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ABSTRACT

A COMPARATIVE STUDY OF COMMON CROSSLINKING AGENTS FOR ELECTROSPUN COLLAGEN SACFFOLDS

by
Pallavi Masih

Cartilage injury is one of the leading causes of knee pain in the world. Over two million Americans suffer from cartilage injury every year, resulting in swelling, pain or joint impairment, causing it difficult to maintain an active life style. Synthetic grafts are used extensively to restore tissue functions. The major drawback limiting successful incorporation of synthetic grafts in body is their lower ability to integrate to natural tissue, poor biocompatibility which often results in triggering immunogenic responses, causing graft rejection. Collagen is thus studied and used excessively as a successful implantable material. The reason being that it is natural in origin, biocompatible, bioresorbable, easily available and very cost effective. The current study involves electrospinning of type I collagen fibers extracted from bovine tendons and to modify their properties by various crosslinking methods using glutaraldehyde, genipin, or N-(3-Dimethyl aminopropyl)-N'-ethyl carbodiimide with and without N-hydroxysulfosuccinimide. The fibers were characterized using both chemical and physical tests to compare the effectiveness of different crosslinker and crosslinking concentrations. The tests involved mechanical testing using instron, determination of thermal stability using DSC, surface and morphological analysis using SEM, measure of free amino acid to determine crosslinking density. The genipin crosslinked samples were comparable in morphology and more thermally stable than EDC crosslinked samples.

**A COMPARATIVE STUDY OF COMMON CROSSLINKING AGENTS FOR
ELECTROSPUN COLLAGEN SACFFOLDS**

**by
Pallavi Masih**

**A Thesis
Submitted to the Faculty of
New Jersey Institute of Technology
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Master of Science in Biomedical Engineering**

Department of Biomedical Engineering

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APPROVAL PAGE

**A COMPARATIVE STUDY OF COMMON CROSSLINKING AGENTS FOR
ELECTROSPUN COLLAGEN SACFFOLDS**

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Dedicated to:

My beloved

Grandparents, parents, sister and friends.

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CHAPTER 1

INTRODUCTION

1.1 Objective

Cartilage injury is one of the leading causes of knee pain in the world. Over two million Americans suffer from cartilage injury every year, resulting in swelling, pain or joint impairment, making it difficult to maintain an active life style. The reparative surgery assures to restore 85% to 90% of stability and joint mobilization but an extrapolation of these results showed a post-operative failure rate of 10% to 15% [2]. The most common causes of chronic knee pain and disability are arthritis, osteoarthritis, rheumatoid arthritis, and traumatic arthritis [3].

Cartilage injury is characterized by mechanical deformations in the connective tissue layer covering the bone surface in joints. The degeneration of tissue may be caused due to swelling in synovial membrane, ACL tear, osteochondral fracture or softening of chondral tissues. A study of 25,124 conducted by W. Widuchowski et al. of knee arthroscopies performed from 1989 to 2004 showed cartilage defect to be cause of knee pain in 60% of the cases, and the numbers are still increasing [44]. The major limitations for the healing of cartilage are avascular, aneural nature of the tissue which limits the supply of blood and nutrients required for new tissue growth and the complex, multidirectional forces which it is subjected to. It has been difficult to tissue engineer a scaffold which can mimic the natural ECM architecture, degrade at a rate that matches new tissue generation rate and physically withstand the load which the initial tissue was subjected to prior to injury. Currently the most commonly employed tissue engineering

approach involves ex-vivo growth of chondrocytes on bioactive scaffolds which can stimulate tissue generation along with providing the base for cell attachment and re-implantation within patient's body. The procedure is known as Autologous Chondrocyte Implantation (ACI). Out of 855 prosthetic ligaments tracked for 15 years, 40-78% of the implants failed due to foreign body inflammation, particulate induced synovitis, wear debris and mechanical limitations [1]

The goal of this project is to develop scaffolds which can mimic the extra-cellular matrix (ECM) architecture within cartilage, and maintain its structural integrity under physiological conditions for a duration needed to regenerate biological tissue. This project will include the fabrication of these scaffolds and enhancement of its physiochemical properties to support cellular responses. Assessment of the scaffold's physicochemical and mechanical properties will be performed.

1.2 Background Information

The articular cartilage is a dense fibrous connective tissue which consists of a highly specialized extra-cellular matrix suspended with cartilage producing cell: chondrocytes, water (about 80%), collagen and proteoglycans (GAGs). The collagen and GAGs offer elasticity, mechanical strength and wear resistance to the tissue. Cartilage has an average breaking load of 2160N and a stiffness of 242 N/mm and absorb 12.8Nm energy during failure [1, 9, 11]. Young's modulus of human ACL was found to be 111MPa and ultimate tensile strength of 38MPa [1]. However, during physiological loading it is typically loaded to only 10–25% of its breaking load [10, 11]. ACL is exposed to tensile forces ranging from 67N to 630N for daily activities [1]. Cartilage is responsible to reduce

friction by lubricating the joint, facilitate movement, withstand mechanical load and provide wear resistance.

Articular cartilage injury can occur because of two reasons, traumatic mechanical destruction which results from direct blow or trauma during sports or other activity causing anterior cruciate ligament tear or osteochondral fracture. Secondly, it can occur as a result of progressive wear and tear due to disease of the chondral tissue which causes softening of cartilage or inflammation and swelling of synovial membrane producing excessive synovial fluid, over-filling the joint space resulting in pain, restricted joint movement and loss of cartilage. It is sometimes possible for the cartilage cells to repair itself depending on the site of injury and extent of damage, but it takes a longer healing time and may lead to formation of scar tissues. Statistics show that in the United States alone sports injuries account for 28% of all knee injuries [2].

The traditional techniques to repair cartilage injury include (a) micro-fracture which involves drilling a hole through the injured cartilage to the underlying bone to enhance the supply of blood which could promote tissue repair; (b) arthroscopic chondroplasty in which the surgeon trims away the loose and damaged cartilage, reduces pain but complete healing is not ensured; (c) osteochondral autograft involving implantation of healthy cartilage from region of lower loading to site of injury within the same patient; (d) autologous cartilage implantation with involves ex-vivo growth of chondrocytes and re-implantation within the same patient; (e) osteochondral allograft involves implantation of freshly donated cadaver cartilage.

1.2.1 Role of Tissue Engineering and Stem Cell Research for Cartilage Repair

“Tissue engineering is an interdisciplinary field which applies the principles of engineering and life sciences towards the development of biological substitutes that can restore, maintain, or improve tissue function” [13]. The three basic approaches adopted for the regeneration of tissues include: (a) isolated cells implanted at the site of injury, (b) a biomaterial scaffold on its own, (c) cells seeded in the scaffold matrix.

The first approach involves extraction of healthy cells from donor site, ex-vivo growth of these cells in physiological fluid and re-implantation of matured cells to the site of injury. The approach is most commonly applied for the repair and regeneration of soft-tissues, as it eliminates any risk of graft rejection due to immune response, which is one of the greatest challenges in the field of tissue engineering.

The second approach involves implantation of a biomaterial scaffold at the site of injury which will provide a ground for cell attachment, migration, proliferation and differentiation and will gradually get replaced by natural tissue over time. In this project, the author aims for the design of an optimum scaffold that mimics natural tissue architecture, mechanical strength and maintains structural integrity in physiological fluid over time. The last approach involves seeding of cells within the scaffold matrix prior to implantation.

A study conducted at the University of Bristol showed that an engineered cartilage tissue can grow and mature when implanted into patients with a knee injury [14]. The study involved re-implantation of cultured cells, harvested from healthy cartilage grown for 14 days on hyaluronic acid scaffold prior to implantation. The material for cell growth and implantation thus plays a critical role in determining the fate

of tissue repair and regeneration. Scientist from Imperial Collage London in 2005, have successfully converted human embryonic stem cells into cartilage cells. The study involved implantation of differentiated embryonic stem cells seeded within a bioactive scaffold in rat model which matured into cartilage cells after 35 days [21]. A similar study was conducted at Rice University in 2007 to successfully convert embryonic stem cells into cartilage cells after seeding on a bioactive scaffold [22].

The demand placed on materials used in tissue engineering varies depending on the implantation site and tissue being replaced [15]. The design of a scaffold that promotes cartilage regeneration requires that (a) the structure replicates the ECM architecture and strength of native tissue, (b) facilitate cell infiltration, (c) and biodegrade at a rate that matches the rate of new tissue formation [11, 15, 16]. The most widely used biomaterials in tissue engineering are polylactic acid (PLA) and polyglycolic acid (PGA) and their copolymers, synthetic in nature and collagen, which is natural in origin. Polymers allows for controlled degradation rate and mechanical properties, as well as surface modification for binding of surface reactive factors to promote cell growth or inhibition.

Collagen is the most commonly used biomaterial for connective tissue repair and regeneration [11, 15, 16, 17, 18, 19, 20]. It is also widely applied in medical and pharmaceutical applications. It is a fibrous protein, natural in origin which has adhesive peptides and integrin-binding domains that promote cell adhesion, migration, proliferation and differentiation. It is biocompatible and hemo-static in nature and can be surface modified to eliminate the surface bound antigens which might elicit an inflammatory immune response causing graft rejection which is one of the major

challenges in tissue engineering.

1.2.2 Collagen Overview

The collagen word derives from Greek word “kolla”, which means “glue”. There are more than 20 different types of collagen identified in the body. It is a fibrous protein, which constitute about 30% of the total body proteins including skin, bones, tendon, cartilage, ligaments and blood vessels.

It is the basic component of all connective tissues and is dispersed in the ECM in large quantities [19]. The specific amino-acid sequences on its surface favors cell adhesion, proliferation and differentiation. It provides flexibility, mechanical strength, wear resistance, resilience to bones and tendons, elasticity to blood vessels and skin and it acts as a scaffold for cellular attachment and tissue growth as a component of the ECM. Although the collagen in skin, blood vessels, bones, tendons and ligaments are all similar in structure and composition, the variation in their properties is caused due to slightly different amino-acid content, amino-acid sequence and their location on the backbone chain.

Collagen is the most abundant protein in animal kingdom and thus is available in abundant supply and can be efficiently extracted from bovine, porcine skin or tendons [19]. The extracted collagen is then purified by enzymatic treatment and chemical washes. The purified collagen can further be processed into fibers, sponges, gels or films by extrusion, lyophilization, casting and other processing techniques. The properties of collagen which make it an efficient biomaterial in medical and pharmaceutical industry are its cell-binding capability, high mechanical strength, non-antigenicity,

biocompatibility and biodegradability.

The resorption rate of collagen depends on its inter- and intra-molecular crosslinking and it can be varied from days to weeks to a non-degrading stable structure by various physical and chemical treatments. The basic framework of collagen [Figure 1.1] constitute a triple helical structure composed of subunit of collagen, known as the pro α -chain, which is coiled together in a right-handed helix. These collagen fibers are assembled together into larger units giving hierarchy to form tendons, ligaments, bones and alike.

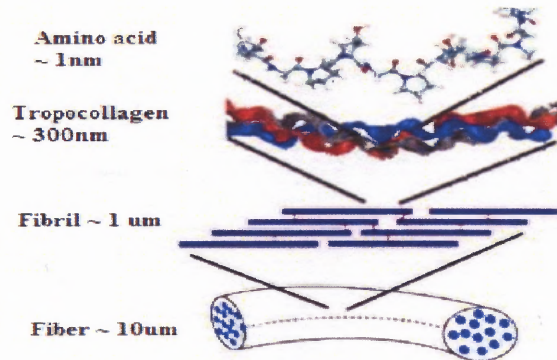


Figure 1.1 The hierarchy of collagen fiber.

[Source: <http://images.google.com/images?hl=en&q=tropocollagen&btnG=Search+Image>]

An individual α -chain is approximately 1050 amino-acid residue in size. It consists of a repeating sequence of glycine-proline-hydroxyproline residues. There are 3.3 amino-acid residues per turn and 2.9°A per amino-acid residue. During the physical or chemical treatment the crosslinks between the amino-acids increases, causing an increase in the tensile strength and denaturation temperature while decreasing the water uptake capacity and degradation rate of collagen.

1.2.3 Collagen Synthesis

The synthesis of collagen begins in the membrane bound ribosome's, which secretes the pro- α chain into the endoplasmic reticulum. Each α -chain consists of a repeating sequence of gly-pro-hydroxy-X amino-acids [Figure 1.2], where 'X' is any amino-acid attached to the side-chain. The glycine being the shortest amino-acid is responsible for the tight bonding between adjacent side-chains. It consists of about one-third of the residue. Proline is an aromatic amino-acid which stabilizes the helical bonding within collagen. It constitutes about 13% of the residue, while hydroxyproline is nearly 10%.

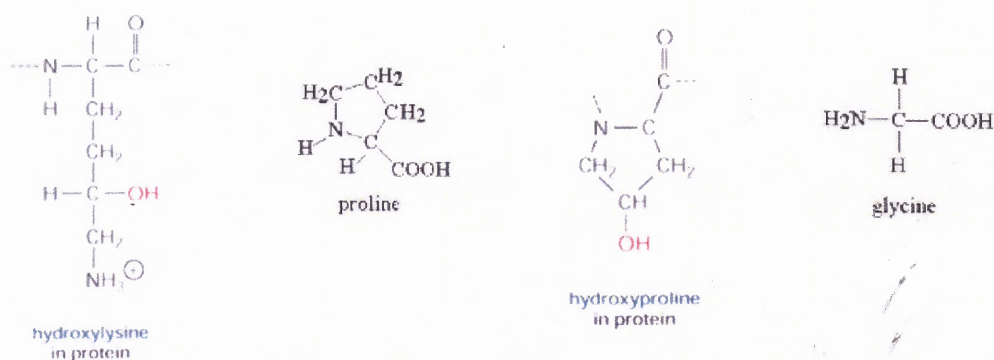


Figure 1.2 Major amino-acids of collagen: hydroxylysine, proline, hydroxyproline and glycine [6].

In the endoplasmic reticulum, the lysine and proline hydroxylate, and depending on the nature of the amino-acid attached at hydroxyl- end, will form hydroxylysine or hydroxyproline. The percentage of proline and hydroxyproline affects the stability of collagen structure. The hydroxyproline molecule thermally stabilizes the collagen molecule. Each pro α -chain sub-unit consists of amino- (N-terminal) and carboxyl- (C-terminal), which are additional amino-acids on either side known as propeptides, with open-ends to form crosslinks. The individual tropocollagen molecule is approximately 300nm in length and 1.5nm in diameter. Each α -chain consists of about 1000 amino-acid

groups [31]. The α -chain combines with other two to form a hydrogen-bonded, right-handed, triple-helical structure, known as procollagen molecule [Figure 1.3].

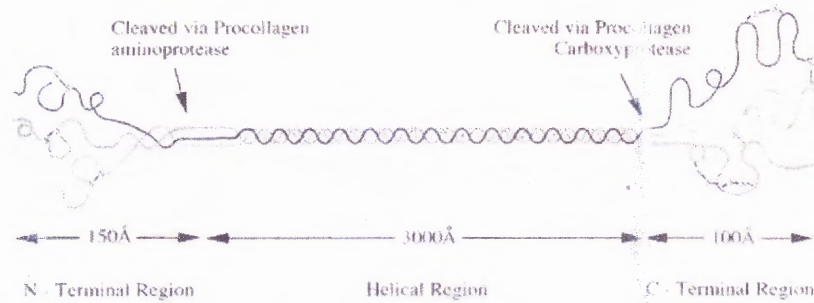


Figure 1.3 The procollagen molecule: building block of collagen fiber [7].

The procollagen molecule is secreted out of the lumen, which converts the procollagen molecule to collagen molecule by the cleavage of propeptides by specific proteolytic enzymes.

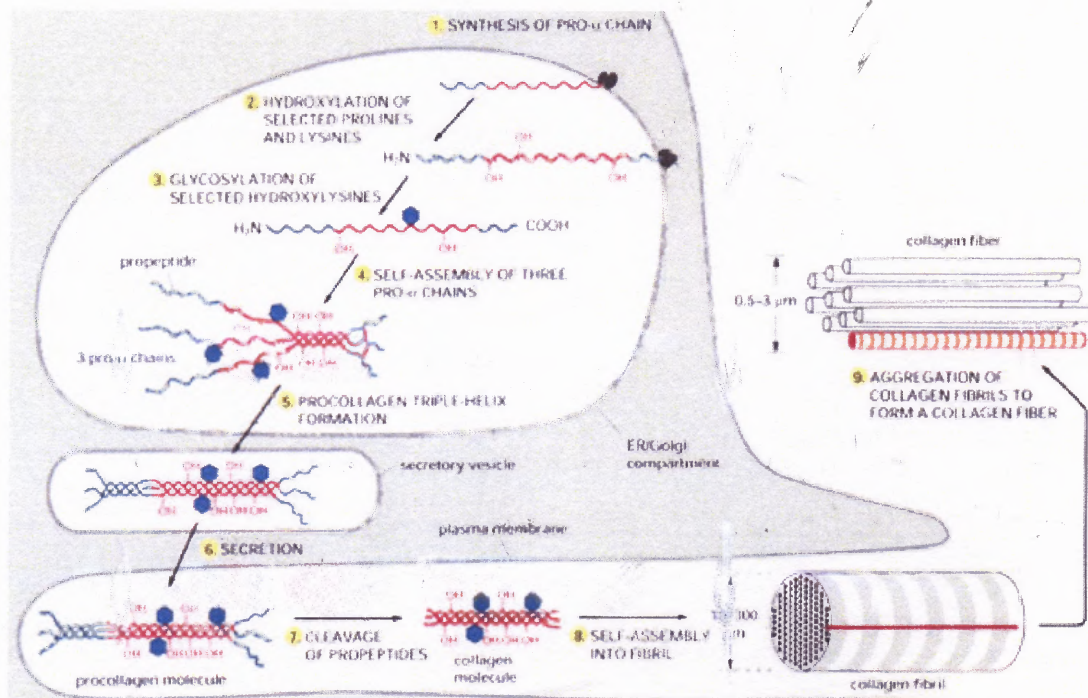


Figure 1.4 The synthesis of collagen fiber from procollagen molecule [7].

The collagen molecules assemble together to form collagen fibril, as shown in Figure 1.4. The structure is stabilized by covalent and hydrogen bonds. The mechanical strength, elasticity, wear resistance of different tissues varies with variation in the extent of crosslinking of collagen fiber within the tissue structure. Figure 1.5 shows the various crosslinks which exist within the native collagen structure providing the mechanical strength, elasticity, stretchability and wear resistance. Intra-helical bonds ($\sim 1.5\text{nm}$) are present between within the helical chain, inter-helical bonds ($\sim 4\text{nm}$) link two or more chains together. Inter-microfibrillar bonds are present over a larger range of $1.3\sim 1.7\text{nm}$ which links neighboring side chains. The type of bond formed affects the physiochemical nature of collagen. Inter- and intra-helical bonds increases the denaturation temperature and resistance against enzymatic degradation, improving the stability of crosslinked structure. The inter-microfibrillar bond improves the mechanical strength. EDC can crosslink fibers within 1nm of range (inter- and intra-helical bonds) while genipin can bond fibers 1.6 to 2.5nm apart [30].

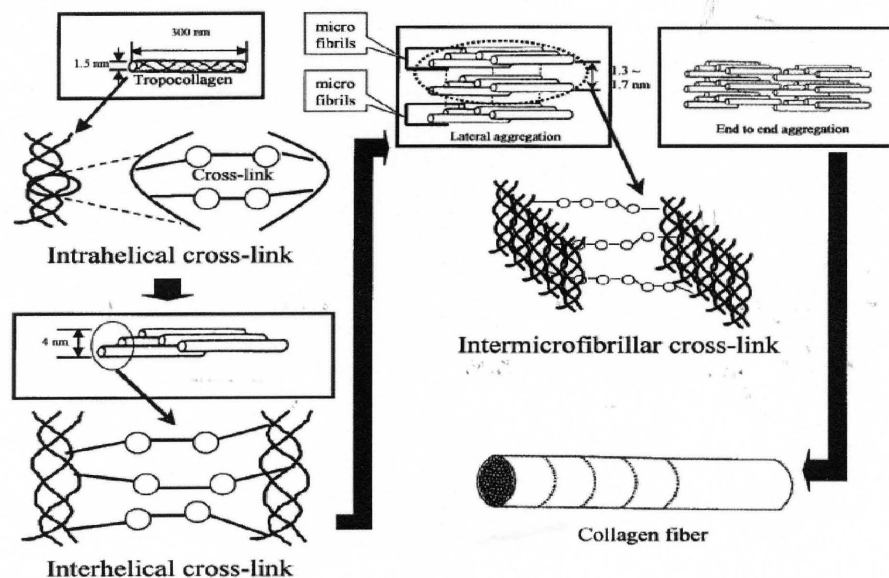


Figure 1.5 The Intra- and Inter-helical crosslinking within collagen molecule [30].

The collagen fiber has a young's modulus of 111MPa, yield stress of 50MPa and 10% yield strain. The strength and architecture of collagen fibers varies within different tissue or organs [Figure 1.6]. They are arranged as long rope-like bundles in tendons and ligaments to offer elasticity and flexibility; meshed web-like structure in the structure of skin and blood vessels to offer elasticity and wear resistance; stacked in form of tiles, one over another in bones and cornea (Figure 1.6). In cartilage the collagen fibers are arranged in form of a meshwork with the glycoaminoglycans (GAGs) dispersed throughout the matrix. The GAGs are responsible for absorbing water like sponges, and releases when loaded, which keeps the joint lubricated and facilitates movement.

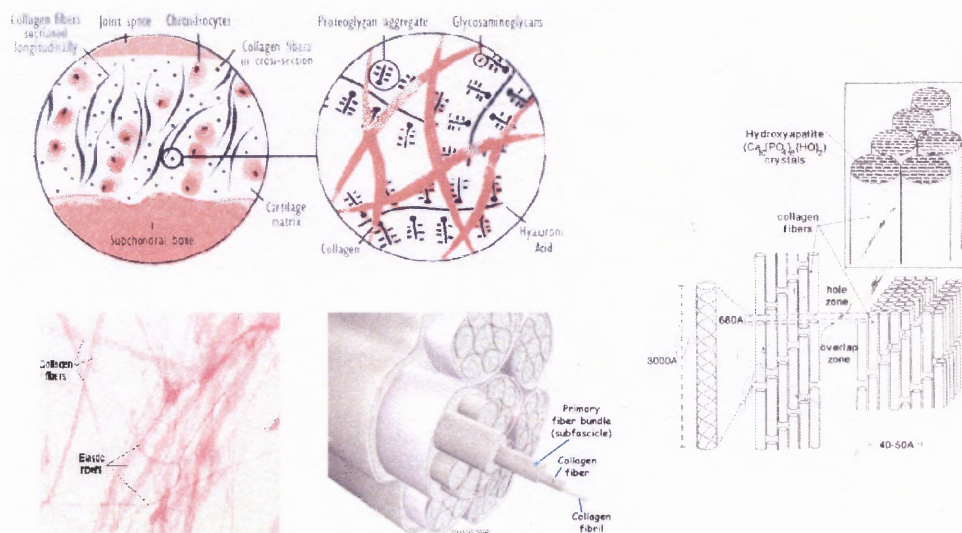


Figure 1.6 The various arrangement of collagen fibers: cartilage (meshed web with GAGs suspended), bones (stacked), skin (web-like), tendon (rope-like). [Source: <http://images.google.com/images?gbv=2&hl=en&q=collagen%2C+tendon&btnG=Search+Images>]

Collagen degrades over time but the rate of turnover varies among different tissues. It might range from few days in skin to approximately 10 years in bone. Collagenase is the naturally occurring enzyme responsible for breakdown of collagen fibers within tissues. Age, injury, genetic disorder stimulates the degeneration rate and

results in slower healing of tissues.

Statistics shows that more than 225,000 people worldwide underwent arthroscopic meniscal repair in 2002, and the number of procedures is expected to have a compound annual growth rate of nearly 5% through 2007 [4]. The continuously increasing demand necessitates the development of new techniques to repair and regenerate tissues to avoid total joint replacement. The goal of this project is to fabricate collagen scaffolds in a web-like architecture which mimics the extra-cellular matrix within native tissue and maintain stable structure to support tissue regeneration.

1.2.4 Collagen Scaffold Fabrication

Collagen scaffolds are of extreme interest in tissue engineering as they provide the base for cellular growth. They mimic the biochemical and structural architecture of the ECM of tissues, which can influence cell behavior [18, 32, 33]. Electrospinning is a processing technique that provides an efficient way to generate a meshed web-like structure of collagen fiber having nanometer to micron diameter scale fibers which would closely resemble the ECM architecture of native tissue [17].

The spinning set-up consists of a syringe-pump, voltage supplier and a metal plate (as shown in Figure 1.7). The syringe is filled with the polymer solution and is mounted on the syringe-pump assembly. The syringe-pump pushes the fluid through syringe at a selected flow rate. A metal plate is placed on the opposite end. The syringe needle and the metal plate are connected to a voltage supplier which can be regulated to maintain a desired potential difference between the two.

The fluid coming out of the needle is pulled towards the metal plate, due to the potential difference applied. The fluid droplet experience a pull outwards and deforms to a pyramidal shape at the needle tip, known as Taylor cone. At a certain level, the droplet overcomes the surface tension of the viscous fluid, and starts drawing towards the metal plate. The droplet thus extends to form a fiber which gets dried in the air due to solvent evaporation, before it reaches the metal plate. The fiber thus formed, gets deposited on the metal plate forming a meshed web-like structure of randomly oriented fibers.

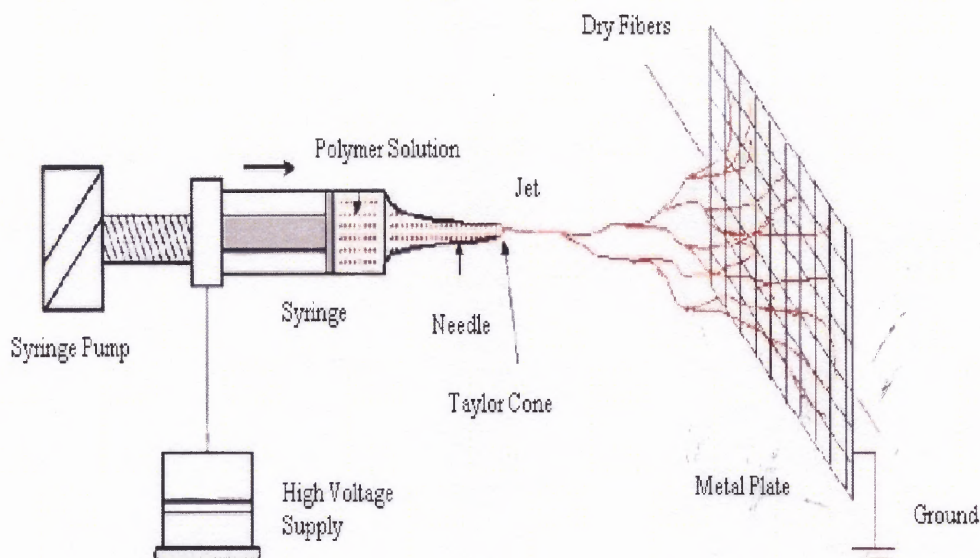


Figure 1.7 The set-up for electrospinning of collagen scaffolds.
[Source: <http://www.che.vt.edu/Wilkes/electrospinning/Slide1.JPG>]

The factors which affect the fiber geometry are: needle diameter, electric voltage, distance between metal plate and needle tip, flow rate and polymer concentration. By varying these factors, the fiber diameter can be varied from nano to micron range. An increase in needle diameter, fluid concentration, flow rate and voltage would increase the fiber diameter size while increasing distance would inversely decrease the fiber diameter.

A few other factors that affect the fiber formation are: humidity, temperature and air flow. A higher humidity would slow the rate of solvent evaporation, leading to beads formation embedded within or on the fibers. The spinning procedure during the project was thus carried out in an environment chamber with vacuum, to minimize formation of any beads and obtain uniform fiber geometry.

A scaffold must mimic the ECM architecture and properties. The structural requirements include high-matrix porosity, stability, small fiber diameter and a three-dimensional structure to allow cell-infiltration [17]. The electrospun collagen fibers consist of reconstituted collagen fibril but are fragile in nature, due to lack of native crosslinks and dissolves instantaneously in water, unlike native collagen which does not dissolve. Thus, collagen fibers need to be physically or chemically modified to increase their stability, reduce the water uptake and lower the degradation rate. Collagen electrospun mats immediately disintegrates and dissolves in an aqueous medium due to bonding of free amines with hydrogen molecule [18, 34].

1.2.5 Physical Modification of Collagen Mats

The most commonly used physical methods to crosslink collagen fibers include dehydroxy-thermal (DHT) treatment and ultra-violet light (UV) exposure. The collagen fibers are heated under atmospheric pressure to a temperature above 105°C for DHT, to dehydrate the structure.

The removal of water molecule forms amide bonds and brings α -chains more closely to one another thereby increasing the crosslinking within the structure. The duration of exposure and heat supplied determines the extent of crosslinking. Irradiation

to UV rays (254nm) offers another approach to physically crosslink collagen scaffold where the degree of crosslinking depends on the extent of penetration of exposed radiations.

Physical crosslinking technique offers advantage of lesser risk for toxins release in the physiological fluid as no chemicals are involved during crosslinking. The physical crosslinking methods increase the mechanical strength of collagen fibers higher than chemical methods but they also induce denaturation of a portion of the collagen molecule which may affect cell attachment, proliferation and differentiation [16, 19].

1.2.6 Chemical Modification of Collagen Mats

Chemical crosslinking has been widely used in leather and pharmaceutical industry since decades. The most commonly used crosslinking reagents for biological tissues are glutaraldehyde, formaldehyde, diisocyanates and epoxy compounds [18, 31]. The chemical crosslinking can be classified on the basis of (a) origin of crosslinker: synthetic/natural and (b) method of crosslinking: vapor/liquid.

Table 1.1 Various Crosslinking Reagents and Methods Used

Crosslinking reagent	Origin	Method for crosslinking
1. Glutaraldehyde	Synthetic	Vapor crosslinking at room temperature
2. EDC	Synthetic	Liquid crosslinking at room temperature
3. EDC with NHS	Synthetic	Liquid crosslinking at room temperature
4. Genipin	Natural	Liquid crosslinking at body temperature

Glutaraldehyde is an oily-colorless liquid at room temperature, miscible in water [16]. It is widely used in manufacturing industries for sterilizing medical and dental equipments, in water treatment or as chemical preservatives. It is highly corrosive and vaporizes easily in atmosphere. Figure 1.8 shows the chemical structure of the glutaraldehyde molecule.

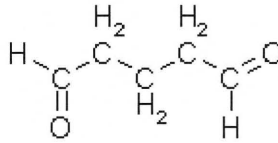


Figure 1.8 Glutaraldehyde molecule [31].

The collagen mats were vapor crosslinked using glutaraldehyde. The crosslinking set-up consisted of a beaker filled with glutaraldehyde solution, covered with an aluminum foil, to avoid any release of glutaraldehyde residues in the air [Figure 1.9]. The mats were placed on the meshed polymer sheet, ensuring complete and uniform exposure to the vapors.

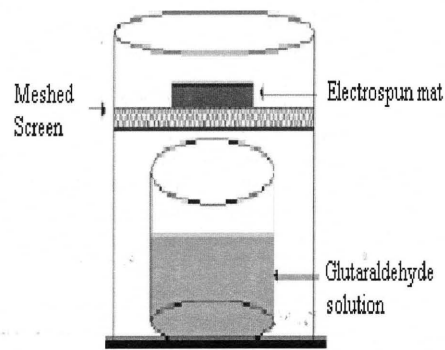


Figure 1.9 Schematic of the vapor crosslinking set-up used in the study [17].

The mats were allowed to crosslink for the desired period of time. The crosslinking was followed by rinsing in DI water to remove un-reacted aldehyde residues. During the glutaraldehyde crosslinking [Figure 1.10], the aldehyde group reacts with amino groups of lysyl and hydroxylysyl residues in collagen, coupled with release of a water molecule and Schiff based intermediates are formed which links further to form larger crosslinked entities [17, 39, 41].

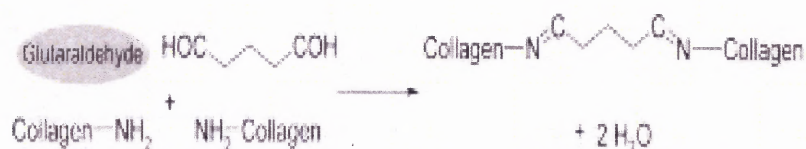


Figure 1.10 The crosslinking mechanism of glutaraldehyde: condensation reaction [Source: <http://arthritis-research.com/content/figures/ar2202-2.jpg>].

Glutaraldehyde offers the advantage of being less expensive, faster reaction time, crosslinks a large number of amino acid groups present in protein molecule over a varying range of distance [18]. The major drawback for the use of glutaraldehyde is that it causes local toxicity at the site of implantation due to release of un-reacted aldehydes [19]. Previous studies have shown a significant increase in mechanical strength and denaturation temperature for tissues crosslinked with glutaraldehyde but have noted calcification over time [18, 31, 39, 41].

The second crosslinker used for this study was N-(3-Dimethyl aminopropyl)-N'-ethyl carbodiimide hydrochloride (EDC) (Figure 1.11). It is a zero-length which means the agent itself is not incorporated in the macromolecule; water-soluble, synthetic reagent

[19]. The protonated carbodiimide links the amine to the carboxylic acid group of glutamic and aspartic amino acid of protein molecule, forming an amine-reactive O-acylisourea intermediate.

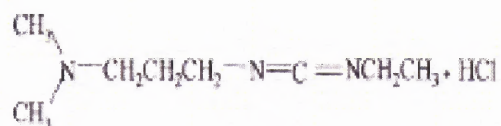


Figure 1.11 EDC (1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide) molecule [30].

The O-acylisourea undergoes a nucleophilic attack by the amine functional group of lysine and hydroxylysine and peptide crosslinks are formed bridging the neighboring polypeptide chains, as shown in figure 1.12. The intermediate formed is in an unstable form and may hydrolyze back to regenerate carboxyl group if not interacted with an amine group.

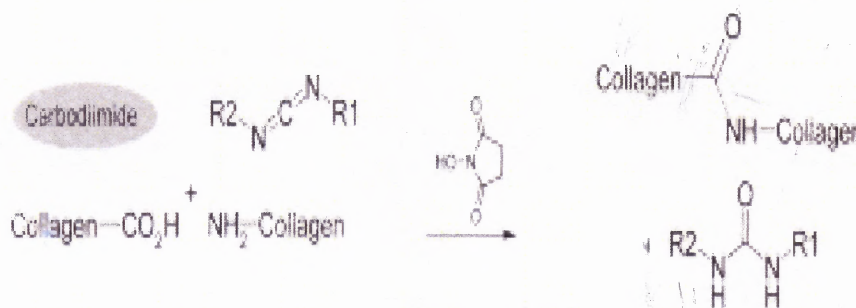


Figure 1.12 Crosslinking mechanism for EDC.

[Source: <http://arthritis-research.com/content/figures/ar2202-2.jpg>].

Secondly, they may rearrange themselves to form N-acylurea group, which is more stable in nature and is attached to the modified peptide, thus lowering the crosslinking efficiency of EDC.

The crosslinking efficiency of EDC can be improved by addition of *N*-hydroxysulfosuccinimide (NHS), shown in figure 1.13, which reacts with the *O*-acylisourea intermediate to form NHS reactive NHS-ester coupled with the release of 1-ethyl-3(3-aminopropyl) urea (EDU) which can be removed by rinsing [18, 34]. The NHS also reacts with the free amino groups and forms peptide crosslinks which are more stable in nature. Figure 1.14 shows the mechanism for crosslinking of collagen with EDC and NHS.

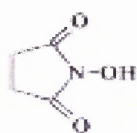


Figure 1.13 (*N*-Hydroxysulfosuccinimide) molecule [30].

The addition of NHS prevents the rearrangement of intermediates and formation of side products. The *O*-acylisourea group is converted into NHS-activated carboxylic acid group which is less susceptible to hydrolysis [30]. Thus, addition of NHS increases the rate of reaction and results in an efficient crosslinking [9, 18, 35].

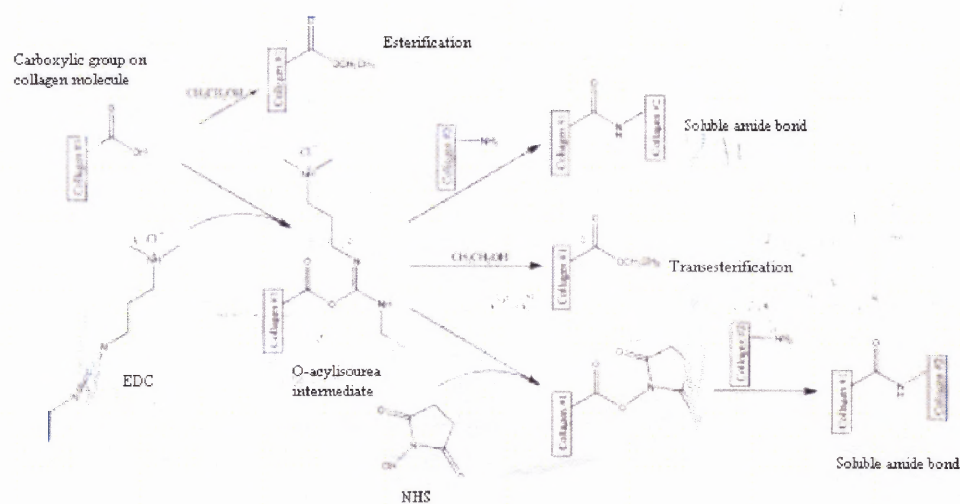


Figure 1.14 The crosslinking mechanism of collagen with EDC and NHS both [18].

