COLLAGEN-BASED MENISCUS TISSUE ENGINEERING: DESIGN AND APPLICATION

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ABSTRACT

COLLAGEN-BASED MENISCUS TISSUE ENGINEERING: DESIGN AND APPLICATION

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Meniscus is a wedge shaped structure, with a convex base attached to a flat tibial surface, and with a concave femoral surface, on which femur and tibia articulate . It has several functions including joint lubrication, shock absorption, load transmission and joint stability. Various methods were tried to treat meniscal tears but each has its own drawbacks. Tissue engineering seems to be a promising solution that avoids all the problems associated with the other approaches. In this study, a three dimensional (3D) collagen-based structure was prepared by tissue engineering to mimic the natural human meniscus. Three different foams prepared under different conditions were combined and nano/microfibrous layers were placed in between them. To mimic the properties of the natural tissue, the top layer was composed of collagen-chondroitin

sulfate-hyaluronic acid (Coll-CS-HA) prepared by freezing at -20°C followed by lyophilization. The middle and bottom layers were made with just collagen after freezing at -20°C and -80°C, respectively and lyophilization. Aligned nano/microfibers were prepared using collagen-poly(L-lactic-co-glycolic acid (Coll-PLGA). Various crosslinking procedures such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/Nhydroxysuccinimide (EDC/NHS), genipin (GP), glutaraldehyde (GLU) either alone or in combination with dehydrothermal treatment (DHT) were used and based on both compressive and tensile properties, the best crosslinker was chosen to be DHT+EDC/NHS. Mechanical properties (compressive, tensile and shear) of the dry foams and the final 3D construct were evaluated. The highest mechanical properties were obtained with the 3D construct. Then all these foams and the 3D construct were seeded with human fibrochondrocytes to study the cell behavior such as attachment, proliferation, and extracellular matrix (ECM) and glucosaminoglycan (GAG) production. Furthermore, the influence of cell seeding on the compressive properties of wet individual foams and the 3D construct was observed. As expected, the highest cell proliferation and compressive properties were obtained with the 3D construct. Finally, the 3D constructs, seeded with fibrochondrocytes, were implanted in New Zealand rabbits after meniscectomy. The initial microscopical examination show that the 3D construct has a significant potential as a meniscus substitute.

Keywords: Meniscus; Tissue engineering; Collagen-based scaffold; Mechanical Properties.

KOLLAJEN TEMELLİ YAPAY MENİSKÜS: TASARIM VE UYGULAMASI

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Menisküs, dış bükeyinde kaval kemiği (tibia) ve iç bükeyinde uyluk kemiğinin (femur) kaydığı yarım ay şeklinde bir yapıdır. Menisküsün eklem kayması, şok emilimi, yükün aktarılması ve eklem dayanıklılığı gibi çeşitli işlevleri vardır. Menisküs yırtıklarını tedavi etmek için çeşitli yöntemler denenmiştir. Ancak, bu yöntemlerin her biri çeşitli açılardan yetersizdir. Doku mühendisliği, diğer yaklaşımlardan kaynaklanan sorunlardan kaçınmak için umut vadeden bir çözüm olarak görünmektedir. Bu çalışmada, doğal insan menisküsünü taklit etmek amacıyla doku mühendisliği yöntemiyle üç boyutlu (3D) kollajen temelli bir yapı üretilmiştir. Farklı koşullarda hazırlanan üç farklı sünger birleştirilmiş ve aralarına nano/mikrofiber katmanlar yerleştirilmiştir. Doğal dokunun yapısını taklit edebilmek için, 3-boyutlu yapının üst

