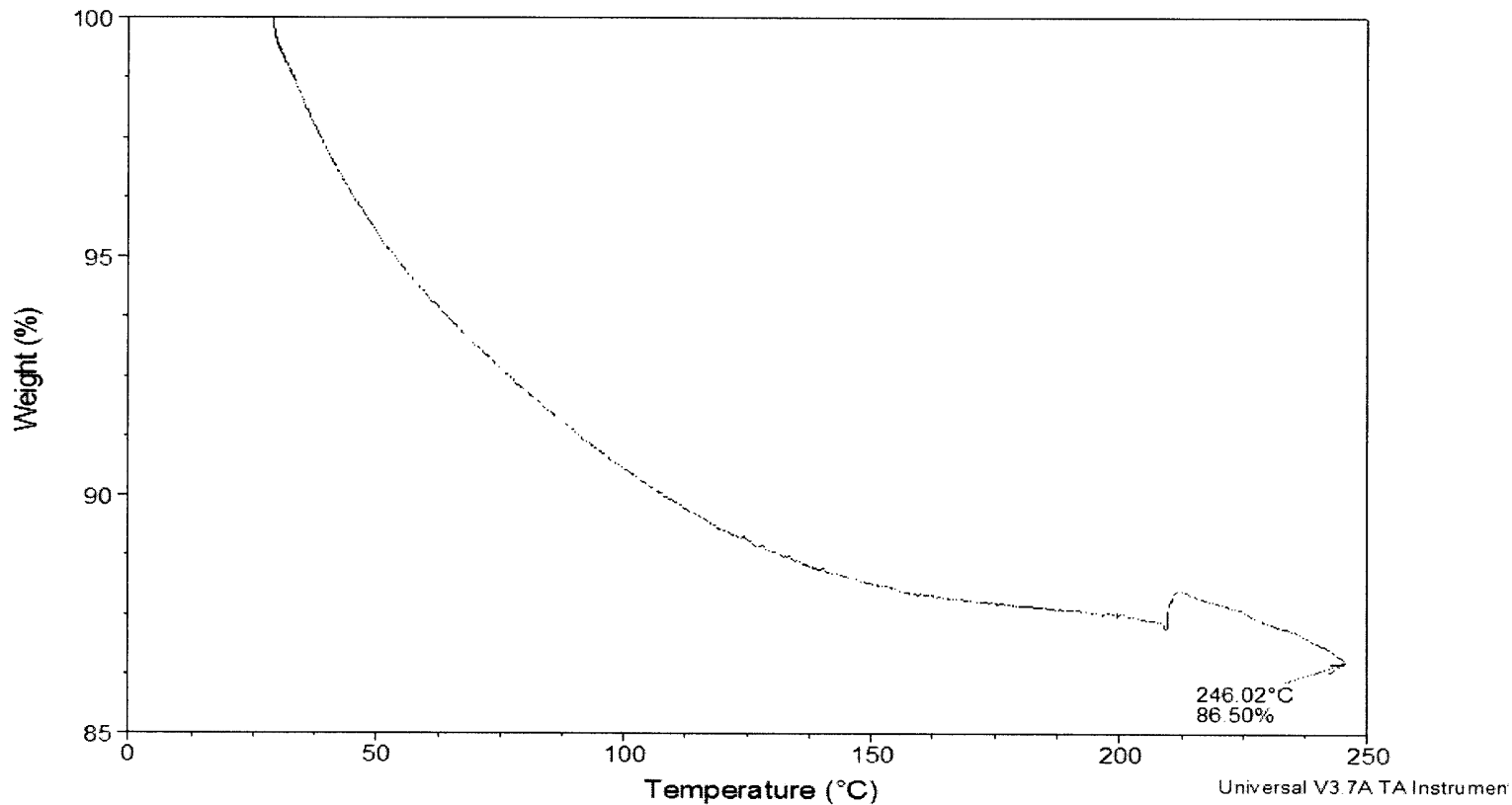
## **APPENDIX B**

The figures below show the TGA, DSC, TMA plots and SEM figures of collagen fibers of different percentage dispersions with and without cross-linking.

Sample: Collagen Fiber 1.6%  
Size: 0.5880 mg  
Method: Ramp  
Comment: with screen

### TGA

File: C:\My Documents\tga\Grace\_TGA\CF1.6%  
Operator: Grace  
Run Date: 18-Nov-03 14:37  
Instrument: TGA Q50 V5.0 Build 164

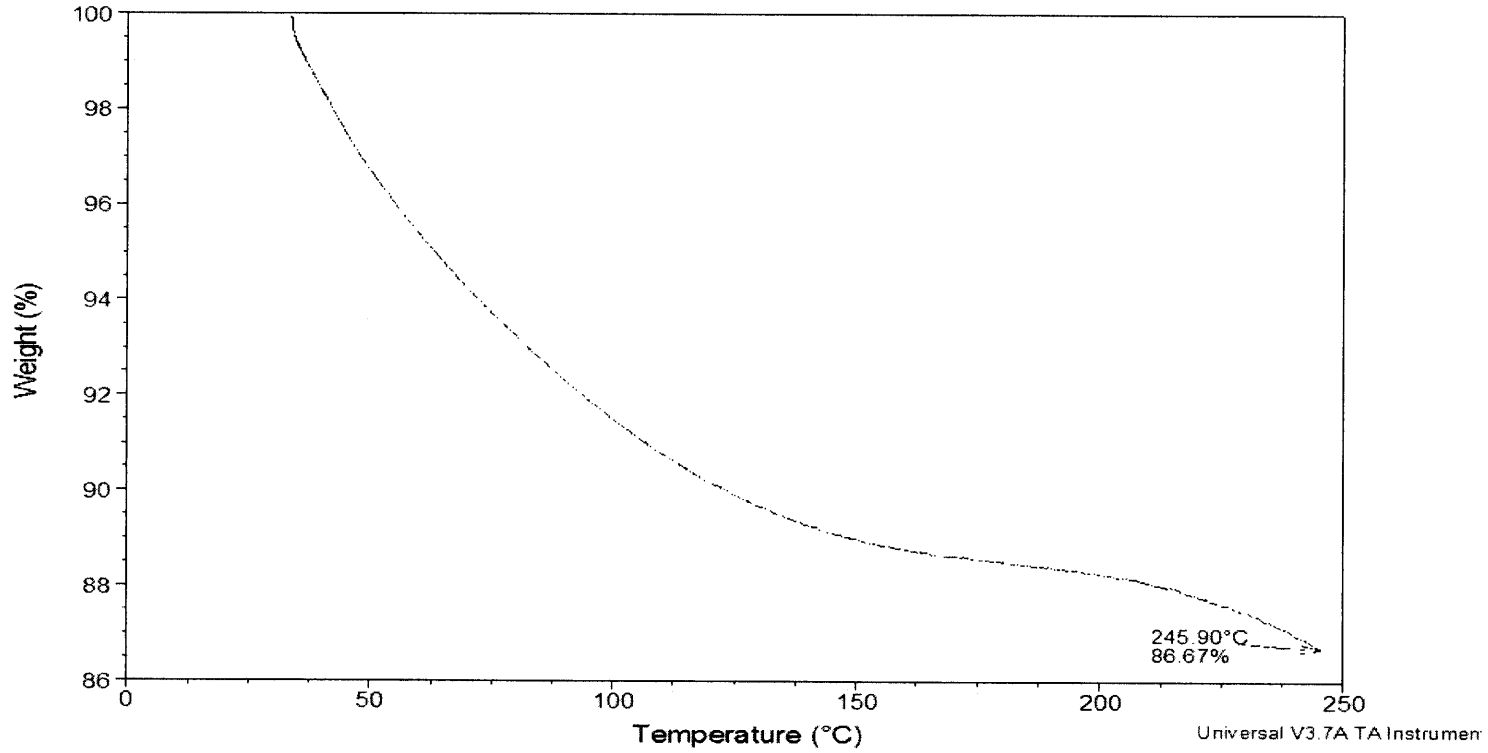


**Figure B.1** Thermogravimetric analysis (TGA) plot of collagen with 1.6% dispersion and no cross-linking.

Sample: Collagen Filber X 1.6%  
Size: 0.8940 mg  
Method: Ramp  
Comment: with screen

### TGA

File: C:\tga\Grace\_TGA\CFX1.6%0  
Operator: Grace  
Run Date: 18-Nov-03 13:41  
Instrument: TGA Q50 V5.0 Build 164

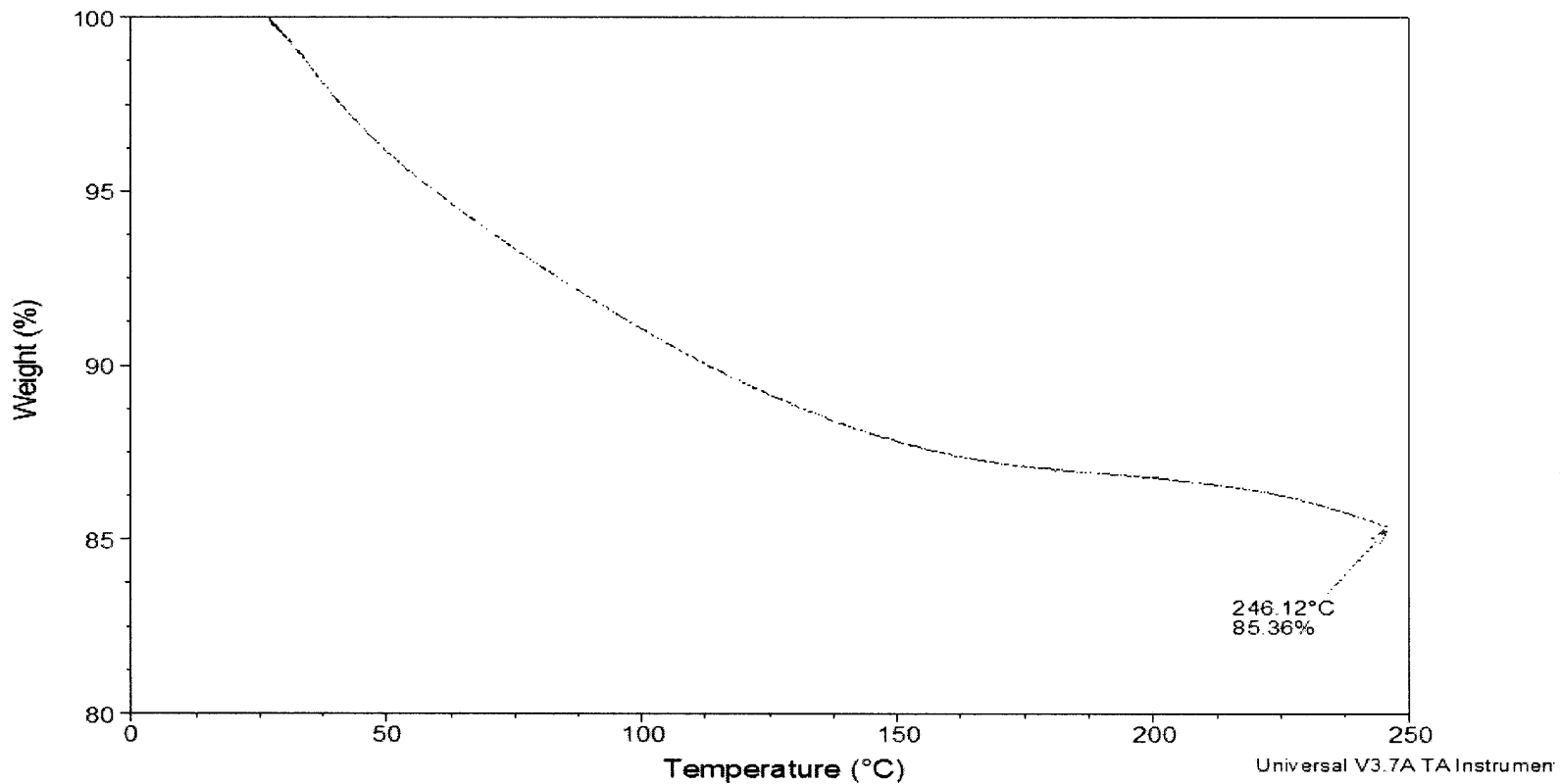


**Figure B.2** Thermogravimetric analysis (TGA) plots of collagen with 1.6 % dispersion and cross-linked.

Sample: Collagen Fiber 2%  
Size: 0.7950 mg  
Method: Ramp  
Comment: with screen

### TGA

File: C:\My Documents\tga\CF 2%.001  
Operator: Grace  
Run Date: 18-Nov-03 15:44  
Instrument: TGA Q50 V5.0 Build 164