The byproduct of EDC is a compound of urea (EDU) which gets eliminated from the body through an enzymatic pathway ensuring no toxic effect on cells or tissues, improving biocompatibility of EDC treated grafts. The carbodiimide can link groups located within 1.0 nm range and thus can form inter- and intra-molecular crosslinking only but not inter-microfibrillar, as the micro-fibrils are too apart (1.3 to 1.7nm) for EDC bridging [30].

Since electrospun collagen dissolves in an aqueous medium, ethanol was used as a common solvent for EDC and EDC with NHS crosslinking. Ethanol is a water-miscible, non-aqueous solvent which has been previously used with collagenous material without denaturation of the collagen or cytotoxicity [18]. The ethyl group reacts through hydrophobic interaction with the collagen surface, while the hydroxyl group binds with other through hydrogen bond forming a polymeric chain, which all together stabilizes the collagen structure and explains the maintenance of its structural integrity with little or no shrinkage [17, 18, 36, 37].

Genipin and proanthocyanidin are some of the natural crosslinkers previously used for biological tissue fixation [39]. The current study employs genipin as the third reagent for collagen fibrous mat crosslinking. Genipin is a naturally occurring crosslinking agent which can be extracted from the fruit of *Gardenia Jasminoides* Ellis [30, 31, 39]. In the plant, it is responsible for germination and growth inhibiting activities. It is colorless in nature but forms a blue color stain when reacted with amino-acid [27]. Figure 1.15 shows the molecular structure of genipin.



Figure 1.15 The molecular structure of genipin [30].

It is used in Chinese medicine to treat ulcer of skin, jaundice, acute conjunctivitis, epistaxis, hematemesis, pyrogenic infections, and externally on sprains and painful swelling due to blood stasis [27, 31, 39]. It is also used as food colorant in East Asia and fingerprinting dye in forensic science [43]. “The structure of genipin was first discovered by Djerassi and his colleagues in 1960’s” [27].

The genipin crosslinking is comparatively slower than glutaraldehyde or carbodiimide crosslinking [30]. The genipin reacts through a nucleophilic reaction between the primary amine groups on C3 carbon on backbone structure [Figure 1.16]. The nucleophilic reaction opens the dihydropyran ring causing crosslinking between aldehyde groups in the secondary amine, followed by dimerization produced as a result of radical reaction to form nitrogen-iridoid which undergoes dehydration to form aromatic monomer [30, 39, 41]. A blue color pigment is produced as an end-product of the reaction of genipin with methylamine, as studied by Touyama et al. [42].

Genipin can bridge peptide chains 1.6 nm to 2.5 nm apart, and thus can form inter-, intra-helical and inter-microfibrillar crosslinks by genipin polymerization [27, 30, 38, 39]. It has been speculated that genipin crosslinked gelatin are 10,000 times less cytotoxic than glutaraldehyde crosslinked gelatin or biological tissues, without any sign of calcification [27, 31, 41].

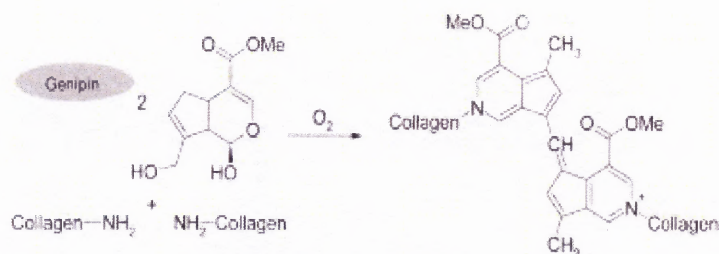


Figure 1.16 The crosslinking mechanism for genipin
 [Source: <http://arthritis-research.com/content/figures/ar2202-2.jpg>].

It is a biodegradable molecule with low cytotoxicity and is actively employed for crosslinking of gelatin for wound dressing, bioadhesives, bone substitutes and nerve guides [27]. Although genipin has been widely used for crosslinking of natural tissues, no previous work demonstrates the use of genipin to crosslink electrospun collagen fibers. The present study documents the generic protocol to crosslink electrospun pure type I collagen fibers with genipin.

The study further proceeded to compare the effectiveness of all three crosslinking reagents with respect to one another. The results were compared on the basis of fiber diameter, denaturation and glass transition temperature, dimensional and weight changes, free amino-acid content and stability in physiological fluid at body temperature over a period of 3 months.

CHAPTER 2

MATERIALS AND METHODS

2.1 Method Optimization

In this project, the author's endeavor was to create a uniform small diameter, porous collagen scaffold with sufficient strength such that it can maintain its structural integrity under physiological conditions for a duration that ensures tissue regeneration *in-vivo*. Collagen fiber mats were fabricated using electrospinning and crosslinked with three different crosslinkers to improve their stability and reduce degradation rate. The parameters for electrospinning of the collagen scaffolds were optimized. The concentrations for the crosslinking agents were also identified. The final samples were tested for physicochemical properties by studying dimensional and weight changes, variation in denaturation and glass transition temperature, porosity, fiber diameter and percent of free amino-acid content. The mechanical strength was analyzed using instron. The mats were incubated in phosphate buffer solution at body temperature to mimic physiological conditions and were harvested at time points of 1 month, 2months and 3 months.

2.1.1 Electrospinning Process

The following electrospinning parameters were optimized to obtain uniform fiber scaffold ranging from few hundred nanometers to 2-3 microns, without formation of any beads. The smaller diameter range fibers would closely mimic the ECM architecture and

could thus enhance cellular attachment, proliferation and differentiation when used for cellular study [4]. The electrospinning parameters used in the current study are: a 20 gauge needle, 42.85% (by weight) collagen solution, 20 KV electric voltage, 0.02 ml/min flow rate, and a 40 cm distance between needle and metal plate.

2.1.2 Optimization of Crosslinking Concentrations

In the study by Cheryl et al., two glutaraldehyde concentrations of 1.5% and 0.5% were used [17]. The 0.5% crosslinked mats maintained better fiber geometry after crosslinking as compared to 1.5%. The crosslinked samples turned yellowish red in color, shrunk and gelled in water based medium. The above finding matched the results found in [17] which concluded 0.5% of glutaraldehyde was a sufficient concentration to crosslink electrospun collagen fibers. Thus, the study proceeded with 0.5% of glutaraldehyde concentration to crosslink collagen fiber mats.

Catherine E. Barnes in her study compared four different EDC concentrations for crosslinking type II collagen electrospun mats used for cartilage repair [18]. The electrospun mats were crosslinked using 20mM and 200mM of EDC compared with 20mM of EDC with 20mM NHS and 200mM of EDC with 200mM NHS in ethanol. The study concluded 20mM EDC crosslinked samples were not statistically different from non-crosslinked samples, while 200mM EDC and 200mM of EDC with 200mM of NHS imparted desirable mechanical strength maintaining fibrous structure of the electrospun mats. Thus, in this study, these two concentrations were further compared on the basis of stability in PBS at 37°C, free amino-acid content, dimensional and weight change, porosity and mechanical strength.

Genipin have been previously used to crosslink biological tissues and collagen gels at concentrations 0.625% to 1% but had not been used until recently for crosslinking of electrospun collagen mats [39, 40, 41]. It has been used previously to crosslink chitosan at concentrations of 5%, 10%, 20% and 30% for disk tissue engineering [42]. The genipin has been of extreme interest for crosslinking because it is natural in origin and has shown no cytotoxic effect on the biological tissues [39]. The current study developed a new technique to crosslink electrospun collagen mat using genipin. The greatest challenge to crosslink collagen mats was maintaining their structural integrity when placed in crosslinking medium. Pure collagen dissolves immediately when placed in water based medium, which is the common solvent for most of the crosslinking reagents. Electrospun collagen fiber scaffold does not dissolve in ethanol [18]. The genipin was thus dissolved in ethanol and the standard crosslinking protocol of immersion for 3 days at body temperature was used as followed in previous studies for crosslinking tissues [40].

The various concentrations tried for crosslinking of collagen mats included: 1% to 10%, 20%, and 30% by weight. The crosslinked mats were air dried and viewed under light microscope for fibers. They were immersed in PBS at room temperature to check for stability of mats in water based medium after crosslinking. The 20% and 30% crosslinked mats showed no distinguishable fibers, probably due to excessive crosslinking, causing fibers to merge into one another. The 1% to 4% crosslinked mats dissolved in PBS within 10 days of immersion.

Based on the above findings, 1%, 5% and 10% were selected to carry out further studies. The immersion study was conducted at body temperature instead of room temperature to more closely mimic the physiological environment and predict the scaffold behavior when placed in cell medium to grow cells. The duration of the immersion study was 3 months with samples harvested after every 1 month.

2.2 Collagen Purification

Pure type I insoluble collagen was purified from bovine tendons by various enzymatic, alkaline and acidic treatments. The frozen bovine tendons (approximately 1000g) were first cleaned manually and later chopped and grinded using an electric deli meat grinder. The grinded tendon was treated with enzyme (ficin) dissolved in potassium phosphate monobasic buffer at a pH of 6.15 ± 0.15 (37°C). Ficin attacks the peptide bond and removes blood or protein components present, which are potent of transferring xeno-graft diseases. The enzyme was deactivated by alkali treatment of sodium hydroxide and anhydrous sodium sulphate solution for 42 hours. The alkali was later washed-off by repeated washing in anhydrous sodium sulphate solution and sulfuric acid at a pH 4.6. The purified collagen bundles were sterilized by isopropanol treatment at 60°C followed by drying in oven overnight at 45°C.

2.3 Electrospinning of Collagen Mats

The electrospinning of collagen involves three basic steps: solvent preparation, spinning of collagen fibers, drying and storage. The solvent was prepared in fume hood, and the spinning was carried out in an environmental chamber with an exhaust and vacuum ON, for safety reasons.

2.3.1 Solvent Preparation

3 g of purified collagen was dissolved in 7 ml of TFA (TriFloro Acetic acid) overnight (42.85% of collagen by weight) [30]. The dissolved collagen solution was mixed homogenously by a magnetic stirrer set at rate of 5 RPM for 15 minutes. 3 ml of final prepared solution was filled in a 20 ml syringe and placed on the syringe pump.

2.3.2 Spinning Set-up

The syringe pump was set at a flow rate of 0.02ml/min. The needle (20 gauges) and metal plate (23 cm X 23 cm) were placed 40 cm apart and an electric voltage of 20 KV was applied across them. The humidity was lowered down to 10% \pm 2% during spinning. The spinning was allowed to run for approximately 120-150 minutes ensuring the generation of a thick, uniform mesh of randomly oriented fibers. The fibers were viewed under scanning electron microscope (SEM) to ensure uniform fiber diameter, no phase separation or presence of un-evaporated solvent.

2.3.3 Drying and Storage of Spun Mats

The spun mats were peeled off the metal plate using a metal blade and allowed to dry on aluminum foil overnight in the fume hood at room temperature to ensure evaporation of any residues of TFA from the mats. The dried mats were foiled and stored in desiccator

for further use. To avoid sticking of mat on the metal plate, the plate can be covered with aluminum foil prior to spinning.

2.4 Crosslinking of Electrospun Mats

The prepared collagen meshes were divided into six batches for 0.5% glutaraldehyde; 200mM EDC only; 200mM of EDC with 200mM of NHS; 1%, 5% and 10% of genipin. The collagen mats were cut into square pieces of 1cm X 1cm dimension, with every batch containing 16 pieces each, for free amino acid, SEM, DSC, TGA, dimensions and weight measurements. Whole mat was crosslinked for mechanical testing separately. The crosslinked samples were air dried in a chemical fume hood and stored in a desiccator.

2.4.1 Glutaraldehyde Crosslinking

In reference to protocols followed by Cheryl et al. study, the prepared samples were vapor crosslinked using 0.5% of glutaraldehyde in DI water for 19 hours at room temperature [17]. The crosslinking set-up was as shown in Figure 1.10. The crosslinked meshes were further washed in distilled water to ensure removal of un-reacted aldehyde residues.

2.4.2 Carbodiimide Crosslinking

The prepared collagen mats were liquid crosslinked with 200 mM of EDC only and 200 mM of EDC with 200 mM of NHS, following previously published protocols [18]. The meshes were crosslinked in 200mM EDC only for 18 hours and 4 hours in 200mM of EDC with 200mM of NHS dissolved in ethanol. After crosslinking the mats were washed

with 0.1 M of sodium phosphate buffer for 2 hours to hydrolyze and remove any O-isoacylurea intermediate. The samples further rinsed with DI water and air dried [11].

2.4.3 Genipin Crosslinking

Three Genipin concentrations of 1%, 5% and 10% by weight dissolved in ethanol were used to crosslink collagen mats for 72hours at 37°C [40]. The samples were later rinsed in DI water and air dried. Change in color was evident of effective crosslinking [42].

2.5 Immersion Study

The crosslinked samples were first sterilized before starting the immersion study in phosphate buffer solution (DPBS) for 3 months. The protocol followed for the sterilization of samples was as follow:

Step 1: Soak in ethanol for 20 minutes.

Step 2: Pipette out the ethanol and add PBS. Soak for 4 minutes.

Step 3: Pipette out the PBS (step 2), add more PBS and soak for 25 minutes.

Step 4: Pipette put the PBS (step 3), add more PBS and soak for 20 minutes.

The samples were sealed in the petri dishes with parafilm and placed in the incubator maintained at 37°C. The temperature was monitored regularly after an interval of every hour for the first day and 1 week later, to ensure maintenance of stable temperature. The samples were harvested after 1 month, 2 months and 3 months of incubation. Samples were air dried under chemical fume hood and stored in desiccator.

2.6 Morphological Studies

The samples were measured for length, width and weight before and after crosslinking and after immersion for 1 month, 2 months and 3 months in PBS. The study was performed on the hypothesis of detecting a noticeable difference in the dimension and weight of the mats before and after crosslinking. More amino-acid bonds are formed during crosslinking which would reduce the dimension of mats by bringing the collagen fibers closer to one another while increasing the mass due to incorporation of crosslinking reagent within and between the collagen molecules. The dimensions and mass of collagen mat was assumed to reduce further after incubation in PBS relative to duration of immersion due to degradation of collagen structure.

2.7 Porosity Measurements

The porosity of scaffold affects the cellular in-growth and matrix deposition. Thus, it plays a critical role in determining the rate of tissue regeneration [20]. The porosity was calculated before and after crosslinking and incubation study using following formula as defined in [5]:

$$P = [1 - \{ (\text{Mass of fiber mat} / \text{Volume of fiber mat}) * 1 / \text{Density of material} \}] * 100$$

Where, density of collagen was found to be 1.3 to 1.4g/cm [43].

2.8 Differential Scanning Calorimetric Measurements

The thermal properties of the electrospun mats can be quantified using thermo-analytical methods. DSC determines the structural change in collagen fiber when subjected to cycle of heat-cool-heat. It determines the thermal stability of the electro-spun mat. The

denaturation temperature is analyzed by heating the sample under high-pressure in aluminum pans and measuring the amount of heat required to raise the temperature of material with respect to reference. The heat is either absorbed or released, depending on nature of reaction if it is exothermic or endothermic, during phase transition. The denaturation temperature is the point of onset for thermo-reversible un-winding of the helical structure of collagen fibril. The hydrogen bonds break and there is a helix-to-coil transition with increase in heat flow, resulting in formation of amorphous polymer known as gelatin. As the temperature is lowered the coil tends to re-wind itself to restore the original collagen helical structure (coil-to-helix transition). The helical structure is not restored completely and contains segments of amorphous polymer phase. This provides gelatin with elasticity and integrity. The glass transition temperature is the onset of increased mobility within the material due to increase in temperature. A phase transition is seen from amorphous to crystalline with change in heat capacity of the material.

The hypothesis of the study was to observe an increase in the glass transition temperature after crosslinking of collagen mats. This could possibly occur as a result of increase in crosslinking density which would require more heat to break the bonds and to initiate or increase mobility within the material. The glass transition temperature can thus be used to quantify the crosslinking density of collagen scaffold [19, 30]. The author also hypothesises to observe a decrease in the glass transition temperature of collagen mats after immersion in PBS as the incubation (37°C) would facilitate the degradation of the collagen mats with time.

The thermal analysis of collagen fiber was carried out with Q100 differential scanning calorimeter; TA instruments New Castle, DE. The uncrosslinked collagen mat was taken as control for the study. Approximately 5mg of sample was weighed and hermetically sealed in aluminum DSC pans. The reference holder consisted of an empty DSC pan, sealed and crimped. The heat-cool-heat cycle was selected to determine the denaturation and glass transition temperature. The heating and cooling rate was 10°C/min. The denaturation of uncrosslinked collagen was found to be about 220°C to 230 °C from previous studies.

2.9 Thermal Gravimetric Analysis

The thermal gravimetric analysis was performed to detect the percent loss in weight with increase in temperature of sample. The analysis was carried out on Q50 Thermogravimetric Analyzer and the results were analyzed using TGA-Q50 software. Non-isothermal experiments were performed in temperature range of 0 to 250°C. The samples were heated at 10°C with nitrogen flow rate of 50 cm³ per min. The average sample size was 3-4mg.

The collagen mat was cut into small pieces using scissors and weighed. An empty pan was tare for reference. The heating rate was selected to be 10 °C/min over a range of 0 °C to 250 °C. The parameters were selected based on previous study on rat tail tendons.

2.10 Determination of Free Amino-Acids

The percent of free amino-acid content of collagen mats was determined using 2,4,6-trinitrobenzenesulphonic acid solution (TNBS-10% w/v in water), as a measure for degree of crosslinking [12, 30]. Crosslinking increases the bonds between amino-acids and thus reduce the free un-bonded amine groups. Hence the number of free amino-acid was expected to decrease after crosslinking. The number would increase if the mat was degraded and the bonds between amino groups were broken.

The sample of approximately 5 mg were incubated in an aqueous solution of sodium carbonate, NaHCO_3 (1 ml, 4% w/v) for 30 minutes. A solution of TNBS (1 ml, 0.5% w/v) was added in the same NaHCO_3 solution and allowed to incubate at 40°C for 2 hours. 3mL of HCl (6M) was added to hydrolyze the samples at 60°C for 90 minutes. The reaction mixture was diluted with 5 ml of DI water and absorbance was measured using spectrophotometer at 420nm range. The blank was prepared following same procedure, only the HCl was added prior to TNBS solution.

2.11 Mechanical Strength Measurements

The samples were tested on instron to determine the tensile strength and modulus of crosslinked samples. The uncrosslinked collagen mat was taken as control. Five pieces were tested for each batch after initial crosslinking (0 month), 1 month, 2 months and 3 months of immersion study. According to ASTM standard D3822-01, the minimum gauge length to effectively test a specimen was found to be 10mm [44]. The mechanical testing was carried out by undergraduate students as their project work. The samples were cut in 10mm X 5mm dimensions. Paper strips were cut of uniform dimension with a

window in the center for same dimensions as sample. This was to prevent the breaking of sample while loading as they were very small. The sample was mounted on the paper mold and loaded on the instron for testing. The extension was applied at 5mm/min with sensitivity for failure set as 20% of maximum load. The samples were hydrated for 2-3 minutes in PBS prior to testing. The results were measured for maximum load and Young's modulus.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Purification of Collagen

Figure 3.1 shows the purified type I collagen extracted from bovine tendons after overnight drying at 45°C in oven. The bundled fibers were separated manually and stored in desiccator to avoid degradation due to atmospheric moisture.

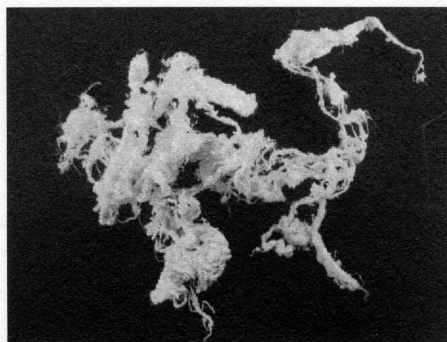


Figure 3.1 Purified type-I collagen.

3.2 Electrospinning of Purified Collagen

The electro-spun collagen mats were viewed under scanning electron microscope to ensure uniform fiber diameter, no phase separation and no presence of any un-evaporated solvent within or on the fibers. The fibers appeared to be uniform in diameter without any phase separation [Figure 3.2]. There was no un-evaporated solvent seen within or over the fibers. The presence of beads might affect the cellular response and cell-tissue regeneration and were thus avoided. The fiber diameter was measured to be approximately 2.50 μm .

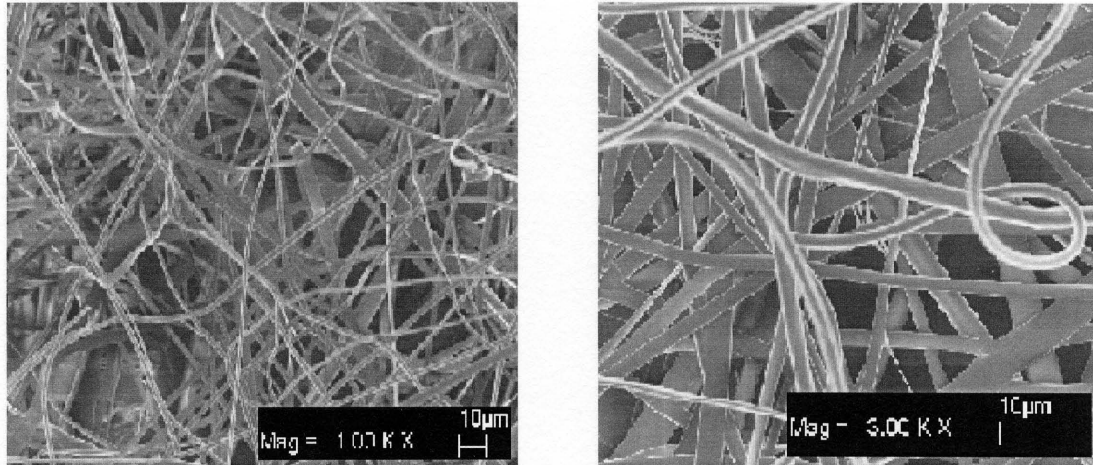


Figure 3.2 SEM image of an electrospun, un-crosslinked collagen scaffold.

3.3 Crosslinking of Electro-spun Collagen Fiber Scaffold

The crosslinking of the electrospun collagen mat with different crosslinkers produced different physical and morphological changes. The genipin crosslinked samples turned greenish-blue in color and shrunk. The EDC and EDC with NHS crosslinked samples stayed colorless and did not change in size noticeably. The glutaraldehyde crosslinked samples turned pale yellow in color, sticky and gelled [See Appendix C for the pictures].

3.4 Immersion Study

The glutaraldehyde and genipin 1% crosslinked samples started to gel after the sterilization process and were completely dissolved following 1 month of immersion in the phosphate buffered saline (PBS) at 37°C. The genipin 5% crosslinked samples started to disintegrate after 2 months of incubation and were very difficult to harvest from the solution [Appendix. C].

The genipin 5% and genipin 10% samples released color during incubation and turned the PBS dark bluish green in color. Genipin 10% samples could be extracted from solution without difficulty at the end of immersion study.

The EDC only and EDC with NHS crosslinked samples did not change color and maintained structural integrity throughout 3 months of incubation without noticeable shrinkage. EDC and EDC with NHS crosslinked samples were soft, but easy to handle than genipin crosslinked mats. There was no disintegration in EDC and EDC with NHS crosslinked mats after 3 months of incubation.

3.5 Dimensions and Weight Measurements

For all the crosslinkers the starting sample size was 1 cm X 1 cm. At each time point, four pieces were harvested and measured for dimensions and weight, of all the concentration and crosslinkers. The average of all the values was taken to compare between different samples (n=4).

The uncrosslinked samples dissolved instantaneously in PBS. The overall dimensions decreased and weight increased after crosslinking. The dimensions of glutaraldehyde and genipin 5% crosslinked samples reduced the greatest as compared to uncrosslinked, genipin 1%, genipin 10%, EDC and EDC with NHS crosslinked samples [Figure 3.3.a]. The dimension of genipin 1% crosslinked samples was found to be greater than genipin 5% and comparable to genipin 10% crosslinked samples. The SEM images of genipin 1% sample shows that the fibers swelled a lot after crosslinking and this might be one of the reasons for not a noticeable decrease in dimension of genipin 1%

crosslinked sample. The dimensions were reduced as compared to uncrosslinked mat [Figure 3.3.a].

The reduction in dimensions of EDC with NHS crosslinked samples was not significantly different from the uncrosslinked collagen mat. The crosslinked samples did not shrink much in dimensions but there was an increase in weight after crosslinking as compared to uncrosslinked samples. The dimensions of the EDC with NHS crosslinked samples were greater than genipin 1%, 5%, 10% and EDC crosslinked samples [Figure 3.3.a]. The EDC crosslinked samples shrunk greater than genipin 10% and EDC with NHS crosslinked samples in dimensions [Figure 3.3.a].

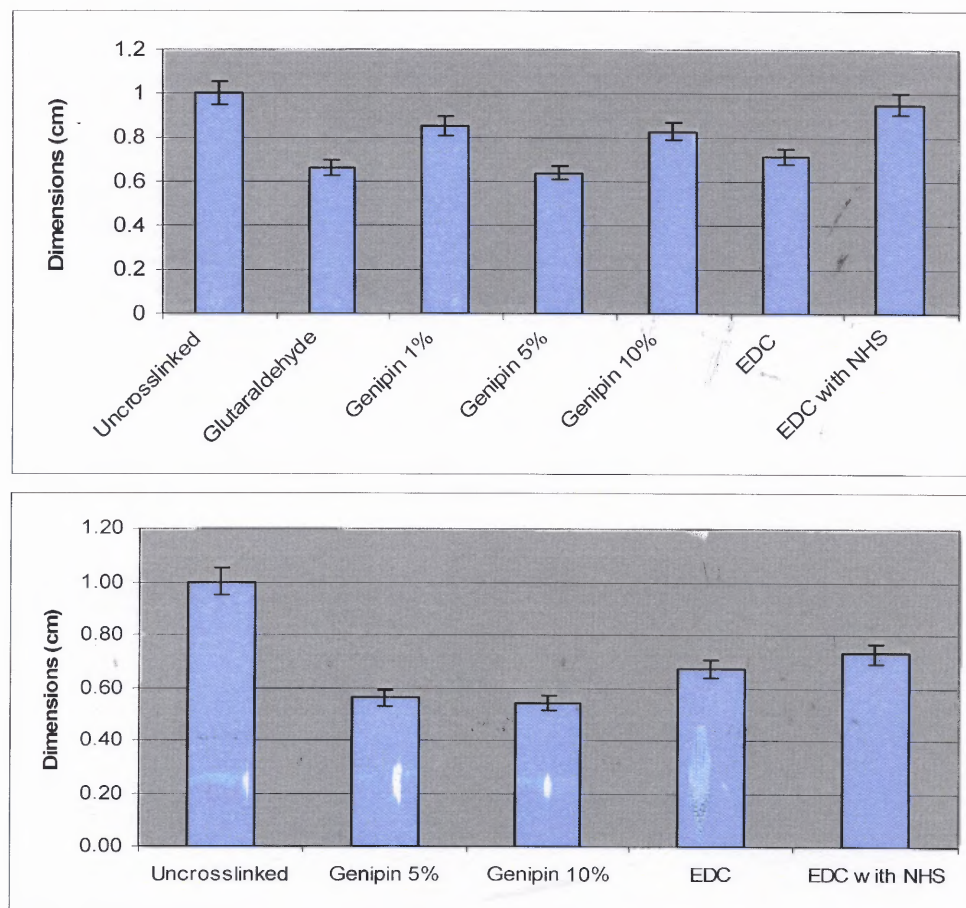
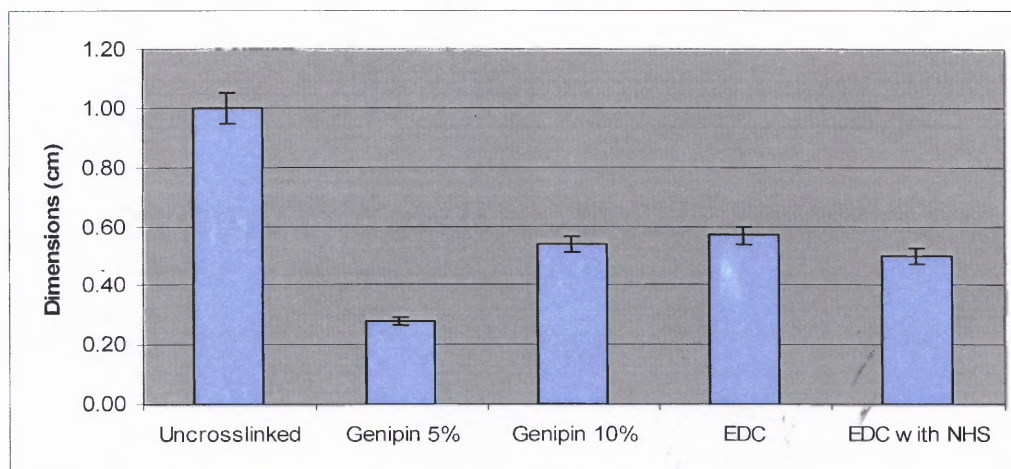
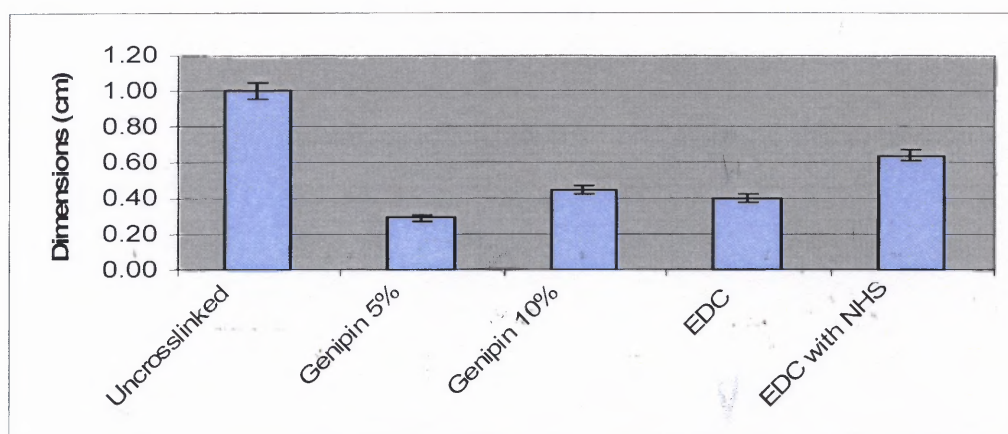


Figure 3.3 Dimensions for all the crosslinked samples at 0 month (a. top) and 1 month (b. bottom) of incubation.

The dimensions of genipin 10% crosslinked samples reduced greatly as compared to starting of incubation study and were comparable in size to genipin 5% after 1 month of incubation [Figure 3.3.b]. There was a decrease in the dimensions of EDC and EDC with NHS crosslinked samples as compared to those before incubation, but they were greater in dimensions than genipin 5% and genipin 10% crosslinked mat [Figure 3.3. b]. The glutaraldehyde and genipin 1% crosslinked samples dissolved after 1 month of incubation [Figure 3.3.b].



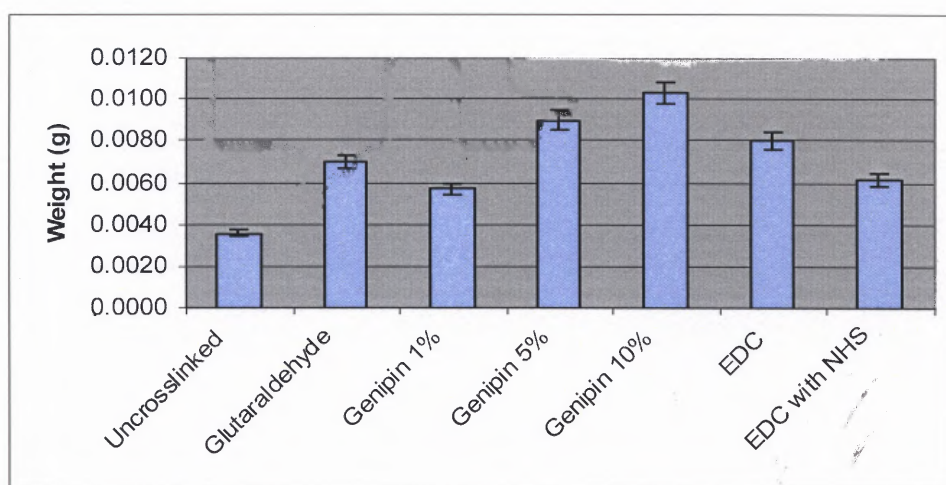
(a)



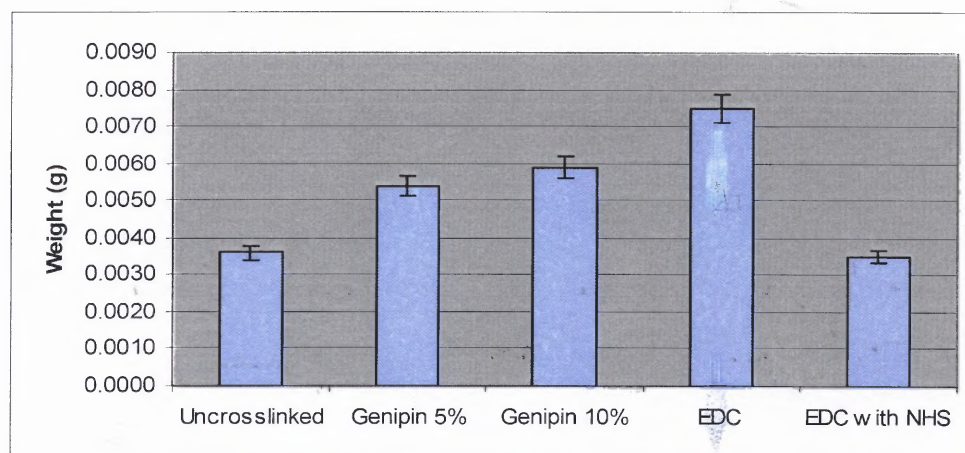
(b)

Figure 3.4 Dimensions for all the crosslinked samples after 2 months (a) and 3 months (b) of incubation.

The dimensions of genipin 5% reduced greatly following 2 months and 3 months of incubation [Figure A.1.3a]. It reduced the greatest among all crosslinkers [Figure.3.4.b]. The dimensions of genipin 10% reduced gradually with incubation [Figure A.1.4a] but was very similar to dimensions of EDC crosslinked mat after 3 months [Figure 3.4.b]. The dimensions of EDC with NHS crosslinked mat were comparably greater than the dimensions of rest of the crosslinkers [Figure 3.4.b].



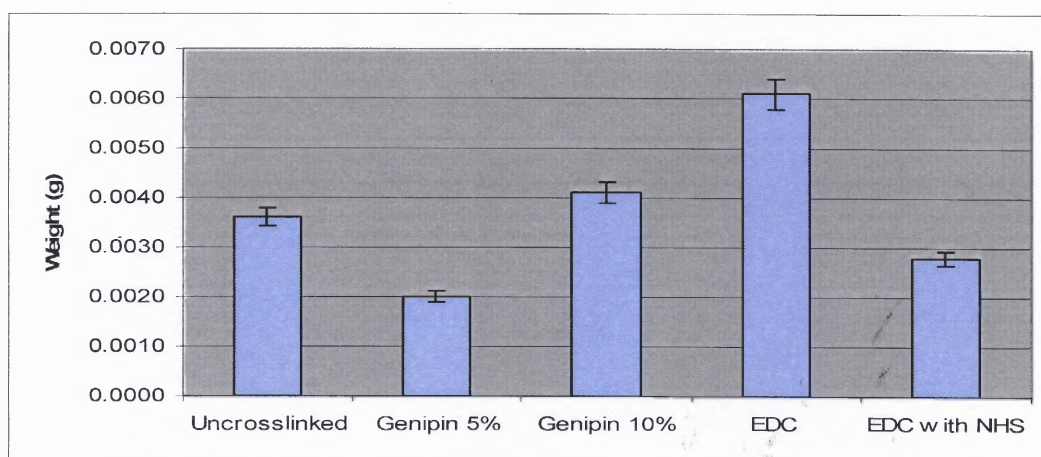
(a)



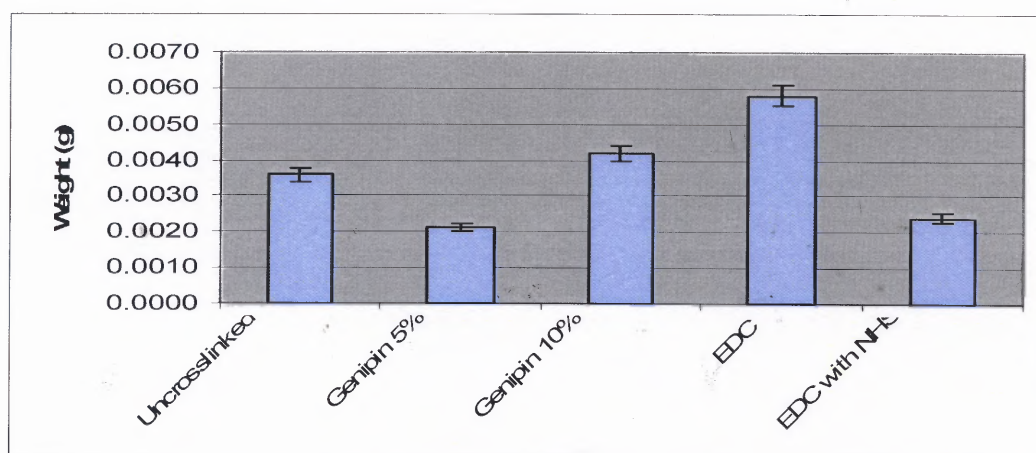
(b)

Figure 3.5 Weights for all the crosslinked samples at 0 month (a) and 1 month (b) of incubation.