tabakası -20°C'de dondurulduktan sonra liyofilize edilmis kollajen-kondroitin sülfathyaluronik asit (Coll-CS-HA) temelli süngerden oluşmuştur. Orta ve alt tabakalar sırayla -20°C ve -80°C'de dondurulup liyofilize edilen kollajen süngerlerle yapılmıştır. Yönlendirilmiş nano/mikrofiberler kollajen-poli(L-laktik-ko-glikolik asit) (Coll-PLGA) ile hazırlanmıştır. 1-etil-3-(3-dimetilaminopropil) karbodiimit/N-hidroksisüksinimit (EDC/NHS), genipin (GP), glutaraldehit (GLU) gibi kimyasal çapraz bağlayıcılar tek başına ya da dehidrotermal (DHT) yöntemiyle birlikte farklı çapraz bağlama işlemleri kullanılmış ve basma ve çekme özelliklerine dayanarak, DHT ile birleştirilmiş EDC/NHS çalışma için çapraz bağlayıcı olarak seçilmiştir. Kuru haldeki süngerlerin ve 3-boyutlu yapının mekanik özellikleri (basma, çekme ve kayma) değerlendirilmiştir. Mekanik açıdan en yüksek değerler 3-boyutlu yapılarla elde edilmiştir. Daha sonra, *in* vitro çalışmalarda hücre yapışması, çoğalması, hücre dışı matris (ECM) ve glikozaminoglikan (GAG) üretimi gibi hücre davranışlarını incelemek amacıyla bütün süngerlere ve 3-boyutlu yapıya fibrokondrosit hücresi ekilmiştir. Ayrıca, hücre varlığının ve çoğalmasının ıslak sünger ve 3-boyutlu yapıların sıkıştırma altındaki özellikleri üzerindeki etkisi incelenmiştir. Beklendiği üzere, en yüksek hücre çoğalması ve sıkıştırma değerleri 3-boyutlu yapılarla elde edilmiştir. Son olarak, hücreli yapılar Yeni Zelanda tavsanlarına meniskektomi sonrası implante edilmiştir. İlk makroskopik sonuçlar bu 3-boyutlu yapının menisküs yırtılmalarında kullanıma uygun olabileceğini göstermiştir.

Anahtar Kelimeler: Menisküs; Doku mühendisliği; Kollajen temelli iskele; Mekanik özellikler.

Dedicated to my lovely family...

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LIST OF ABBREVIATIONS

Bovine Achilles' Tendon Collagen Type I
Collagen
Collagen-Chondroitin sulfate-Hyaluronic acid
Carbon fibers
Chondroitin Sulfate
Confocal Laser Scanning Microscope
Diaminobenzidine
4',6-Diamidino-2-phenylindole dihydrochloride
Dulbecco's Modified Eagle Medium/Ham F12
Dimethyl Sulphoxide
Young's Modulus
Linear Elastic Modulus
Extracellular Matrix
1-Ethyl-3-(3-Dimethylaminopropyl) Carbodiimide
Fetal Bovine Serum
Fluorescein isothiocyanate
Shear Modulus
Glucosaminoglycan
Glutaraldehyde
Genipin
Hyaluronic acid
Acetic Acid
1,1,1,3,3,3-Hexafluoroisopropanol

HRP	Horseraddish Peroxidase
MTT	Thiazolyl Blue Tetrazolium Bromide
NaCl	Sodium Chloride
NGS	Normal Goat Serum
NHS	N-Hydroxysuccinimide
PE	Phycoerythrin
PBS	Phosphate Buffer Saline
PLGA	Poly(L-lactic-co-glycolic acid)
PI	Propidium Iodide
PLLA	Poly(L-lactic acid)
PP	Polypropylene
PTFE	Polytetrafluoroethylene
PU	Polyurethane
RTC I	Rat Tail Collagen Type I
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SEM	Scanning Electron Microscope
SDS-PAGE	Sodium Dodecyl Polyacrylamide Gel Electrophoresis
UHMWPE	Ultra high molecular weight polyethylene
ϵ^{*}_{el}	Elastic Collapse Strain
σ^*_{el}	Elastic Collapse Stress

CHAPTER 1

INTRODUCTION

Meniscal tears are the most common injuries nowadays and can occur either as a result of various sport activities or normal tissue degeneration as the age increases. Since these injuries influence the life quality it is of utmost importance that these incidences should be reduced as much as possible. According to some statistical data taken from British Orthopaedic Sports Trauma Association (B.O.S.T.A), 61 of 100000 people suffer from meniscus problems. Furthermore, more than 400000 surgeries are performed per year in Europe [van der Bracht et. al., 2007]. The Stone Clinic reports that around 1.4 million surgeries are performed per year in USA. All these numbers show how serious the situation is and that a fast solution should be found. Depending on the degree of trauma, various treatment methods like meniscectomy, meniscus repair, meniscal transplants, either allografts or autografts can be used to treat these injuries. Meniscal repair has been shown to have a success rate of 60-70%. Since in patients that underwent partial or total meniscectomy osteoarthritis was observed after a period of time, meniscal transplantation has been chosen as a better solution. In terms of taking away pain, transplantation has revealed a success rate of 80%. However, a reduction in efficiency has been observed after 7-8 years of transplantation and since there is no evidence that transplantation eliminates osteoarthritis, it is not preferred for long term applications. Tissue engineering appears as a better and more promising solution for meniscus replacement. To our knowledge, there are only few products for this aim and there is no any tissue engineered one. The aim of this study was to design a 3D tissue engineered total (or partial) meniscus that would mimic best the structure and function of the natural tissue.