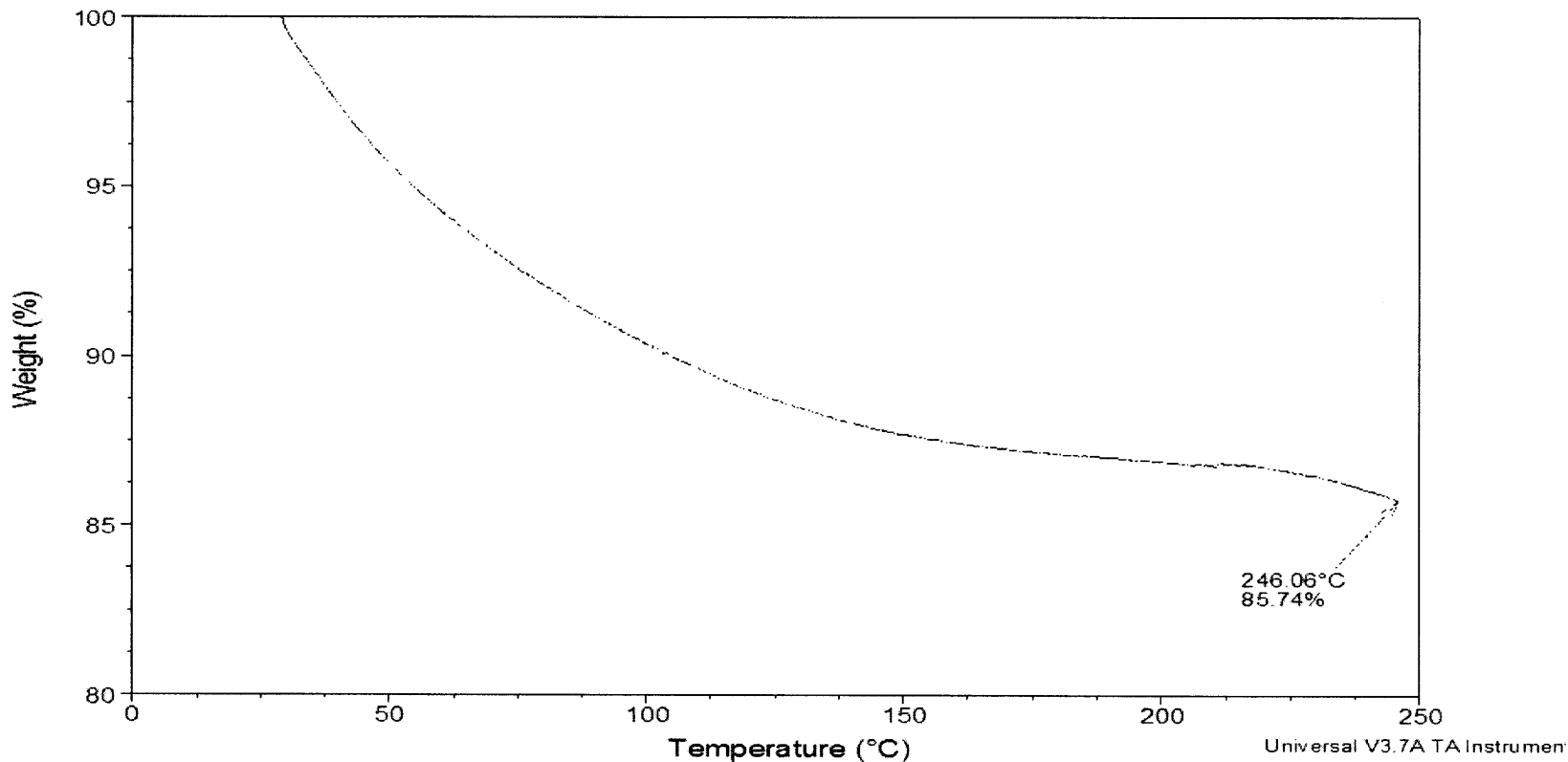


**Figure B.3** Thermogravimetric analysis (TGA) plot of collagen with 2% dispersion and no cross-linking.

Sample: Collagen Fiber X 2%  
Size: 1.4450 mg  
Method: Ramp  
Comment: with screen

### TGA

File: C:\My Documents\tga\CF X 2%.001  
Operator: Grace  
Run Date: 18-Nov-03 16:45  
Instrument: TGA Q50 V5.0 Build 164

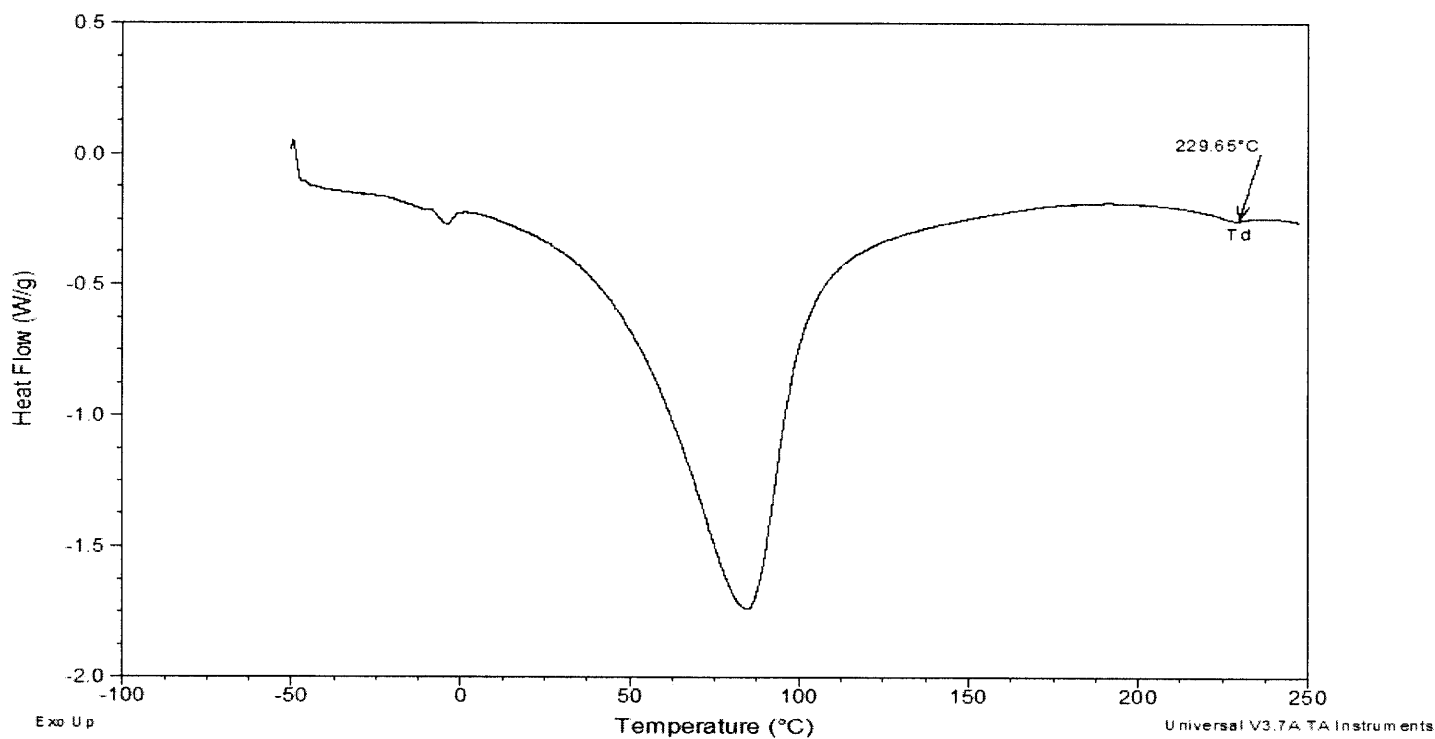


**Figure B.4** Thermogravimetric analysis (TGA) plot of collagen with 2% dispersion and with cross-linking.

Sample: collagen fiber (X) 1%  
Size: 4.5000 mg  
Method: Ramp

DSC

File: C:\DSC1\CF1\Grace\CF1(X).002  
Operator: Grace  
Run Date: 10-Dec-03 13:30  
Instrument: DSC Q100 V7.0 Build 244



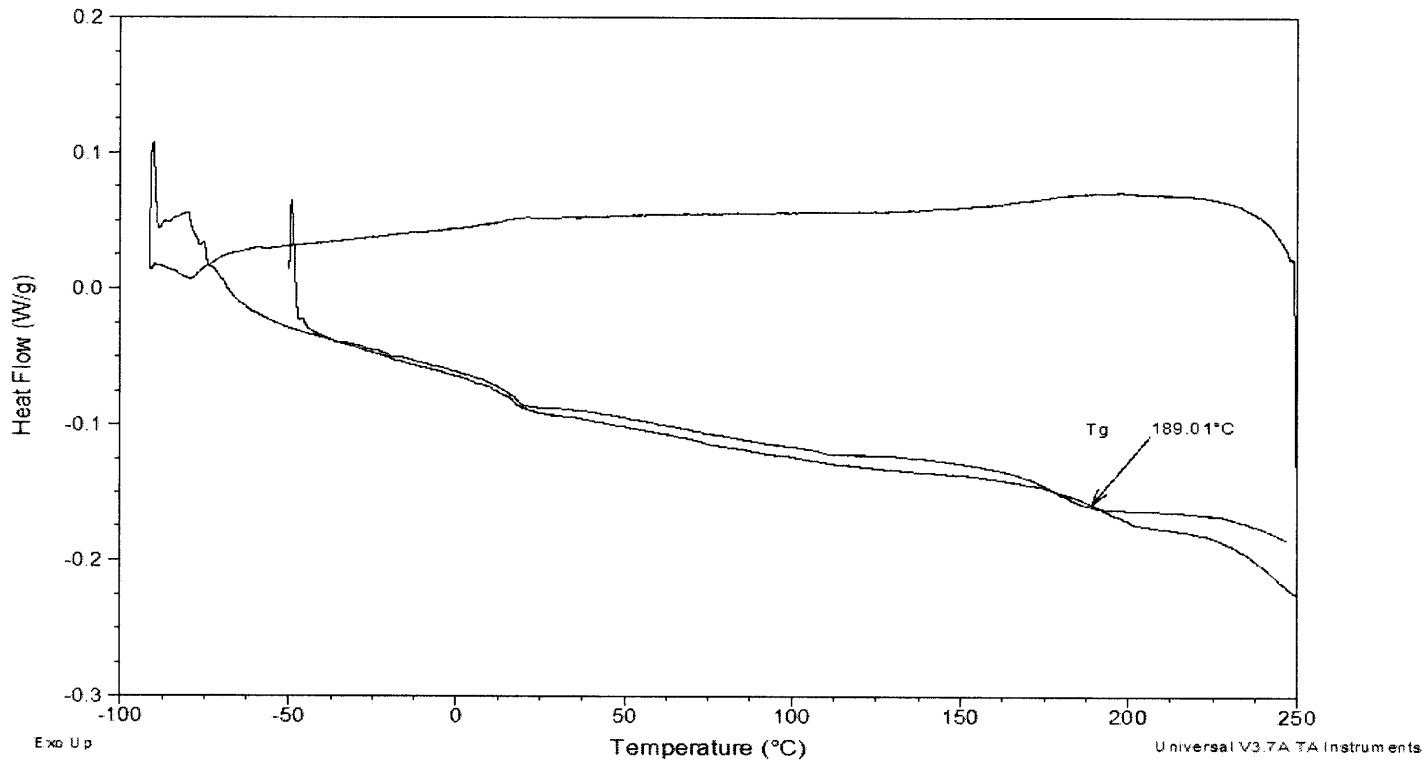
**FigureB.5** DSC plot above shows the heating cycle for 1% collagen fiber with cross links.



Sample: collagen fiber (X) 1%  
Size: 4.5000 mg  
Method: Heat/Cool/Heat

### DSC

File: C:\DSC1\CF1\Grace\CF1(X).003  
Operator: Grace  
Run Date: 10-Dec-03 15:12  
Instrument: DSC Q100 V7.0 Build 244

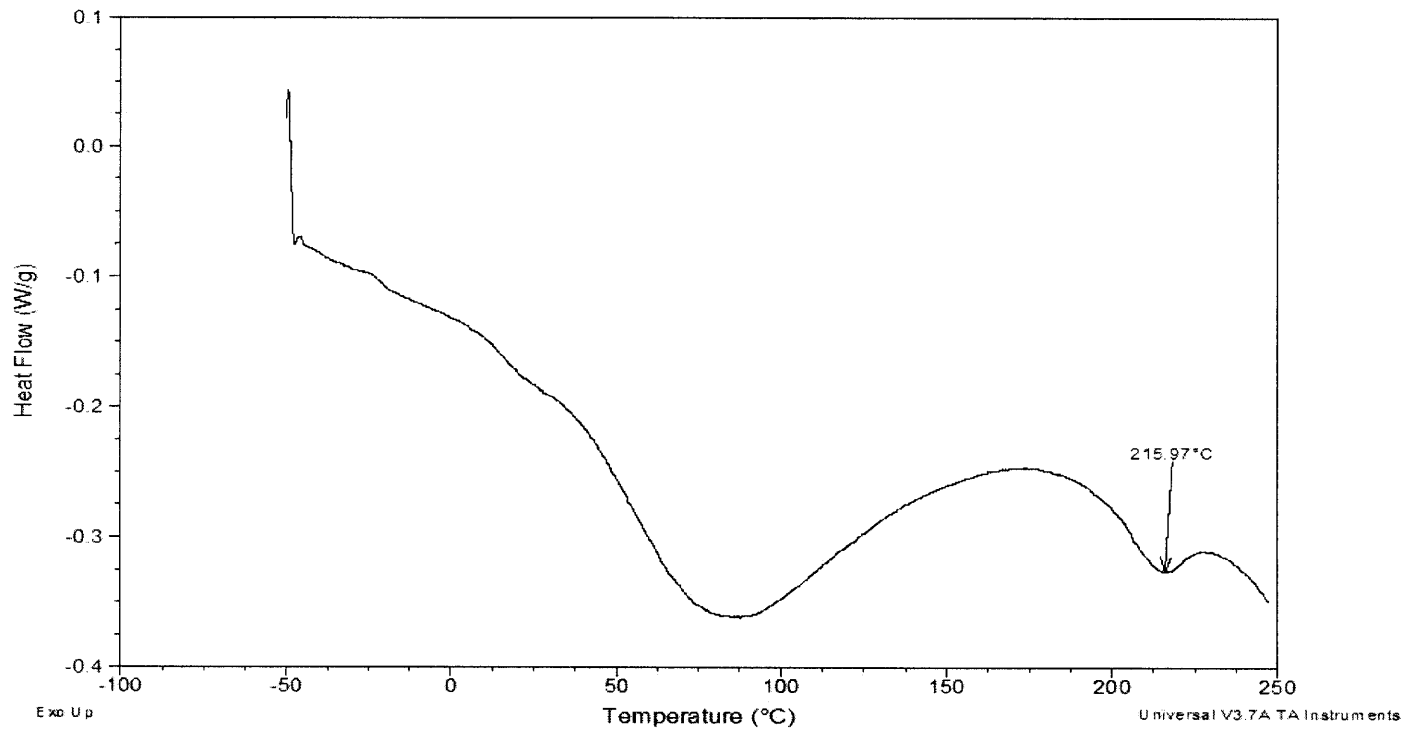


**Figure B.6** DSC plot above shows the heat/cool/heat cycle for 1% collagen fiber with cross linking.

Sample: collagen fiber 1.6%  
Size: 3.5000 mg  
Method: Ramp

DSC

File: C:\DSC\Cf01\Grace\CFR1.600  
Operator: Grace  
Run Date: 09-Dec-03 10:51  
Instrument: DSC Q100 V7.0 Build 244