The increase in weight after crosslinking was found to be highest for genipin 10% concentration [Figure 3.5a]. The weight of EDC crosslinked mat increased greater than genipin crosslinked samples which shows that the water uptake for EDC crosslinked mat was greater. Genipin might be crosslinking collagen mat more efficiently than EDC. Similarly the percent increase in weight of EDC with NHS crosslinked samples was lesser with incubation and among all the crosslinker, showing NHS improved the crosslinking efficiency of EDC, thus reducing the water uptake.



(a)



(b)

Figure 3.6 Weights for crosslinked samples at 2 month (a) and 3 month (b) of incubation.

The weights of genipin 5% [Figure A.1.3b] and EDC with NHS [Figure A.1.6b] crosslinked gradually decreased with increase in incubation time. Weight of genipin 5% decreased most among all crosslinkers [Figure 3.6b]. Genipin 10% and EDC crosslinked samples showed a similar behavior as genipin 5%, the percent reduction in weight for 2nd and 3rd month of study was not significantly different from each other. It reduced more than the EDC crosslinked mats. The weight for EDC was greater than EDC with NHS crosslinked mat, suggesting that the EDC with NHS crosslinked mat was degrading faster than the EDC only crosslinked mat during incubation.

3.6 Free Amino-Acid Measurements

The free amino-acid count was used to determine the crosslinking extent of crosslinked samples. N=5 for each crosslinker and for every time point (150 μ l).

Table 3.1 Free Amino-Acid Count for all the Crosslinkers

Sample	Weight (g)	Absorbance					Average Absorbance	Standard Deviation
		A1	A2	A3	A4	A5		
Control 1	0.0000	0.000	-0.001	0.003	0.002	-0.002	0.000	0.00
Control 2	0.0000	0.006	-0.003	0.001	0.002	-0.003	0.000	0.00
Uncrosslinked	0.0024	0.403	0.434	0.420	0.412	0.436	0.419	0.01
Glutaraldehyde	0.0024	0.563	0.537	0.636	0.587	0.597	0.579	0.04
Genipin 1%- 0 month	0.0024	0.361	0.331	0.360	0.331	0.370	0.351	0.02
Genipin 5%- 0month	0.0024	0.323	0.318	0.337	0.353	0.330	0.326	0.02
Genipin 10%- 0month	0.0024	0.311	0.321	0.342	0.322	0.353	0.325	0.01
EDC- 0 month	0.0025	0.030	0.029	0.027	0.026	0.028	0.029	0.00
EDC with NHS- 0 month	0.0024	0.205	0.202	0.203	0.214	0.212	0.203	0.01
Genipin 5%- 1month	0.0024	0.173	0.186	0.187	0.200	0.180	0.182	0.01
Genipin 10%- 1month	0.0024	0.178	0.199	0.201	0.208	0.211	0.193	0.01
EDC- 1 month	0.0026	0.105	0.099	0.100	0.093	0.099	0.101	0.00
EDC with NHS- 1 month	0.0026	0.006	0.059	0.068	0.075	0.088	0.044	0.03
Genipin 5%- 2 months	0.0026	0.143	0.157	0.153	0.148	0.158	0.151	0.01
Genipin 10%- 2 months	0.0024	0.113	0.128	0.125	0.129	0.129	0.122	0.01
EDC- 2months	0.0026	0.131	0.138	0.138	0.144	0.139	0.136	0.01
EDC with NHS- 2 months	0.0026	0.095	0.105	0.103	0.107	0.097	0.101	0.01
Genipin 5%- 3 months	0.0025	0.339	0.353	0.361	0.344	0.357	0.351	0.01
Genipin 10%- 3 months	0.0026	0.289	0.299	0.299	0.303	0.295	0.296	0.01
EDC- 3 months	0.0026	0.162	0.173	0.193	0.206	0.201	0.176	0.02
EDC with NHS- 3 months	0.0026	0.105	0.095	0.096	0.095	0.101	0.099	0.00

For the 0 month of incubation, immediately after crosslinking, the free amino group count of EDC was lowest as compared to all other crosslinkers, suggesting EDC crosslinked the mat to the greatest extent [Figure 3.7]. The free amino count for the genipin 1%, 5% and 10% crosslinked samples immediately after crosslinking was not significantly different. It was greater than EDC and EDC with NHS crosslinked samples

[Figure 3.7]. There was no significant difference seen in the count for genipin 5% and 10% after 1 month of incubation [Figure 3.8].

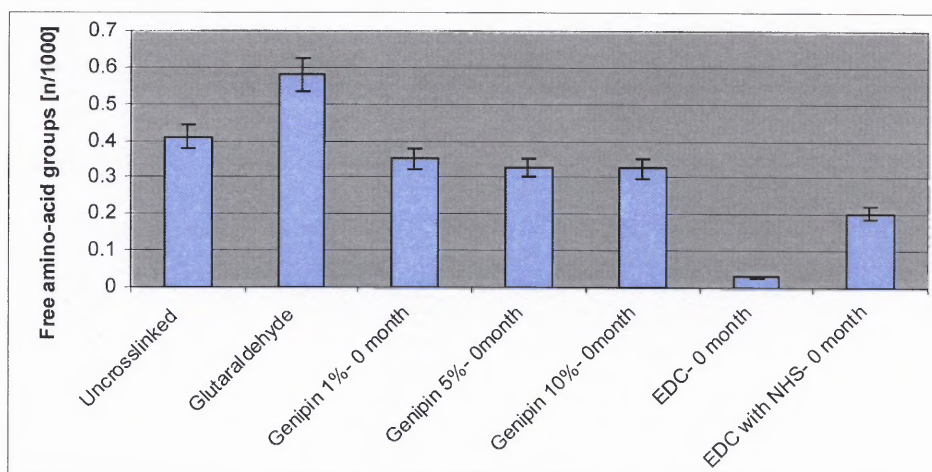


Figure 3.7 Free amino-acid count for all crosslinked samples before incubation.

The EDC with NHS crosslinked samples had lower free amino group count than genipin crosslinked samples but higher than the EDC crosslinked samples [Figure 3.8]. After 1 month of incubation, the free amino acid group for EDC was lower than the genipin 5% and 10% concentration, suggesting the genipin crosslinked mat degraded faster than EDC and EDC with NHS crosslinked mats [Figure 3.8].

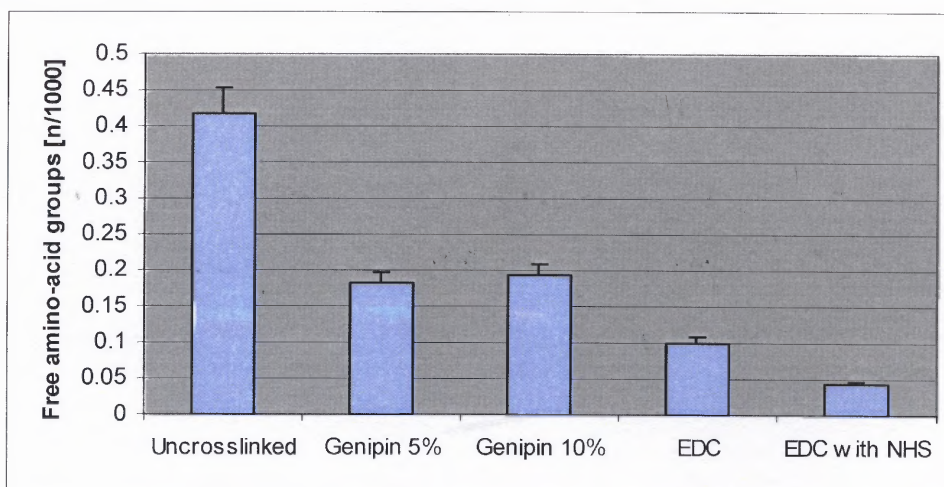


Figure 3.8 Free amino-acid count for all crosslinked samples after 1 month of incubation.

The count for EDC with NHS was found to be lower than EDC suggesting the degradation of EDC with NHS crosslinked mat was slower than the EDC crosslinked mat. This was supported by the observation that the dimension for EDC with NHS crosslinked samples was greater than EDC samples after 1 month of incubation [Figure 3.8]. Following 2 months of incubation, the free amino acid count for EDC was higher than the EDC with NHS crosslinked samples and was comparable to the genipin 5% and 10% concentrations [Figure 3.9].

The free amino acid count for genipin 10% was lesser than the genipin 5% crosslinked samples after 2 months of incubation. The finding can be supported with the observation that the greatest decrease in dimensions and weight was seen for genipin 5% crosslinked samples than rest of the crosslinker after 2 months of incubation [Figure 3.9].

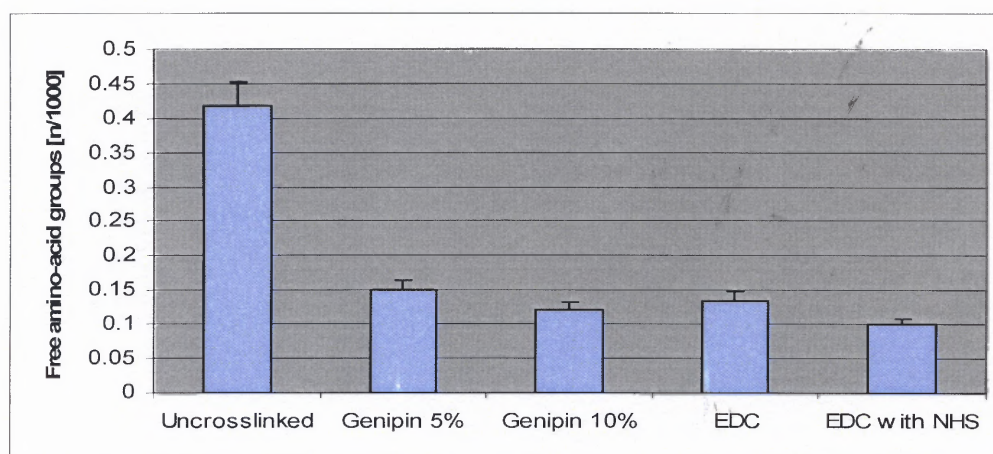


Figure 3.9 Free amino-acid count for all crosslinked samples after 2 months of incubation.

The count for EDC was comparable to the genipin 5% crosslinked sample after 2 months of incubation [Figure 3.9]. The free amino count greatly increased after 3 months of incubation as compared to 1 month and 2 months of data. The highest count was found

for genipin 5% which was very closer to the control value, suggesting the mat completely degrade following 3 months of incubation [Figure 3.10].

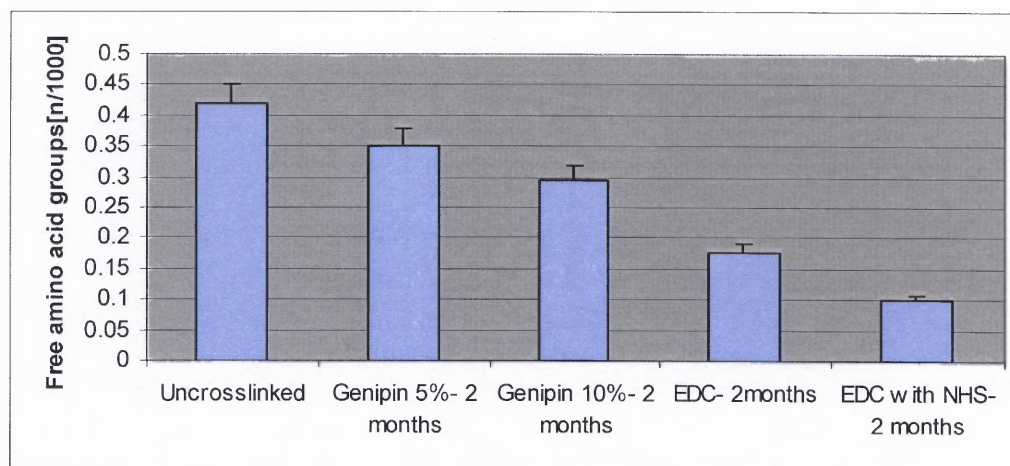


Figure 3.10 Free amino-acid counts for all crosslinkers after 3 months of incubation.

The free amino-acid count for EDC with NHS crosslinked samples was found to lower than the genipin 10% crosslinked samples after 3 months of incubation but was greater than the EDC with NHS crosslinked samples, suggesting the EDC with NHS crosslinked samples were more resistant to degradation than any of the other crosslinkers [Figure 3.10].

The free amino acid count was lower for all the crosslinkers as compared to the uncrosslinked sample, suggesting all the reagents effectively crosslinked the mat except glutaraldehyde for which the count was found higher than the control. This could be due to denaturation of fibrous mat during crosslinking as glutaraldehyde was mixed with water to vapor crosslink the samples and moisture degrades collagen. The free amino acid groups genipin 5% crosslinked samples decreased for the first two months of incubation. The count was increased greatly for third month of incubation. The decrease in free amino acid count indicates continued crosslinking [Figure A.1.2.1].

A similar pattern was observed for genipin 10% crosslinked samples too. During incubation, the PBS for genipin 5% and 10% turned blue in color, suggesting that even after washes and sterilization of samples prior to incubation, there was some crosslinker left unwashed which was released when immersed in PBS. As genipin crosslinking was done initially at 37°C, the incubation conditions favored additional crosslinking of the mat, which was evident from the lesser free amino acid count and further reduction in dimensions of the mat with incubation. The mat slowly started to degrade after 2 months of incubation seen by an increase in the free amino groups which suggests lesser availability of crosslinking molecules in the solution. The increase in free amino-acid count for genipin 5% and genipin 10% was the indication for start of degradation of crosslinked mat [Figure A.1.2.2]

The free amino groups for EDC crosslinked samples was very less for the 0 month of incubation (just after crosslinking), suggesting EDC effectively crosslinked the collagen mat structure. The free amino group count increased, with increase of incubation time suggesting the mat degraded slowly with time [Figure A.1.2.3].

The free amino groups was lower than the uncrosslinked samples showing EDC with NHS effectively crosslinked the collagen mat. The count decreased after the first month of incubation and then increased steadily the following 2 months and 3 months of incubation in PBS [Figure A.1.2.4].

3.7 SEM Measurements

The SEM images were taken using Leo 1350 VP. The images were analyzed to detect the presence of fibers, study the morphology of fiber (uniformity, diameter) and scaffold, to measure the change in diameter of fiber before and after crosslinking and following incubation. The fiber diameter was analyzed using imageJ software. The average fiber diameter of uncrosslinked electrospun type I collagen fibrous mat was found to be approximately 2.50 μm .

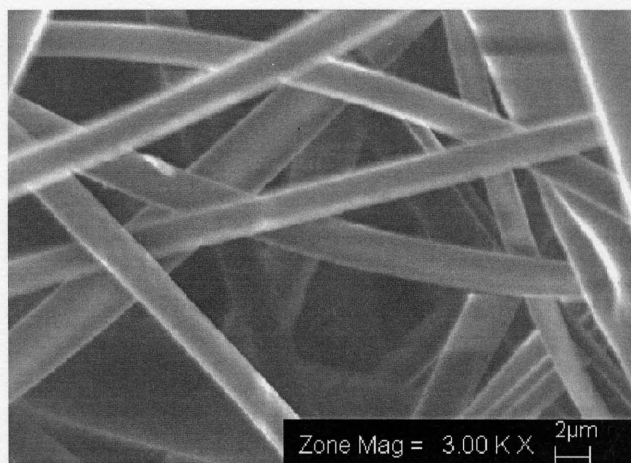


Figure 3.11 SEM image of uncrosslinked, electrospun collagen fiber mat.

No fibers were seen for the glutaraldehyde crosslinked samples. The fibers were fused together, gelled to form a film-like structure [Figure 3.12a]. The fibers were swelled for genipin 1%, genipin 5% and genipin 10% crosslinked mat [Figure 3.12b, 3.12c, 3.12d]. With the EDC and EDC with NHS crosslinked samples, the fibers were easily distinguishable and pores appeared between the fibers [Figure 3.12e, 3.12f].

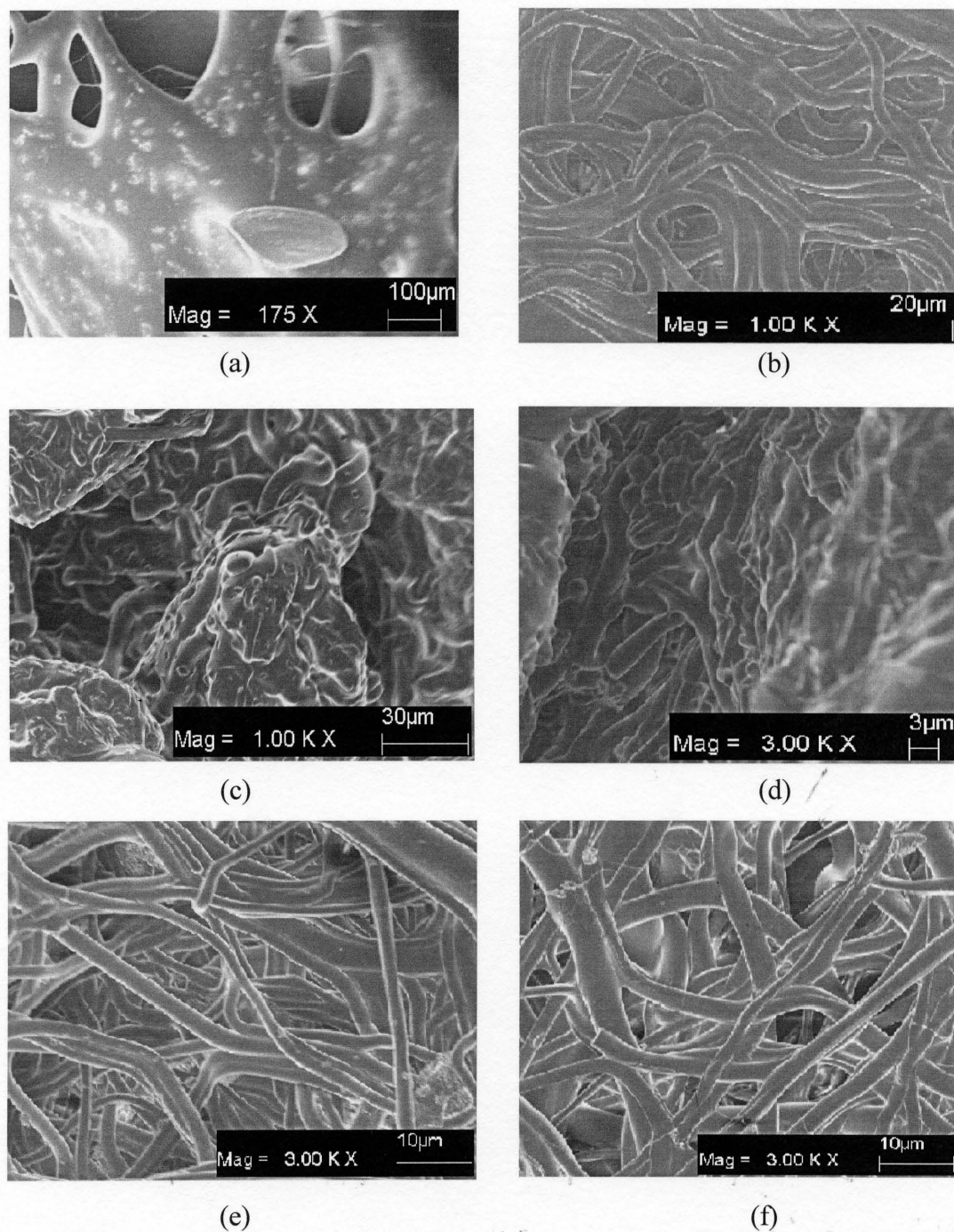


Figure 3.12 SEM images for glutaraldehyde (a), genipin 1% (b), genipin 5% (c), genipin 10% (d), EDC 200mM (e) and EDC with NHS 200 mM each (f) crosslinked samples prior to incubation.

* SEM images for all the crosslinkers at every time point is available in Appendix A.3.

After measurement of fiber diameter using imageJ software, the fiber diameter of each crosslinked mat was compared to fiber diameter of its specific control mat to calculate the percent change in fiber diameter before and after crosslinking and at three different time points of incubation study. The results (n=1) can be summarized as follow:

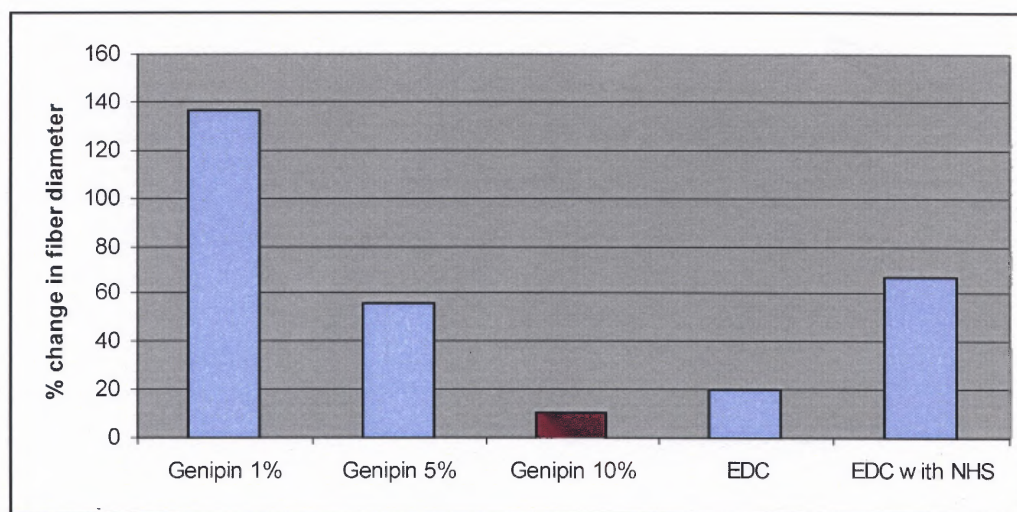


Figure 3.13 The percent change in fiber diameter as compared to their control mats after crosslinking (0 month). Note: The magenta color shows a negative value.

The percent fiber diameter change for genipin 1% crosslinked sample was increased by 136.61 from 2.54 μ m (control) to 6.01 μ m [Table A.3.3 b]. The fiber diameter for genipin 5% crosslinked samples increased from 2.75 μ m (control) to 4.28 μ m having a percent increase in fiber diameter by 55.63% [Table 3.4 b]. The diameter for genipin 10% decreased after crosslinking from 2.75 μ m to 2.45 μ m, having a negative value for change in diameter of -10.90% [Table 3.5 d]. The fiber diameter for EDC increased from 2.14 μ m to 2.58 with 20.44% increase in the fiber diameter [Table 3.6.e].

* All the measurements for fiber diameter of crosslinked sample, control and percent change in fiber diameter is available in Appendix A.3.

Similarly, diameter for EDC with NHS crosslinked fibers increased from $2.39\ \mu\text{m}$ to $3.99\ \mu\text{m}$, having 66.94% increase in diameter value [Table 3.7.e]. Genipin 1% crosslinked fibers swelled the greatest among all the crosslinkers followed by EDC with NHS. EDC swelled the least while genipin 10% crosslinked fibers shrunk the greatest among all crosslinked samples [Figure 3.13].

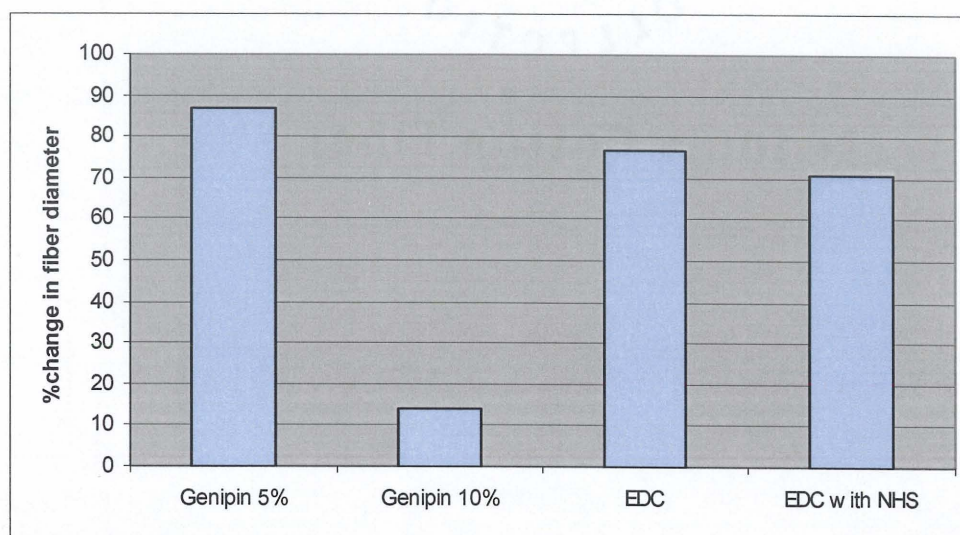


Figure 3.14 The percent change in fiber diameter as compared to their control mats after 1 month incubation in PBS.

Genipin 5% crosslinked samples had the highest percent increase in fiber diameter after 1 month of incubation. The fibers swelled in dimensions too [Figure 3.14]. The fiber diameter for genipin 10% increased (positive value) [Figure 3.14]. The EDC crosslinked fibers swelled greater than the EDC with NHS crosslinked samples. NHS improved the crosslinking efficiency of EDC, thus reducing the water uptake [Figure 3.14]. The percent increase in diameter after 1 month of incubation was least for genipin 10%, and highest by genipin 5%. The difference in water uptake might be a factor of difference in crosslinking extent due to higher concentration of crosslinker.

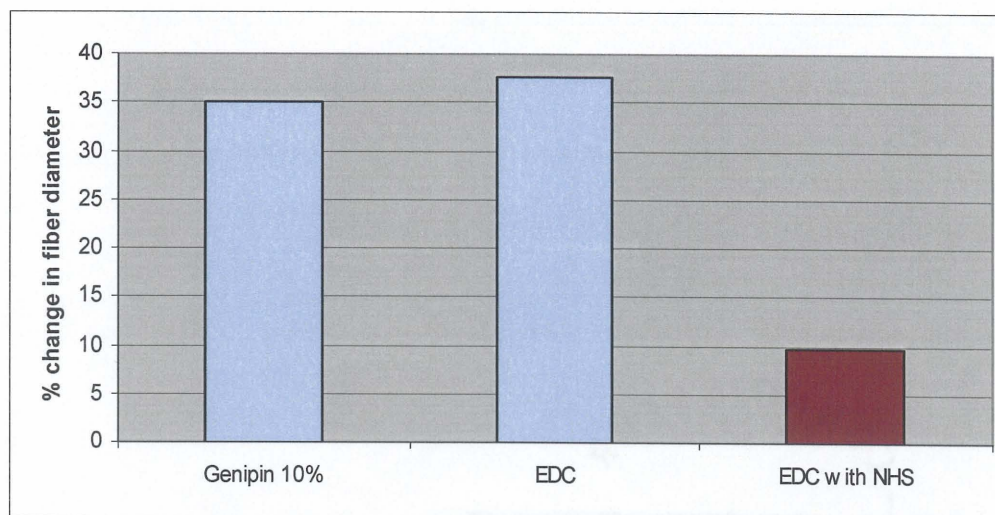


Figure 3.15 The percent change in fiber diameter as compared to their control mats after 2 months incubation. Note: The magenta color shows a negative value.

The fiber geometry of genipin 5% crosslinked mat collapsed after 2 months of incubation and no fibers were visible. The genipin 10% swelled sharply as compared to 1 month of incubation. The values for EDC crosslinked sample also increased after 2 months of incubation whereas the EDC with NHS crosslinked fibers started to shrink with time [Figure 3.15]. The decrease in fiber geometry shows degradation of the crosslinked mat during immersion in PBS at body temperature over time.

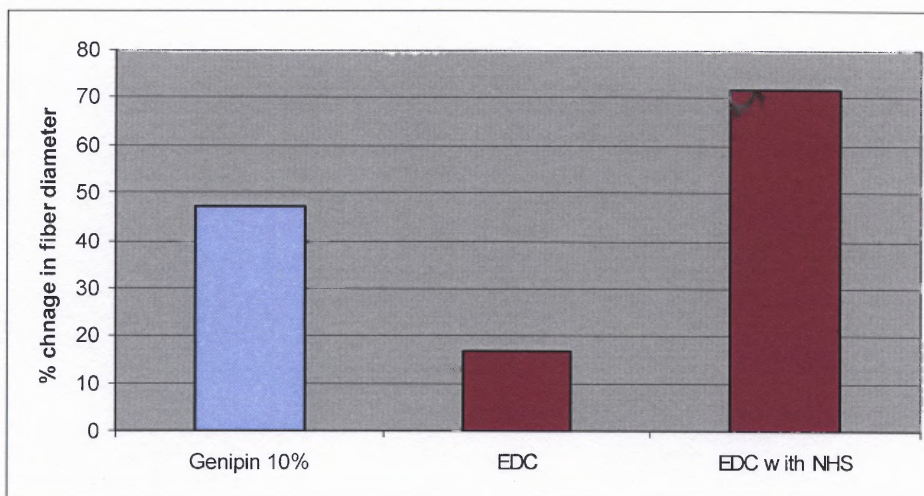


Figure 3.16 The percent change in fiber diameter as compared to their control mats after 3 months of incubation. Note: The magenta color shows a negative value.

Following 3rd month of incubation in PBS, the fiber diameter for genipin 10% continued to increase. The EDC and EDC with NHS crosslinked samples fiber diameter decreased with incubation time [Figure 3.16]. The EDC with NHS crosslinked fibers shrunk more than the EDC crosslinked fibers. The EDC with NHS crosslinked mat degraded faster than EDC crosslinked mat.

On summarizing all the results, the overall fiber diameter of genipin 5% crosslinked samples increase for the first month of incubation and then continued to swell until gelled out to form film like structure. EDC crosslinked fibers swelled for the first 2 months of incubation and then started to shrink. EDC with NHS crosslinked samples swelled only for the first month of incubation and then gradually decreased. The fiber diameter in genipin decreased with increase in concentration of crosslinker, as more bonds would have formed causing greater crosslinking within the collagen structure.

3.8 Porosity Measurements

The porosity was calculated for all the crosslinked samples using:

$$P = [1 - \{ (\text{Mass of fiber mat} / \text{Volume of fiber mat}) * 1 / \text{Density of material} \}] * 100$$

Where, density of collagen was found to be 1.3 to 1.4g/cm [4]. Resulted are listed in

Table 3.1. All the samples were cut with blade to equal dimensions before measurement.

Table 3.2 Porosity for Uncrosslinked and Crosslinked Mats (n=1).

Samples	Dimensions				Avg length	weight	Porosity	Control porosity	% decrease
Glutaraldehyde	2.78	2.85	2.14	2.63	2.60	5.5	75.94	99.72	23.85
G1	8.69	8.57	8.79	8.14	8.55	6.8	99.16	99.99	0.83
G5-0	2.61	2.79	2.55	2.95	2.73	3.2	87.40	99.55	12.20
G5-1	2.37	2.52	2.23	2.59	2.43	0.8	95.69	99.55	3.87
G5-2	2.80	2.73	2.72	2.70	2.74	2.6	90.25	99.55	9.34
G5-3	2.78	3.51	2.98	3.48	3.19	5.0	88.12	99.55	11.48
G10-0	5.38	5.42	5.34	4.91	5.26	6.0	96.83	99.50	2.68
G-10-1	3.62	3.35	3.61	3.56	3.54	2.3	95.99	99.50	3.52
G10-2	5.28	4.68	5.26	4.25	4.87	5.7	96.14	99.50	3.37
G10-3	5.62	5.19	5.91	5.77	5.62	7.8	96.62	99.50	2.89
E-0	5.66	5.40	5.83	5.55	5.61	3.2	98.60	99.31	0.71
E-1	5.83	5.21	5.69	5.11	5.46	2.9	98.62	99.31	0.69
E-2	5.50	6.36	6.02	5.38	5.82	7.1	97.22	99.31	2.10
E-3	6.41	5.80	6.22	5.98	6.10	4.7	98.40	99.31	0.91
EN-0	9.26	8.88	9.25	8.94	9.08	3.8	99.60	99.65	0.05
EN-1	4.08	3.67	4.03	3.29	3.77	3.2	95.39	99.65	4.27
EN-2	6.22	6.28	6.03	6.08	6.15	3.9	98.71	99.65	0.94
EN-3	4.58	5.16	4.52	4.99	4.81	0.8	99.44	99.65	0.21

The measured weight was in milligrams and dimensions in millimeters.

On plotting the results for decrease in porosity after crosslinking before incubation:

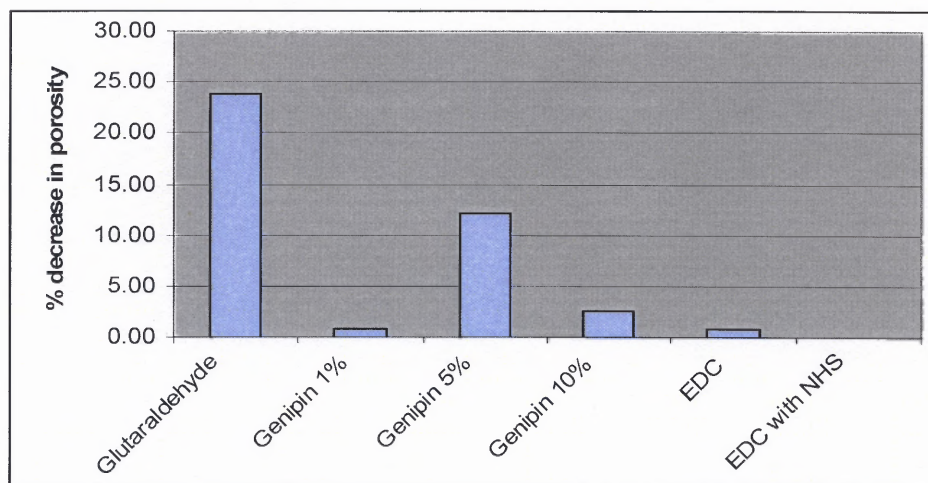


Figure 3.17 The percent decrease in the porosity of samples as compared to control after crosslinking and before incubation. Note: The values are negative in number.

The glutaraldehyde crosslinked samples porosity decreased by 23.85%, indicative of crosslinking, reduction in porous volume as the fibers were drawn closer [Figure 3.17]. The percent decrease in porosity for genipin 1% crosslinked samples was found to be very small (-0.83%), lesser than genipin 10% and comparable to EDC.

Genipin 5% porosity decreased by 12% approximately, after crosslinking and increased following incubation for first month of incubation. The decrease in porosity might be caused due to swelling of fibers or additional crosslinking [Figure 3.17]. The SEM images supported the first phenomena of increase in fiber diameter [Table A.3.4b] after crosslinking.

Genipin 10% the porosity decreased after crosslinking but was lesser than genipin 5% and glutaraldehyde crosslinked samples possibly caused due to shrinkage of fiber [Table A.3.5a] as also shown in SEM images [Figure 3.17]. However even after 1 months

of incubation the porosity continued to decrease, evident of additional crosslinking during incubation [Figure 3.18]. It started to increase after 2 months of incubation [Figure 3.19a] probably due to exhaustion of crosslinker in the solution, and the mat started to degrade [Figure 3.19b].

Similar phenomena were observed for genipin 5% crosslinked mats after 1 month of incubation [Figure 3.18]. Porosity increased from second month, suggesting there might not be enough crosslinker after 1 month to cause more crosslinking and shrinkage of mat. Thus, the mat starts to degrade after 1st month of incubation. The decrease in porosity of EDC and EDC with NHS crosslinked mat was not as high as other crosslinkers [Figure 3.17], suggesting lesser shrinkage of the mat after crosslinking but with increase in weight as noticed in dimensions and weight measurements [Table A.1.5 and A.1.6].

Key notes:

- ❖ Porosity might decrease either due to shrinkage of mat or swelling of fibers.
- ❖ Porosity increases as a result of shrinkage of fibers or degradation of mat.

Plotting of results for 1 month of incubation showed that:

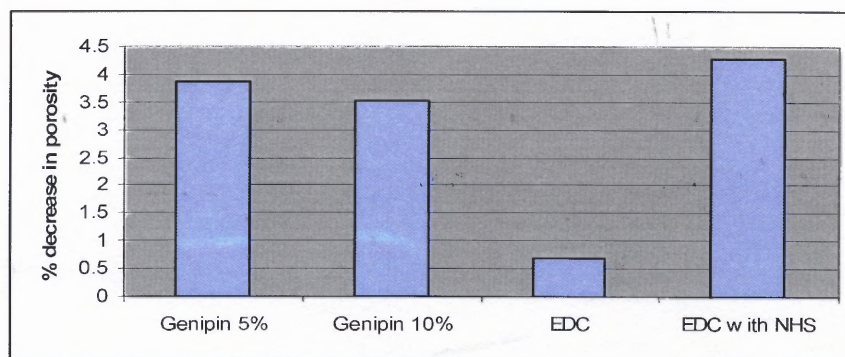


Figure 3.18 The percent decrease in the porosity of samples as compared to their control after 1 month of incubation. Note: The values are negative in number.