1.1. Meniscus Structure and Function

Meniscus is a wedge-shaped fibrocartilaginous tissue present between the tibial and femoral surfaces of the knee joint. It has a concave superior surface in order to be consistent with the shape of femur condyles and a flat inferior surface to sit well on the tibial plateau. There exists two types of meniscal tissue, namely the medial and the lateral meniscus, the former being more semilunar and the latter more semicircular in shape (*Fig. 1*). They are attached to each other by the transverse ligament. Some other ligaments such as anterior and posterior cruciate ligaments and medial and lateral collateral ligaments are present; all of which help in restricting bone movement and maintaining functionality of the knee joint [Athanasiou *et. al.*, 2009]. Ligaments of Humphrey and Wrisberg are located in the anterior and posterior regions of posterior cruciate ligament, respectively.

Typical dimensions for a human medial meniscus are: 25-27 mm in width and 40.5-45.5 mm in length. Those for lateral meniscus are: 26.6-29.3 mm in width and 32.4-35.7 in length [Shaffer *et. al.* 2000; McDermott *et. al.* 2004]. While the width of anterior horn of medial meniscus is about 6 mm, for the posterior horn it is about 12 mm. In the lateral meniscus they are practically equal (10 mm) in the anterior to posterior direction. As it is seen in *Figure 1*, the medial meniscus is a slightly more open C shape with a wide posterior horn and it is larger than the lateral one. Due to its attachment to medial collateral ligament it is less mobile and more rigid than the lateral; thus, it is much more prone to injury. Earlier it was thought to be a functionless tissue, however, later on it was realized that it has very important roles in the knee biomechanics. The main functions reported are load bearing and transmission, shock absorption, joint lubrication

and stability within the knee joint [McDermott et. al., 2006; Angele et. al., 2007; Sanchez-Adams et. al., 2009].



Figure 1. Superior and lateral views of the knee. (A) Superior [http://biokineticist.com/knee%20-%20meniscus.htm], and (B) lateral view [Sanchez-Adams *et. al.*, 2009].

1.1.1. Meniscus Development

Unlike hyaline cartilage, meniscus is a highly cellular and fibrous tissue. In their initial stages of development both are fully cellularized structures; however, it was shown that meniscus vascularity and cellularity change with time (*Fig. 2*) [Sweigart *et. al.*, 2001; Sanches-Adams *et. al.*, 2009]. At 3.5 months of gestation, meniscus is a fully vascularized tissue with a low amount of extracellular matrix. The tissue continues to be fully vascular for a further 3 months while the amount of extracellular matrix (ECM) increases. As the fetus gets larger, amount of ECM keeps increasing; the nucleus to

cytoplasmic ratio decreases and the collagen orientation becomes predominant. Three months after birth the tissue is still vascularized but this is more concentrated in the outer region and this becomes more distinct by 9 months. This decrease in the amount of vascularization continues until age 11 [Clark *et. al.*, 1983; Athanasiou *et. al.*, 2009].



Figure 2. Meniscus development. (A) Schematic presentation of meniscus vascularization [van der Bracht et. al., 2007], and (B) change in vascularization as the tissue develops [Sweigart et. al., 2001].

The main factor influencing the degree of vascularity of meniscus is endostatin, a 20 kDa portion of collagen XVIII that inactivates vascular endothelial growth factor (VEGF). It is initially present throughout all the meniscal tissue, however, in time it is concentrated in the inner portion of the meniscus. Thus, the tissue becomes avascular in the inside and vascular on the outside. An adult medial meniscus has a vascularity of 10-30% whereas that of the lateral one is 10-25% [Pufe *et. al.*, 2004; Sanchez-Adams *et. al.*, 2009].

As a result of this varying vascularization, meniscus is divided into three parts, the redred zone, the red-white zone and the white-white zone. As the name implies, the whitewhite zone has no blood supply (no vascularization), and receives its nutrition by means of diffusion only. The middle part, the red-white zone is poor in vascularization and gets its nutrients from both the synovial fluid and the blood vessels present there. The red-red zone is composed more of connective tissue with blood vessels, that provide its nutrients, and fibrocytes. [Drengk *et. al.*, 2008].

1.1.2. Cell Types

The degree of vascularization as well as the cell type, size and morphology change as the tissue matures. At the very beginning of development steps the cells are similar in shape (mostly round), and size, and they have a high nuclear to cytoplasmic volume ratio. Later, the cells show different behaviors in different parts of the tissue. In the superficial layer a fibroblast-like morphology is observed; they are more oval or fusiform with less cytoplasm. However, as you go toward the interior of the meniscus, the cells become more round or polygonal shaped, and chondrocyte-like with a high amount of cytoplasm and organelles (*Fig. 3*). Due to this morphological change, various names like fibroblasts, fibrocytes, chondrocytes and fibrochondrocytes, have been used to refer to the meniscal cells [McDevitt *et. al.*, 2002; Athanasiou *et. al.*, 2009].