**Figure B.7** DSC plot above shows the ramp heating of collagen fiber with 1.6% dispersion.

Sample: collagen fiber 1.6%  
Size: 3.5000 mg  
Method: Heat/Cool/Heat

DSC

File: C:\DSC\Cf01\Grace\CFH1.6%0  
Operator: Grace  
Run Date: 09-Dec-03 11:33  
Instrument: DSC Q100 V7.0 Build 244

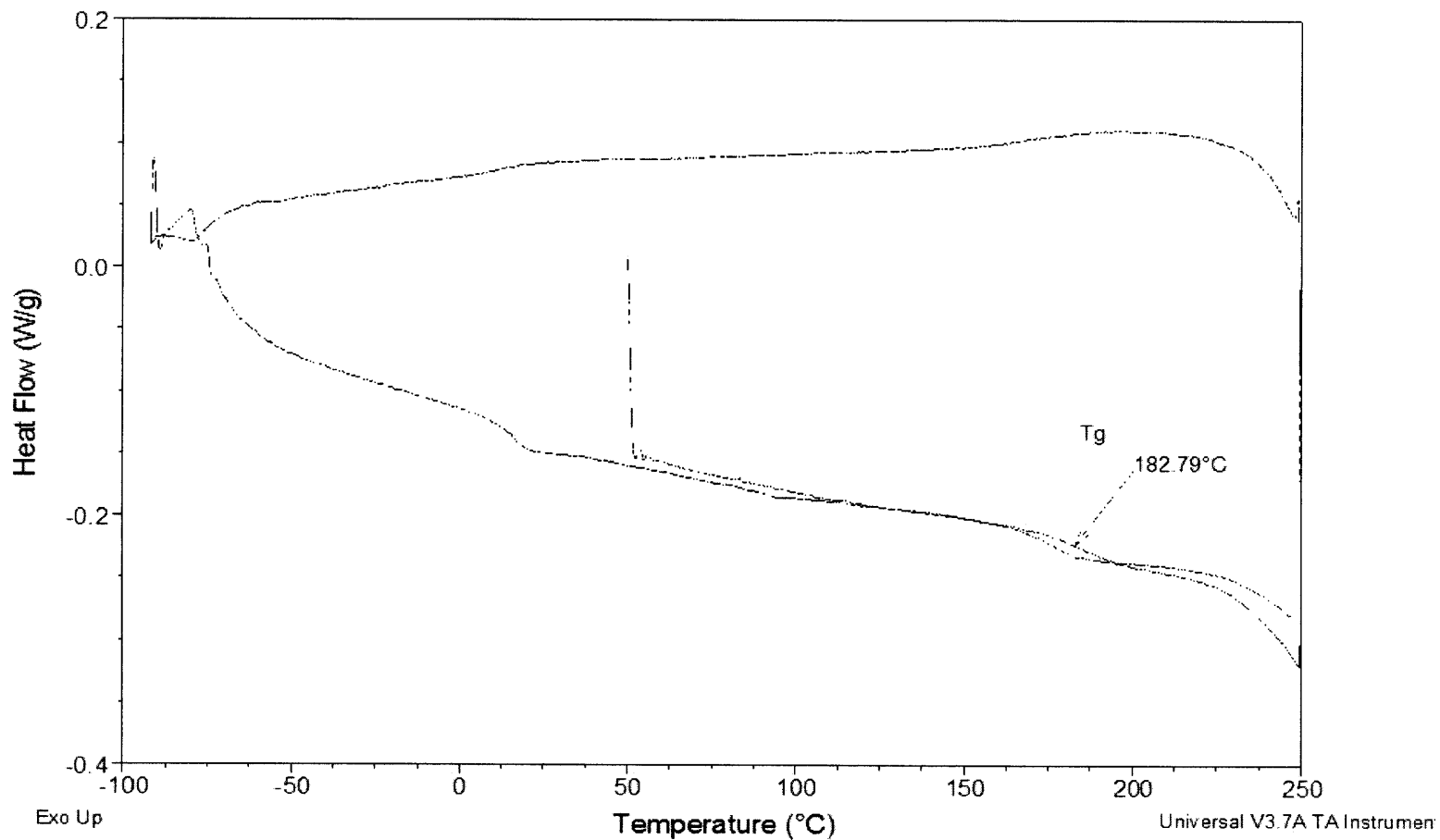


Figure B.8 DSC plot above shows the heat/cool/heat cycle of collagen fiber with 1.6 % dispersion.

Sample: collagen fiber (X) 1.6%  
Size: 3.5000 mg  
Method: Ramp

DSC

File: C:\DSC1\CF1\CF1.6 (X).001  
Operator: Grace  
Run Date: 11-Dec-03 09:59  
Instrument: DSC Q100 V7.0 Build 244

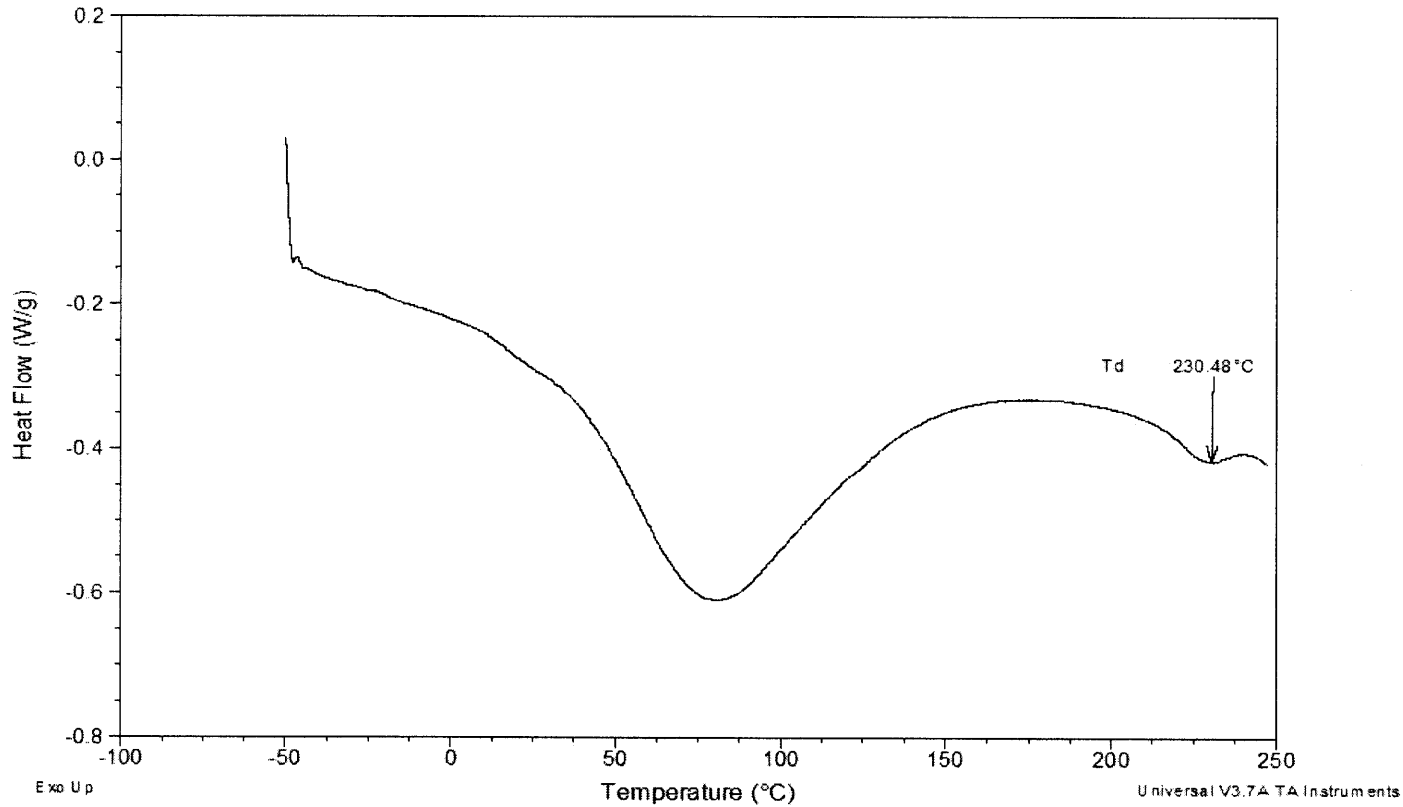


Figure B.9 DSC plot above shows the ramp heating of collagen fiber with 1.6% dispersion and cross linked.

Sample: collagen fiber (X) 1.6%  
Size: 3.5000 mg  
Method: Heat/Cool/Heat

DSC

File: C:\...\CFH1\CFH1.6 (X).002  
Operator: Grace  
Run Date: 11-Dec-03 10:57  
Instrument: DSC Q100 V7.0 Build 244