The porosity for genipin 5%, genipin 10% and EDC with NHS decreased greatly after 1 month of incubation [Figure 3.18] caused either due to shrinkage of mat as a result of crosslinking for genipin 10% [Figure 3.14] or due to increase in fiber diameter, for EDC with NHS [Figure 3.14].

The decrease in porosity was least for EDC crosslinked samples [Figure 3.18]. The porosity was decreased as compared to that prior to incubation [Figure 3.17], suggesting EDC crosslinked fibers swelled during first month of incubation [Figure 3.14].

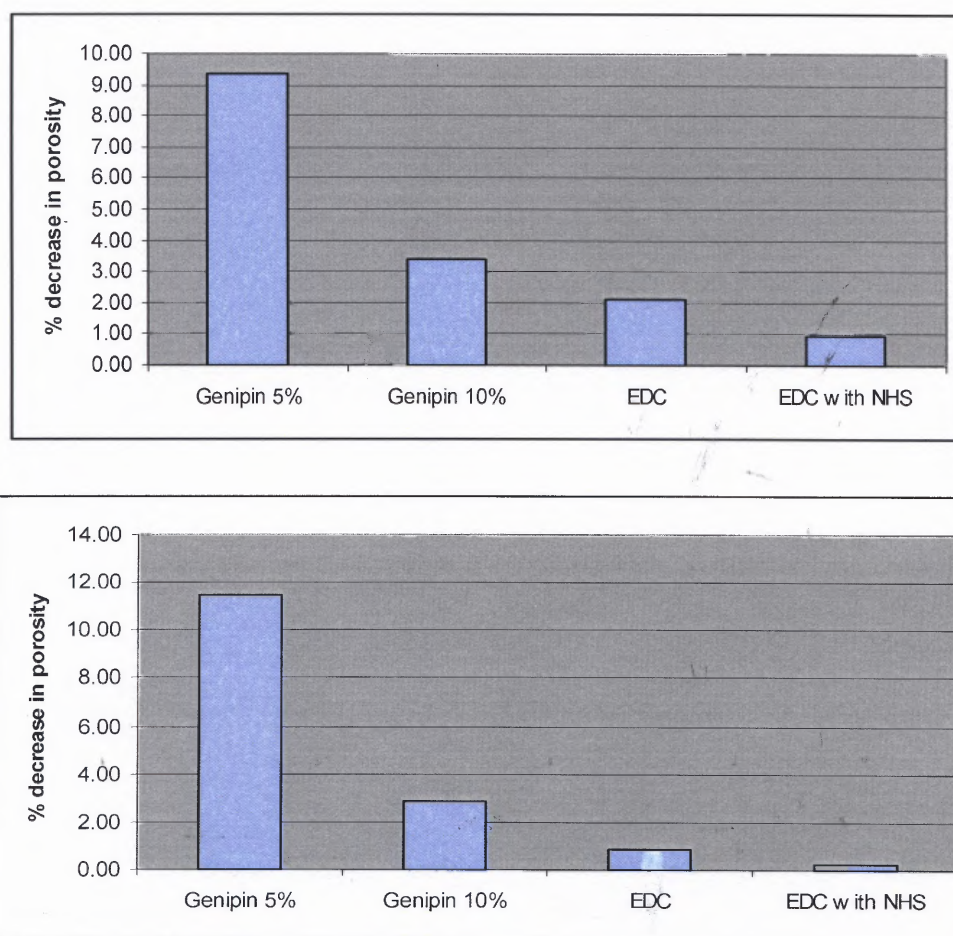


Figure 3.19 The percent decrease in the porosity of samples as compared to their control after crosslinking and incubation for 2 months (top), 3 months (bottom). Note: The values are negative in number.

The porosity of genipin 5% crosslinked samples decreased greatly following 2 months and 3 months of incubation [Figure 3.19a, 3.19b], indicating greater shrinkage of mat, also shown by SEM [Table 3.4a, 3.4b], that the fibers continued to swell collapsing the fiber architecture after 2nd month of incubation and the dimensions decreased greatly showing faster degradation of mat [Figure 3.3a to 3.4b].

The porosity for genipin 10% decreased for first 2 months [Figure 3.17, 3.19a] and then started to increase during 3rd month of incubation [Figure 3.19b], possibly caused due to crosslinking of mat for the first 2 months and then the mat started to degrade following third month of incubation. Porosity increased for EDC and EDC with NHS crosslinked mats [Figure 3.19a, 3.19b] indicating the degradation of mat following incubation.

On summarizing all the results for porosity the genipin 5% mat continued to crosslink for the first month of incubation and then started to degrade while genipin 10% mats continued to crosslink for 2 months before starting to degrade supported by the SEM and dimensions and weight measurement results too.

No such behavior was observed for EDC and EDC with NHS crosslinked samples, they did not continue to crosslink during incubation instead started to degrade following first month of incubation. The decrease in porosity for the first month of incubation could be contributed to swelling of fiber after immersion in PBS, shown in SEM images too.

3.9 TGA Analysis

The Thermal Gravimetric Analysis was performed to study the percent weight loss with increase in temperature of the samples. TGA was done for uncrosslinked collagen mat, collagen mat held over water for 19 hours and for EDC crosslinked mat, to see the effect of moisture and crosslinking.

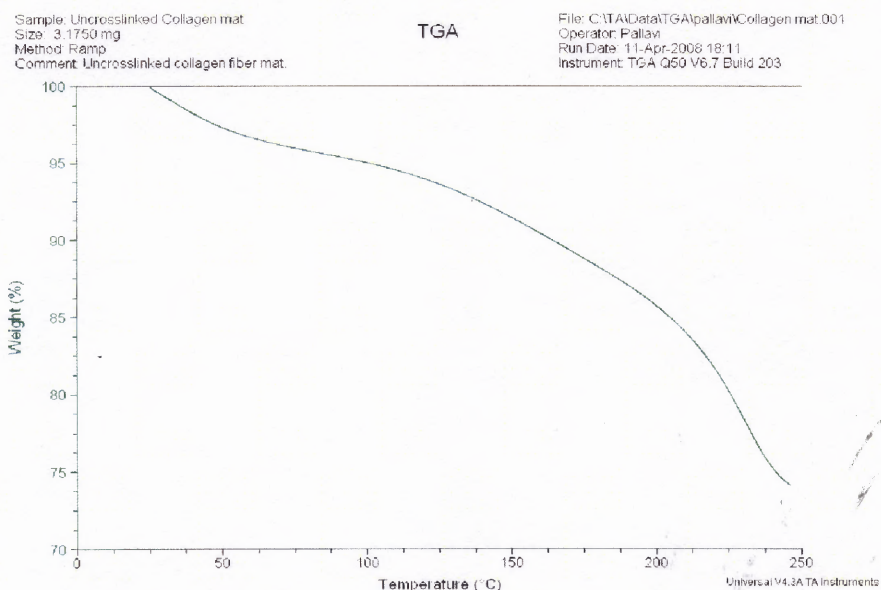


Figure 3.20 TGA plot for uncrosslinked collagen mat. The mat was stored in dessicator to prevent absorption of moisture.

The loss of water was seen over a temperature range of 47°C to 110 °C [Figure 3.20, 3.21]. The loss of moisture was more prominent for collagen mat held over water for 19 hours, separated by a poly mesh film.

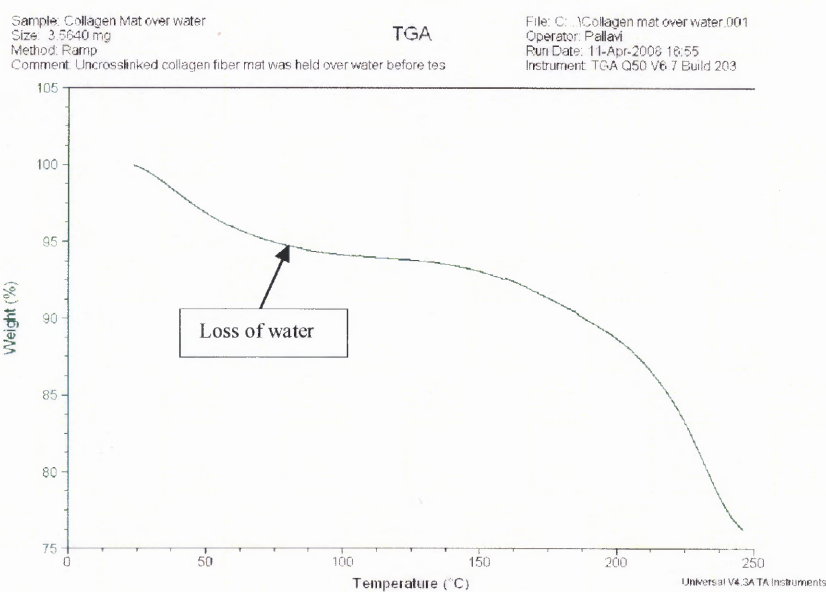


Figure 3.21 TGA plot for collagen mat held over water for 19 hours.

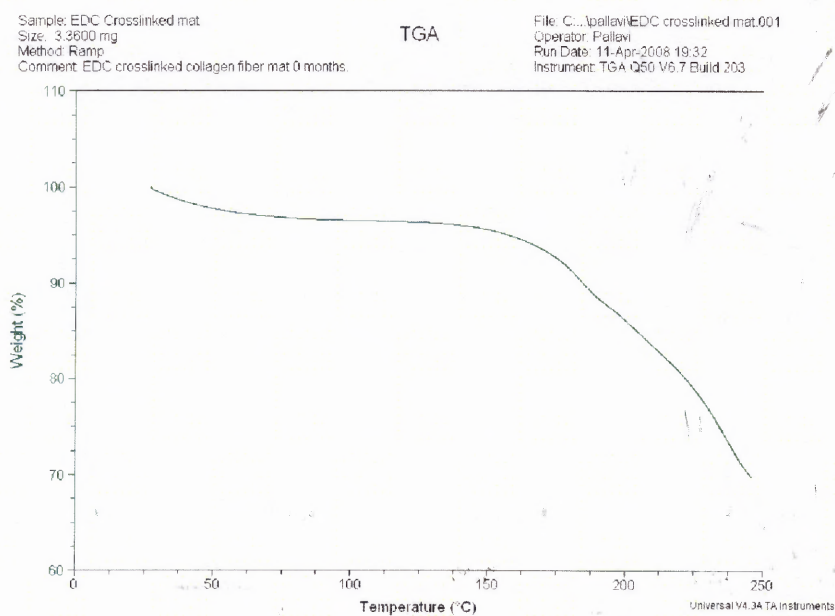


Figure 3.22 TGA plot for EDC crosslinked collagen mat without incubation.

The moisture can accelerate the degradation of collagen, possibly the reason for early dissolution of glutaraldehyde crosslinked mat as similar protocol was followed during crosslinking. The TGA for crosslinked collagen mat showed retention of water for a longer duration as compared to uncrosslinked mat [Figures 3.21, 3.22], which is evident for crosslinking of the structure.

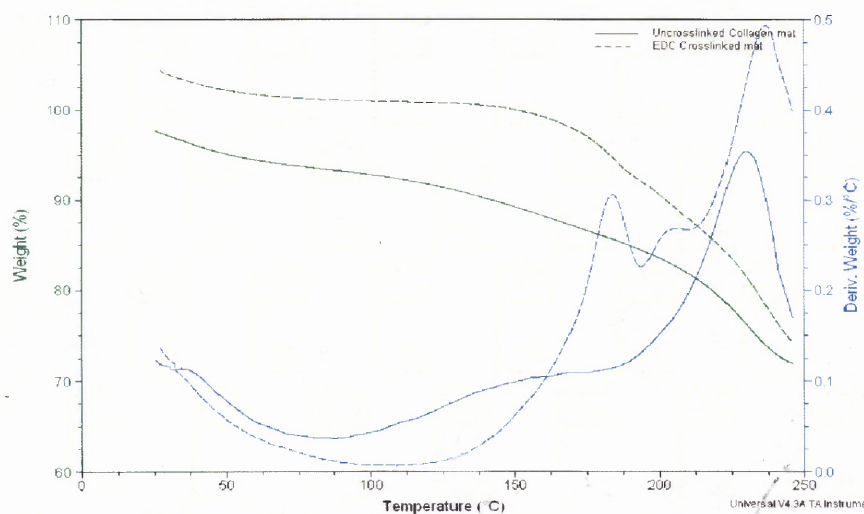


Figure 3.23 TGA plots for uncrosslinked mat (solid line) and EDC crosslinked mat (dashed line).

There was also noticeable change found in the pattern of weight loss for the EDC crosslinked samples as compared to the uncrosslinked mat [Figure 3.23]. All the results from TGA plots were used to better analyze the DSC data.

3.10 DSC Measurements

The Differential Scanning Calorimetric measurement was performed to analyze the thermal behavior of collagen scaffolds by studying their denaturation and glass transition temperature peaks. The denaturation temperature is the unwinding of the triple helical structure of native collagen by breaking of the native bonds which stabilizes the structure. The glass transition temperature is the point where the mobility of particles increases within the structure gained by the thermal heating, and the material change from solid rigid phase to a plastic, rubbery phase.

A denaturation temperature is seen for native collagen while glass transition temperature is seen for denatured collagen, gelatin. Collagen or pure protein does not have a glass transition temperature. The native collagen does not dissolve in water because the bonds present in the native triple helical structure stabilizes the structure, whereas, it is seen that electrospun collagen fibers dissolve instantaneously in aqueous medium, which necessitates the physical and chemical treatment of these structures to improve their stability. The curiosity to understand the reason for dissolution of electrospun mat in water over native collagen, while both are same material, lead to design of new method by the author. The DSC was ran for electrospun collagen fiber without crosslinking, up to 150°C and held at this temperature for 3 minutes to evaporate all the moisture. The temperature was lowered down to 0 °C and ramped up to 250 °C at a heating rate of 10 °C/min. The DSC plot obtained is shown in Figure 3.24.

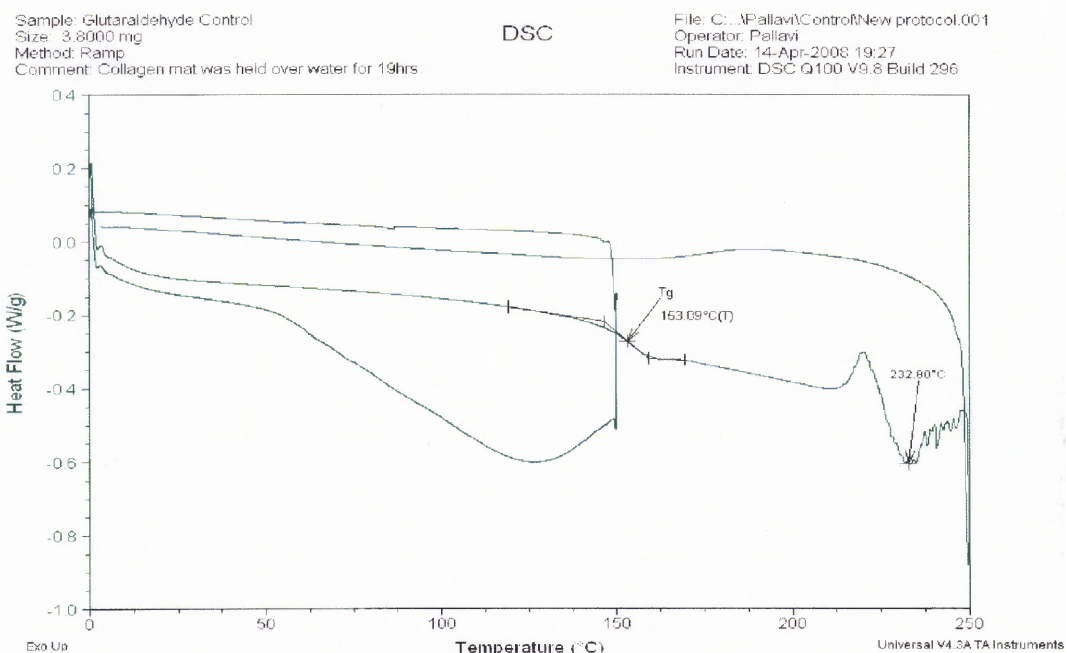


Figure 3.24 DSC plot for the electrospun collagen mat without crosslinking.

The DSC plot showed both a glass transition temperature at 153.09 °C and a denaturation temperature at 232 °C which showed that the electrospun collagen mat consisted of both native and denatured collagen. The native collagen has to be dispersed in the gel structure, as the mat dissolves in water, which showed that the native collagen are not assembled together to hold the structure together and prevent dissolution, as occurs for non electrospun pure collagen.

Thus, electrospinning of collagen reassembles the collagen fibrils together in fiber structure. There is not bonding present between the helical structure and the native collagen is dispersed within denatured collagen structure which causes the electrospun mat to dissolve in water as the gel swells and disintegrate.

DSC was ran for all the samples with heat-cool-heat cycle from 0 °C to 250 °C at 10 °C heating and cooling rate. Controls were prepared for all the crosslinker by treating them similar to crosslinked samples without addition of crosslinker.

- ❖ Glutaraldehyde control: mat was held over water for 19 hours separated by poly mesh.
- ❖ Genipin control: mat was immersed in ethanol and incubated for 3 days at 37 °C.
- ❖ EDC control: mat was immersed in ethanol for 18 hours at room temperature.
- ❖ EDC with NHS control: mat was immersed in ethanol for 4 hours at room temperature.

Table 3.3 Tg and Td for Uncrosslinked Collagen Mat, Control and Crosslinked Mat Before and After Incubation

Samples	Glass Transition Temperature (Tg)	Denaturation Temperature (Td)
Uncrosslinked	165.83	238.11
Glutaraldehyde control	178.19	231.33
Glutaraldehyde	183.57	230.71
Genipin control	186.88	230.41
Genipin 1%- 0 month	194.00	230.71
Genipin 5%- 0month	200.27	234.41
Genipin 5%- 1month	210.99	235.03
Genipin 5%- 2 months	203.25	*
Genipin 5%- 3 months	206.90	*
Genipin 10%- 0month	208.62	*
Genipin 10%- 1month	203.35	*
Genipin 10%- 2 months	208.13	*
Genipin 10%- 3 months	210.05	*
EDC control	182.55	230.71
EDC- 0 month	173.63	228.86
EDC- 1 month	187.27	200.81
EDC- 2months	182.18	*
EDC- 3 months	184.57	207.90
EDC with NHS control	184.19	228.56
EDC with NHS- 0 month	158.83	231.83
EDC with NHS- 1 month	181.70	*
EDC with NHS- 2 months	189.95	201.12
EDC with NHS- 3 months	191.18	203.89

* The denaturation temperature was not clearly seen. All the DSC graphs are shown in Appendix B.

The DSC data showed that the denaturation of native collagen was about 230°C. The denaturation temperature was not seen for some of the crosslinked samples and was not taken as an indicator to determine crosslinking extent, instead the glass transition temperature was considered. The glass transition temperature shifts up with increase in crosslinking density and vice versa.

The Tg of control increased after crosslinking for glutaraldehyde and genipin crosslinked samples, indicative of effective crosslinking. For EDC and EDC with NHS crosslinked mat the Tg lowered down. The Tg for genipin crosslinked samples increased with increase in concentration for crosslinking.

Tg of genipin crosslinked samples was found to be greater than glutaraldehyde treated mats, showing genipin can crosslink collagen mat more effectively than glutaraldehyde. The glutaraldehyde treated mats were not effectively crosslinked and thus continued to uptake water, swell, until dissolved.

Genipin 1% and 5% were able to crosslink the collagen mat, seen by increase in Tg, but the crosslinking density was not enough to resist the swelling of fibers due to water uptake and thus the mat eventually dissolved after 1 month and 2 months of incubation.

Tg for genipin 10% crosslinked mat decreased during first month of incubation, probably due to degradation of mat. The author hypothesis that during incubation the degrading mat released un-reacted crosslinker in PBS which turned the color of solution blue. This crosslinker continued to crosslink the collagen mat additionally as the temperature favored the mechanism too. This hypothesis was proven by an increase in Tg

seen with increase in incubation time. Thus, genipin 10% crosslinked mat continued to crosslink even during incubation.

The T_g for EDC and EDC with NHS was found to be lower than its control. This could possibly due to chemical nature of the compound. A dual peak was seen in EDC crosslinked mat without incubation, before the denaturation of collagen [Figure 3.25]. This could be due to early breaking of the crosslinker molecule before denaturation of collagen. The EDC might not be as thermally stable as genipin and glutaraldehyde. These are chemical reagents and they might react under the testing conditions for DSC which may affect the results.

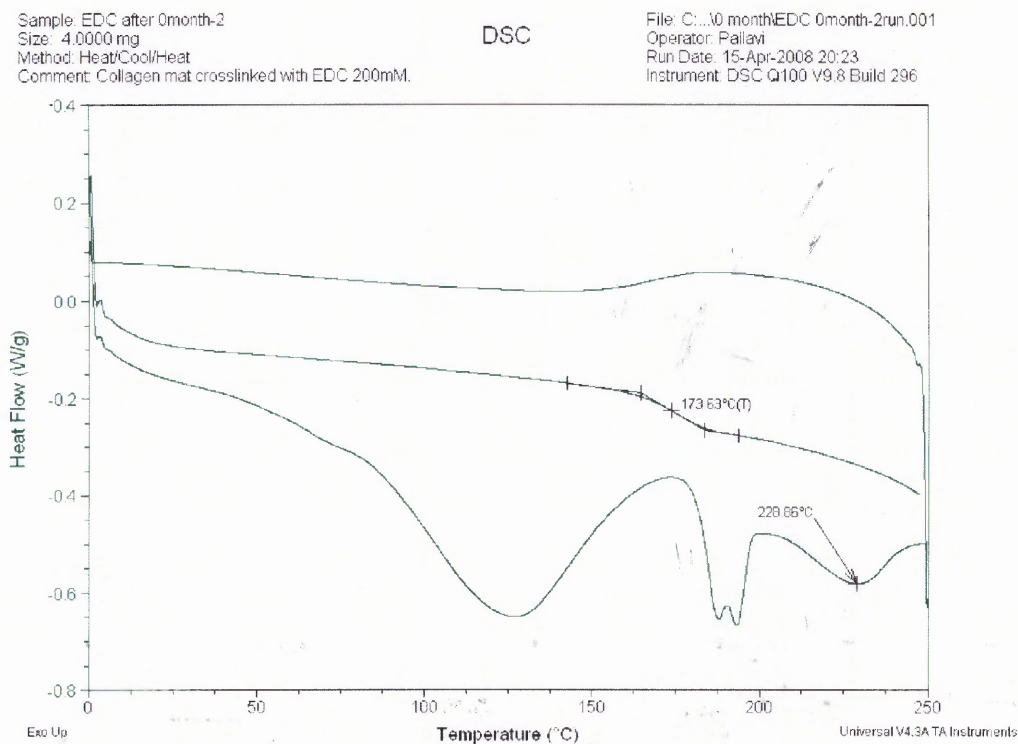


Figure 3.25 DSC plot for EDC crosslinked mat. Dual peaks are seen before the denaturation of collagen which might be breaking down of crosslinker molecule.

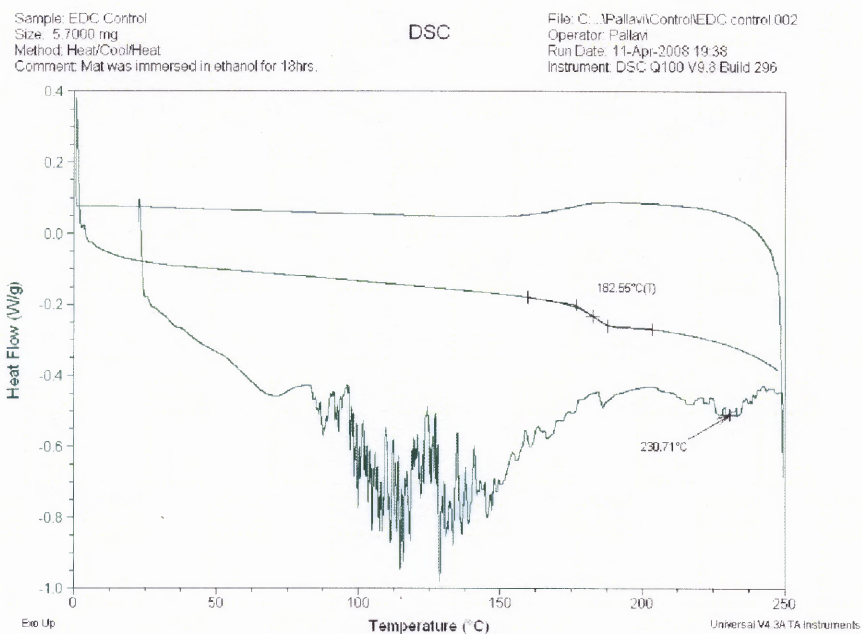


Figure 3.26 DSC plot for EDC control.

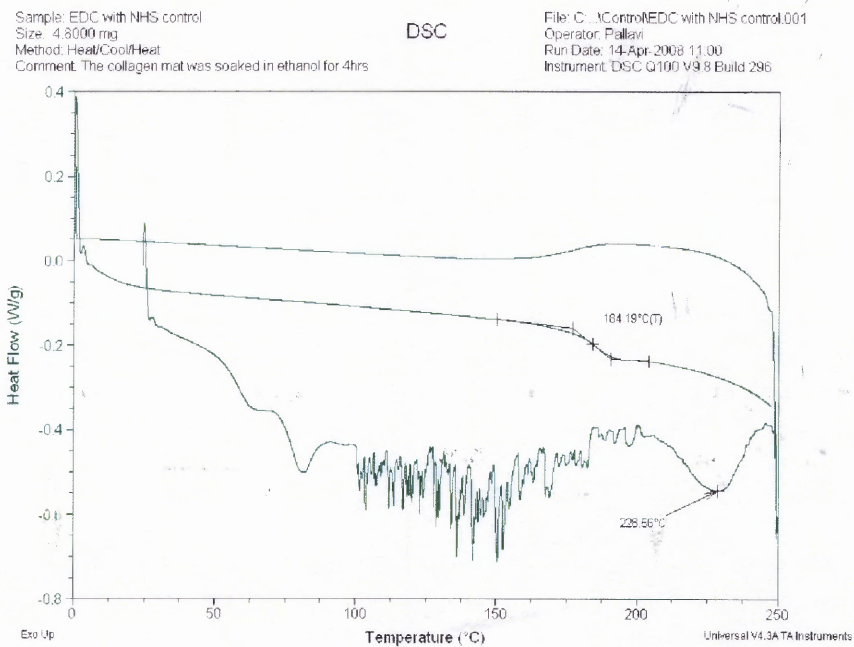


Figure 3.27 DSC plot for EDC with NHS control.

Another effect to be considered was the environment the samples were subjected to during incubation. The control was immersed in ethanol while the incubation was performed in PBS. The degradation was taking place in water media and water have found to plasticize the Tg lowering the denaturation temperature.

The distortions seen in the EDC and EDC with NHS control mat DSC plots were not clear and could possibly be due to vaporization or decomposition of ethanol [Figures 3.26, 3.27]. These behaviors might also affect the breakdown of EDC or release of crosslinker molecule which may lower the Tg. These are chemically reactive agents and they might be reacting at high temperature.

Additionally, variation in the crosslinking extent could contribute to variation in values to Tg than expected. Collagen mats were immersed in ethanol, crosslinker solution but depending on the variation for diffusion of crosslinker within the scaffold matrix would affect the crosslinking degree. This would in turn depend on the uniformity and thickness of the electrospun mats. These distortions in DSC plots were not seen for genipin control [Figure B.3]. They were also treated differently (3 days incubation, body temperature). These treatments might affect the thermal behavior of material all together.

CHAPTER 4

CONCLUSION

The vapor and liquid crosslinking mechanism followed in this project can be used to effectively crosslink electrospun collagen mat. The author developed a new method to successfully crosslink electrospun collagen fibers with genipin. The author also explained the possible reason for dissolution of electrospun collagen in water, which necessitates the need for crosslinking of collagen scaffold.

On comparing all the crosslinkers and different concentration used in the current study, it can be concluded that genipin is an effective crosslinker. It was found to be more thermally stable than EDC and does not break down at higher temperature. Following incubation for 3 months at body temperature gave a better understanding of possible behavior of crosslinked mats *in-vivo*. The EDC had the lowest free amino acid count after crosslinking and the number continued to be lower than rest of the crosslinker, after incubation also, which showed that EDC can effectively crosslink collagen mats. NHS showed to enhance the crosslinking efficiency of EDC but not significantly. EDC and EDC with NHS treated scaffolds were able to maintain fiber architecture till end of study (3 months in PBS) while other crosslinked mats either dissolved or disintegrated except for genipin 10% for which fibers were detected clearly. The fiber diameter, dimensions, free amino acid count decreased with increase in genipin concentration while Tg increased with increase in crosslinker concentration. The authors goal to effectively crosslink electrospun type I collagen scaffold and to maintain the structure in PBS during incubation, was successfully achieved and fulfilled. The choice of crosslinker would depend on its application and cellular response to crosslinked mat.

CHAPTER 5

FUTURE WORK

In this project my endeavor was to create scaffolds with uniform fiber diameter and improve their physical and chemical properties by chemical treatment while considering the physiochemical effects of cytotoxicity and inflammation in account. Future work can include generating fibers in nanometer range as previous studies have shown that nano-fibers can mimic the ECM more closely.

An *in-vitro* cell study would definitely give a better insight to possible tissue regeneration mechanism when using one of the different crosslinkers used in the current study. This would provide a better understanding of the cellular response to the crosslinked scaffold which were compared only on the physio-chemical basis in the current study.

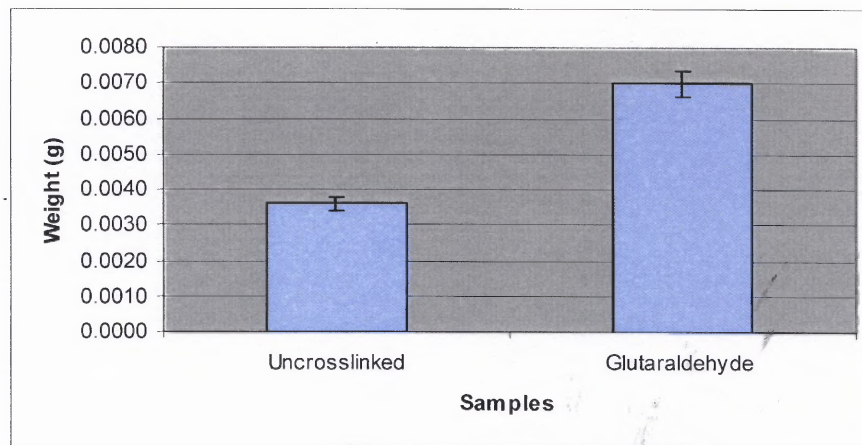
New method can be developed to generate crosslinked electrospun fibers. Genipin can be dissolved in common solvent for collagen, for instance, HFIP with ethanol can be used to dissolve genipin and collagen and than electrospin the solution to generate crosslinked scaffold. Method optimization might be needed to ensure the solution is not completely crosslinked before spinning but is crosslinked enough, as genipin is a slow crosslinker and require body temperature to crosslink. This study would give a biological viability to further characterize and generate more effective scaffolds for tissue engineering application.

APPENDIX A

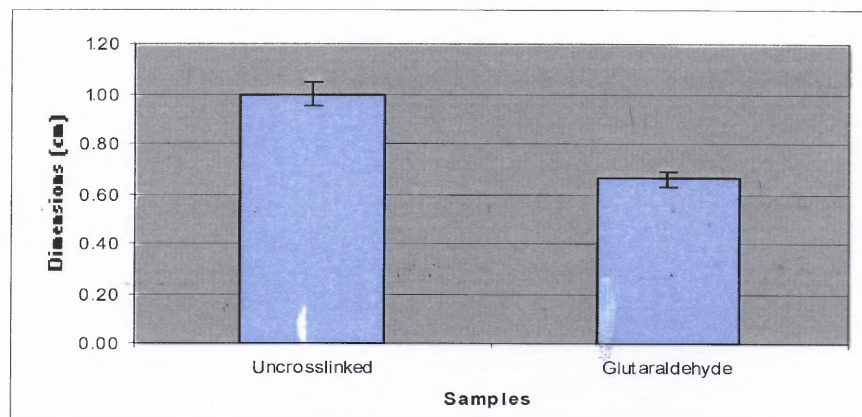
DIMENSIONS, WEIGHT, FREE AA MEASUREMENTS AND SEM IMAGES

A.1 Dimensions and Weights

The dimensions for all the samples was calculated after crosslinking and 1 month, 2 months and 3 months of incubation in PBS at body temperature. For all the measurements, n=4 at each time point and for every crosslinker. All the dimensions are in centimeter and weight is in grams.



(a)



(b)

Figure A.1.1 Weight and dimension for glutaraldehyde crosslinked and control.