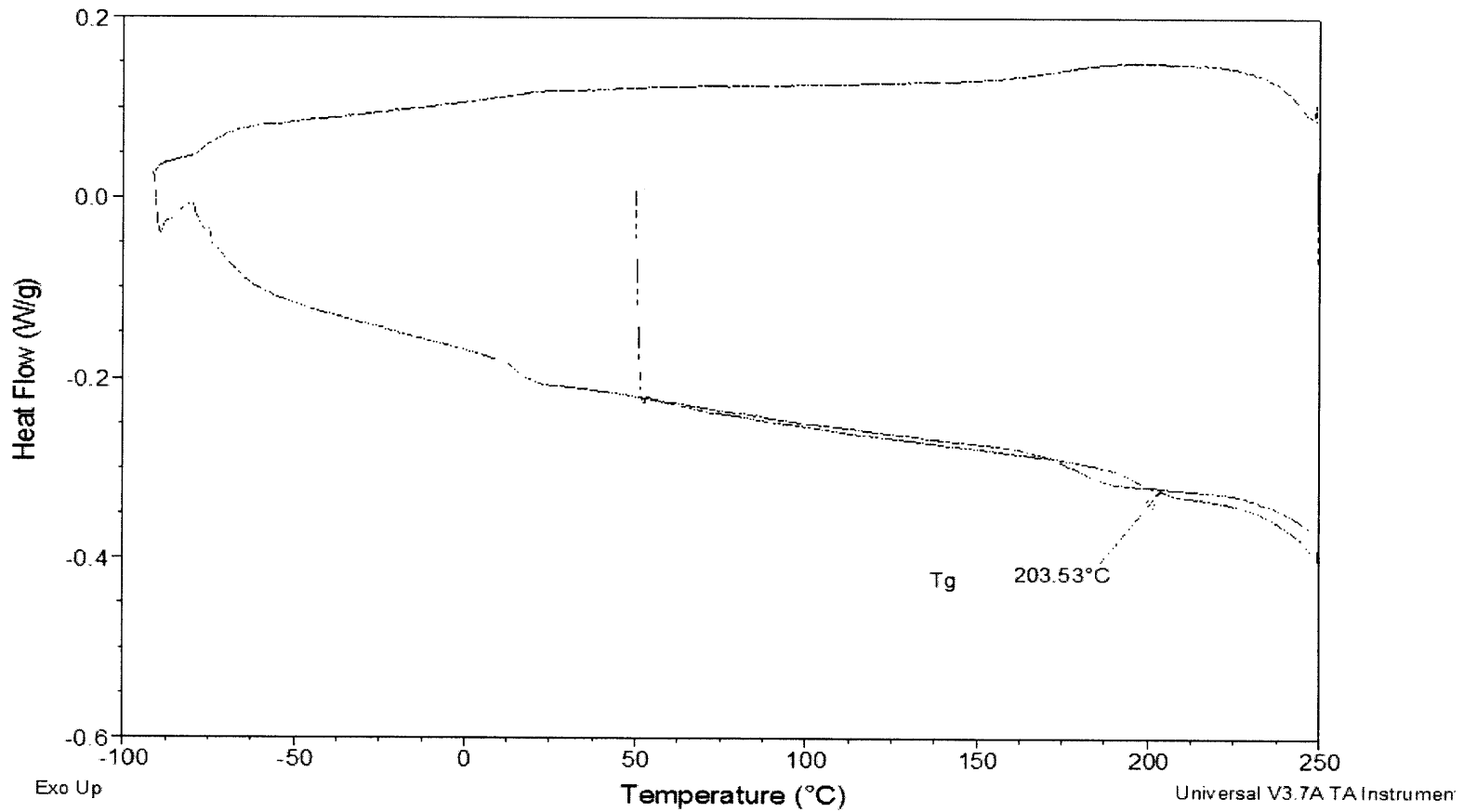
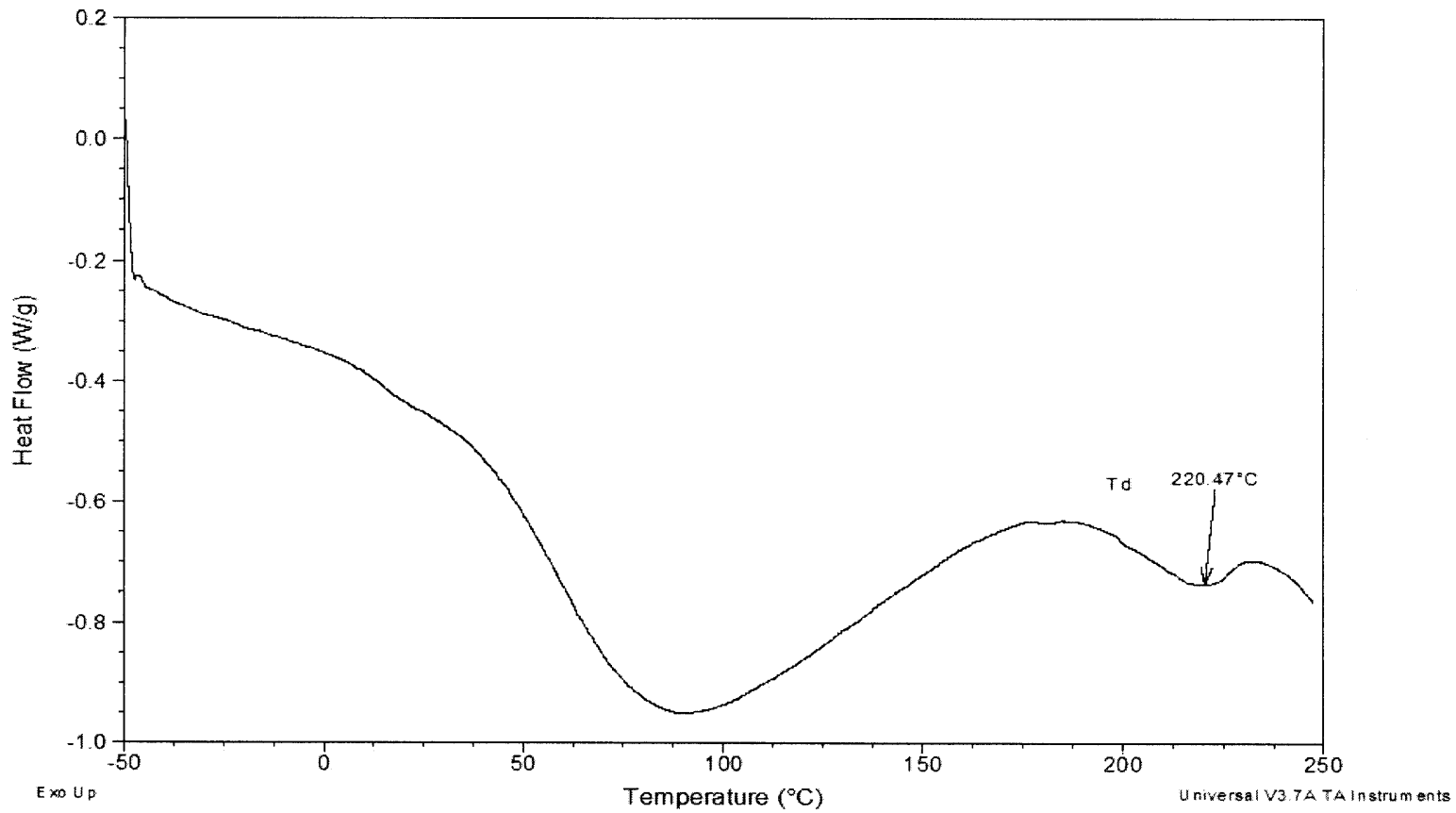


Figure B.10 DSC plot above shows the ramp heating of collagen fiber with 1.6% dispersion with cross links .

Sample: CF 2%  
Size: 2.1000 mg  
Method: Ramp

### DSC

File: C:\...My Documents\CF2.001  
Operator: Grace  
Run Date: 02-Dec-03 11:56  
Instrument: DSC Q100 V7.0 Build 244

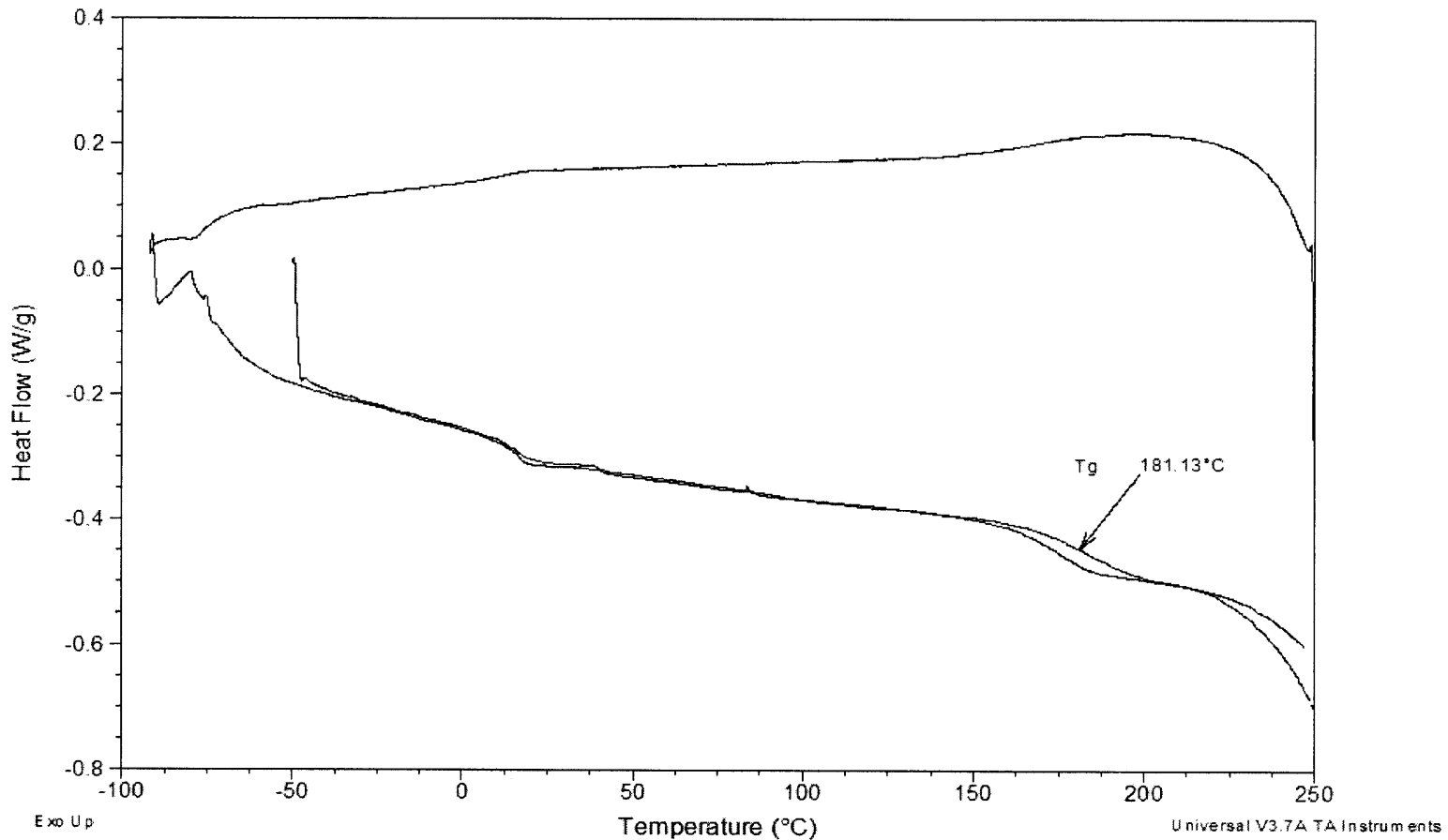


**Figure B.11** DSC plot above shows the ramp heating of collagen fiber with 2% dispersion.

Sample: CFH 2%  
Size: 2.1000 mg  
Method: Heat/Cool/Heat

DSC

File: C:\DSC\Cf01\Grace\CFH2.001  
Operator: Grace  
Run Date: 02-Dec-03 12:45  
Instrument: DSC Q100 V7.0 Build 244



**Figure B.12** DSC plot above shows the heat/cool/heat cycle of collagen fiber with 2 % dispersion.

Sample: Collagen Fiber (X) 2%  
Size: 5.2000 mg  
Method: Ramp

DSC

File: C:\DSC\Cf01\Grace\CFX2.001  
Operator: Grace  
Run Date: 10-Nov-03 12:33  
Instrument: DSC Q100 V7.0 Build 244

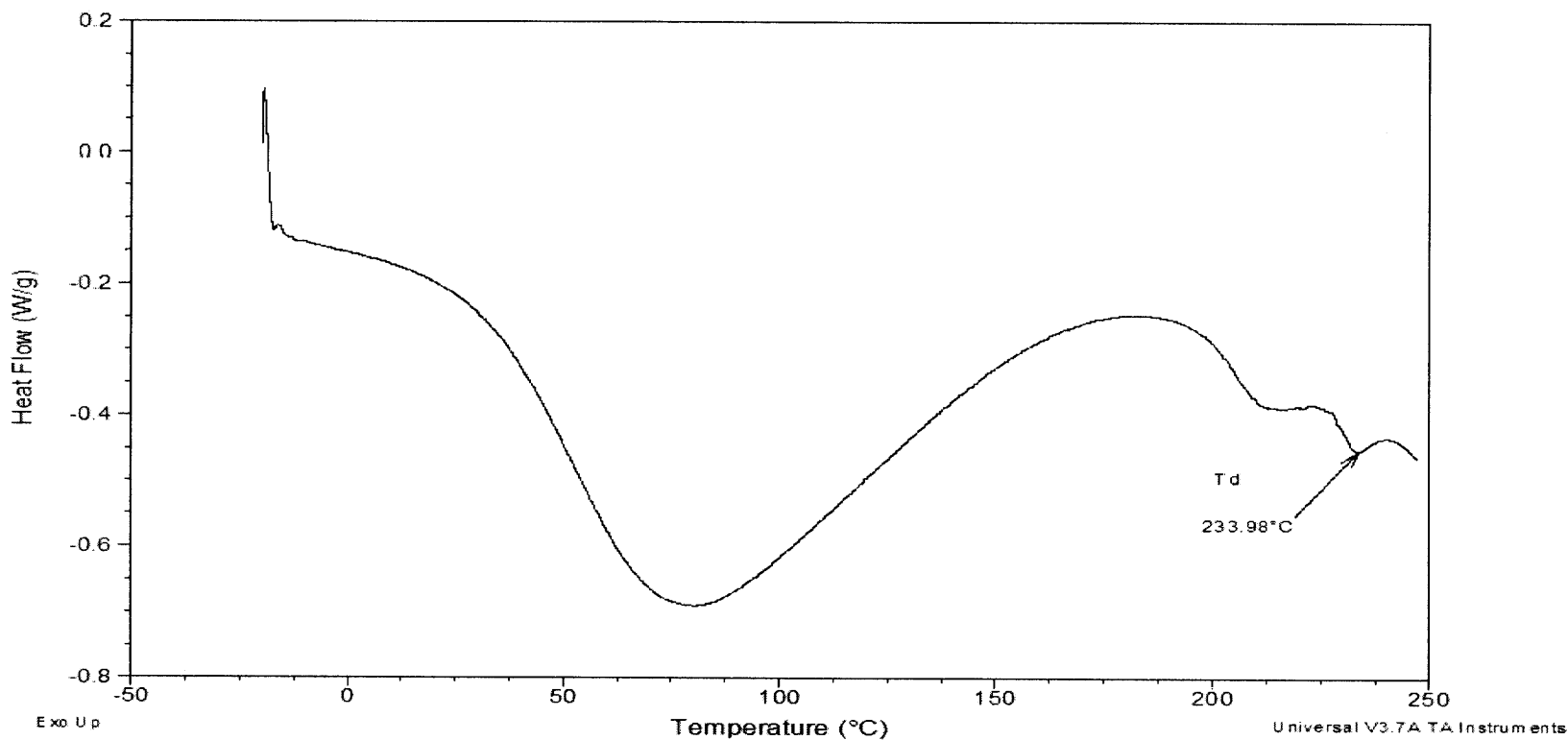


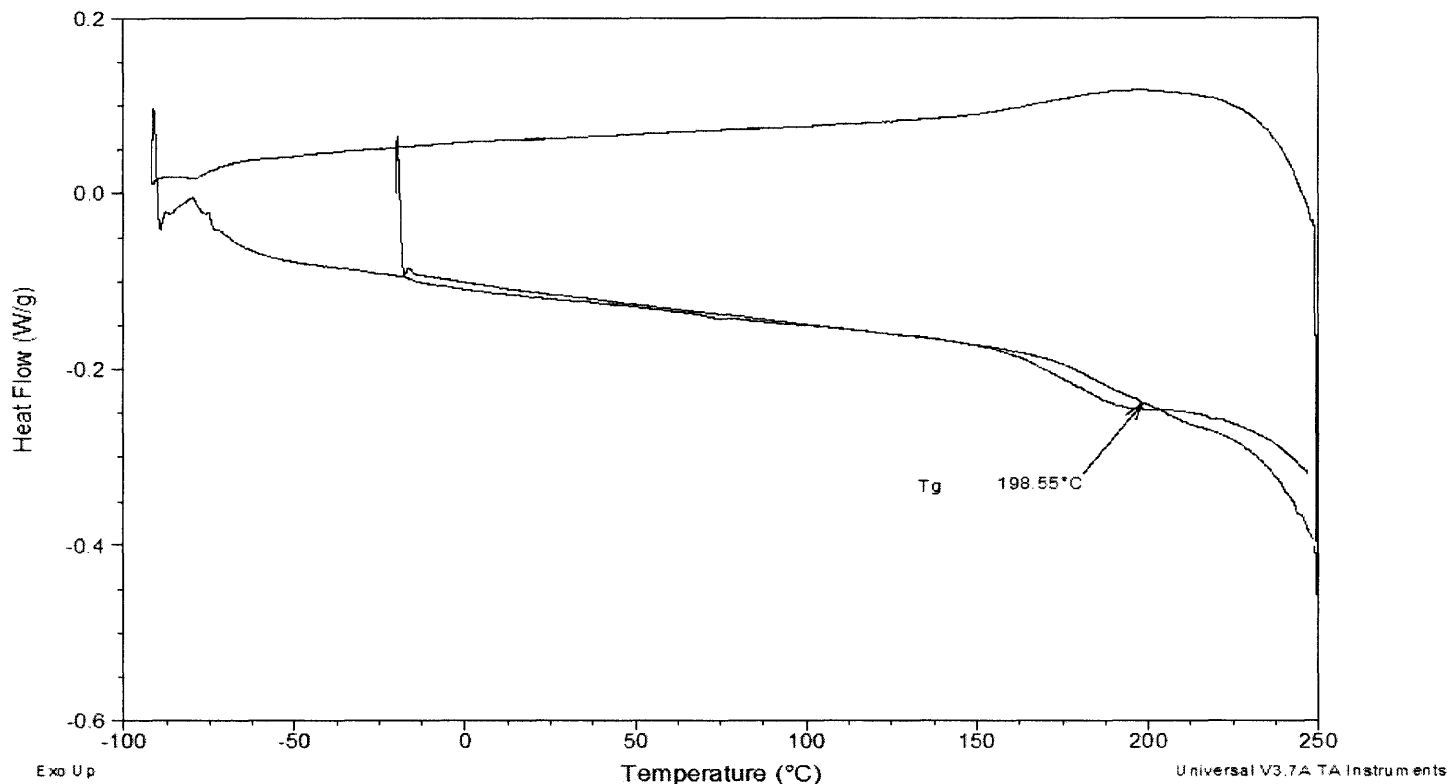
Figure B.13 DSC plot above shows the ramp heating of collagen fiber with 2 % dispersion with cross linking.



Sample: Collagen Fiber (X) 2%  
Size: 5.2000 mg  
Method: Heat/Cool/Heat

DSC

File: C:\DSC1\Dsc\Grace\CFX2.004  
Operator: Grace  
Run Date: 10-Nov-03 13:26  
Instrument: DSC Q100 V7.0 Build 244



**Figure B.14** DSC plot above shows the heat/cool/heat cycle of collagen fiber with 2 % dispersion and cross-linking .

Sample: Collagen Fiber 1.6%  
Size: 12.9039 mm  
Method: Ramp

TMA

File: C:\...Desktop\TMA Files\Grace.007  
Operator: Grace  
Run Date: 13-Nov-03 17:30  
Instrument: 2940 TMA V2.4E

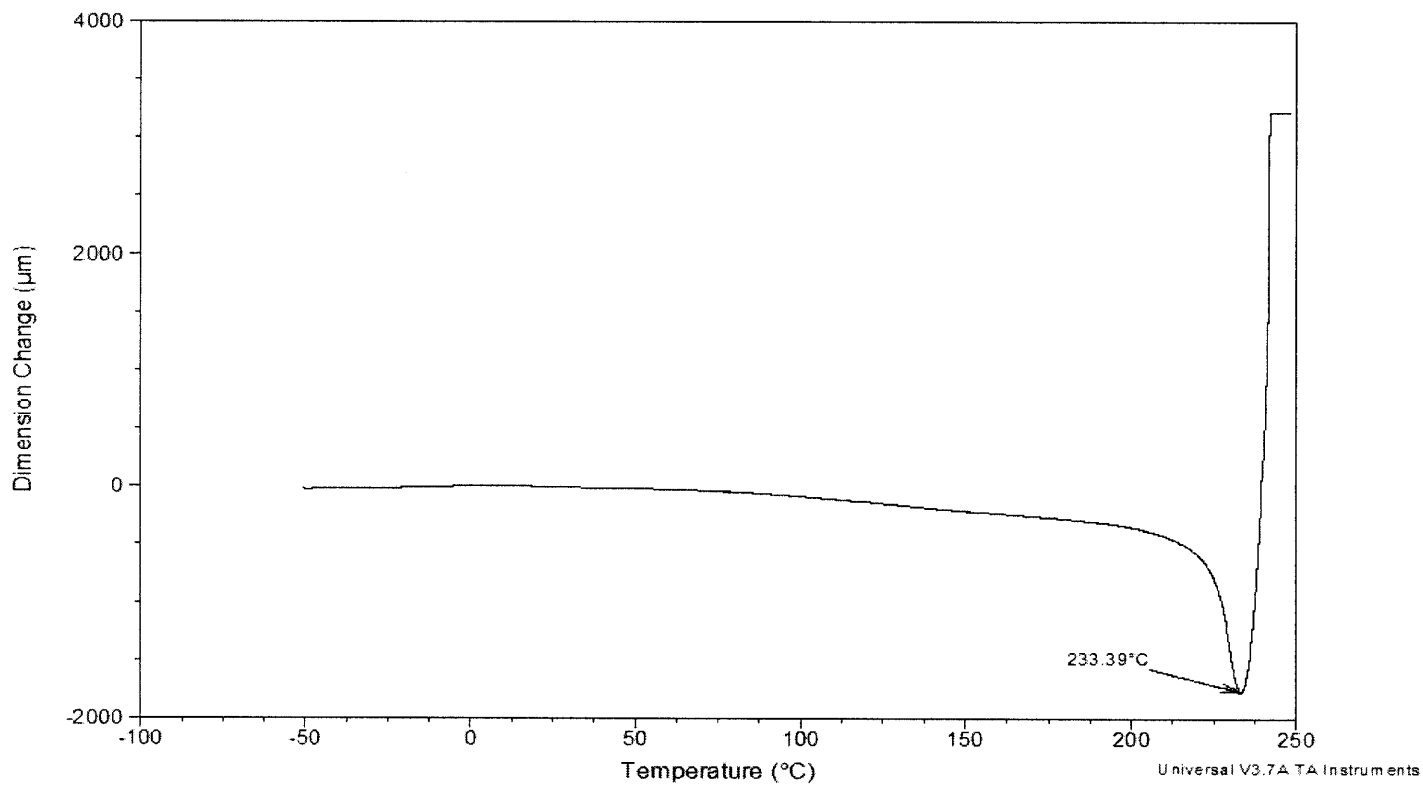
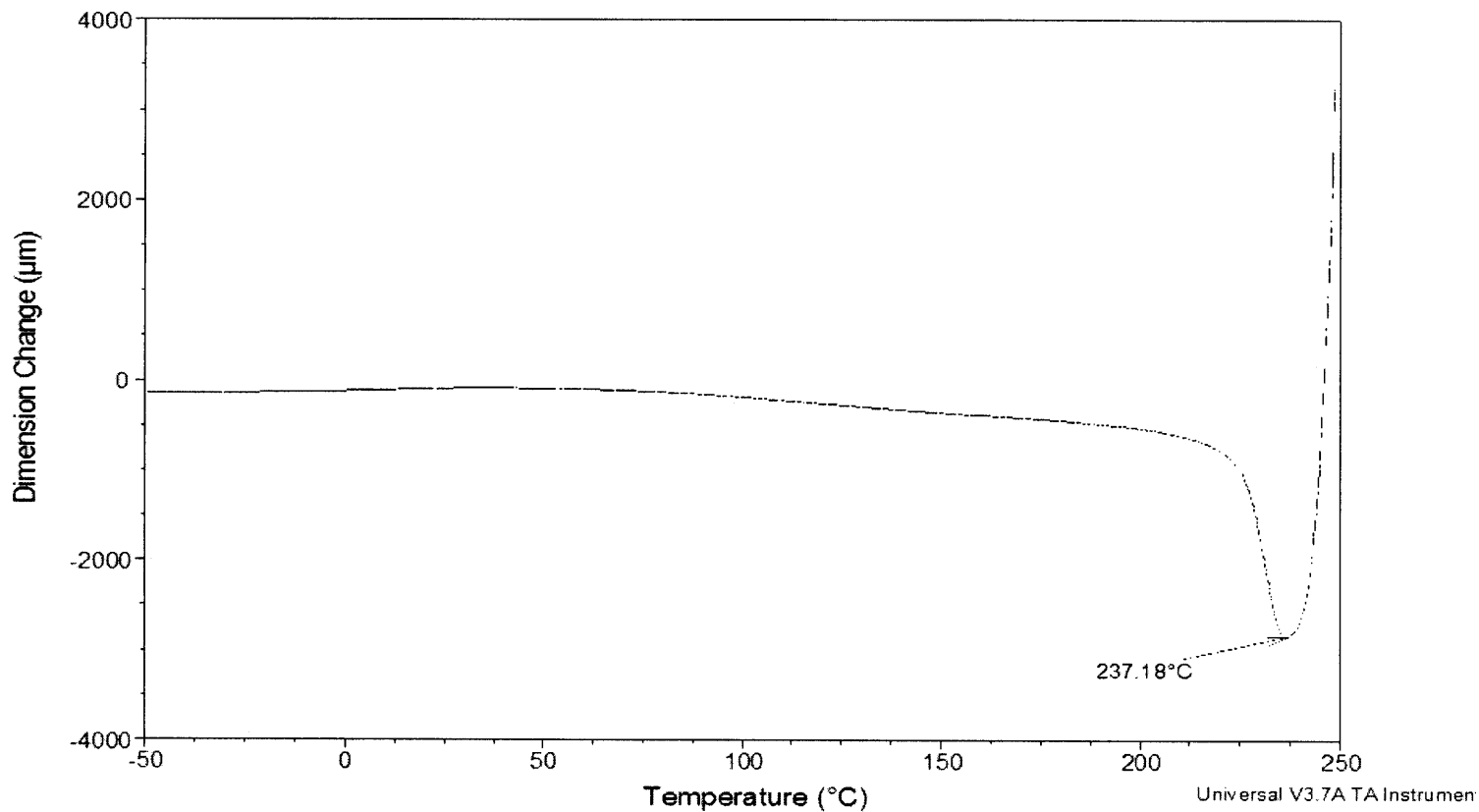


Figure B.15 TMA plot above shows the contraction and break point of collagen fiber with 1.6 % dispersion.