Table A.1.1 Dimensions and Weights for Glutaraldehyde Crosslinked Mat

		Dimensions								Weight		
		L1	L2	L3	L4	Average length	S.D	A.L. of whole batch	Avg. SD	Weight	Average wt.	S.D
Batch #1	Before crosslinking	1	1	1	1	1	0	1	0	0.0025	0.0031	0.0011
		1	1	1	1	1	0			0.0044		
		1	1	1	1	1	0			0.002		
		1	1	1	1	1	0			0.0036		
	After crosslinking (0month)	1.0	1.0	1.0	0.8	0.95	0.10	0.67	0.26	0.0047	0.0056	0.0014
		0.5	0.4	0.4	0.4	0.43	0.05			0.004		
		0.6	0.7	0.8	0.4	0.63	0.17			0.0067		
		0.9	0.5	1.0	0.3	0.68	0.33			0.0068		
Batch #2	Before crosslinking	1	1	1	1	1	0	1	0	0.0038	0.0032	0.0005
		1	1	1	1	1	0			0.0029		
		1	1	1	1	1	0			0.0031		
		1	1	1	1	1	0			0.0028		
	after crosslinking	0.9	1.0	0.9	0.8	0.90	0.08	0.66	0.32	0.0052	0.0073	0.0032
		0.8	0.8	0.8	0.3	0.68	0.25			0.0046		
		0.9	0.8	0.2	0.2	0.53	0.38			0.0117		
		0.2	0.9	0.9	0.1	0.53	0.43			0.0076		
Batch #3	Before crosslinking	1	1	1	1	1	0	1	0	0.0081	0.0046	0.0024
		1	1	1	1	1	0			0.0041		
		1	1	1	1	1	0			0.0027		
		1	1	1	1	1	0			0.0034		
	after crosslinking	1.2	0.3	1.2	0.6	0.83	0.45	0.72	0.24	0.0039	0.0080	0.0033
		0.8	0.8	0.5	0.5	0.65	0.17			0.0111		
		0.7	0.6	0.7	0.5	0.63	0.10			0.0102		
		0.9	0.8	0.8	0.6	0.78	0.13			0.0068		
Batch #4	Before crosslinking	1	1	1	1	1	0	1	0	0.0030	0.0035	0.0009
		1	1	1	1	1	0			0.0035		
		1	1	1	1	1	0			0.0047		
		1	1	1	1	1	0			0.0028		
	After crosslinking	0.67	0.2	0.8	0.33	0.50	0.28	0.60	0.24	0.0075	0.0070	0.0011
		0.66	0.9	0.45	0.21	0.56	0.29			0.0081		
		0.7	0.8	0.3	0.7	0.63	0.22			0.0068		
		0.8	0.47	0.58	0.98	0.71	0.23			0.0055		

Table A.1.2 Dimensions and Weights for Genipin 1% Crosslinked Mat

		Dimensions								Weight		
		L1	L2	L3	L4	average length	S.D	A.L. batches	S.D batches	Weight	Average wt.	S.D
Batch #1	Before crosslinking	1	1	1	1	1	0.00	1	0.00	0.0060	0.0048	0.0011
		1	1	1	1	1	0.00			0.0045		
		1	1	1	1	1	0.00			0.0033		
		1	1	1	1	1	0.00			0.0052		
	After crosslinking (0month)	0.90	0.90	0.90	0.80	0.88	0.05	0.88	0.09	0.0051	0.0050	0.0020
		0.90	1.00	0.90	1.00	0.95	0.06			0.0070		
		1.00	0.80	1.00	0.80	0.90	0.12			0.0023		
		0.80	0.80	0.80	0.70	0.78	0.05			0.0055		
Batch #2	Before crosslinking	1	1	1	1	1	0.00	1	0.00	0.0064	0.0063	0.0005
		1	1	1	1	1	0.00			0.0067		
		1	1	1	1	1	0.00			0.0055		
		1	1	1	1	1	0.00			0.0065		
	after crosslinking	0.80	0.90	0.80	0.80	0.83	0.05	0.85	0.06	0.0037	0.0069	0.0027
		0.90	0.80	0.90	0.70	0.83	0.10			0.0070		
		0.90	0.90	0.90	0.80	0.88	0.05			0.0066		
		0.90	0.90	0.90	0.80	0.88	0.05			0.0102		
Batch #3	Before crosslinking	1	1	1	1	1	0.00	1	0.00	0.0039	0.0048	0.0020
		1	1	1	1	1	0.00			0.0069		
		1	1	1	1	1	0.00			0.0025		
		1	1	1	1	1	0.00			0.0060		
	after crosslinking	0.80	0.80	0.80	0.80	0.80	0.00	0.82	0.08	0.0061	0.0054	0.0014
		0.80	0.90	0.80	0.80	0.83	0.05			0.0035		
		1.00	0.70	1.00	0.70	0.85	0.17			0.0067		
		0.80	0.80	0.80	0.80	0.80	0.00			0.0052		
Batch #4	Before crosslinking	1	1	1	1	1	0.00	1	0.00	0.0068	0.0049	0.0016
		1	1	1	1	1	0.00			0.0045		
		1	1	1	1	1	0.00			0.0030		
		1	1	1	1	1	0.00			0.0051		
	after crosslinking	0.90	0.80	0.90	1.00	0.90	0.08			0.0070	0.0055	0.0014
		0.85	0.9	0.78	0.8	0.83	0.05			0.0060		
		0.9	0.9	1	0.8	0.90	0.08			0.0052		
		0.8	0.9	0.9	0.85	0.86	0.05			0.0038		

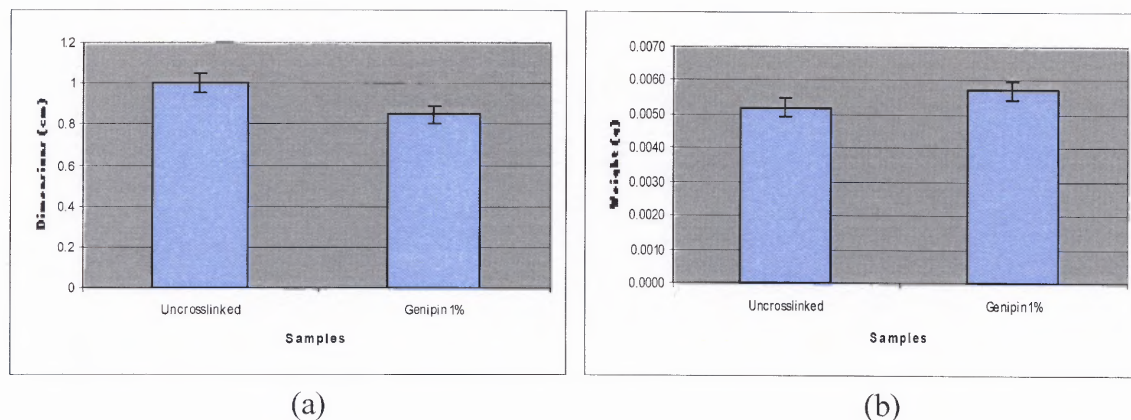
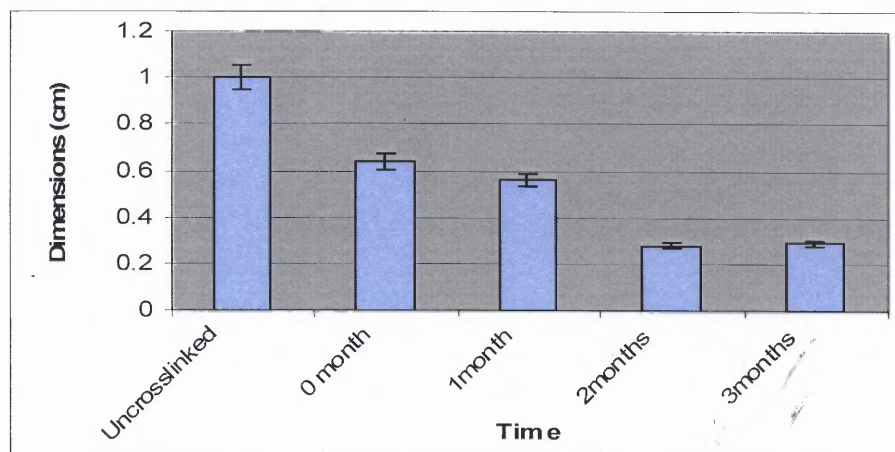
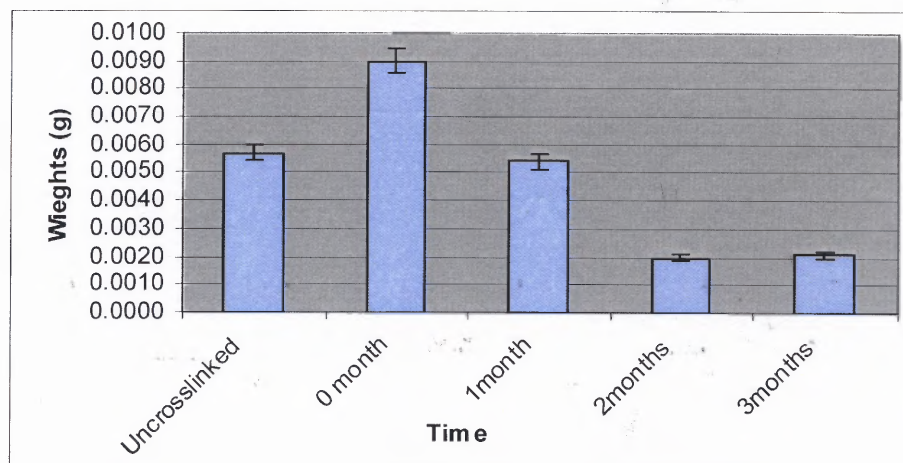


Figure A.1.2 Dimension and weight for genipin 1% crosslinked and uncrosslinked mat.



(a)

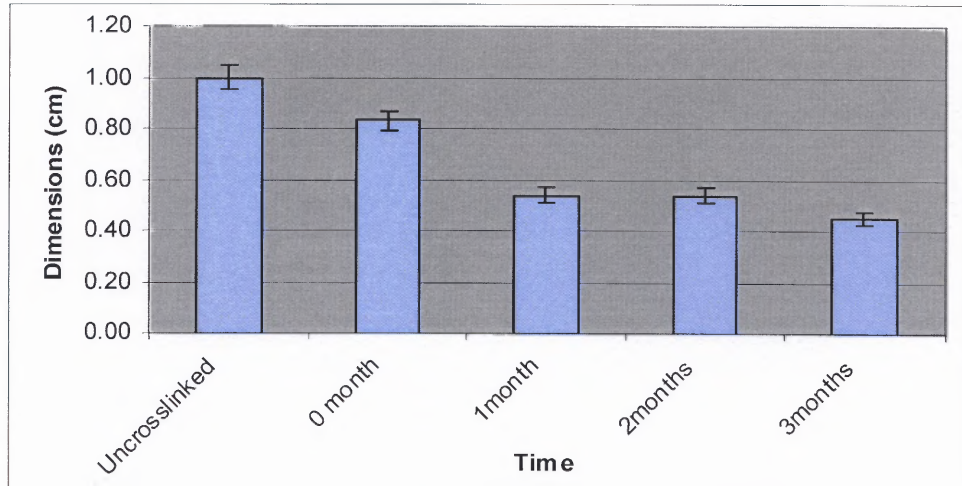


(b)

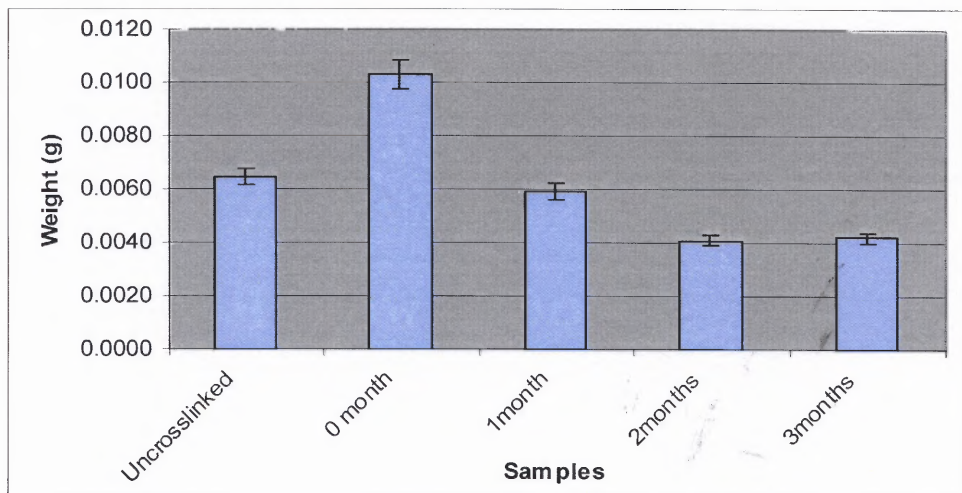
Figure A.1.3 Dimension and weight for genipin 5% crosslinked mat compared to control.

Table A.1.3 Dimensions and Weights for Genipin 5% Crosslinked Mat

	Dimensions								Weight		
	L1	L2	L3	L4	Average length	S.D	A.L. in batch	S.D in batch	Weight	Average wt.	S.D
Batch #1	1	1	1	1	1	0	1	0	0.0044	0.0052	0.0010
Before crosslinking	1	1	1	1	1	0			0.0045		
	1	1	1	1	1	0			0.0053		
	1	1	1	1	1	0			0.0065		
After crosslinking (0 month)	0.70	0.40	0.65	0.25	0.50	0.21	0.69	0.30	0.0074	0.0100	0.0035
	1.20	0.80	0.90	0.95	0.96	0.17			0.0100		
	0.40	0.30	0.45	0.30	0.36	0.07			0.0075		
	1.00	0.90	1.00	0.85	0.94	0.07			0.0149		
Batch #2	1	1	1	1	1	0	1	0	0.0044	0.0056	0.0014
Before crosslinking	1	1	1	1	1	0			0.0075		
	1	1	1	1	1	0			0.0048		
	1	1	1	1	1	0			0.0056		
after crosslinking	0.90	0.60	0.85	0.65	0.75	0.15	0.70	0.19	0.0118	0.0096	0.0018
	0.60	0.40	0.65	0.34	0.50	0.15			0.0083		
	0.70	0.70	1.00	1.00	0.85	0.17			0.0080		
	0.60	0.50	0.80	0.85	0.69	0.17			0.0101		
After 1 month immersion	0.54	0.31	0.52	0.19	0.39	0.17	0.57	0.31	0.0071	0.0056	0.0037
	0.42	0.25	0.40	0.19	0.31	0.11			0.005		
	0.35	0.45	0.29	0.46	0.39	0.08			0.0013		
	0.49	0.19	0.49	0.42	0.40	0.14			0.0021		
Batch #3	1	1	1	1	1	0	1	0	0.0050	0.0059	0.0006
Before crosslinking	1	1	1	1	1	0			0.0061		
	1	1	1	1	1	0			0.0064		
	1	1	1	1	1	0			0.0062		
after crosslinking	0.70	0.50	0.80	0.50	0.63	0.15	0.60		0.0086	0.0106	0.0019
	0.70	0.60	0.85	0.73	0.72	0.10			0.0113		
	0.70	0.50	0.45	0.82	0.62	0.17			0.0096		
	0.50	0.40	0.37	0.44	0.43	0.06			0.0129		
After 2 month immersion	0.35	0.21	0.41	0.16	0.28	0.12	0.29	0.10	0.002	0.0019	0.0005
	0.46	0.31	0.41	0.14	0.33	0.14			0.002		
	0.36	0.26	0.36	0.17	0.29	0.09			0.0011		
	0.33	0.31	0.14	0.34	0.28	0.09			0.0022		
	0.33	0.35	0.34	0.16	0.29	0.09			0.0023		
Batch #4	1	1	1	1	1	0	1	0	0.0096	0.0062	0.0031
Before crosslinking	1	1	1	1	1	0			0.0078		
	1	1	1	1	1	0			0.0040		
	1	1	1	1	1	0			0.0032		
after crosslinking	0.70	0.33	0.48	0.66	0.54	0.17	0.57	0.13	0.0101	0.0106	0.0025
	0.6	0.5	0.55	0.42	0.52	0.08			0.0096		
	0.7	0.69	0.70	0.52	0.65	0.09			0.0083		
	0.70	0.55	0.59	0.35	0.55	0.15			0.0142		
after 3 months immersion	0.28	0.38	0.29	0.39	0.34	0.06	0.29	0.09	0.0033	0.0021	0.0011
	0.47	0.25	0.45	0.24	0.35	0.12			0.0027		
	0.26	0.23	0.25	0.23	0.24	0.02			0.0008		
	0.17	0.26	0.18	0.28	0.22	0.06			0.0016		



(a)

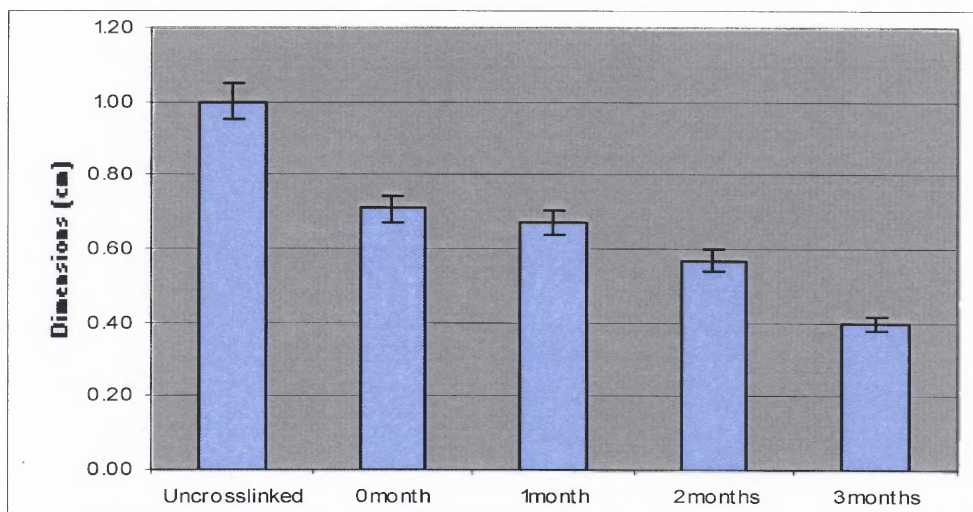


(b)

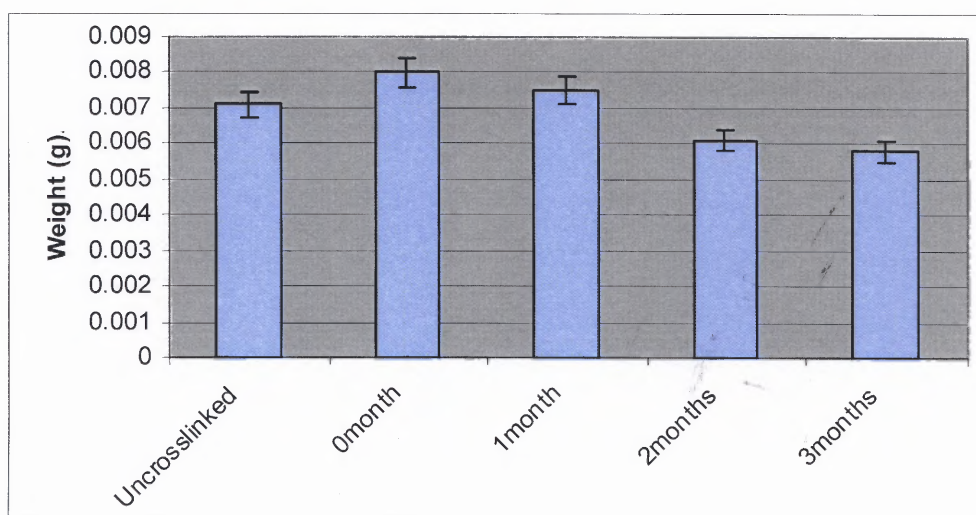
Figure A.1.4 Dimensions (a) and weights (b) for genipin 10% crosslinked samples after 1 month, 2 months and 3 months of incubation.

Table A.1.4 Dimensions and Weights for Genipin 10% Crosslinked Mat

	Dimensions						A.L. in batch	S.D in batch	Weight		
	L1	L2	L3	L4	AL	S.D			Weight	Average wt.	S.D
Batch #1	1	1	1	1	1	0	1	0	0.0054	0.0064	0.0007
Before	1	1	1	1	1	0			0.0072		
crosslinking	1	1	1	1	1	0			0.0063		
	1	1	1	1	1	0			0.0065		
After	0.80	0.90	0.70	0.60	0.75	0.13	0.72	0.13	0.0103	0.0081	0.0019
crosslinking	0.70	0.50	0.70	0.50	0.60	0.12			0.0070		
(0month)	0.80	0.80	0.90	0.90	0.85	0.06			0.0088		
	0.70	0.70	0.70	0.60	0.68	0.05			0.0061		
Batch #2	1	1	1	1	1	0	1	0	0.0049	0.0066	0.0018
Before	1	1	1	1	1	0			0.0086		
crosslinking	1	1	1	1	1	0			0.0074		
	1	1	1	1	1	0			0.0053		
after	0.90	0.90	0.90	0.80	0.88	0.05	0.81	0.10	0.0120	0.0116	0.0025
crosslinking	0.80	0.60	0.80	0.60	0.70	0.12			0.0134		
	0.80	0.80	0.80	0.70	0.78	0.05			0.0080		
	0.90	0.90	0.80	0.90	0.88	0.05			0.0131		
After	0.68	0.64	0.72	0.64	0.67	0.04	0.54	0.16	0.0076	0.0059	0.0018
1 month	0.45	0.39	0.47	0.36	0.42	0.05			0.0034		
immersion	0.66	0.34	0.70	0.39	0.52	0.19			0.006		
	0.64	0.32	0.77	0.47	0.55	0.20			0.0065		
Batch #3	1	1	1	1	1	0	1	0	0.0078	0.0066	0.0021
Before	1	1	1	1	1	0			0.0079		
crosslinking	1	1	1	1	1	0			0.0035		
	1	1	1	1	1	0			0.0072		
after	1.00	1.00	1.00	0.90	0.98	0.05	0.93	0.15	0.0126	0.0117	0.0036
crosslinking	1.00	0.90	1.00	1.00	0.98	0.05			0.0146		
	1.00	1.00	0.90	1.00	0.98	0.05			0.0064		
	1.00	0.60	1.00	0.50	0.78	0.26			0.0133		
After	0.59	0.55	0.60	0.51	0.56	0.04	0.54	0.18	0.0054	0.0041	0.0011
2 month	0.64	0.35	0.63	0.39	0.50	0.15			0.0029		
immersion	0.93	0.47	0.96	0.42	0.69	0.29			0.0035		
	0.33	0.43	0.40	0.48	0.41	0.06			0.0046		
Batch #4	1	1	1	1	1	0	1	0	0.0064	0.0064	0.0020
Before	1	1	1	1	1	0			0.0055		
crosslinking	1	1	1	1	1	0			0.0045		
	1	1	1	1	1	0			0.0091		
after	1.00	0.90	0.89	0.85	0.91	0.06	0.87		0.0111	0.0099	0.0030
crosslinking	0.79	0.93	1.00	0.6	0.83	0.18			0.0085		
	1.00	0.8	0.70	1.00	0.88	0.15			0.0134		
	0.90	0.78	1	0.9	0.88	0.09			0.0065		
After	0.55	0.50	0.62	0.62	0.57	0.06	0.45	0.11	0.0088	0.0042	0.0032
3 month	0.57	0.47	0.55	0.3	0.48	0.12			0.0039		
immersion	0.33	0.41	0.5	0.3	0.40	0.08			0.0025		
	0.58	0.23	0.47	0.3	0.40	0.15			0.0017		



(a)

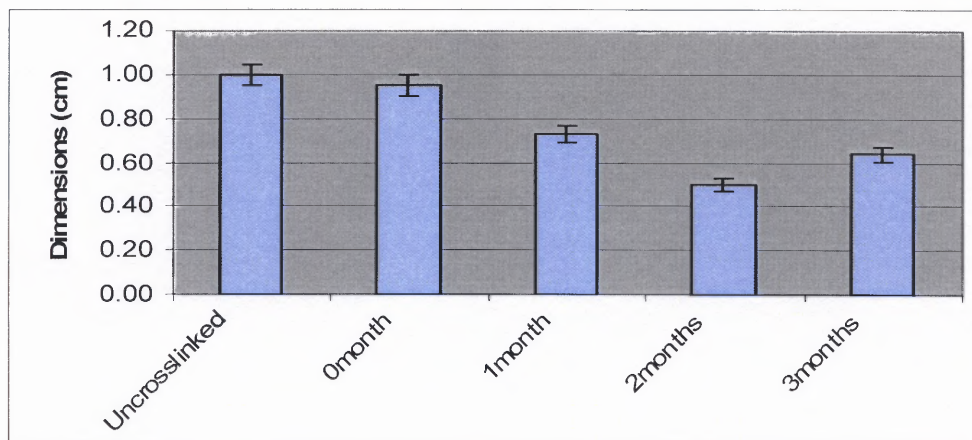


(b)

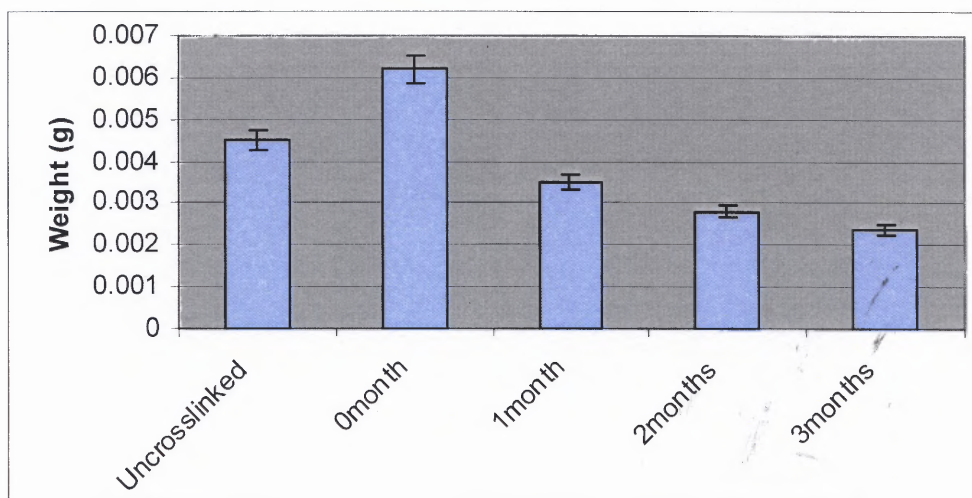
Figure A.1.5 Dimensions (a) and weights (b) for EDC crosslinked samples after 1 month, 2 months and 3 months of incubation.

Table A.1.5 Dimensions and Weights for EDC Crosslinked Mat

	Dimensions						A.L. in batch	S.D in batch	Weight		
	L1	L2	L3	L4	Average length	S.D			Weight	Average	S.D
Batch #1	1	1	1	1	1	0	1	0	0.008	0.0070	0.0031
Before	1	1	1	1	1	0			0.0077		
crosslinking	1	1	1	1	1	0			0.0098		
	1	1	1	1	1	0			0.0026		
After	0.70	0.69	0.90	1.00	0.82	0.15	0.74	0.15	0.0079	0.0073	0.0016
crosslinking (0month)	0.80	0.50	0.70	0.85	0.71	0.15			0.0076		
	0.55	0.79	0.97	0.69	0.75	0.18			0.005		
	0.45	0.77	0.70	0.73	0.66	0.14			0.0088		
Batch #2	1	1	1	1	1	0	1	0	0.005	0.0070	0.0015
Before	1	1	1	1	1	0			0.0083		
crosslinking	1	1	1	1	1	0			0.008		
	1	1	1	1	1	0			0.0068		
after	1	0.7	1	0.7	0.85	0.17	0.70	0.22	0.0079	0.0072	0.0025
crosslinking	0.9	0.9	1	0.7	0.88	0.13			0.0085		
	0.7	0.4	0.7	0.5	0.58	0.15			0.009		
	0.7	0.3	0.6	0.4	0.50	0.18			0.0035		
After	0.65	0.76	0.63	0.77	0.70	0.07	0.67		0.0109	0.0075	0.0028
1 month immersion	0.903	0.83	0.89	0	0.66	0.44			0.0066		
	0.96	0.5	0.93	0.51	0.72	0.26			0.0028		
	0.78	0.45	0.43	0.79	0.61	0.20			0.0026		
Batch #3	1	1	1	1	1	0	1	0	0.0081	0.0073	0.0015
Before	1	1	1	1	1	0			0.0052		
crosslinking	1	1	1	1	1	0			0.0072		
	1	1	1	1	1	0			0.0085		
after	0.5	0.6	0.7	0.8	0.65	0.13	0.73	0.10	0.0084	0.0106	0.0015
crosslinking	0.8	0.8	0.8	0.8	0.80	0.00			0.0109		
	0.8	0.6	0.8	0.8	0.75	0.10			0.0114		
	0.7	0.7	0.6	0.8	0.70	0.08			0.0115		
After	0.58	0.60	0.60	0.67	0.61	0.04	0.57	0.17	0.0055	0.0061	0.0012
2 month immersion	0.64	0.43	0.69	0.46	0.55	0.13			0.0052		
	0.86	0.22	0.76	0.27	0.53	0.33			0.0079		
	0.51	0.64	0.57	0.66	0.59	0.07			0.0058		
	0.68	0.37	0.77	0.36	0.55	0.21			0.0085		
	0.62	0.27	0.65	0.26	0.45	0.21			0.0027		
Batch #4	1	1	1	1	1	0	1	0	0.0086	0.0072	0.0016
Before	1	1	1	1	1	0			0.0051		
crosslinking	1	1	1	1	1	0			0.007		
	1	1	1	1	1	0			0.0082		
after	0.7	0.4	0.7	0.4	0.55	0.17	0.69	0.21	0.0072	0.0070	0.0017
crosslinking	1	0.7	1	0.3	0.75	0.33			0.0076		
	0.6	0.6	0.8	0.6	0.65	0.10			0.0046		
	0.9	0.6	0.9	0.8	0.80	0.14			0.0085		
after	0.40	0.38	0.37	0.40	0.39	0.01	0.40	0.02	0.0094	0.0058	0.0025
3 months immersion	0.43	0.35	0.43	0.36	0.39	0.04			0.0053		
	0.41	0.40	0.39	0.40	0.40	0.01			0.004		
	0.41	0.40	0.41	0.39	0.40	0.01			0.0045		



(a)



(b)

Figure A.1.6 Dimensions (a) and weights (b) for EDC with NHS crosslinked samples after 1 month, 2 months and 3 months of incubation.

Table A.1.6 Dimensions and Weights for EDC with NHS Crosslinked Mat

	Dimensions						A.L. batch	S.D batch	Weight		
	L1	L2	L3	L4	average	S.D			Weight	Average	S.D
Batch #1	1	1	1	1	1	0	1	0	0.0031	0.0035	0.0008
Before crosslinking	1	1	1	1	1	0			0.0045		
	1	1	1	1	1	0			0.0039		
	1	1	1	1	1	0			0.0026		
After crosslinking (0month)	0.98	1.00	1.00	1.00	1.00	0.01	0.97	0.04	0.0069	0.0046	0.0016
	0.95	0.92	1.00	1	0.97	0.04			0.0045		
	0.90	1	0.94	0.88	0.93	0.05			0.0032		
	1.00	0.98	1	1	0.99	0.01			0.0037		
Batch #2	1	1	1	1	1	0	1	0	0.0102	0.0068	0.0029
Before crosslinking	1	1	1	1	1	0			0.0056		
	1	1	1	1	1	0			0.0035		
	1	1	1	1	1	0			0.0078		
after crosslinking	1	1	1	1	1.00	0.00	0.92	0.21	0.0085	0.0114	0.0127
	1	1	1	0.9	0.98	0.05			0.0024		
	1	1	1	1	1.00	0.00			0.0045		
	1	0.5	0.3	1	0.70	0.36			0.03		
After 1 month immersion	0.45	0.38	0.45	0.37	0.41	0.05	0.73	0.33	0.006	0.0035	0.0015
	0.44	0.39	0.39	0.44	0.42	0.03			0.0029		
	0.41	1.08	0.92	0.38	0.70	0.36			0.0018		
	0.76	0.25	0.75	0.40	0.54	0.26			0.0038		
Batch #3	1	1	1	1	1	0	1	0	0.003	0.0036	0.0010
Before crosslinking	1	1	1	1	1	0			0.0024		
	1	1	1	1	1	0			0.0044		
	1	1	1	1	1	0			0.0045		
after crosslinking	1	0.4	0.4	1.4	0.80	0.49	0.94	0.27	0.004	0.0041	0.0012
	1.4	0.8	1	0.8	1.00	0.28			0.0039		
	1	1	1	0.9	0.98	0.05			0.0057		
	1	1	1	1	1.00	0.00			0.0028		
After 2 month immersion	0.96	0.37	0.95	0.37	0.66	0.34	0.50	0.28	0.006	0.0028	0.0019
	0.98	0.40	0.10	0.37	0.46	0.37			0.0023		
	0.30	0.40	0.35	0.35	0.35	0.04			0.001		
	0.60	0.32	0.59	0.38	0.47	0.15			0.0025		
	0.90	0.19	0.87	0.20	0.54	0.40			0.0024		
Batch #4	1	1	1	1	1	0	1	0	0.0035	0.0042	0.0017
Before crosslinking	1	1	1	1	1	0			0.0068		
	1	1	1	1	1	0			0.0031		
	1	1	1	1	1	0			0.0034		
after crosslinking	1.4	1.5	1.3	1	1.30	0.22	0.98	0.25	0.0040	0.0047	0.0014
	1	0.8	0.8	1	0.90	0.12			0.0033		
	1	1	0.8	0.8	0.90	0.12			0.0048		
	1	0.6	0.6	1	0.80	0.23			0.0065		
after 3 months immersion	1.03	0.66	1.07	0.72	0.87	0.21	0.64	0.23	0.0038	0.0024	0.0011
	0.62	0.63	0.82	0.82	0.72	0.11			0.0021		
	0.71	0.69	0.49	0.33	0.56	0.18			0.0023		
	0.49	0.32	0.5	0.33	0.40	0.09			0.0012		

A.2 Free Amino-Acid Count

The free amino-acid was counted as a measure for crosslinking extent for various crosslinked mats. N=5 for all the crosslinked samples at each time point.

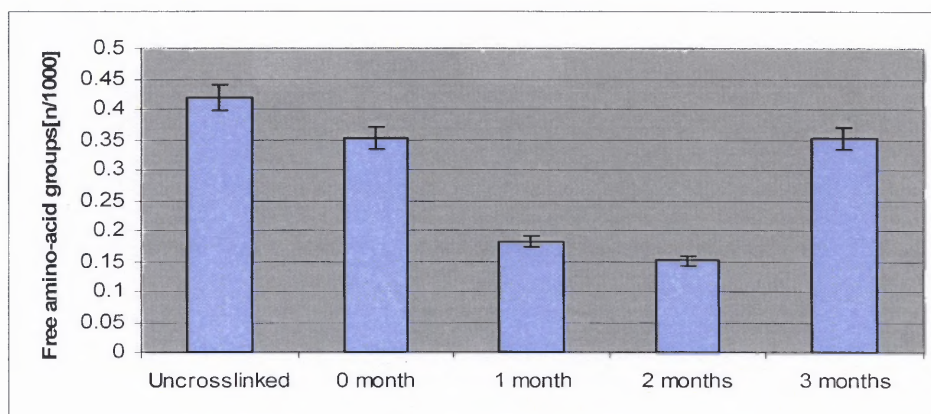


Figure A.2.1 Free amino-acid count for genipin 5% crosslinked mat after no incubation (0 month), 1 month, 2 months and 3 months of incubation.

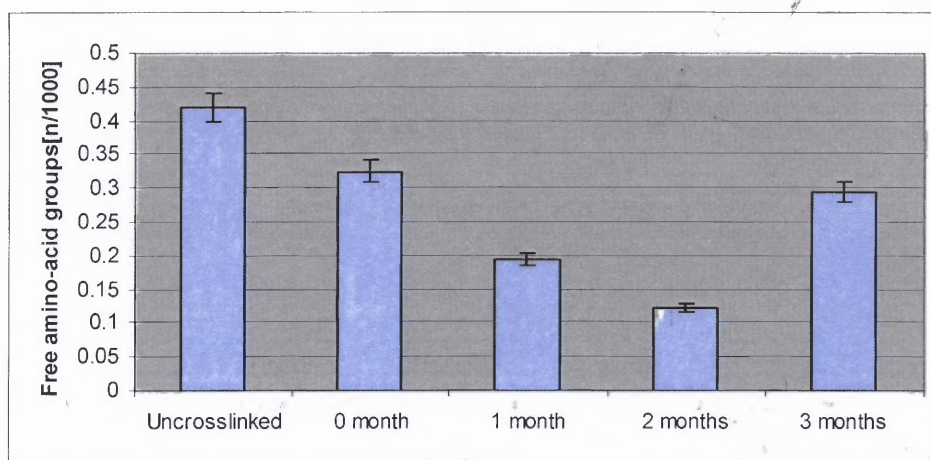


Figure A.2.2 Free amino-acid count for genipin 10% crosslinked mat after no incubation (0 month), 1 month, 2 months and 3 months of incubation.

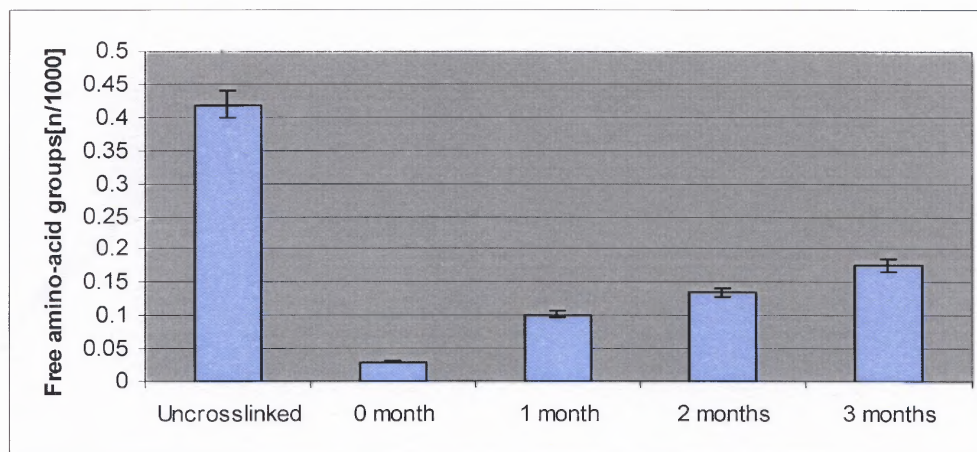


Figure A.2.3 Free amino-acid count for EDC crosslinked mat after no incubation (0 month), 1 month, 2 months and 3 months of incubation.

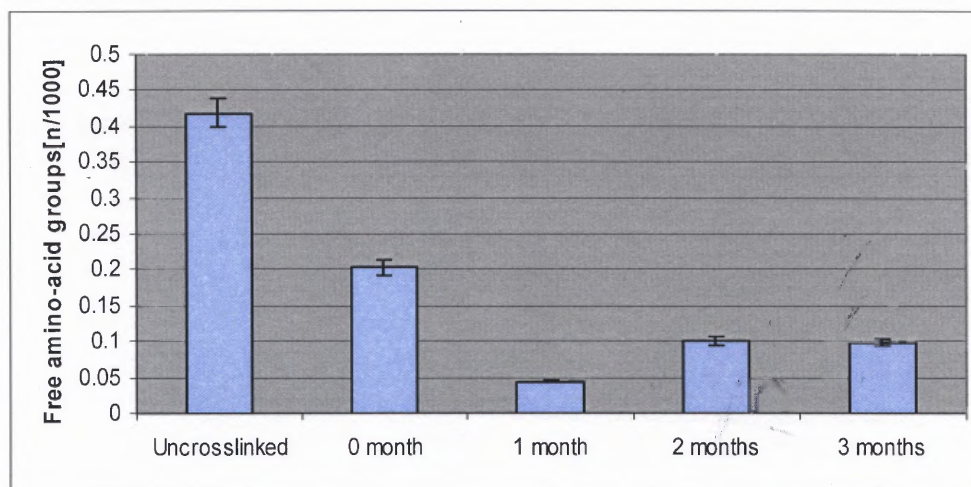
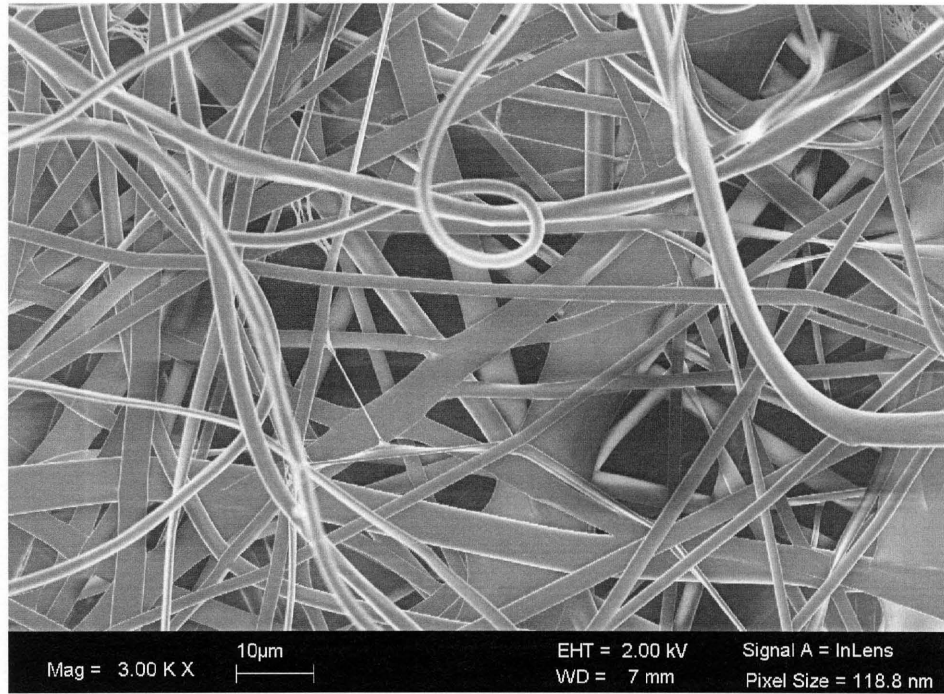


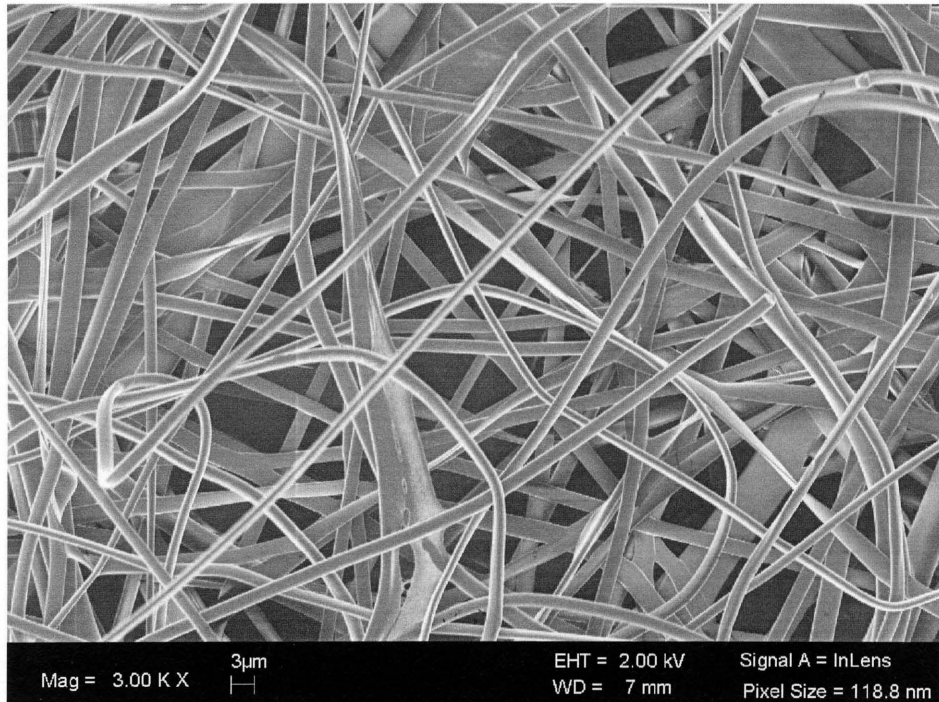
Figure A.2.3 Free amino-acid count for EDC with NHS crosslinked mat after no incubation (0 month), 1 month, 2 months and 3 months of incubation.