Sample: Collagen Fiber X 1.6 %  
Size: 13.3146 mm  
Method: Ramp

### TMA

File: C:\...Desktop\TMA Files\CF 1.7%  
Operator: Grace  
Run Date: 18-Nov-03 15:23  
Instrument: 2940 TMA V2.4E

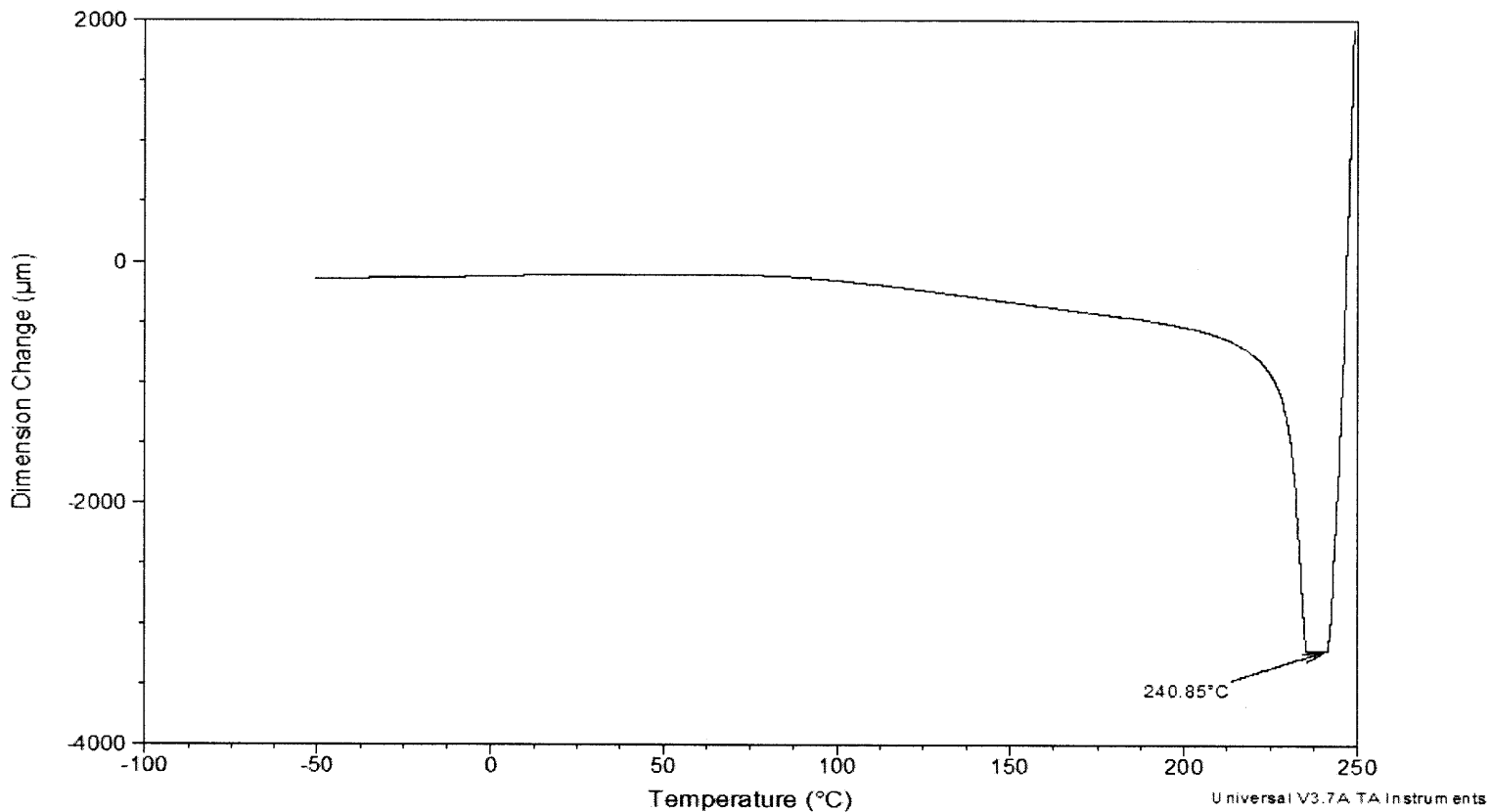


**Figure B.16** TMA plot above shows the contraction and break point of collagen fiber with 1.6 % dispersion and cross-linked.

Sample: Collagen Fiber 2 %  
Size: 12.7627 mm  
Method: Ramp  
Comment: Test 3

### TMA

File: C:\...Desktop\CF 2 %.001  
Operator: Grace  
Run Date: 18-Nov-03 16:27  
Instrument: 2940 TMA V2.4E

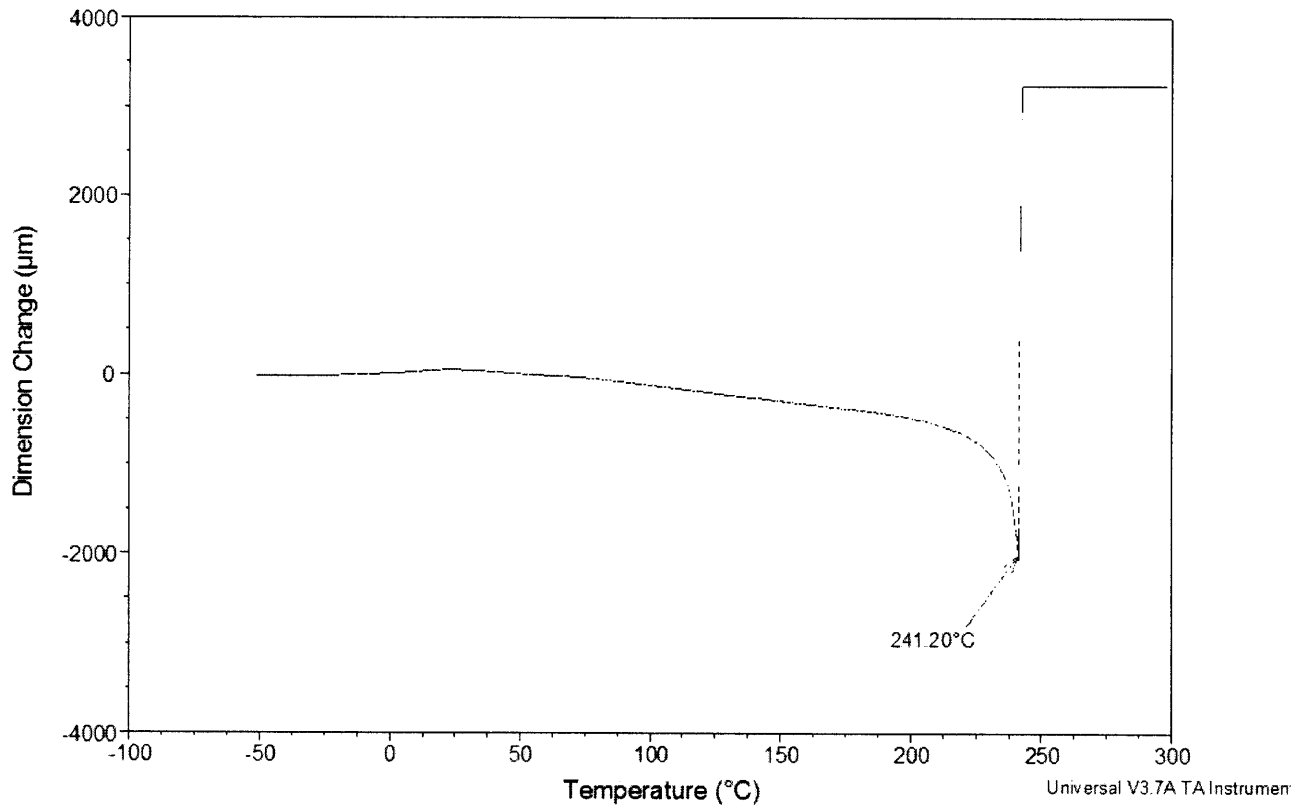


**Figure B.17** TMA plot above shows the contraction and break point of collagen fiber with 2% dispersion.

Sample: Collagen Fiber (X) 2 %  
Size: 12.7065 mm  
Method: Ramp

TMA

File: C:\Desktop\TMA Files\Grace.010  
Operator: Grace  
Run Date: 14-Nov-03 17:26  
Instrument: 2940 TMA V2.4E

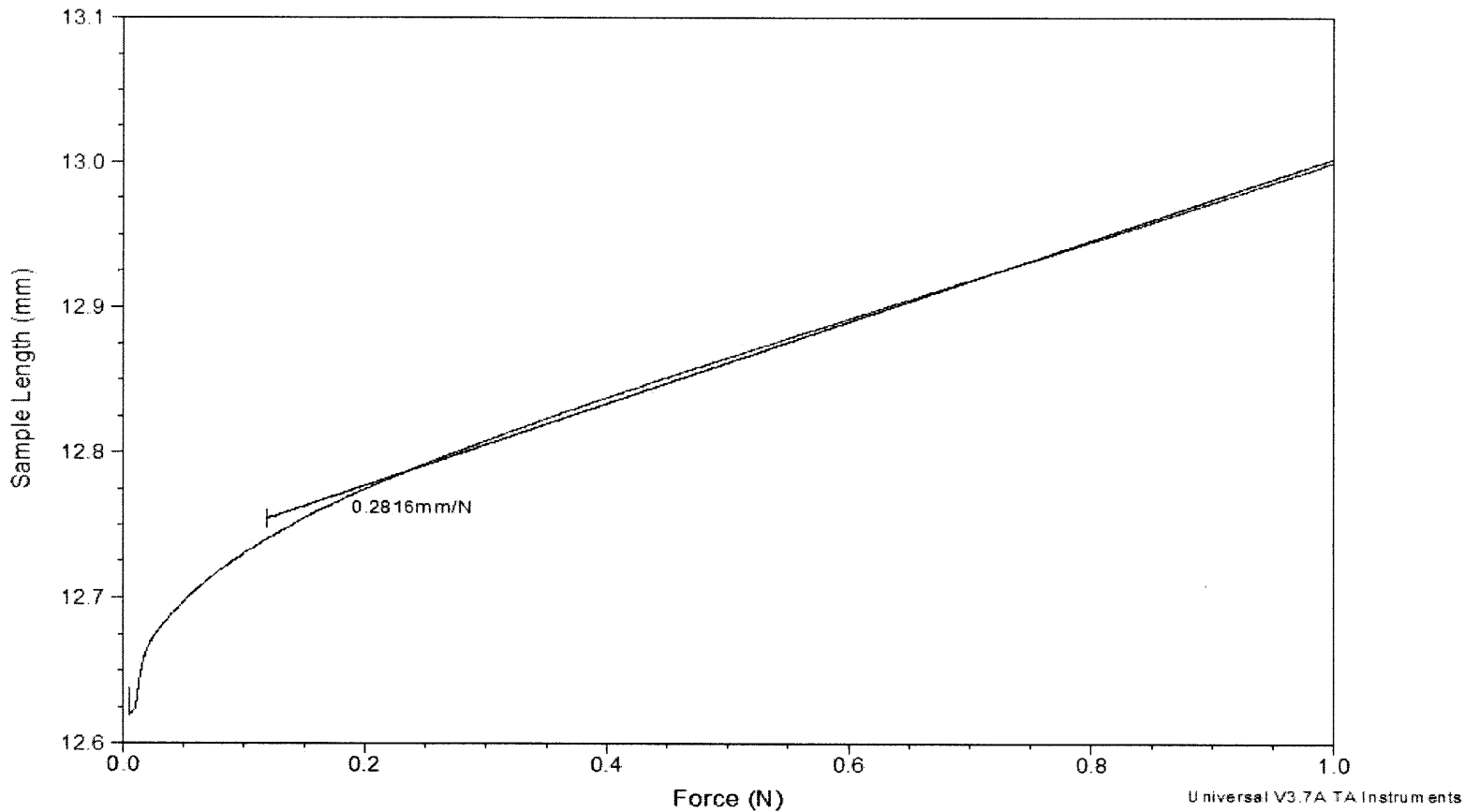


**Figure B.18** TMA plot above shows the contraction and break point of collagen fiber with 2% dispersion and cross-linked.

Sample: Collagen Fiber 1.6%  
Size: 12.5925 mm  
Method: Stress/Strain  
Comment: Test1

TMA

File: C:\...Desktop\ts\CF1[1]6.003f  
Operator: Grace  
Run Date: 29-Dec-03 11:41  
Instrument: 2940 TMA V2.4E