A.3 SEM Images and Fiber Diameter Analysis

The SEM images were analyzed using imageJ software online and the fiber diameter of crosslinked and incubated samples was compared to control to obtain the percent change in fiber diameter. N=1 for all the crosslinked samples at each time-point.

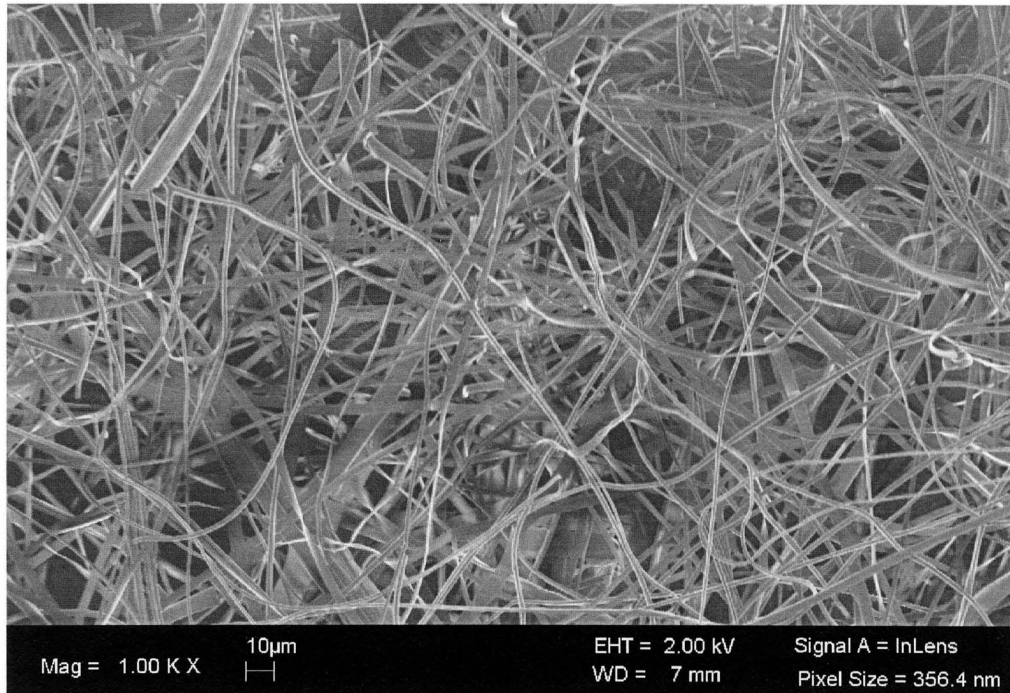


(a)

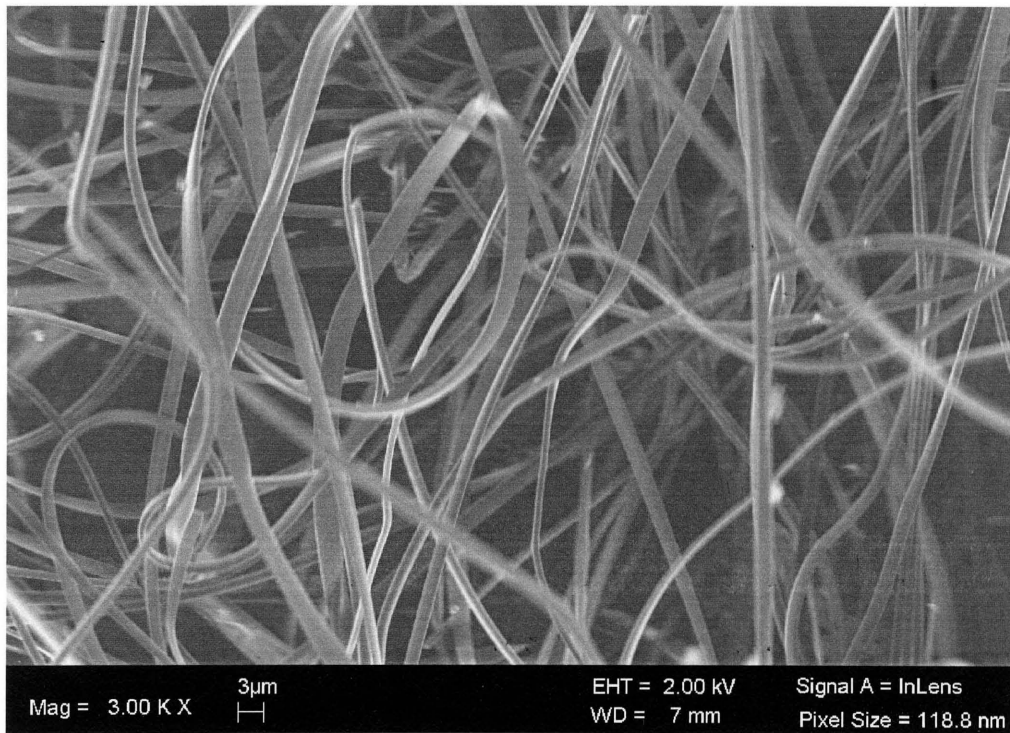


(b)

Figure A.3.1a SEM image of uncrosslinked, electrospun collagen scaffold at 3KX magnification.



(c)



(d)

Figure A.3.1b SEM image of uncrosslinked, electrospun collagen scaffold at 1KX (c) and 3KX (d) magnification.

Table A.3.1a Fiber Diameter for Uncrosslinked Collagen Fiber Scaffold

Image a

	Mean	Min	Max	Angle	Length
1	141.35	117	177.667	35.538	3.226
2	155.845	135.961	193.452	-97.496	3.536
3	150.493	122	184.289	15.255	2.095
4	145.899	137.267	163.778	57.095	1.908
5	156.601	119.667	195.667	14.036	1.941
Mean	150.038	126.379	182.97	4.886	2.541
SD	6.512	9.52	12.935	59.872	0.777
Min	141.35	117	163.778	-97.496	1.908
Max	156.601	137.267	195.667	57.095	3.536

Image b

	Mean	Min	Max	Angle	Length
1	144.551	132.422	198.111	-31.759	2.995
2	161.711	139	227.249	4.236	3.179
3	162.506	71.556	252.093	58.782	4.628
4	158.418	115.889	214.163	53.13	2.953
5	128.933	97.971	207	30.964	2.07
6	100.567	72.667	116.375	-68.199	1.918
7	159.551	140.292	227.227	13.392	2.618
8	172.852	156.667	225.528	9.462	2.088
9	159.476	124.222	207.617	34.38	2.731
10	146.349	131	187.667	-90	2.381
Mean	149.491	118.169	206.303	1.439	2.756
SD	21.07	28.803	36.375	49.939	0.786
Min	100.567	71.556	116.375	-90	1.918
Max	172.852	156.667	252.093	58.782	4.628

Table A.3.1b Fiber Diameter for Uncrosslinked Collagen Fiber Scaffold (continued)

Image c

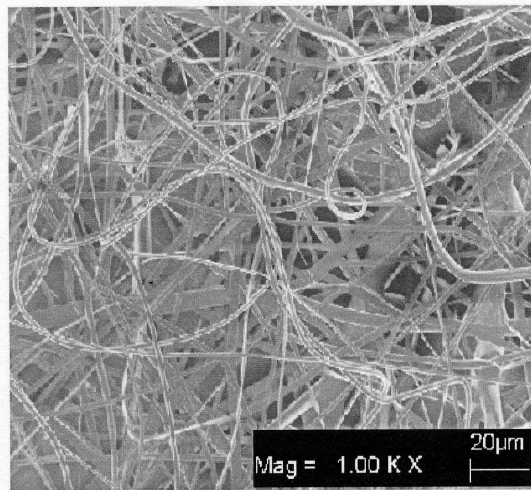
	Mean	Min	Max	Angle	Length
1	149.106	116.222	176.867	2.862	2.386
2	91.08	86.619	102	29.358	2.205
3	157.293	130.909	214.727	90	1.27
4	92.477	79.333	131.667	0	4.286
5	142.7	125.877	167.185	-63.435	2.13
6	150.802	121.333	220.306	-84.56	2.559
7	141.866	127	202	-23.199	1.858
Mean	132.189	112.47	173.536	-6.996	2.385
SD	28.089	20.776	44	58.224	0.936
Min	91.08	79.333	102	-84.56	1.27
Max	157.293	130.909	220.306	90	4.286

Image d

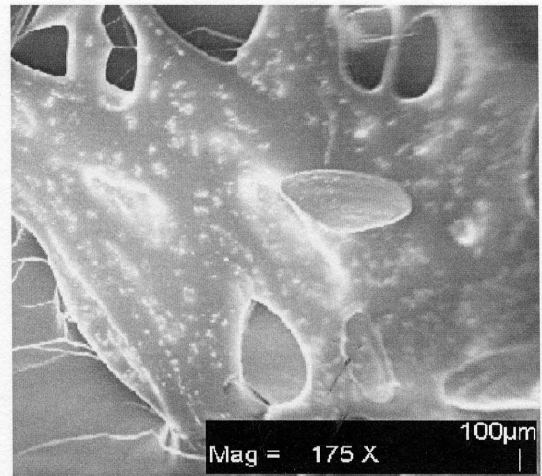
	Mean	Min	Max	Angle	Length
1	131.513	105.111	160.383	0	3.175
2	84.646	77.706	101.541	-10.176	4.671
3	118.619	84.667	135.048	60.945	2.559
4	179.14	136.421	228.526	0	2.222
5	132.593	124.571	152.693	0	2.54
6	135.157	125.999	147	81.573	3.21
7	145.99	132.667	156.833	86.424	1.911
Mean	136.674	114.109	163.063	27.823	2.755
SD	28.818	22.364	42.757	40.993	0.936
Min	84.646	77.706	101.541	-10.176	1.753
Max	179.14	136.421	228.526	86.424	4.671

Table A.3.2 Fiber Diameter for Glutaraldehyde Control Mat

	Mean	Min	Max	Angle	Length
1	115.579	101.222	126.667	-90	5.294
2	117.478	113	126.704	-90	2.353
3	104.055	93	111.129	-90	2.706
4	96.571	93.152	103.333	0	2.941
5	122.615	111	128.27	0	1.882
Mean	111.26	102.275	119.221	-54	3.035
SD	10.653	9.507	11.305	49.295	1.324
Min	96.571	93	103.333	-90	1.882
Max	122.615	113	128.27	0	5.294



(a)



(b)

Figure A.3.2 SEM image for glutaraldehyde control (a) and glutaraldehyde crosslinked sample (b).

Table A.3.3a Fiber Diameter for Genipin 1% Control Mat and Crosslinked Mat

Glutaraldehyde Control

	Mean	Min	Max	Angle	Length
1	141.35	117	177.667	35.538	3.226
2	155.845	135.961	193.452	-97.496	3.536
3	150.493	122	184.289	15.255	2.095
4	145.899	137.267	163.778	57.095	1.908
5	156.601	119.667	195.667	14.036	1.941
Mean	150.038	126.379	182.97	4.886	2.541
SD	6.512	9.52	12.935	59.872	0.777
Min	141.35	117	163.778	-97.496	1.908
Max	156.601	137.267	195.667	57.095	3.536

Genipin1-01

	Mean	Min	Max	Angle	Length
1	165.666	147	221	38.66	5.277
2	154.466	146.667	184.333	10.204	6.101
3	160.031	144.167	211.333	82.875	5.739
4	158.681	144.002	215.605	-16.699	4.945
5	154.439	144.422	187.667	58.299	4.687
Mean	158.657	145.252	203.988	34.668	5.35
SD	4.647	1.456	16.815	39.164	0.575
Min	154.439	144.002	184.333	-16.699	4.687
Max	165.666	147	221	82.875	6.101

Genipin1-0

	Mean	Min	Max	Angle	Length
1	155.321	142.686	188.046	0	6.19
2	157.387	139.307	217.613	0	9.048
3	158.696	139.191	207.222	42.879	6.734
4	158.473	143.882	199.504	23.629	6.19
5	157.133	140.952	186.159	-45	5.062
Mean	157.402	141.204	199.709	4.302	6.645
SD	1.344	2.067	13.198	32.899	1.475
Min	155.321	139.191	186.159	-45	5.062
Max	158.696	143.882	217.613	42.879	9.048

Table A.3.3b Fiber Diameter for Genipin 1% Crosslinked Mat and Percent Increase in Fiber Diameter

Genipin1-02

	Mean	Min	Max	Angle	Length
1	161.937	144.667	218.333	85.236	7.149
2	152.96	140.894	199	45	6.699
3	161.363	145.432	197.605	63.435	5.296
4	165.407	145	219	45	4.466
5	163.148	142	204	90	4.737
6	153.342	140.074	185.667	-54.462	5.055
Mean	159.693	143.011	203.934	45.701	5.567
SD	5.255	2.31	12.903	52.676	1.097
Min	152.96	140.074	185.667	-54.462	4.466
Max	165.407	145.432	219	90	7.149

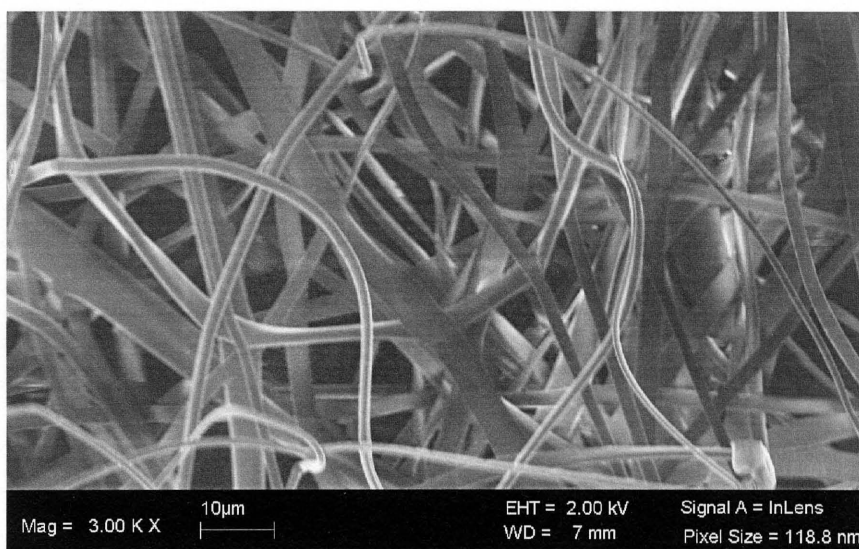
Genipin1-03

	Mean	Min	Max	Angle	Length
1	164.809	147.059	196.927	56.31	5.07
2	172.676	144.332	232.602	87.397	7.983
3	162.204	140.833	228.444	37.405	7.688
4	163.93	145.524	217.667	-45	4.983
5	167.938	143.159	232	-6.009	6.579
Mean	166.311	144.181	221.528	26.021	6.461
SD	4.122	2.364	15.002	52.163	1.41
Min	162.204	140.833	196.927	-45	4.983
Max	172.676	147.059	232.602	87.397	7.983

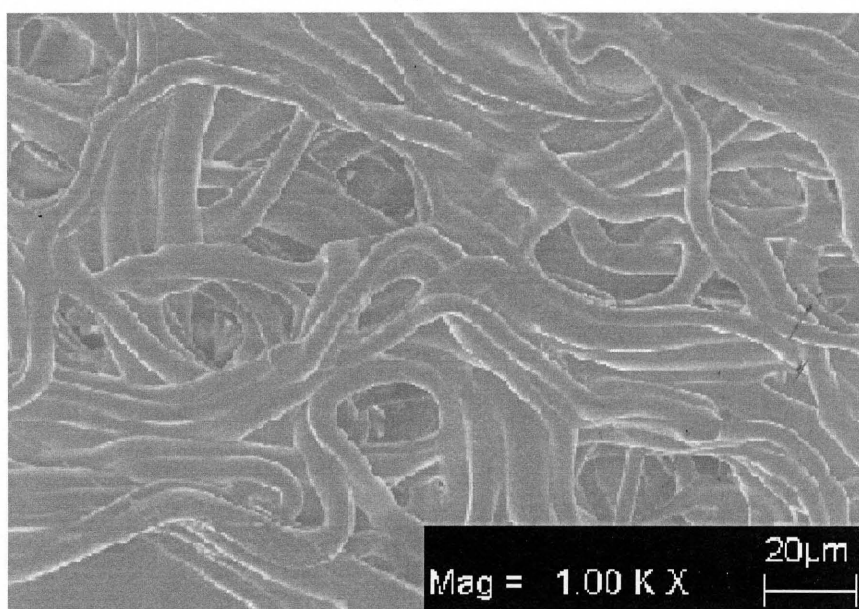
Average

Length	Average Length	SD
5.35	6.01	0.64
6.645		
5.567		
6.461		

sample	fiber diameter	% change in fiber diameter
Control	2.54	
0 month	6.01	136.61



(a)



(b)

Figure A.3.3 SEM image for genipin 1% control (a) and genipin 1% crosslinked sample (b) before incubation.

Table A.3.4a Fiber Diameter for Genipin 5% Control Mat and Crosslinked Mat Without Incubation

Control	Mean	Min	Max	Angle	Length
1	131.513	105.111	160.383	0	3.175
2	84.646	77.706	101.541	-10.176	4.671
3	118.619	84.667	135.048	60.945	2.559
4	179.14	136.421	228.526	0	2.222
5	132.593	124.571	152.693	0	2.54
6	135.157	125.999	147	81.573	3.21
7	145.99	132.667	156.833	86.424	1.911
Mean	136.674	114.109	163.063	27.823	2.755
SD	28.818	22.364	42.757	40.993	0.936
Min	84.646	77.706	101.541	-10.176	1.753
Max	179.14	136.421	228.526	86.424	4.671

Genipin05

	Mean	Min	Max	Angle	Length
1	113.026	91.667	172	14.036	4.39
2	102.306	84	135.667	-29.745	2.777
3	137.462	103	225.667	14.036	4.39
4	90.526	63.278	129.861	-50.194	5.734
5	94.726	75.333	128.111	0	3.81
Mean	107.609	83.456	158.261	-10.373	4.22
SD	18.751	15.189	41.708	28.549	1.073
Min	90.526	63.278	128.111	-50.194	2.777
Max	137.462	103	225.667	14.036	5.734

Genipin06

	Mean	Min	Max	Angle	Length
1	112.2	90.156	152.578	-90	5.237
2	94.744	82.667	128.667	-90	4.285
3	86.998	77.395	104.111	-144.462	3.049
4	124.092	78	184.389	-79.38	5.792
5	97.206	81.641	124.333	-45	3.367
Mean	103.048	81.972	138.816	-89.769	4.346
SD	14.894	5.106	30.755	35.72	1.176
Min	86.998	77.395	104.111	-144.462	3.049
Max	124.091	90.156	184.389	-45	5.792

Length (μm)	Average Length	SD
4.22	4.28	0.09
4.346		

Table A.3.4b Fiber Diameter for Genipin 5% Mat after 1 Month of Incubation and Percent Change in Fiber Diameter

G5-10

	Mean	Min	Max	Angle	Length
1	150.048	132.178	222.222	0	5.116
2	154.744	117.333	232	0	4.186
3	132.132	119.57	175.333	5.194	3.75
4	128.811	116.113	158.333	60.945	3.75
5	131.01	108.889	196	3.576	5.601
6	174.01	130.262	251.867	59.036	6.064
Mean	145.126	120.724	205.959	21.459	4.745
SD	17.806	8.903	35.653	29.922	0.99
Min	128.811	108.889	158.333	0	3.75
Max	174.01	132.178	251.867	60.945	6.064

G5-19

	Mean	Min	Max	Angle	Length
1	163.204	127.196	242.333	0	6.19
2	136.193	109.602	233.288	-60.642	6.615
3	150.627	125.627	214	4.086	4.786
4	151.293	114.593	226.613	81.87	5.324
5	137.92	110.408	183.333	-45	4.714
Mean	147.847	117.485	219.913	-3.937	5.526
SD	11.066	8.384	22.91	55.553	0.848
Min	136.193	109.602	183.333	-60.642	4.714
Max	163.204	127.196	242.333	81.87	6.615

Average

Length (μm)	Average Length	SD
4.745	5.14	0.55
5.526		

Time	fiber diameter	% change in fiber diameter
Control	2.75	0.00
0 month	4.28	55.63
1 month	5.14	86.90

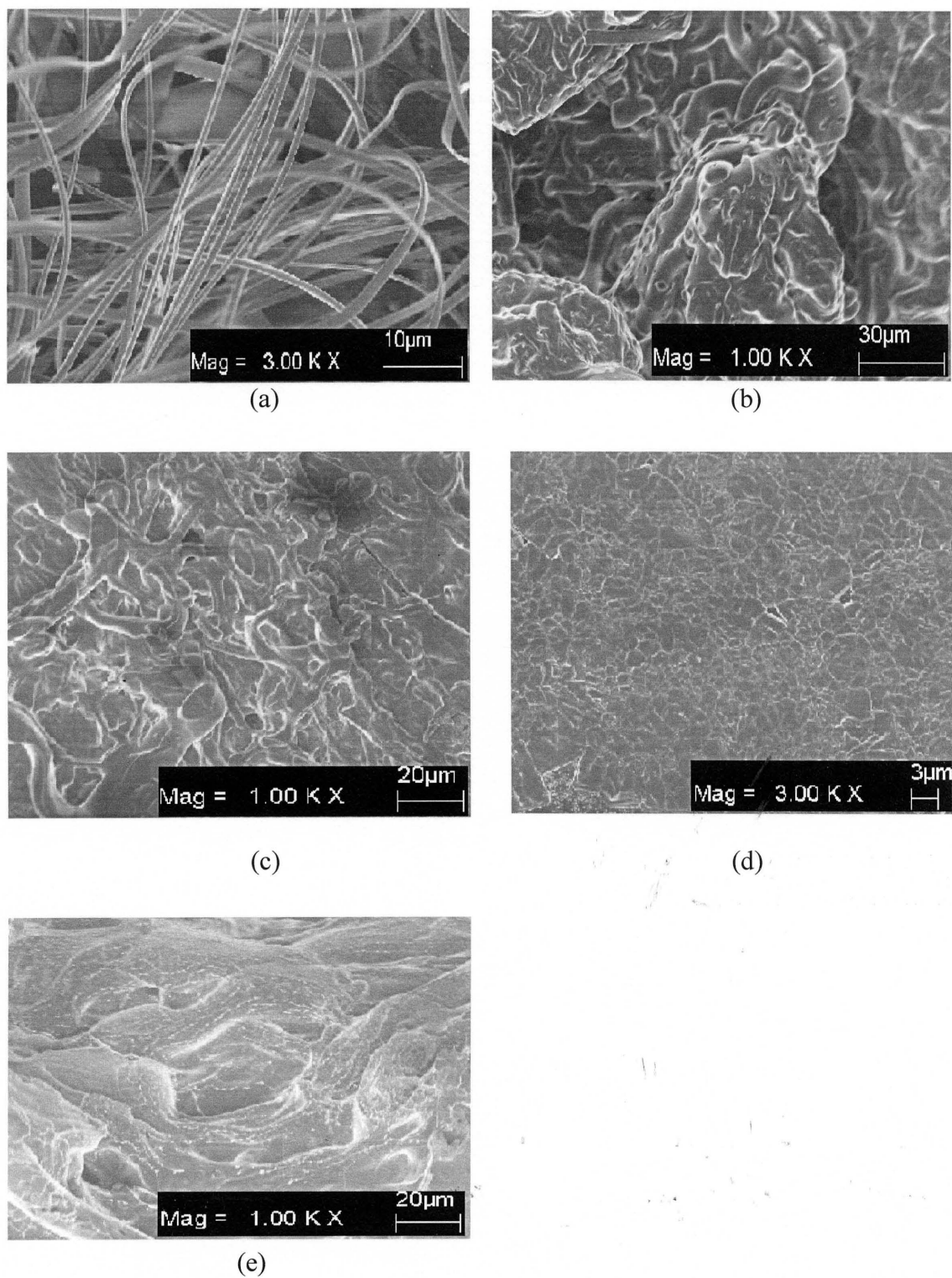


Figure A.3.4 SEM image for genipin 5% control (a), genipin 5% crosslinked sample (b) before incubation, genipin 5% crosslinked sample after 1 month (c), 2 months (d) and 3 months (e) of incubation.

Table A.3.5a Fiber Diameter for Genipin 10% Control Mat and Crosslinked Mat Without Incubation

Control	Mean	Min	Max	Angle	Length
1	144.551	132.422	198.111	-31.759	2.995
2	161.711	139	227.249	4.236	3.179
3	162.506	71.556	252.093	58.782	4.628
4	158.418	115.889	214.163	53.13	2.953
5	128.933	97.971	207	30.964	2.07
6	100.567	72.667	116.375	-68.199	1.918
7	159.551	140.292	227.227	13.392	2.618
8	172.852	156.667	225.528	9.462	2.088
9	159.476	124.222	207.617	34.38	2.731
10	146.349	131	187.667	-90	2.381
Mean	149.491	118.169	206.303	1.439	2.756
SD	21.07	28.803	36.375	49.939	0.786
Min	100.567	71.556	116.375	-90	1.918
Max	172.852	156.667	252.093	58.782	4.628

G10-01

	Mean	Min	Max	Angle	Length
1	159.697	146.815	200.667	-87.614	2.862
2	183.785	153.4	252.2	40.914	2.36
3	169.1	145.111	224.947	-90	3.175
4	182.193	153.543	245.249	45	2.02
Mean	173.694	149.717	230.766	-22.925	2.604
SD	11.418	4.391	23.159	76.099	0.514
Min	159.697	145.111	200.667	-90	2.02
Max	183.785	153.543	252.2	45	3.175

G10-03

	Mean	Min	Max	Angle	Length
1	96.757	79.36	141.627	0	3
2	100.431	84.19	141.222	-45	2.456
3	99.873	89	148.667	0	2.368
4	94.982	83.333	129.627	-32.905	2.41
5	96.394	84.35	140.444	-33.69	1.273
Mean	97.687	84.047	140.317	-22.319	2.302
SD	2.354	3.432	6.829	20.929	0.63
Min	94.982	79.36	129.627	-45	1.273
Max	100.431	89	148.667	0	3

Table A.3.5a Fiber Diameter for Genipin 10% Control Mat and Crosslinked Mat Without Incubation (continued)

Average

Length (μm)	Average Length	SD
2.302	2.45	0.21
2.604		

Table A.3.5b Fiber Diameter for Genipin 10% Crosslinked Mat after 1 Month of Incubation

G5-10

	Mean	Min	Max	Angle	Length
1	150.048	132.178	222.222	0	5.116
2	154.744	117.333	232	0	4.186
3	132.132	119.57	175.333	5.194	3.75
4	128.811	116.113	158.333	60.945	3.75
5	131.01	108.889	196	3.576	5.601
6	174.01	130.262	251.867	59.036	6.064
Mean	145.126	120.724	205.959	21.459	4.745
SD	17.806	8.903	35.653	29.922	0.99
Min	128.811	108.889	158.333	0	3.75
Max	174.01	132.178	251.867	60.945	6.064

G5-19

	Mean	Min	Max	Angle	Length
1	163.204	127.196	242.333	0	6.19
2	136.193	109.602	233.288	-60.642	6.615
3	150.627	125.627	214	4.086	4.786
4	151.293	114.593	226.613	81.87	5.324
5	137.92	110.408	183.333	-45	4.714
Mean	147.847	117.485	219.913	-3.937	5.526
SD	11.066	8.384	22.91	55.553	0.848
Min	136.193	109.602	183.333	-60.642	4.714
Max	163.204	127.196	242.333	81.87	6.615

Average

Length (μm)	Average Length	SD
4.745	5.14	0.55
5.526		

Table A.3.5c Fiber Diameter for Genipin 10% Crosslinked Mat after 2 Months of Incubation

Genipin 10-20

	Mean	Min	Max	Angle	Length
1	134.698	91.01	249.667	0	4.127
3	138.174	94.032	234.587	71.565	3.363
4	143.077	90.714	238.205	20.323	3.419
5	134.157	89.324	237	0	4.444
6	138.77	97.314	230.143	0	4.127
Mean	134.694	91.749	232.089	0.73	3.697
SD	8.206	3.376	15.687	51.36	0.649
Min	119.286	88.101	202.936	-87.51	2.703
Max	143.077	97.314	249.667	71.565	4.444

Genipin 10-28

	Mean	Min	Max	Angle	Length
1	117.289	81.333	139.309	-43.727	3.706
2	101.252	75.312	204.889	0	3.651
3	113.106	90.578	196.556	74.932	3.119
4	105.133	81.245	201.333	1.909	3.496
5	107.677	85.299	151.111	-15.524	2.311
6	101.695	85.697	143.111	0	1.27
Mean	107.692	83.244	172.718	2.932	2.925
SD	6.409	5.188	31.246	39.293	0.961
Min	101.252	75.312	139.309	-43.727	1.27
Max	117.289	90.578	204.889	74.932	3.706

Average

Length (μm)	Average Length	SD
3.697	3.31	0.55
2.925		

Table A.3.5d Fiber Diameter for Genipin 10% Crosslinked Mat after 3 Months of Incubation And Percent Change in Fiber Diameter

Genipin10-32

	Mean	Min	Max	Angle	Length
1	167.829	153.852	216.136	-38.29	2.782
2	174.269	151.167	249.556	8.881	3.802
3	153.937	147.208	208.667	24.146	3.776
4	183.592	154.047	249.275	-40.03	3.881
5	182.563	149.055	252.479	17.354	3.953
6	173.536	151.235	251.333	-62.103	4.542
Mean	172.621	151.094	237.908	-15.007	3.789
SD	10.907	2.669	19.933	36.158	0.569
Min	153.937	147.208	208.667	-62.103	2.782
Max	183.592	154.047	252.479	24.146	4.542

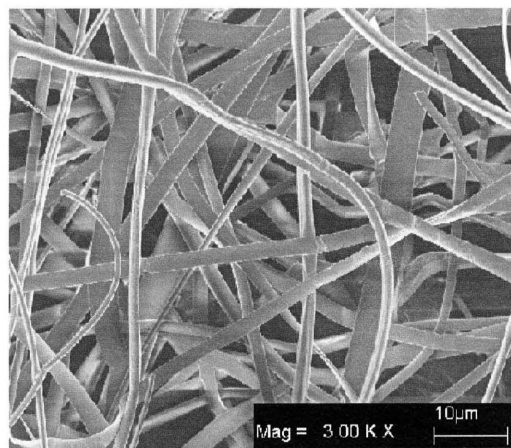
Genipin 10-33

	Mean	Min	Max	Angle	Length
1	173.806	157.001	235.613	-4.574	3.119
2	176.321	156.667	234.259	-62.354	2.933
3	158.947	148.996	216.556	0	3.607
4	178.793	153	251	0	3.443
5	185.199	157.111	253.939	-57.381	3.684
6	165.952	145.645	245.839	0	3.77
Mean	173.17	153.07	239.534	-20.718	3.426
SD	9.392	4.826	13.784	30.417	0.333
Min	158.947	145.645	216.556	-62.354	2.933
Max	185.199	157.111	253.939	0	3.77

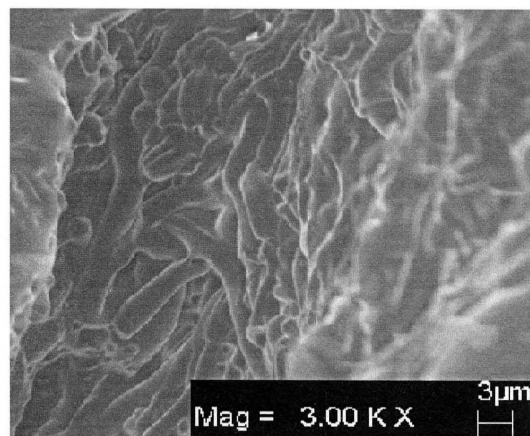
Average

Length (μm)	Average Length	SD
3.789	3.61	0.26
3.426		

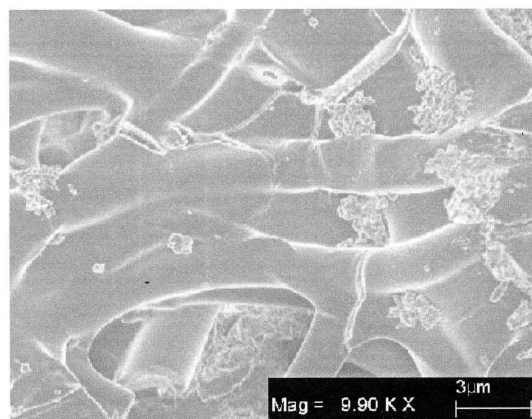
Time	Fiber diameter	% increase in diameter
Control	2.75	0.00
0 month	2.45	-10.90
1 month	2.79	13.87
2 months	3.31	35.10
3 months	3.61	47.34



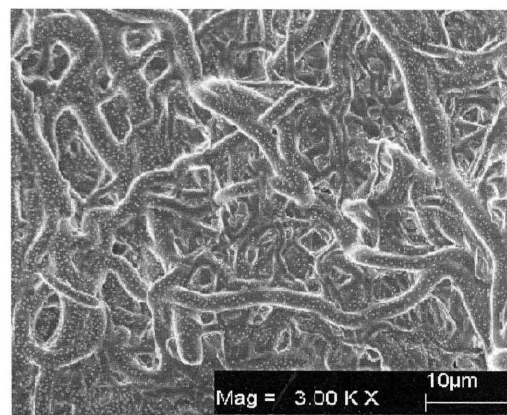
(a)



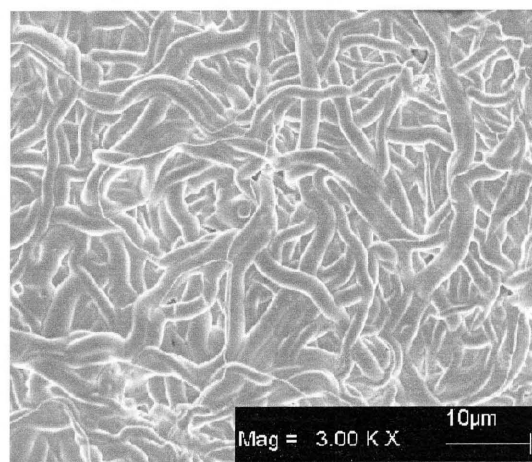
(b)



(c)



(d)



(e)

Figure A.3.5 SEM image for genipin 10% control (a), genipin 10% crosslinked sample (b) before incubation, genipin 10% crosslinked sample after 1 month (c), 2 months (d) and 3 months (e) of incubation.

Table A.3.6a Fiber Diameter for EDC Control Mat and Crosslinked Mat Without Incubation

Control	Mean	Min	Max	Angle	Length
1	145.313	90.444	175.249	0	2.286
2	126.788	82.222	138.646	25.017	1.726
3	145.027	92.667	164.444	-29.745	2.623
4	160.028	98.333	203.531	-10.008	1.879
5	134.731	115.053	167.111	2.49	2.433
6	136.581	127.864	145.667	3.013	2.005
7	123.452	81.333	139.649	23.962	2.045
Mean	138.846	98.274	162.042	2.104	2.142
SD	12.471	17.294	23.253	19.014	0.318
Min	123.452	81.333	138.646	-29.745	1.726
Max	160.028	127.864	203.531	25.017	2.623

EDC-02

	Mean	Min	Max	Angle	Length
1	149.1	123.03	233.111	-4.086	1.753
2	155.418	117.632	250	0	2.222
3	116.539	101.227	161.111	-159.228	3.716
4	159.867	119.404	245.563	-85.764	3.179
5	154.011	125	229	-90	3.333
6	129.003	100.251	173.795	3.814	1.753
7	147.818	118.491	214.614	0	2.222
8	148.749	119.022	233.471	-71.565	1.156
Mean	145.063	115.507	217.583	-50.853	2.417
SD	14.714	9.444	32.892	60.087	0.899
Min	116.539	100.251	161.111	-159.228	1.156
Max	159.867	125	250	3.814	3.716

EDC-03

	Mean	Min	Max	Angle	Length
1	139.106	101.444	243.667	39.401	4.266
2	143.117	115.493	239.333	0	3
3	129.07	104.053	221.997	-46.469	3.24
4	146.854	115.086	250.099	25.346	2.471
5	145.125	120.694	239	38.991	3.255
6	132.971	106.283	219.556	-68.749	2.199
7	134.784	106.667	212.667	0	1.895
8	134.855	99	198.333	0	1.579
Mean	138.235	108.59	228.081	-1.435	2.738
SD	6.34	7.644	17.751	38.885	0.873
Min	129.07	99	198.333	-68.749	1.579
Max	146.854	120.694	250.099	39.401	4.266

Table A.3.6a Fiber Diameter for EDC Control Mat and Crosslinked Mat Without Incubation (continued)

Average

Length	Average Length	SD
2.417	2.58	0.23
2.738		

Table A.3.6b Fiber Diameter for EDC Crosslinked Mat after 1 Month of Incubation

EDC-1-04

	Mean	Min	Max	Angle	Length
1	134.929	114.359	218	0	4.762
2	123.239	104.778	134.778	0	4.286
3	111.846	80	199	0	4.286
4	118.294	75.493	214.667	71.565	3.467
5	122.531	98.778	170.333	-45	2.694
6	120.846	99.5	158.944	14.036	2.897
Mean	121.947	95.485	182.62	6.767	3.732
SD	7.578	14.893	33.309	37.6	0.839
Min	111.846	75.493	134.778	-45	2.694
Max	134.929	114.359	218	71.565	4.762

EDC-10

	Mean	Min	Max	Angle	Length
1	127.963	106.091	164.192	0	3.81
2	117.607	103	169	0	3.333
3	116.613	106.056	139	68.199	5.754
4	117.725	106	143.182	0	3.81
5	125.577	103.605	184.556	0	3.333
Mean	121.097	104.95	159.986	13.64	4.008
SD	5.265	1.52	18.877	30.499	1.005
Min	116.613	103	139	0	3.333
Max	127.963	106.091	184.556	68.199	5.754

EDC-11

	Mean	Min	Max	Angle	Length
1	122.588	106.333	166.333	0	5.714
2	133.742	114.44	175	-11.31	1.963
3	120.803	107.949	160.889	0	3.81
4	124.507	103.133	160.978	48.814	3.719
5	122.543	109.444	132	-20.556	3.012
Mean	124.837	108.26	159.04	3.39	3.644
SD	5.148	4.174	16.173	26.813	1.373
Min	120.803	103.133	132	-20.556	1.963
Max	133.742	114.44	175	48.814	5.714

Average

Length (μm)	Average Length	SD
3.732	3.79	0.19
4.008		
3.644		

Table A.3.6c Fiber Diameter for EDC Crosslinked Mat after 2 Months of Incubation

EDC-23

	Mean	Min	Max	Angle	Length
1	140.129	106.803	203.051	-90	1.587
2	107.423	83.113	194.333	59.036	2.777
3	121.938	87.556	197.198	26.565	4.259
4	123.03	97.068	200.889	-88.668	5.082
5	101.931	80.677	194	55.008	2.953
6	124.832	95.484	197.889	-52.431	2.033
7	122.682	98.242	206.667	-5.711	1.28
Mean	120.281	92.706	199.147	-13.743	2.853
SD	12.468	9.304	4.652	64.081	1.397
Min	101.931	80.677	194	-90	1.28
Max	140.129	106.803	206.667	59.036	5.082

EDC-24

	Mean	Min	Max	Angle	Length
1	112.43	87.73	190.889	-71.565	3.363
2	110.099	86.41	171	-67.38	1.563
3	140.959	103.52	197	6.009	2.228
4	107.064	80.9	209.667	-55.923	4.93
5	138.207	97	240	0	3.333
6	129.455	94.692	204	0	1.587
Mean	123.036	91.709	202.093	-31.476	2.834
SD	15.017	8.211	22.874	37.096	1.301
Min	107.064	80.9	171	-71.565	1.563
Max	140.959	103.52	240	6.009	4.93

EDC-26

	Mean	Min	Max	Angle	Length
1	127.902	95.333	189.333	0	1.935
2	136.928	104.7	233.069	-56.689	5.01
3	155.826	132.236	204.991	32.619	3.625
4	151.746	104.458	197.837	-78.111	2.309
5	107.893	82.537	158.655	-37.875	2.645
6	108.241	91.667	147.333	0	3.387
Mean	131.423	101.822	188.536	-23.343	3.152
SD	20.699	17.072	31.406	41.338	1.112
Min	107.893	82.537	147.333	-78.111	1.935
Max	155.826	132.236	233.069	32.619	5.01

Average

Length (μm)	Average Length	SD
2.853	2.95	0.18
2.834		
3.152		

Table A.3.6d Fiber Diameter for EDC Crosslinked Mat after 3 Months of Incubation

EDC-35

	Mean	Min	Max	Angle	Length
1	164.652	135.03	236.778	-90	1.27
2	176.341	132.432	250.444	-90	1.111
3	169.412	127.565	238.222	-33.69	1.717
4	167.703	125.718	245.634	0	4.444
5	155.047	111	218.481	61.39	1.42
6	155.604	132.481	218.333	-90	1.111
Mean	164.793	127.371	234.649	-40.383	1.846
SD	8.278	8.731	13.528	62.32	1.293
Min	155.047	111	218.333	-90	1.111
Max	176.341	135.03	250.444	61.39	4.444

EDC-36

	Mean	Min	Max	Angle	Length
1	145.667	129.617	180.506	7.595	1.775
2	162.761	126.93	243.655	0	2.222
3	163.726	126.467	227.704	0	1.746
4	157.838	128	220	9.462	1.437
5	170.478	127.967	238.876	14.931	1.81
Mean	160.094	127.796	222.148	6.398	1.798
SD	9.238	1.215	25.059	6.433	0.28
Min	145.667	126.467	180.506	0	1.437
Max	170.478	129.617	243.655	14.931	2.222

EDC-37

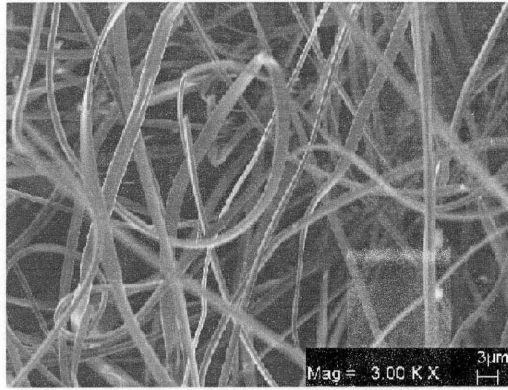
	Mean	Min	Max	Angle	Length
1	166.479	134.066	223.444	12.529	1.122
2	187.74	146.972	253.444	-68.199	1.918
3	152.504	130.523	223.667	19.983	1.356
4	173.699	139.531	254	0	3.175
5	167.231	127	242	-90	1.429
6	160.101	127.975	236.778	0	1.111
Mean	167.959	134.345	238.889	-20.948	1.685
SD	12.063	7.688	13.598	46.205	0.787
Min	152.504	127	223.444	-90	1.111
Max	187.74	146.972	254	19.983	3.175

Average

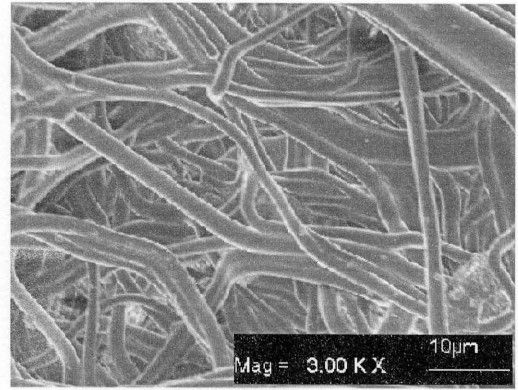
Length (μm)	Average Length	SD
1.846	1.78	0.08
1.798		
1.685		

Table A.3.6e Percent Change in Fiber Diameter of EDC Crosslinked Mat Over Incubation for 3 Months

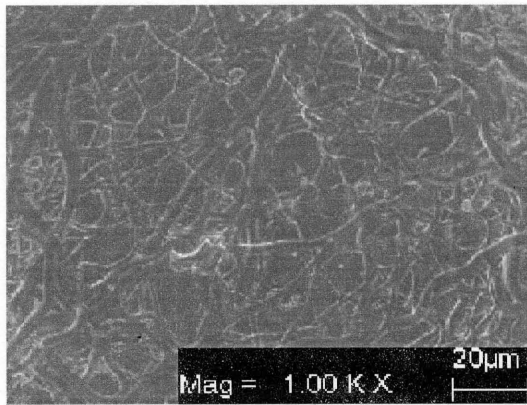
Time	Fiber diameter	% increase in diameter
Control	2.14	0
0 month	2.58	20.44
1 month	3.79	76.93
2 month	2.95	37.72
3 months	1.78	-16.9



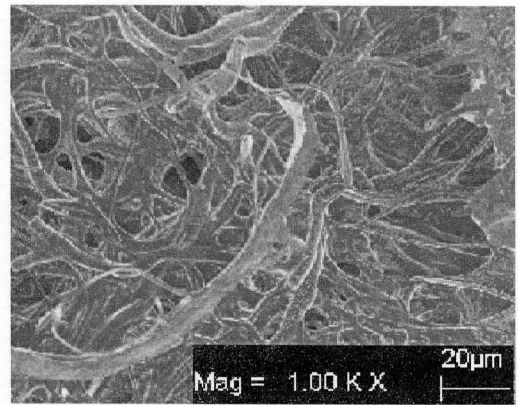
(a)



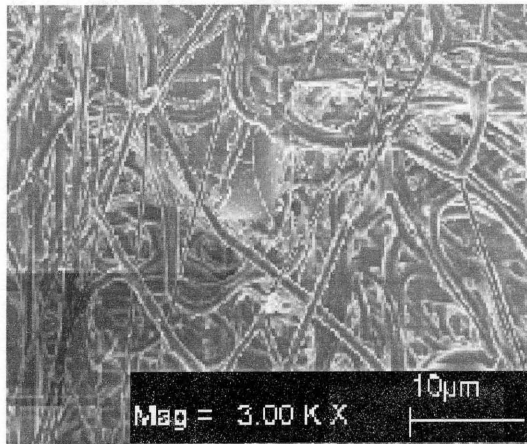
(b)



(c)



(d)



(e)

Figure A.3.6 SEM image for EDC control (a), EDC crosslinked sample (b) before incubation, EDC crosslinked sample after 1 month (c), 2 months (d) and 3 months (e) of incubation.

Table A.3.7a Fiber Diameter for EDC with NHS Control Mat and Crosslinked Mat Without Incubation

Control	Mean	Min	Max	Angle	Length
1	149.106	116.222	176.867	2.862	2.386
2	91.08	86.619	102	29.358	2.205
3	157.293	130.909	214.727	90	1.27
4	92.477	79.333	131.667	0	4.286
5	142.7	125.877	167.185	-63.435	2.13
6	150.802	121.333	220.306	-84.56	2.559
7	141.866	127	202	-23.199	1.858
Mean	132.189	112.47	173.536	-6.996	2.385
SD	28.089	20.776	44	58.224	0.936
Min	91.08	79.333	102	-84.56	1.27
Max	157.293	130.909	220.306	90	4.286

NHS-06

	Mean	Min	Max	Angle	Length
1	137.779	119.488	206.391	0	8.095
2	137.407	121.778	194.963	0	3.333
3	129.364	119.248	162.282	0	4.762
4	154	126	208.667	0	2.857
5	153.434	121.467	213	53.13	3.719
Mean	142.397	121.596	197.061	10.626	4.553
SD	10.869	2.711	20.553	23.761	2.101
Min	129.364	119.248	162.282	0	2.857
Max	154	126	213	53.13	8.095

NHS-07

	Mean	Min	Max	Angle	Length
1	140.232	121.477	219.667	-48.504	4.047
2	149.086	123.352	234.052	52.125	2.603
3	171.9	123.833	245.392	-6.009	2.228
4	133.336	118.107	220.333	19.093	3.214
5	138.536	118.222	230.325	48.013	3.257
6	147.705	122.927	243.553	37.694	3.397
Mean	146.799	121.32	232.22	17.069	3.124
SD	13.629	2.568	11.025	38.596	0.637
Min	133.336	118.107	219.667	-48.504	2.228
Max	171.9	123.833	245.392	52.125	4.047

Table A.3.7b Fiber Diameter for EDC with NHS Crosslinked Mat Without Incubation

NHS-08

	Mean	Min	Max	Angle	Length
1	142.611	120.938	245.222	0	5.079
2	130.407	114.356	226.667	0	5.397
3	131.2	119.333	194.667	0	5.238
4	138.912	123.035	207.778	6.911	3.997
5	149.777	120.355	243	51.34	3.049
6	148.824	115.267	244	-50.194	1.81
7	167.236	122.749	240.038	-3.366	2.07
Mean	144.138	119.433	228.767	0.67	3.806
SD	12.734	3.421	20.167	29.471	1.517
Min	130.407	114.356	194.667	-50.194	1.81
Max	167.236	123.035	245.222	51.34	5.397

Average

Length (μm)	Average Length	SD
4.262	3.99	0.24
3.888		
3.806		

Table A.3.7c Fiber Diameter for EDC with NHS Crosslinked Mat after 1 Month of Incubation

NHS-10

	Mean	Min	Max	Angle	Length
1	111.969	92.385	165	-90	4.762
2	101.381	87.03	141.667	-90	3.81
3	123.56	88.771	200.49	0	6.19
4	109.056	94.889	155.333	19.654	5.574
5	107.926	87.667	168	90	2.857
6	115.857	93.952	163	0	2.381
Mean	111.625	90.782	165.582	-11.724	4.262
SD	7.559	3.386	19.537	69.082	1.51
Min	101.381	87.03	141.667	-90	2.381
Max	123.56	94.889	200.49	90	6.19

NHS-11

	Mean	Min	Max	Angle	Length
1	113.984	90.752	165.444	-90	4.762
2	112.625	89.157	180.111	-29.055	3.839
3	149.67	127.433	180.556	15.945	2.428
4	126.105	89.074	206	-85.236	4.312
5	122.041	87.22	174.234	52.125	4.096
Mean	124.885	96.727	181.269	-27.244	3.888
SD	14.942	17.211	15.111	62.188	0.883
Min	112.625	87.22	165.444	-90	2.428
Max	149.67	127.433	206	52.125	4.762

Average

Length (μm)	Average Length	SD
4.262	4.08	0.26
3.888		

Table A.3.7d Fiber Diameter for EDC with NHS Crosslinked Mat after 2 Months of Incubation

NHS-21

	Mean	Min	Max	Angle	Length
1	133.255	118.506	175.778	26.565	1.28
2	154.379	126.083	239.667	69.444	2.063
3	150.494	123.207	224.883	4.97	2.703
4	132.952	110.667	248.667	0	2.381
5	139.603	117.521	228	86.987	2.228
Mean	142.137	119.197	223.399	37.593	2.131
SD	9.866	5.907	28.259	38.902	0.531
Min	132.952	110.667	175.778	0	1.28
Max	154.379	126.083	248.667	86.987	2.703

NHS-22

	Mean	Min	Max	Angle	Length
1	151.774	126.94	224.991	0	1.587
2	140.476	113	215.053	0	2.222
3	126.36	117.786	160.222	0	1.587
4	136.359	117	187.222	78.69	2.428
5	153.646	115.167	224	109.983	2.862
Mean	141.723	117.979	202.298	37.735	2.137
SD	11.287	5.338	28.046	52.842	0.553
Min	126.36	113	160.222	0	1.587
Max	153.646	126.94	224.991	109.983	2.862

NHS-24

	Mean	Min	Max	Angle	Length
1	158.847	115.489	220.741	0	1.746
2	169.327	147.667	229.778	-90	1.905
3	128.184	112.333	200	-9.462	2.897
4	141.593	113.444	205.889	-9.462	2.897
5	131.458	118.752	203	-90	1.587
Mean	145.882	121.537	211.881	-39.785	2.206
SD	17.73	14.81	12.794	46.002	0.64
Min	128.184	112.333	200	-90	1.587
Max	169.327	147.667	229.778	0	2.897

Average

Length	Average Length	SD
2.131	2.16	0.04
2.137		
2.206		

Table A.3.7e Fiber Diameter for EDC with NHS Crosslinked Mat after 3 Months of Incubation And Percent Change in Fiber Diameter

NHS-34

	Mean	Min	Max	Angle	Length
1	182	155.778	248.222	-20.556	0.506
2	167.542	147.299	217.667	-51.843	1.088
3	160.012	147.013	211	-77.471	0.582
4	169.324	162.444	183.472	70.346	0.862
5	165.609	150.669	218.333	-17.354	1.012
6	169.599	147.321	239.333	-24.444	0.716
7	167.671	150.596	216.333	-66.038	0.609
8	176.594	152.565	252.398	45	0.792
9	177.119	157.333	248.333	-79.695	0.645
10	171.569	152.935	223.667	3.814	0.884
Mean	170.704	152.395	225.876	-21.824	0.77
SD	6.384	4.989	21.416	50.496	0.192
Min	160.012	147.013	183.472	-79.695	0.506
Max	182	162.444	252.398	70.346	1.088

NHS-35

	Mean	Min	Max	Angle	Length
1	140.264	129.839	174.621	0	0.657
2	147.121	131.552	179.609	0	0.657
3	165.165	137.062	244	-39.806	0.531
4	143.475	132.104	188.111	48.576	0.508
5	150	134	184.976	-51.34	0.573
6	148.847	121.667	194.118	-82.235	0.511
7	164.774	142.021	243	-59.036	0.522
8	138.031	129	158.28	66.801	0.682
9	145.24	130	205	-34.992	0.538
Mean	149.213	131.916	196.857	-16.892	0.576
SD	9.718	5.642	29.418	49.933	0.07
Min	138.031	121.667	158.28	-82.235	0.508
Max	165.165	142.021	244	66.801	0.682

Average

Length (μm)	Average length	SD
0.77	0.67	0.14
0.576		

Time	fiber diameter	% change in fiber diameter
Control	2.39	0.00
0 month	3.99	66.94
1 month	4.08	70.71
2 month	2.16	-9.62
3 months	0.67	-71.96

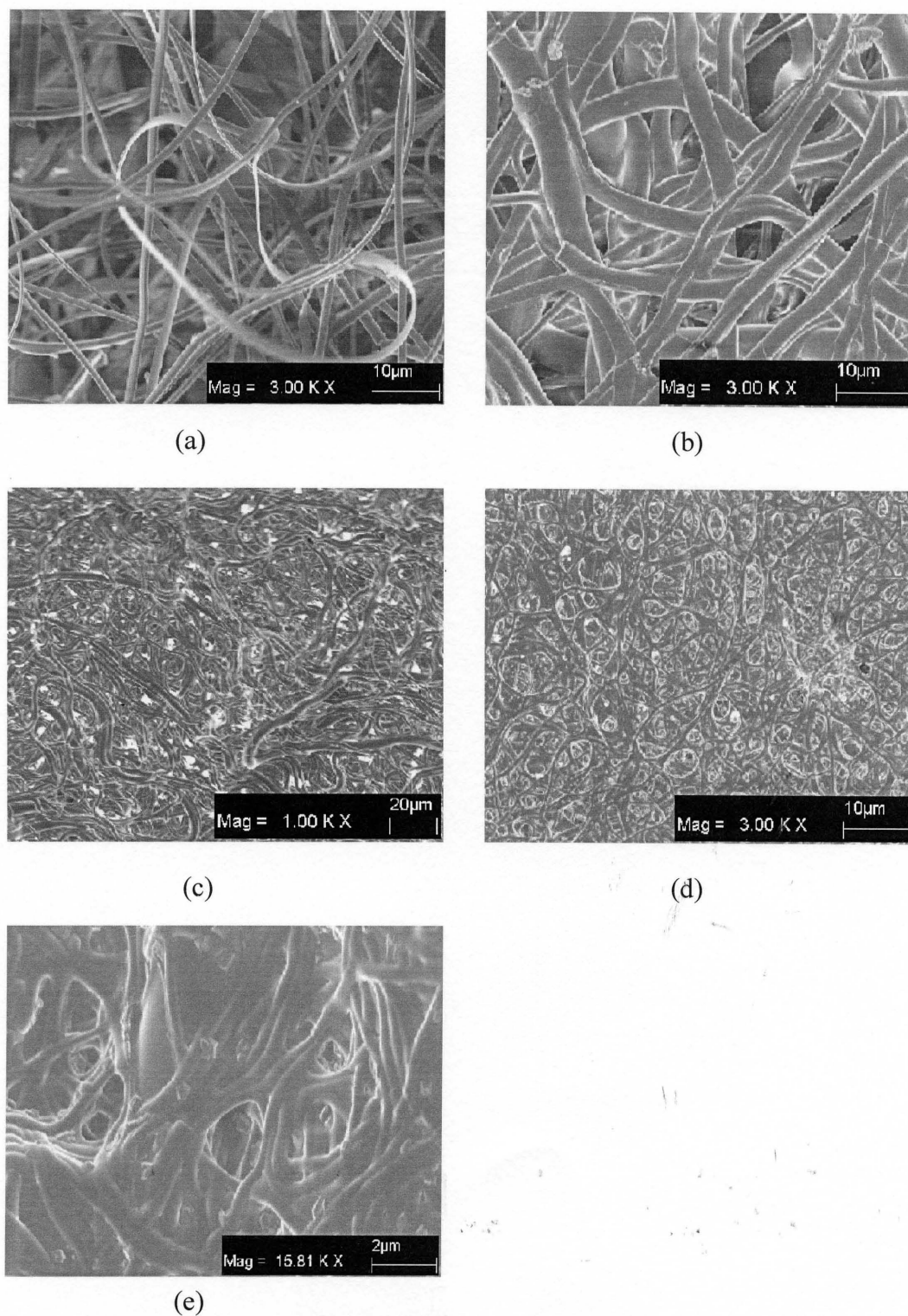


Figure A.3.7 SEM image for EDC with NHS control (a), EDC with NHS crosslinked sample (b) before incubation, EDC with NHS crosslinked sample after 1 month (c), 2 months (d) and 3 months (e) of incubation.

APPENDIX B

DSC PLOTS

Differential Scanning Calorimetric analysis provides an understanding of the structural changes occurring in collagen when subjected to thermal heating and stress.

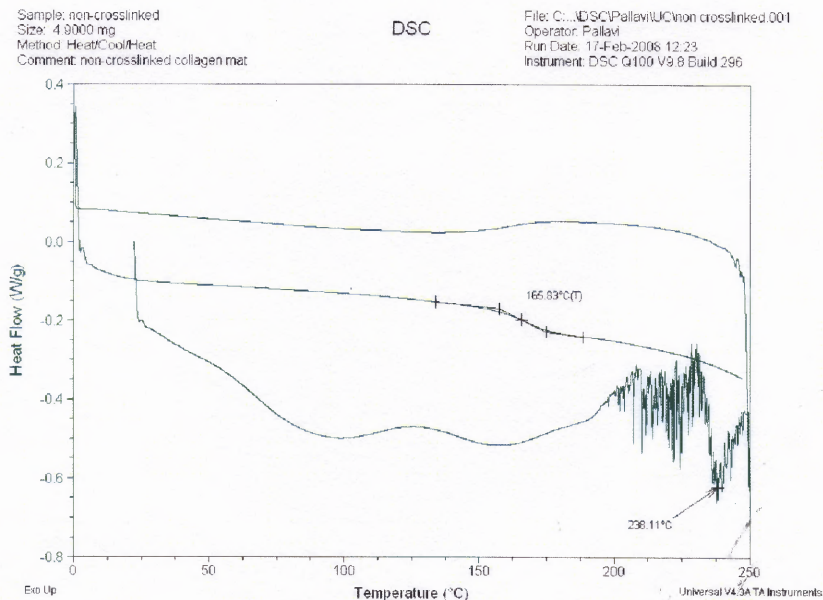


Figure B.1 DSC plot for uncrosslinked electrospun collagen scaffold.

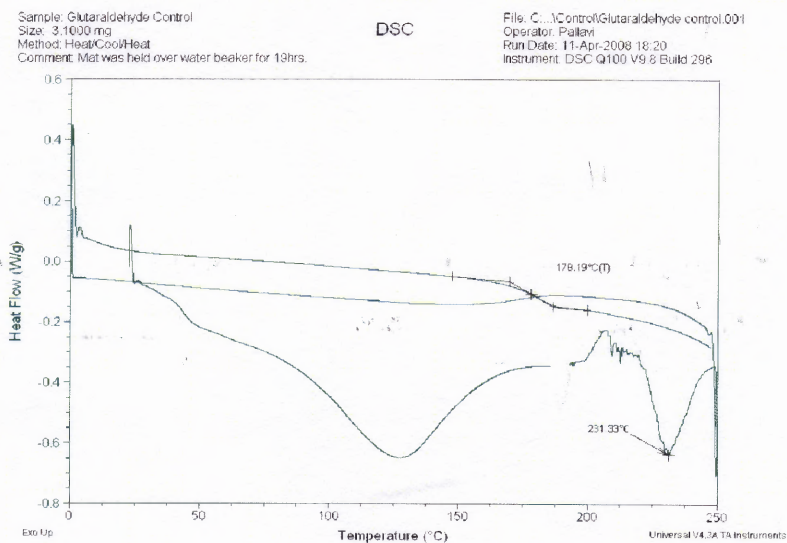


Figure B.2 DSC plot for glutaraldehyde control.

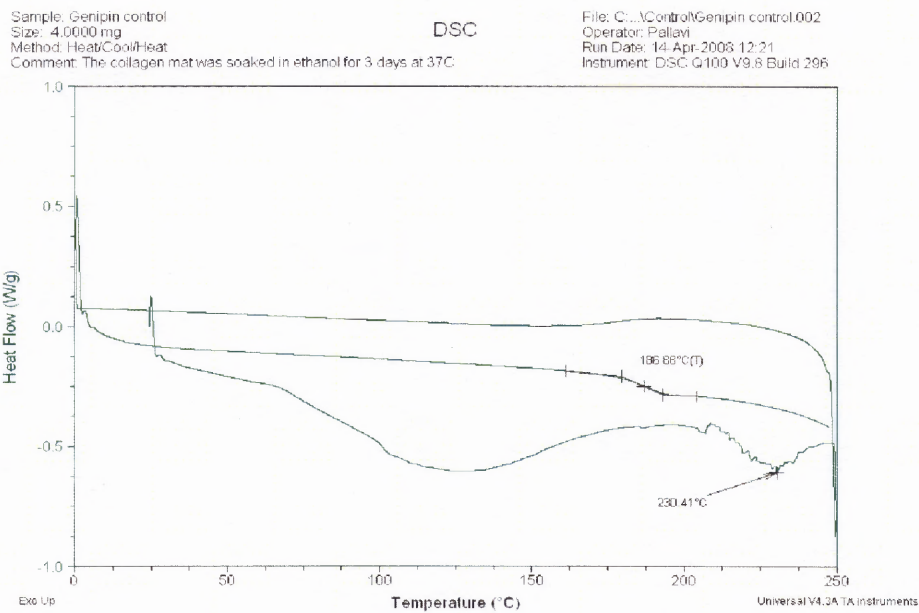


Figure B.3 DSC plot for genipin control.

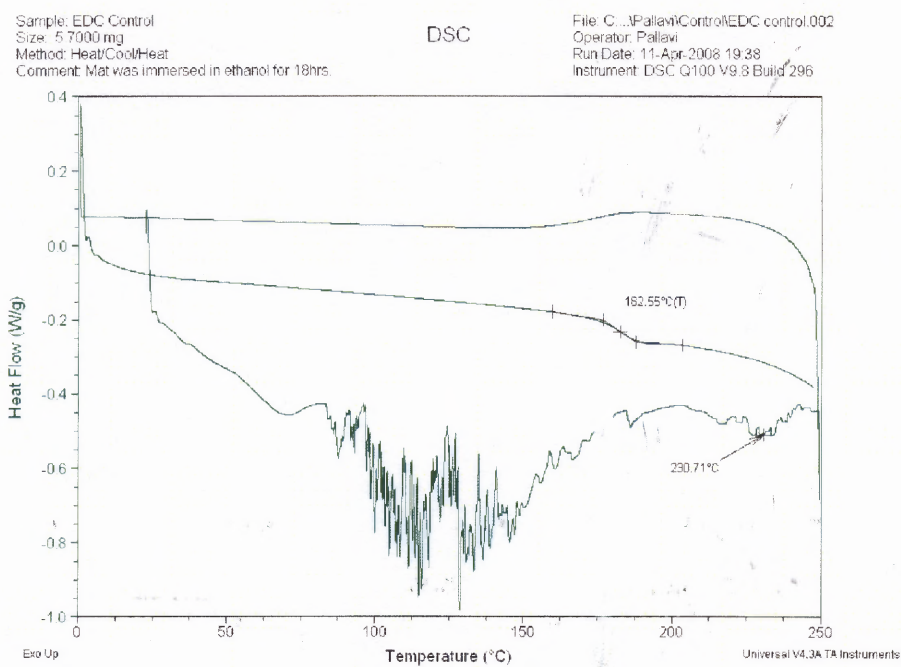


Figure B.4 DSC plot for EDC control.

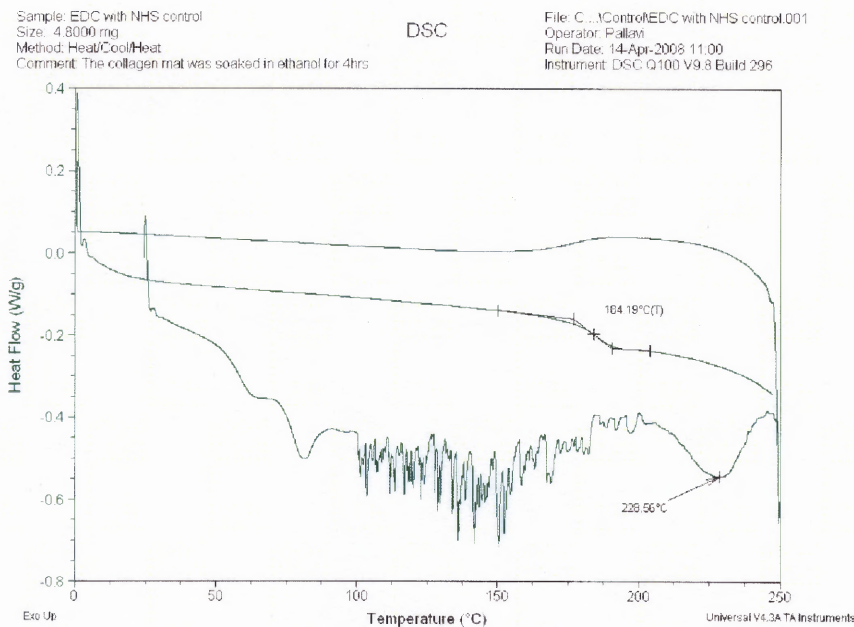


Figure B.5 DSC plot for EDC with NHS control.

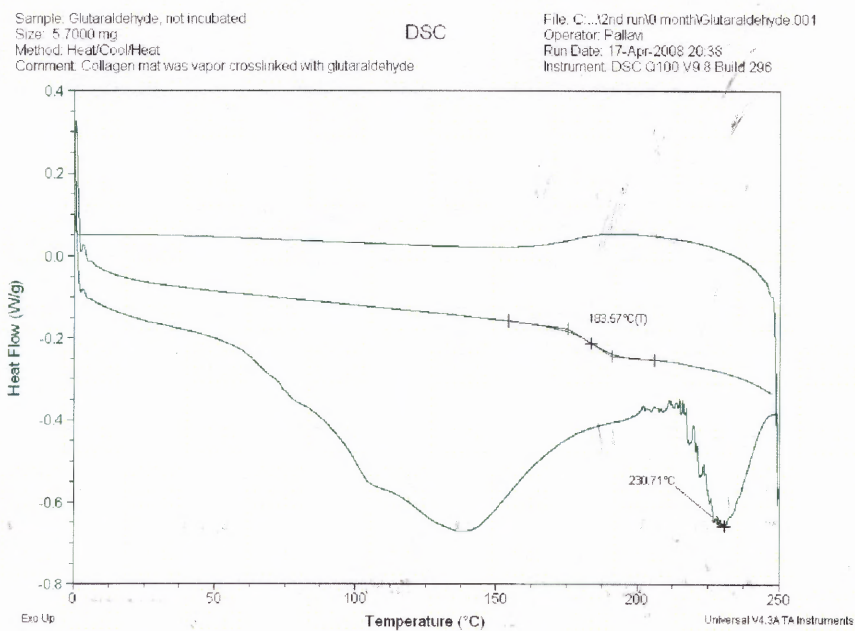


Figure B.6 DSC plot for glutaraldehyde crosslinked collagen scaffold without incubation.

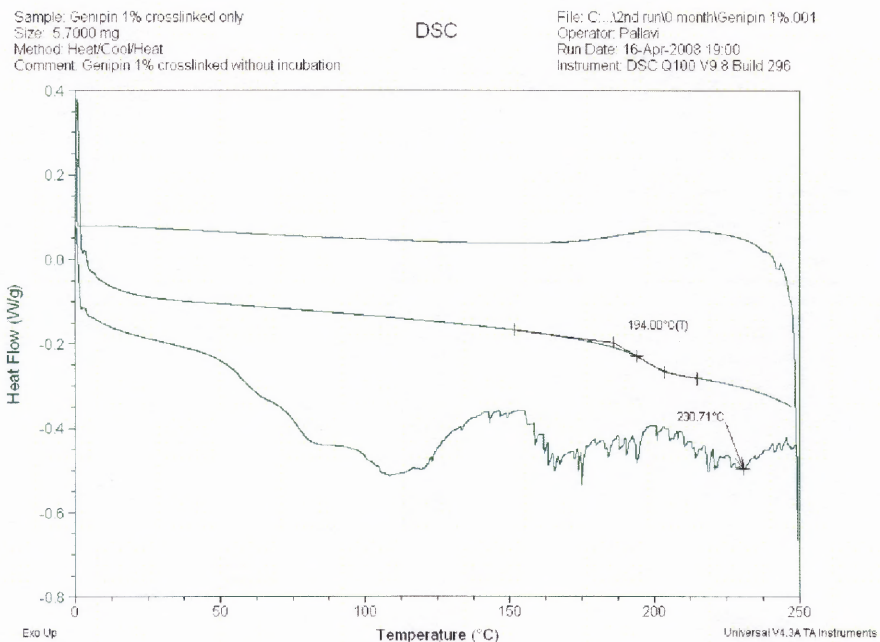


Figure B.7 DSC plot for genipin 1% crosslinked collagen scaffold without incubation.

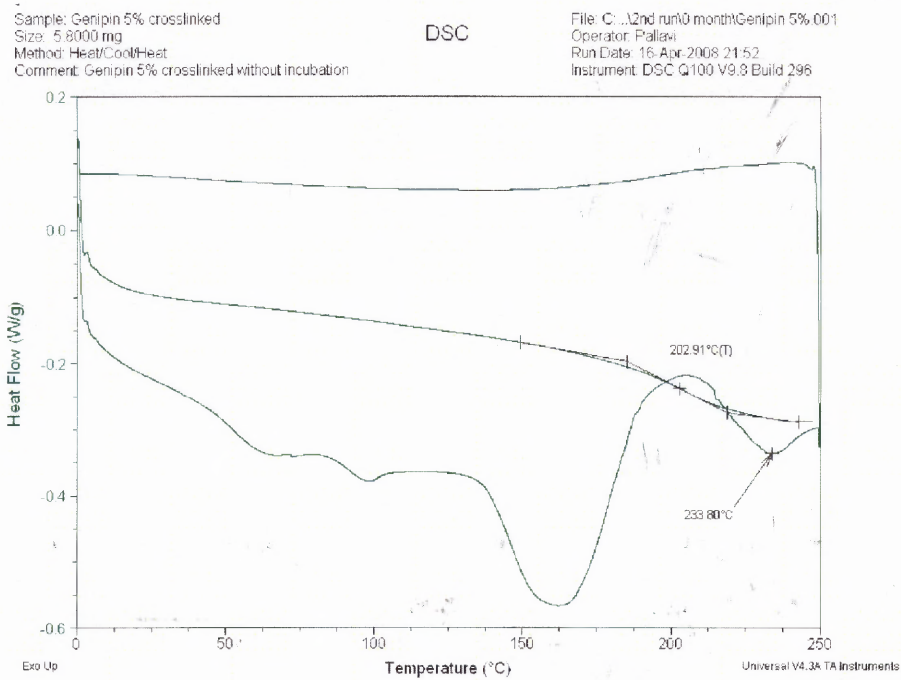


Figure B.8 DSC plot for genipin 5% crosslinked collagen scaffold without incubation.