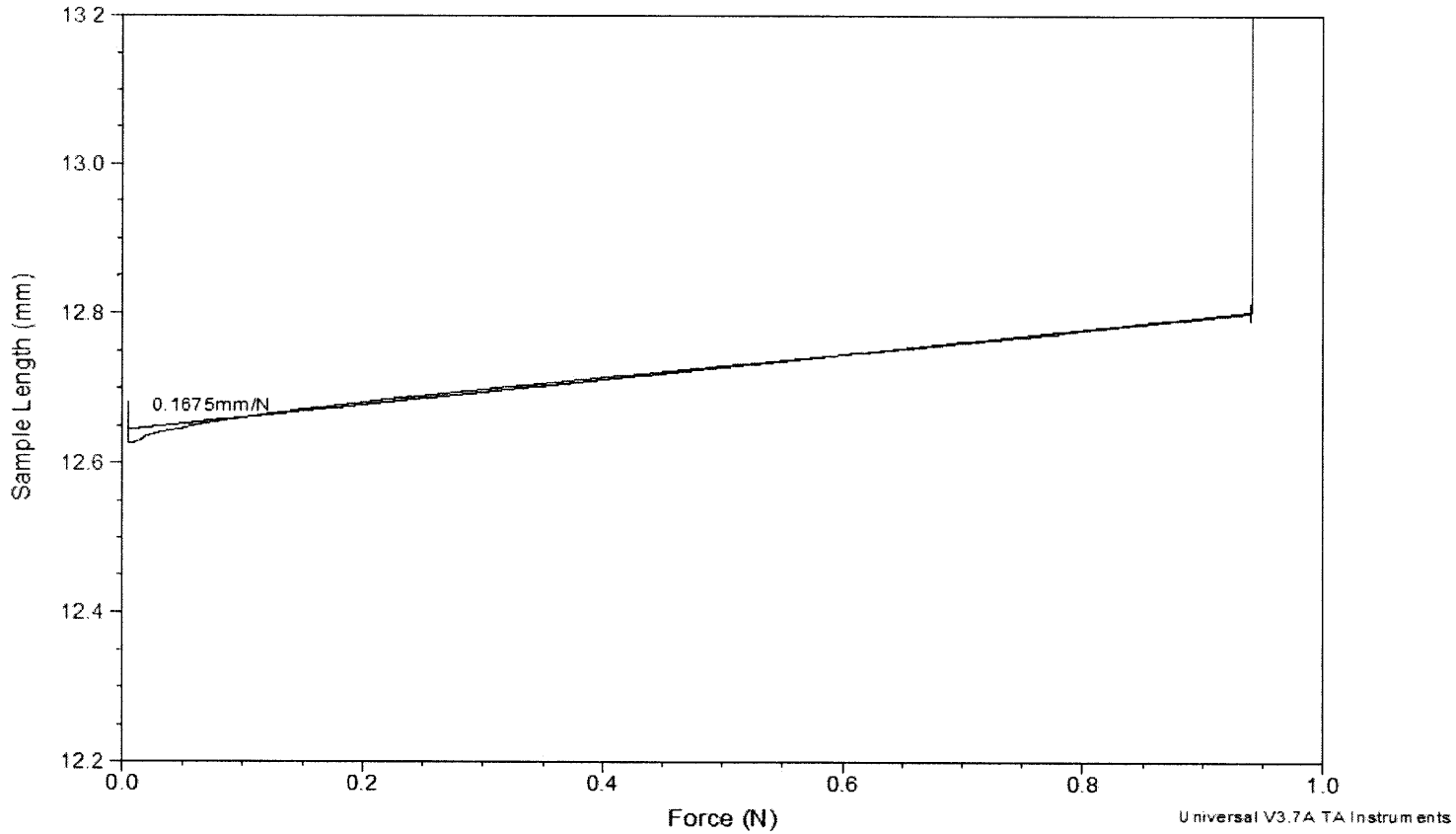


**Figure B.19** This plot above shows the slope of the stress strain curve of collagen film with 1% dispersion.

Sample: CFX1.6  
Size: 12.7236 mm  
Method: Stress/Strain  
Comment: Test1

### TMA

File: C:\Desktop\ts\CFX1.6f  
Operator: Grace  
Run Date: 19-Dec-03 13:54  
Instrument: 2940 TMA V2.4E

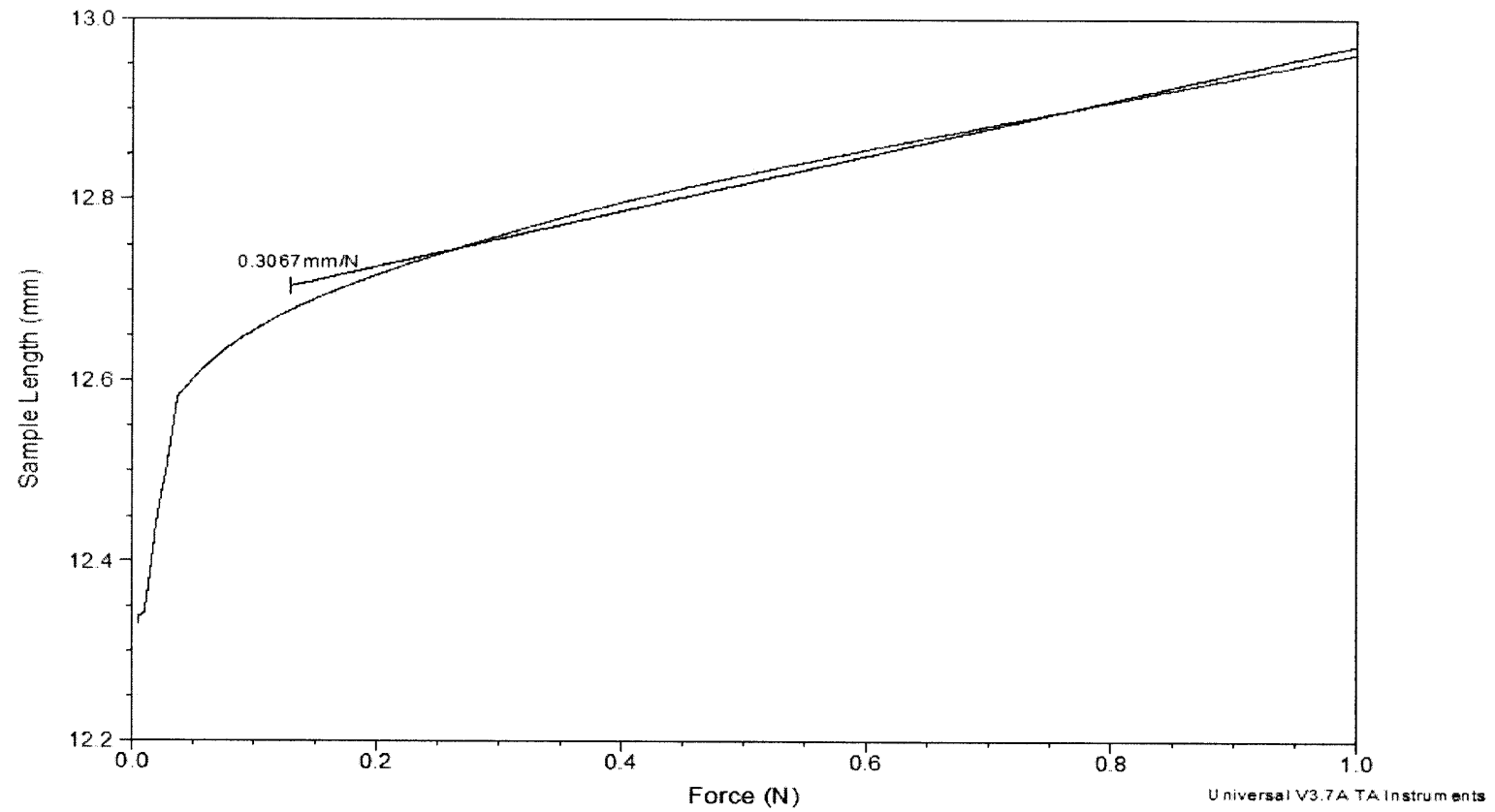


**Figure B.20** This plot above shows the slope of the stress strain curve of collagen film with 1.6 % dispersion cross-linked.

Sample: Collagen Fiber 2%  
Size: 12.3392 mm  
Method: Stress/Strain  
Comment: Test1

### TMA

File: C:\Desktop\its\CF2.001f  
Operator: Grace  
Run Date: 23-Dec-03 14:28  
Instrument: 2940 TMA V2.4E



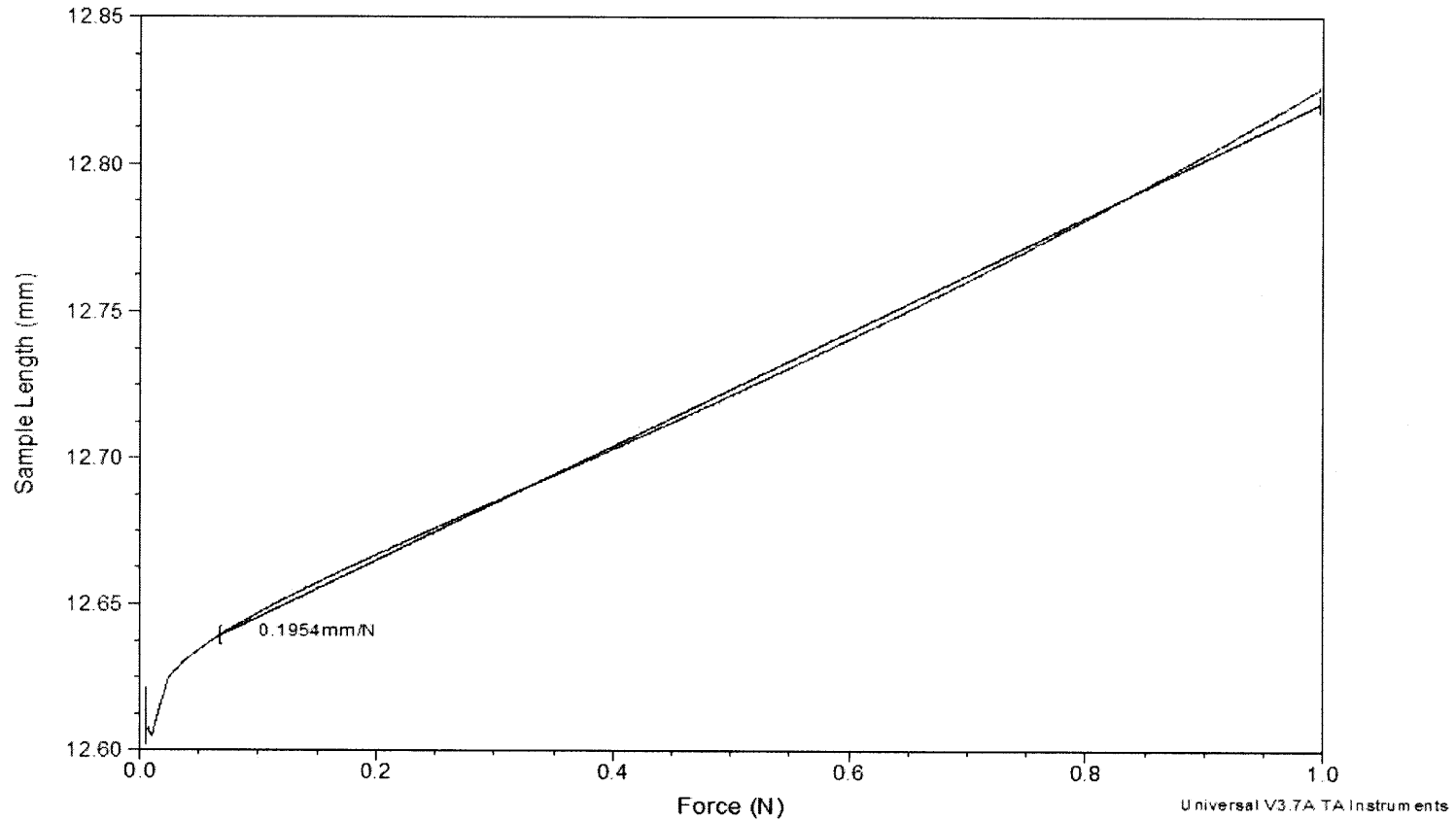
**Figure B.21** This plot above shows the slope of the stress strain curve of collagen film with 2% dispersion.



Sample: Collagen Fiber 2% (X)  
Size: 12.6221 mm  
Method: Stress/Strain  
Comment: Test1

TMA

File: C:\Desktop\ts\CF2(X).001f  
Operator: Grace  
Run Date: 23-Dec-03 14:52  
Instrument: 2940 TMA V2.4E



**Figure B.22** This plot above shows the slope of the stress strain curve of collagen film with 2 % dispersion and cross-linked.