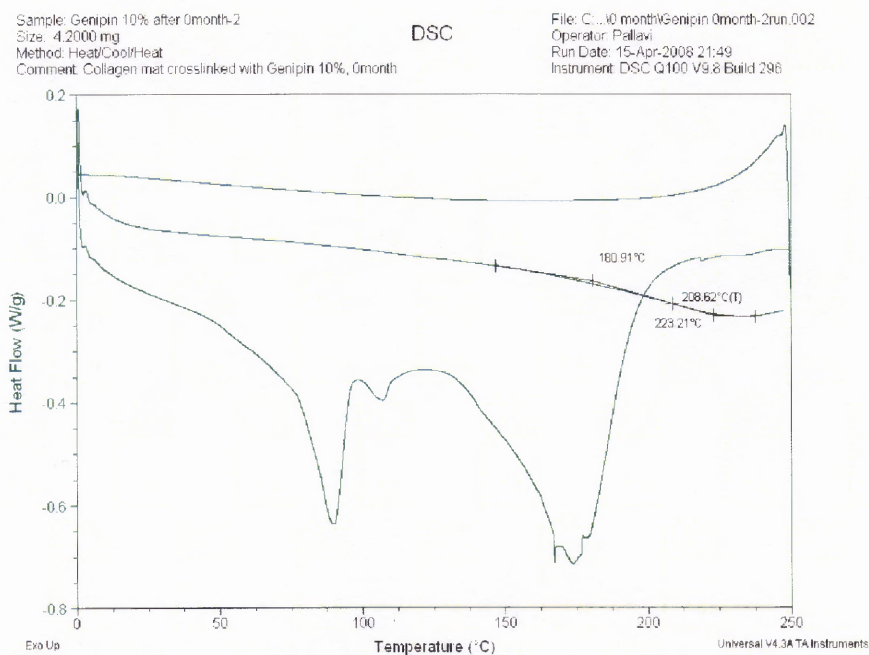


Figure B.9 DSC plot for genipin 10% crosslinked collagen scaffold without incubation.

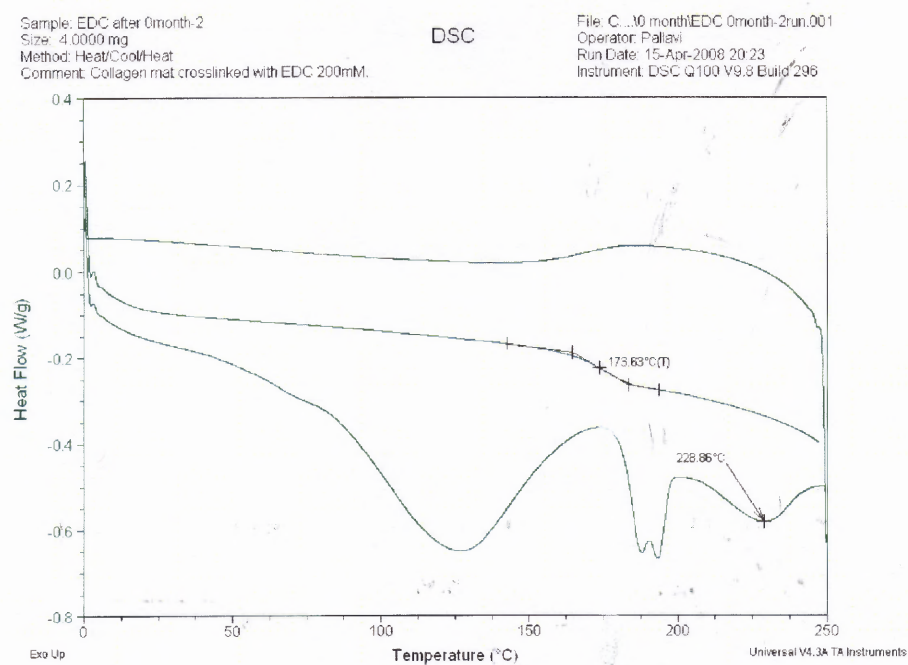


Figure B.10 DSC plot for EDC crosslinked collagen scaffold without incubation.

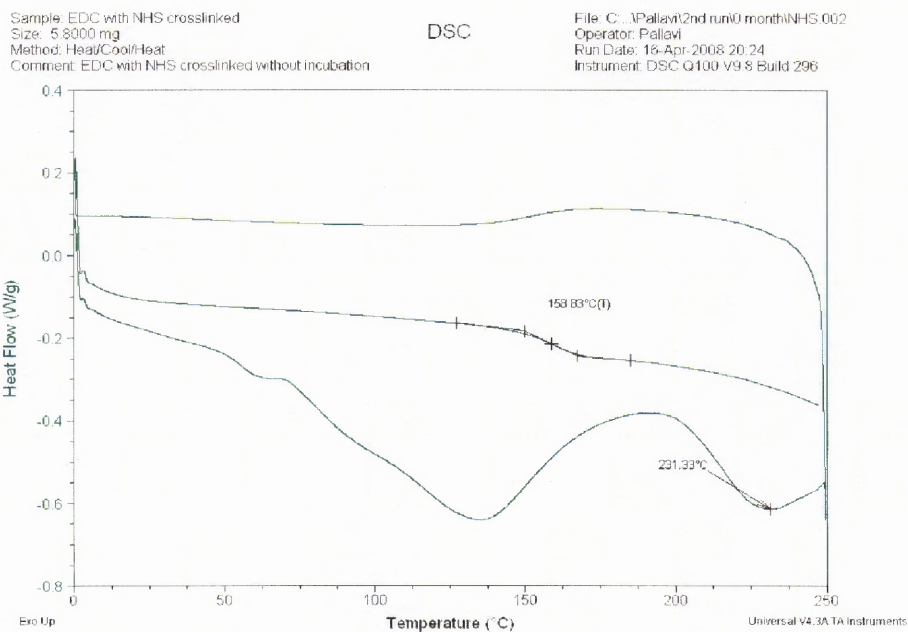


Figure B.11 DSC plot for EDC with NHS crosslinked collagen scaffold without incubation.

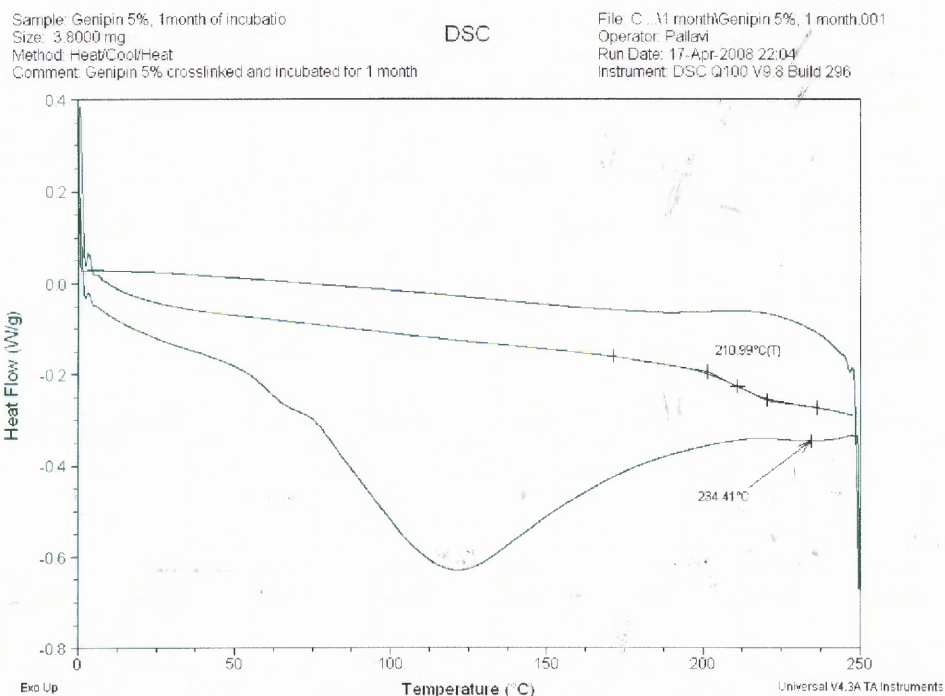


Figure B.12 DSC plot for genipin 5% crosslinked collagen scaffold after 1 month of incubation in PBS at 37°C.

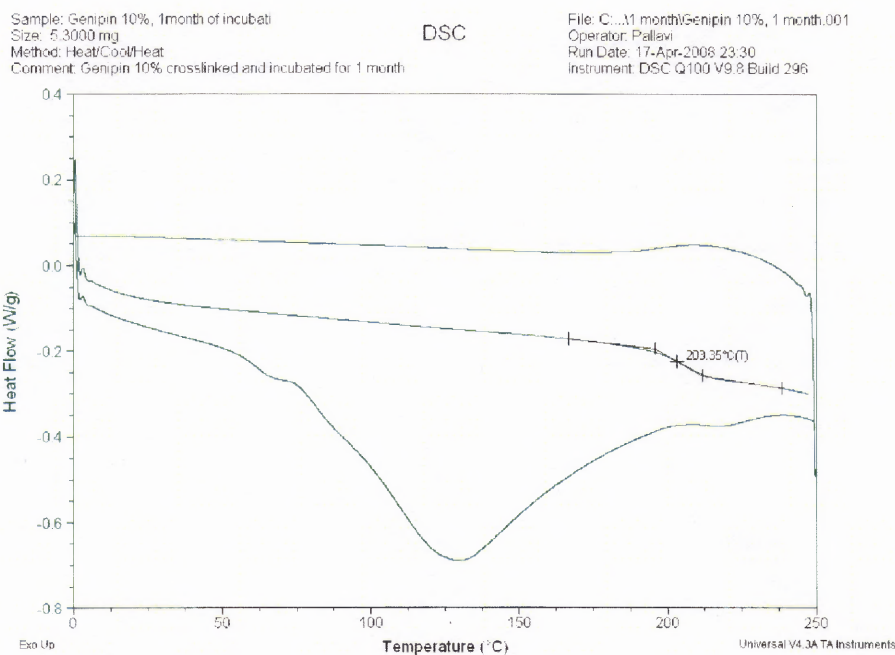


Figure B.13 DSC plot for genipin 10% crosslinked collagen scaffold after 1 month of incubation in PBS at 37°C.

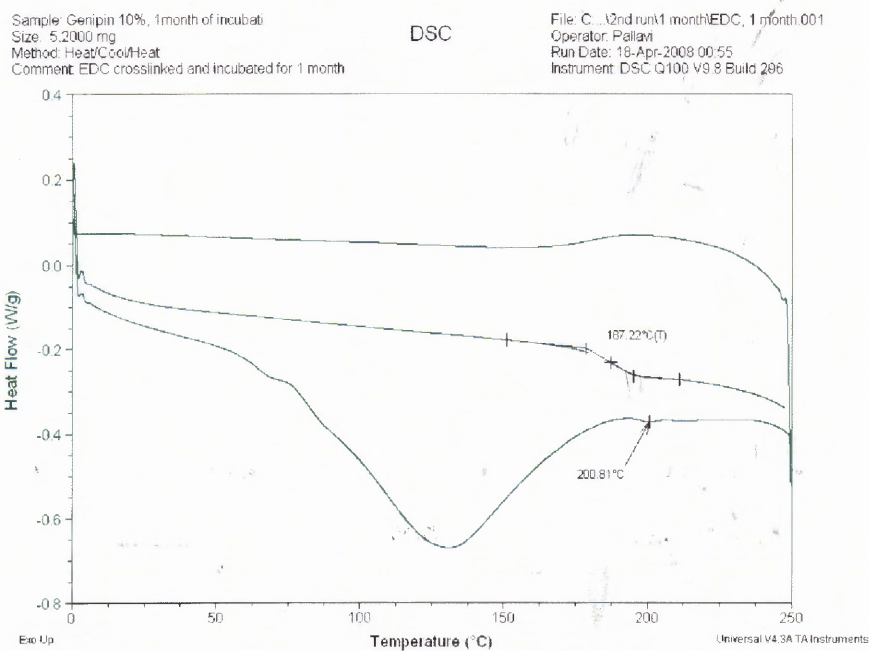


Figure B.14 DSC plot for EDC crosslinked collagen scaffold after 1 month of incubation in PBS at 37°C.

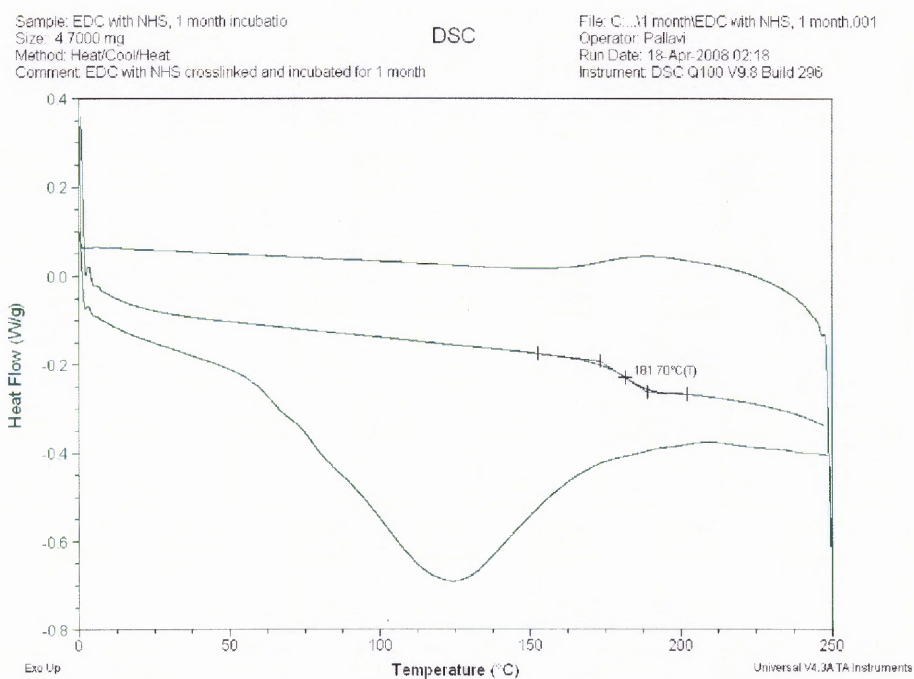


Figure B.15 DSC plot for EDC with NHS crosslinked collagen scaffold after 1 month of incubation in PBS at 37°C.

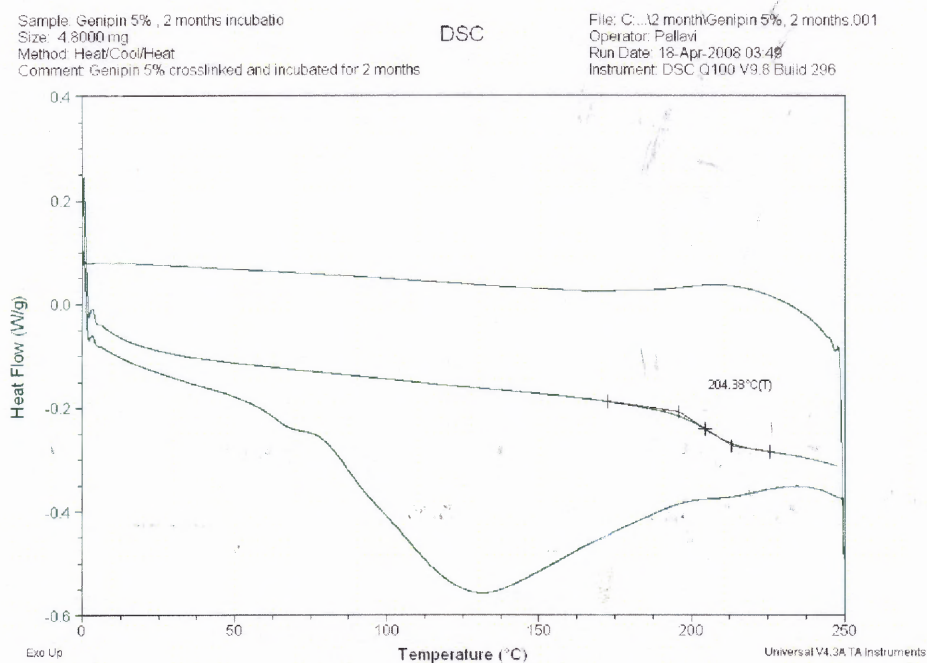


Figure B.16 DSC plot for genipin 5% crosslinked collagen scaffold after 2 months of incubation in PBS at 37°C.

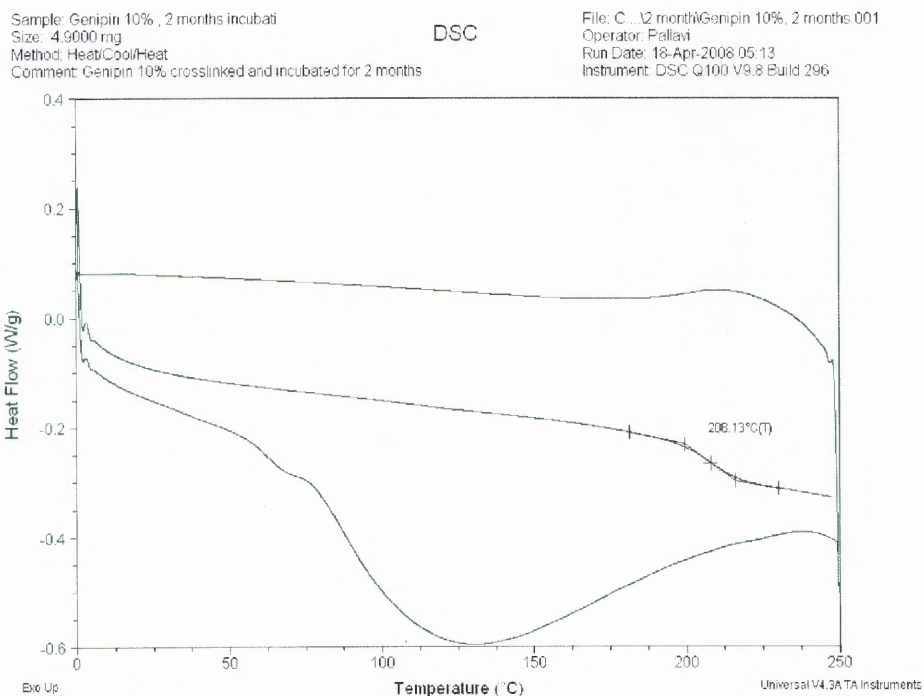


Figure B.17 DSC plot for genipin 10% crosslinked collagen scaffold after 2 months of incubation in PBS at 37°C.

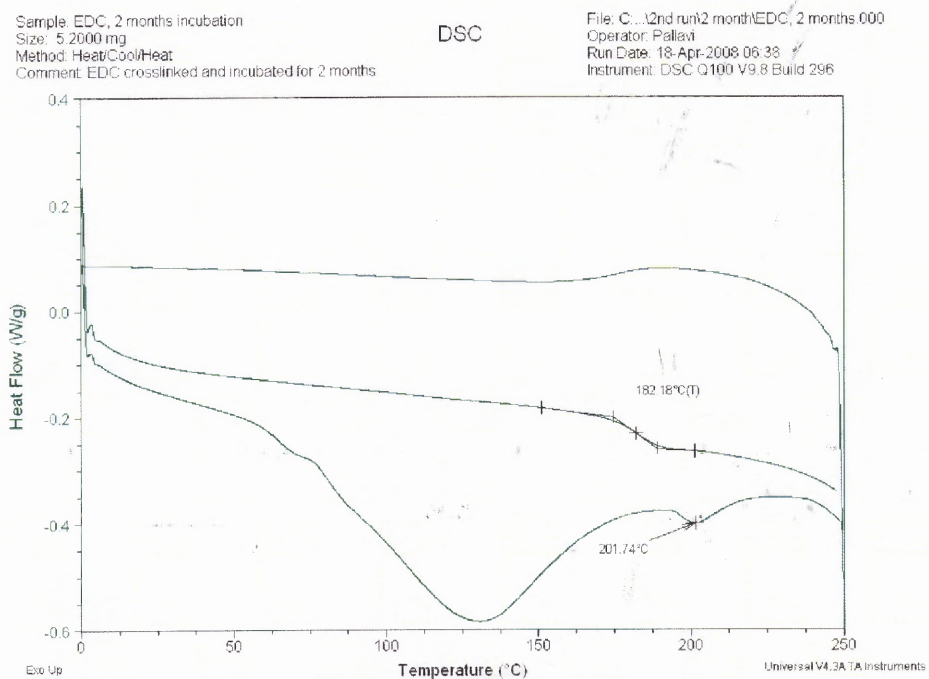


Figure B.18 DSC plot for EDC crosslinked collagen scaffold after 2 months of incubation in PBS at 37°C.

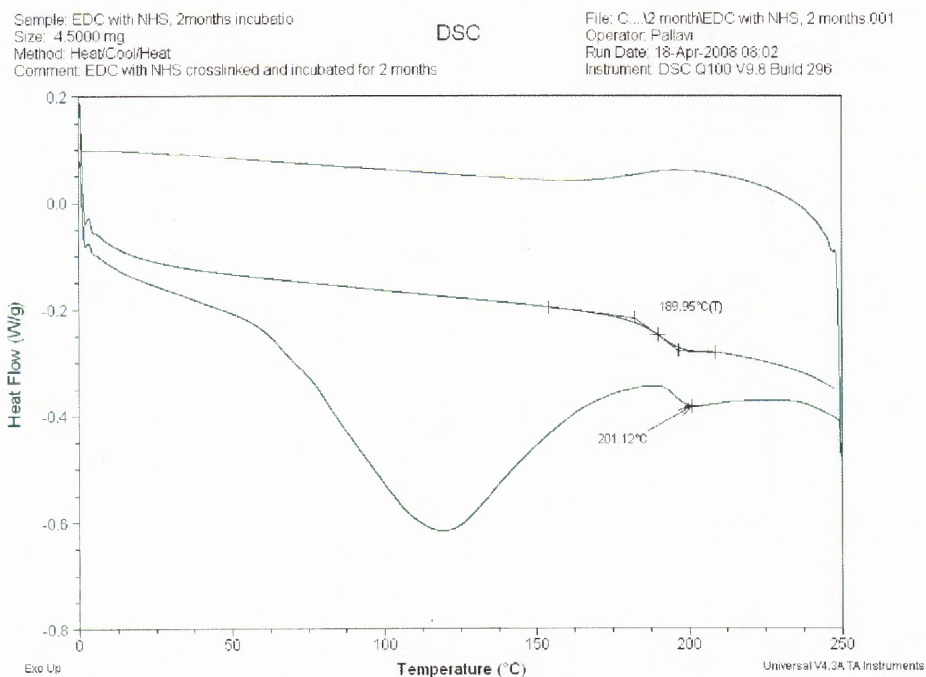


Figure B.19 DSC plot for EDC with NHS crosslinked collagen scaffold after 2 months of incubation in PBS at 37°C.

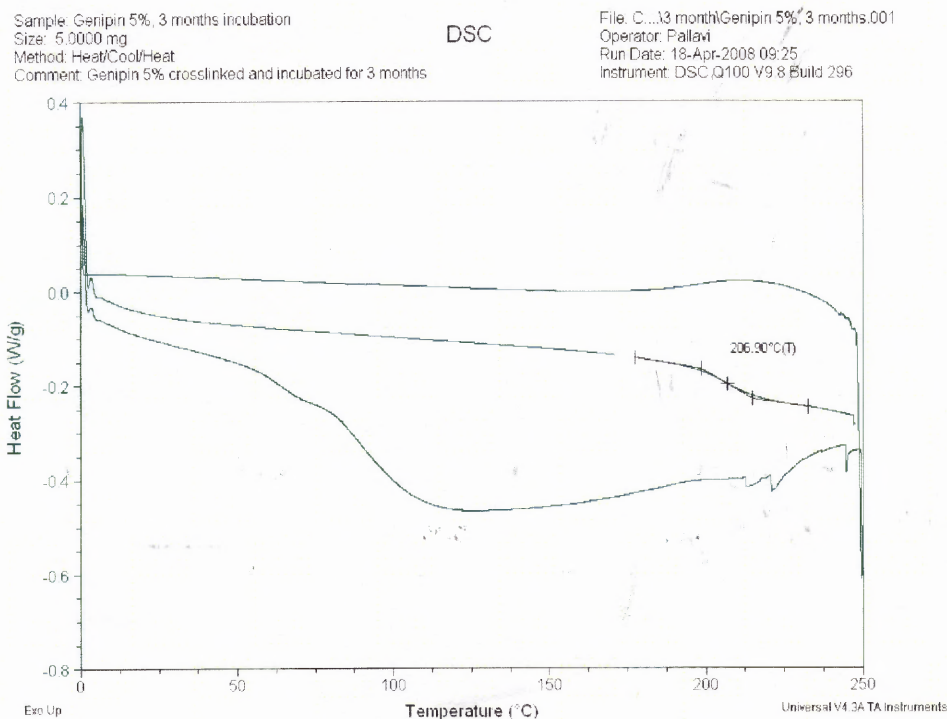


Figure B.20 DSC plot for genipin 5% crosslinked collagen scaffold after 3 months of incubation in PBS at 37°C.

Sample: Genipin 10%, 3 months incubatio
 Size: 3.4000 mg
 Method: Heat/Cool/Heat
 Comment: Genipin 10% crosslinked and incubated for 3 months

DSC

File: C:_13 month\Genipin 10%, 3 months.000
 Operator: Pallavi
 Run Date: 18-Apr-2008 10:50
 Instrument: DSC Q100 V9.8 Build 296

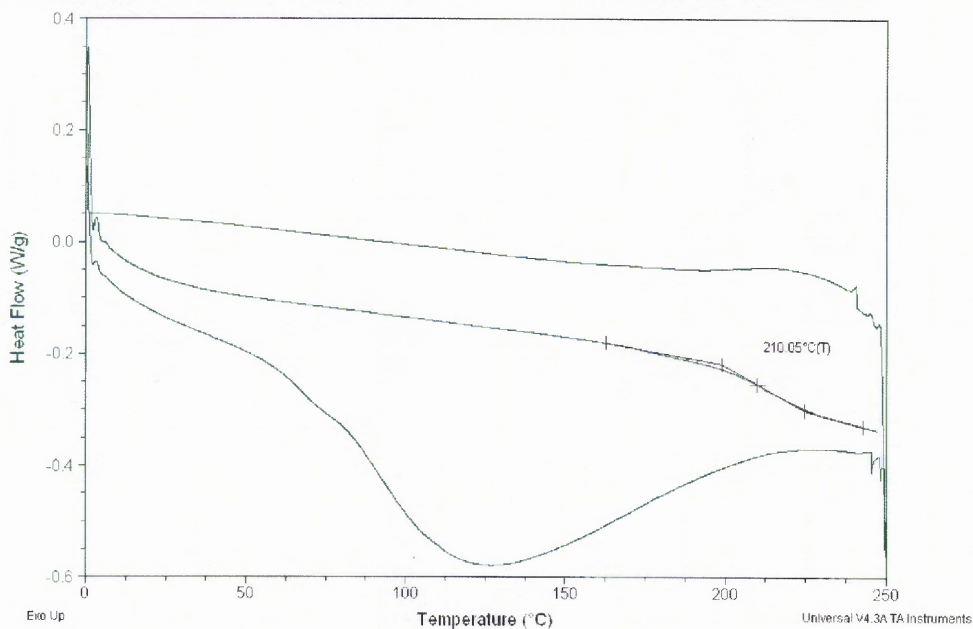


Figure B.21 DSC plot for genipin 10% crosslinked collagen scaffold after 3 months of incubation in PBS at 37°C.

Sample: EDC, 3 months incubatio
 Size: 3.8000 mg
 Method: Heat/Cool/Heat
 Comment: EDC crosslinked and incubated for 3 months

DSC

File: C:_12nd run\3 month\EDC, 3 months.001
 Operator: Pallavi
 Run Date: 18-Apr-2008 12:29
 Instrument: DSC Q100 V9.8 Build 296

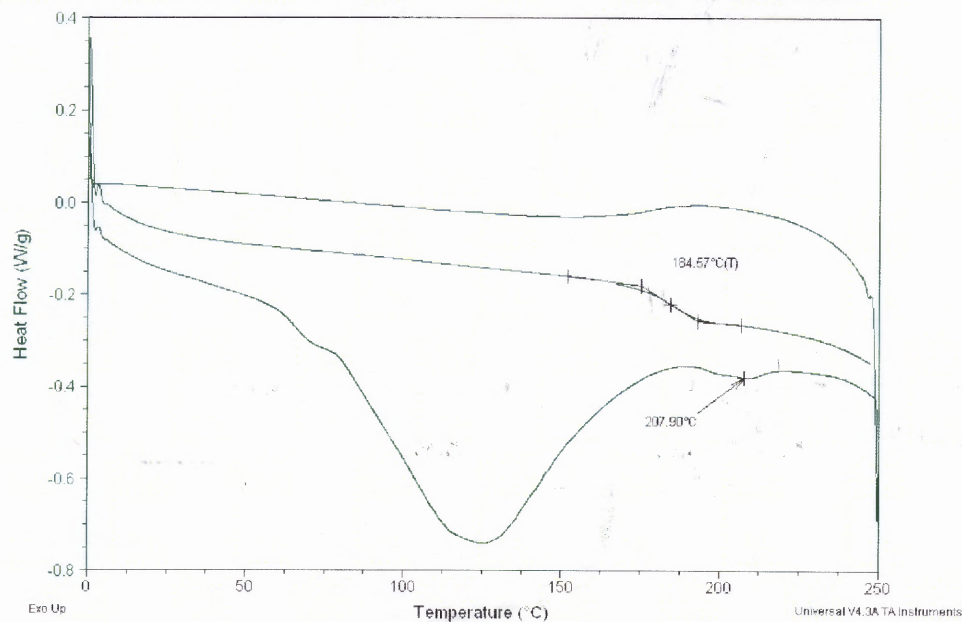


Figure B.22 DSC plot for EDC crosslinked collagen scaffold after 3 months of incubation in PBS at 37°C.

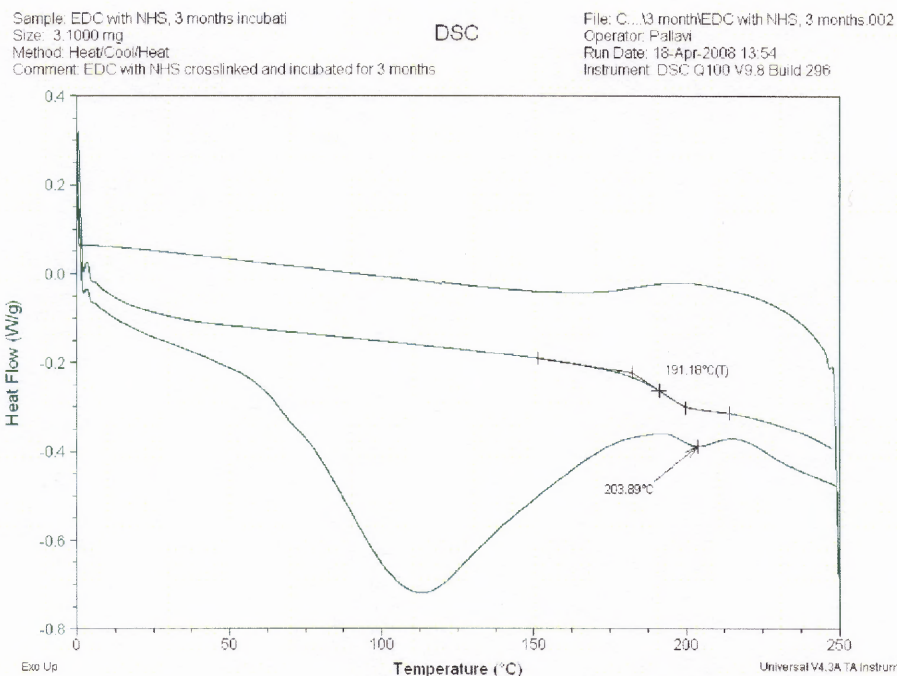


Figure B.23 DSC plot for EDC with NHS crosslinked collagen scaffold after 3 months of incubation in PBS at 37°C.

APPENDIX C
PICTURES OF SAMPLES

The samples as they appeared before crosslinking, after crosslinking, after incubation for 1 month, 2 months and 3 months are shown below:

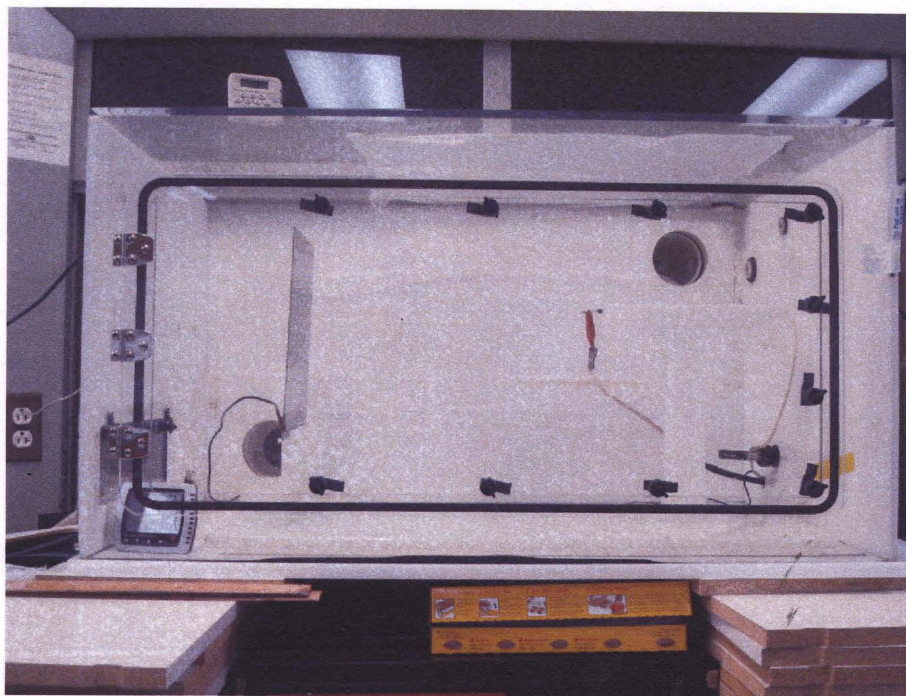


Figure C.1 Electrospinning hood used for generate collagen scaffold.



Figure C.2 Uncrosslinked electrospun type I collagen scaffold.

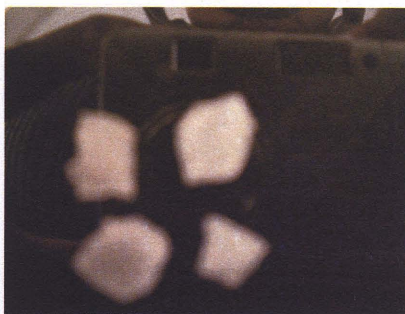


Figure C.3 Uncrosslinked collagen scaffold cut into small pieces 1 cm X 1 cm.

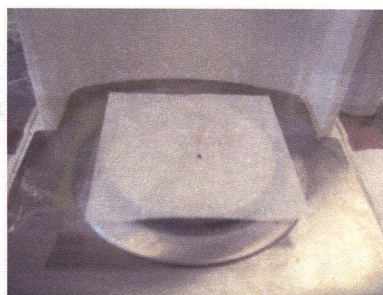


Figure C.3 Method used to measure the samples dimensions and weights.

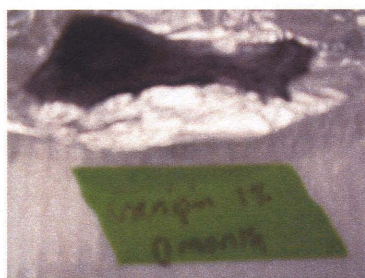


Figure C.4 Genipin 1% crosslinked sample before (left) and after (right) incubation.

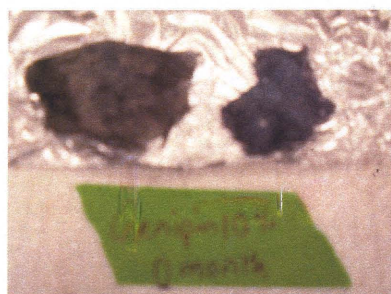


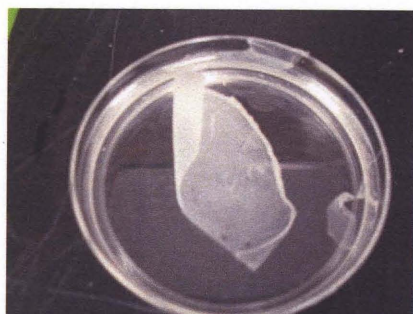
Figure C.5 Genipin 10% crosslinked sample before (left) and genipin 5% crosslinked mat after (right) 3 months incubation. The genipin 5% crosslinked mat looked similar to genipin 10% crosslinked mat.



(a)



(b)



(c)

Figure C.6 EDC and EDC with NHS crosslinked samples before incubation (a) and EDC crosslinked (b), EDC with NHS crosslinked (c) samples after 3 months of incubation.

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