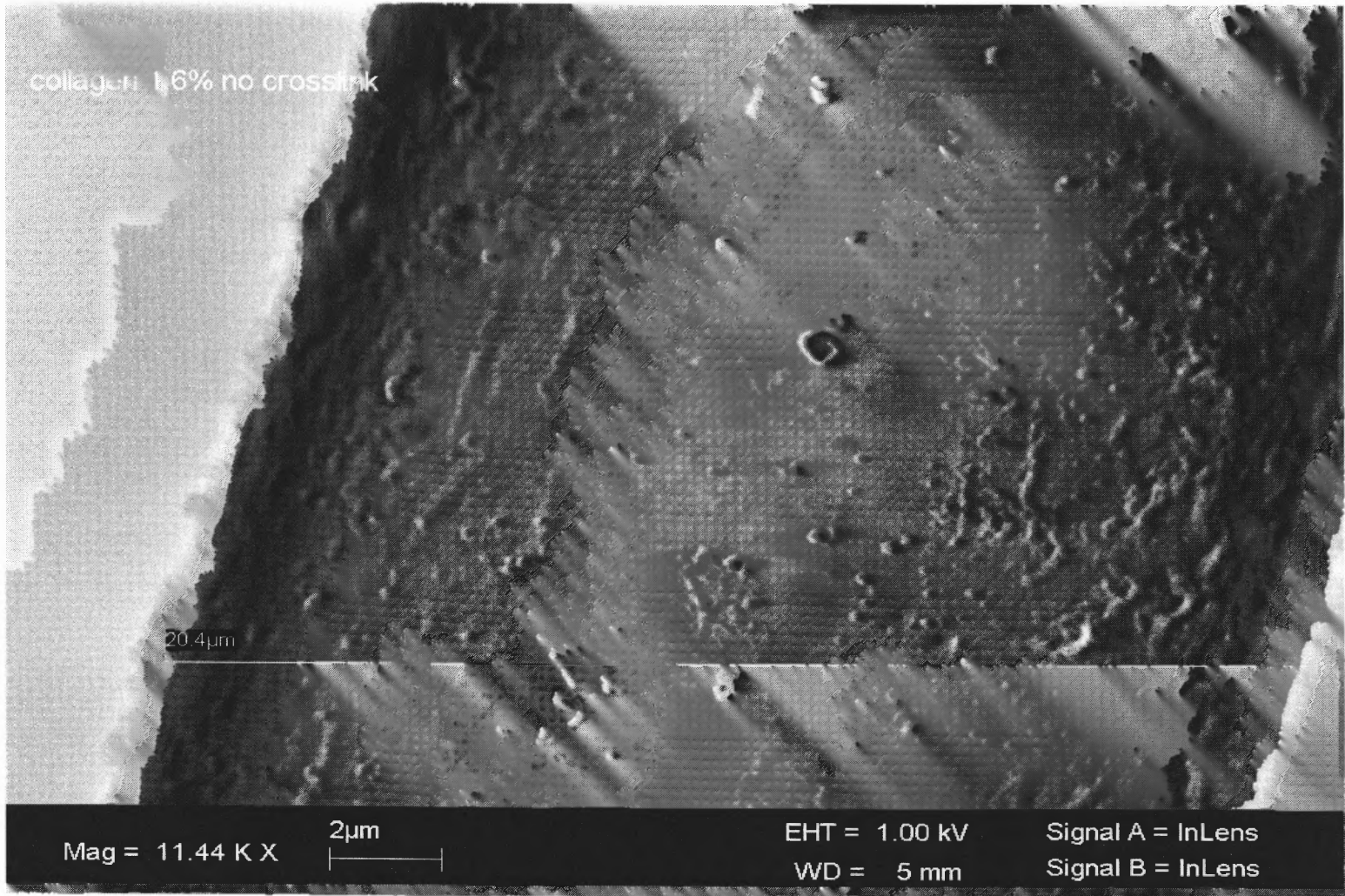


Figure B.23 Collagen fiber with 1.6% dispersion and non cross-linked,

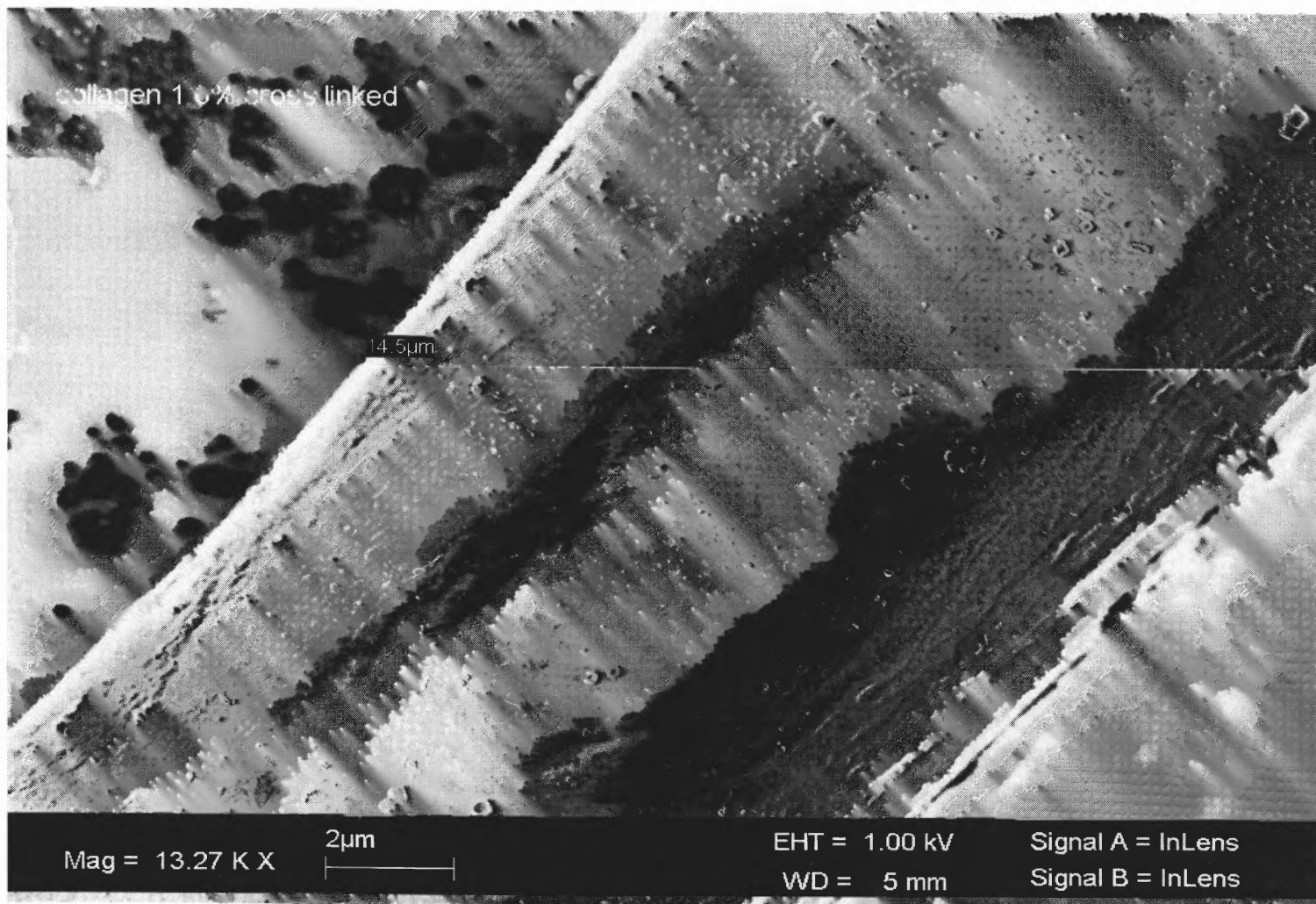
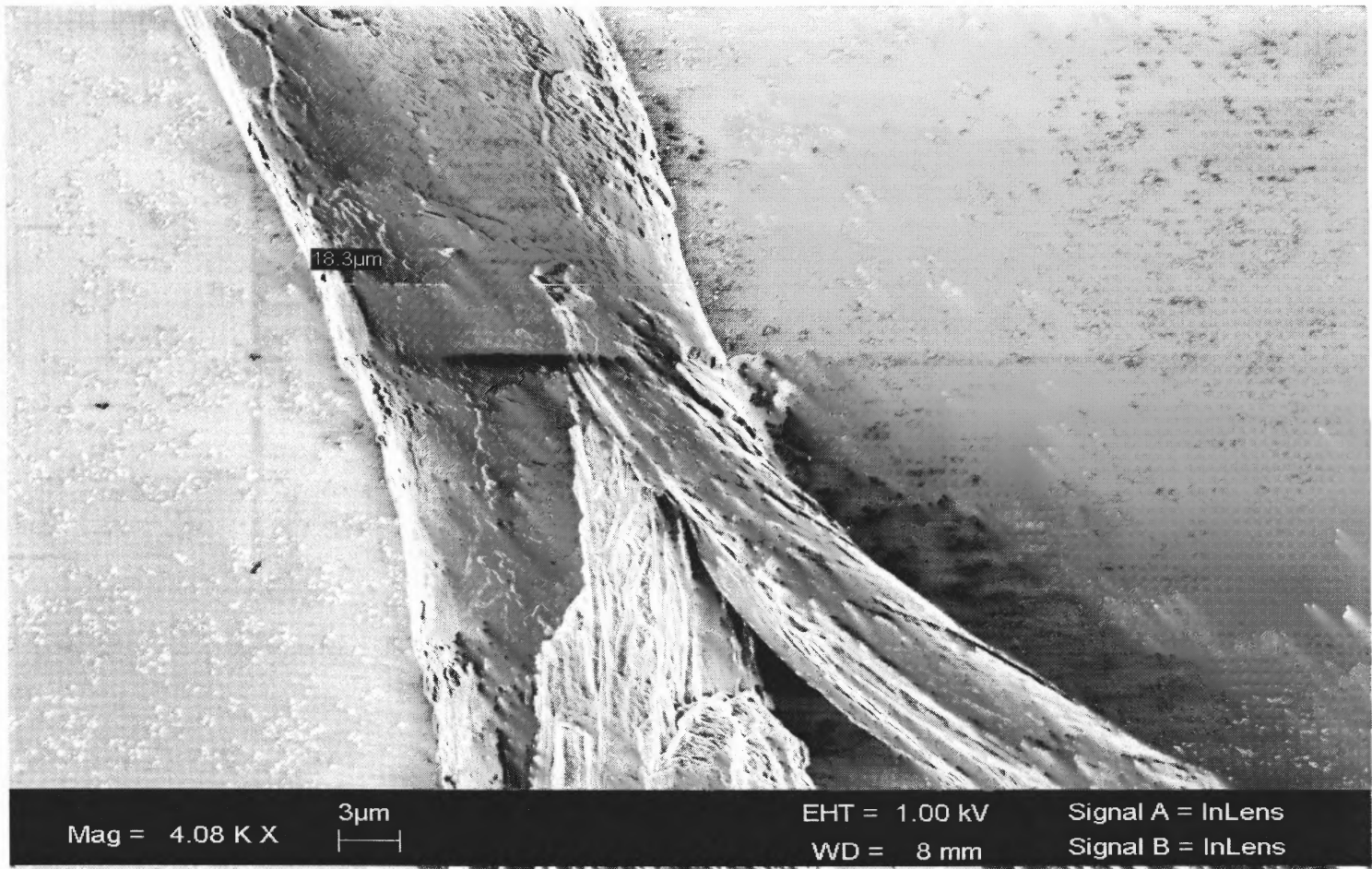
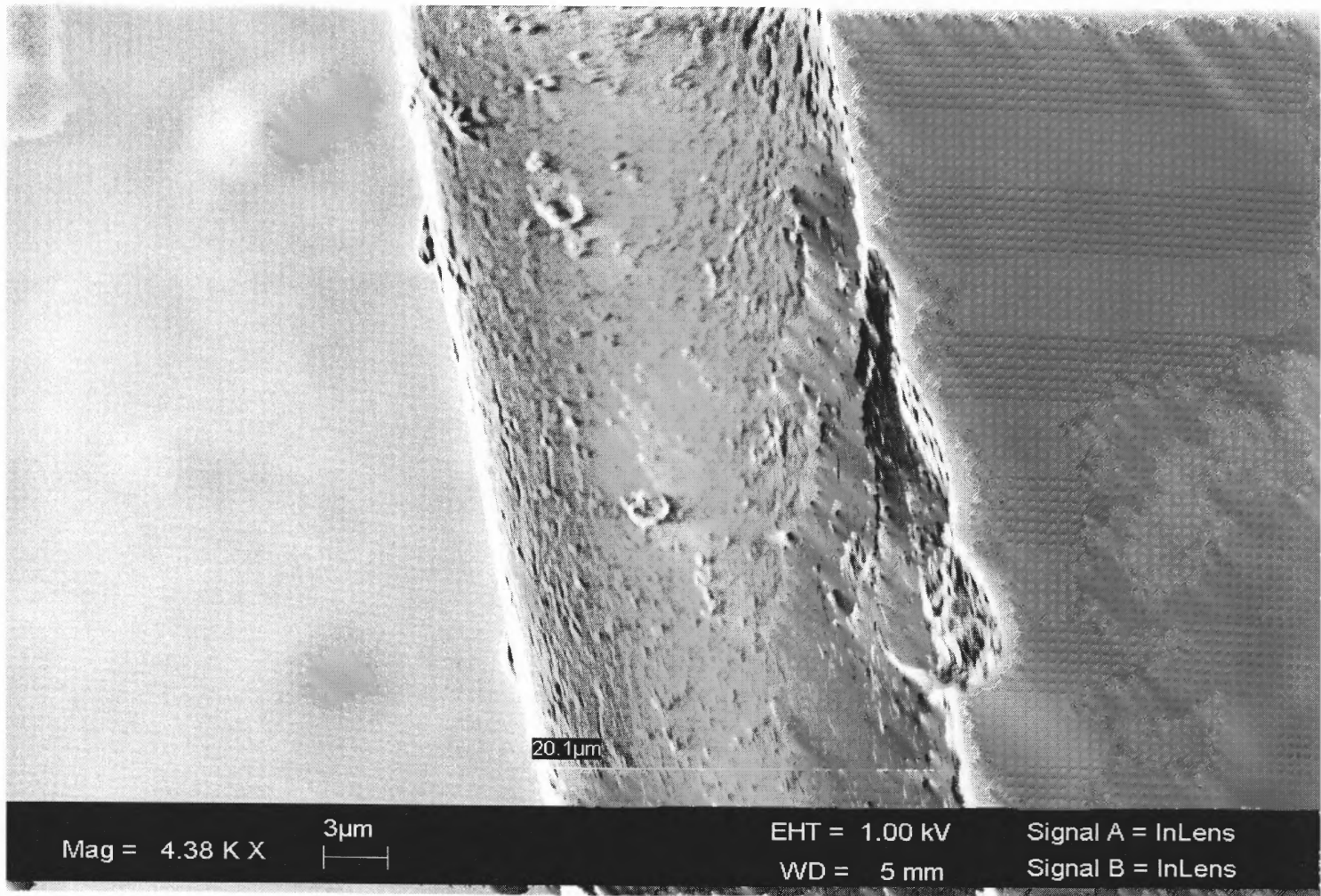


Figure B.24 Collagen fiber with 1.6% dispersion and cross links.



**Figure B.25** Collagen fiber with 2% dispersion and non cross-linked.





**Figure B.26** Collagen fiber with 2% dispersion and cross-linked.

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