While many researchers in general call the meniscus cells fibrochondrocytes, some others prefer to divide them in 4 groups based on their shape; location and function [McDevitt *et. al.* (2002)]: Fibrochondrocytes are the cells present in the inner half of meniscus. They are round or oval in shape and produce type I collagen mainly in addition to types II, III and VI to a lower extent. It is important to mention that these cells are present where compressive forces are more predominant. The second type is fibroblast-like cells located in the outer region of the tissue where tensile forces are more

important. The third group is represented with cells in the superficial zone, which are in contact with the synovial fluid. They are fusiform in shape and have no cytoplasmic projections. And finally in group 4, there are some cells with intermediate morphology between that of the fibroblast-like and the fibrochondrocyte [McDevitt *et. al.*, 2002].



Figure 3. Morphological differences of the various meniscus cell types [Sanchez-Adams et. al., 2009].

Another important difference is that not only the shape of the cells changes but also their synthetic profiles vary. Where fibroblast-like cells are present (the exterior), collagen Type I is predominant, and in the interior of the meniscus collagen Type II is abundant [van der Bracht *et. al.*, 2007; Schoenfeld *et. al.*, 2007; Sanchez-Adams *et. al.*, 2009].

1.1.3. Extracellular Matrix of Meniscus

Meniscus tissue shows regional variations in its extracellular matrix (ECM) components. In dry state, unlike articular cartilage, meniscus consists of mainly collagen Type I (\geq 90%), which makes it a fibrous and cartilageneous tissue. To a lesser degree it has collagen types II, III, V and VI. When wet it is composed mostly of water (72%) and fibrillar components (22%), proteoglycans and some adhesion glycoproteins [Angele et. al., 2007; van der Bracht et. al., 2007]. Collagen type I is more predominant in the redred region of the tissue, whereas the white-white region is composed of both types I and II. Thus, the inner portion of meniscus is more articular-cartilage like and the outer region is more fibrous [Athanasiou et. al., 2009]. Elastin, with less than 1% of the dry weight, is the other fibrillar component present and it is found in both its mature and immature forms [Sweigart et. al., 2001]. Glucosaminoglycans (GAGs) like chondroitin-6-sulphate (40%), chondroitin-4-sulphate (10-20%), keratin sulphate (15%) and dermatan sulphate (20-30%) make up the remaining parts of the meniscus dry weight. They play a crucial role in the viscoelastic behavior, compressive strength and hydration of the tissue due to their negative charge [Herwig et. al., 1984; Sweigart et. al., 2001; Buma et. al., 2004; Hoben et. al., 2006]. In addition, decorin, biglycan and fibromodulin are some of the other GAGs that are in the complex architecture [Sweigart et. al., 2001; van der Bracht et. al., 2007]. Decorin has been shown to act in collagen fibril organization, whereas biglycans are important for cell protection during the load applied by the body weight [Scott et. al., 1997]. Adhesion glycoproteins like thrombospondin, fibronectin and collagen type VI are molecules that function in cell-to-cell and cell-tomatrix binding since they have the Arginine-Glycine-Aspartic acid (RGD) amino acid sequence in their composition [McDevitt et. al., 1990]. This tripeptide is present in many ECM molecules such as collagen, fibrinogen, vibronectin, tenascin and some other proteins also [Scaffner et. al. 2003]. They have been shown to have a great influence on cell adhesion mechanism due to the fact that during binding some cell adhesion receptors present on the cell membrane recognize these sequences [Cutler et. al., 2003;

Garcia *et. al.*, 2005]. Based on this fact, many researchers work on the modification of surfases with these tripeptides.

1.1.4. Biomechanics of the Knee Meniscus

Meniscus has several crucial functions and due to these functions as well as the tissue geometry it is under the stress of various forces, namely compressive, tensile and shear. A meniscus tissue is considered a porous composite made up of a water-saturated organic solid matrix. The load carrying ability of the tissue comes from the organization of collagen fibers and proteoglycans. Even though they constitute only 1% of meniscus dry weight, proteoglycans are crucial due to their ability to take up water approximately 50 times of their weight [Hansen et. al., 2006]. Meniscus has been shown to be a viscoelastic tissue whose mechanical properties depend on the type and rate of loading. Responsiveness to creep and the stress relaxation ability of the tissue has been shown by McDermott et. al. (2008) who state that under a constant load the tissue shows immediately an elastic-like behavior. The tissue then continues to deform under the load but to a lower extent since the fluid phase present in meniscus is expelled and this continues until the resistance to stress is only due to the matrix alone. This is called the 'creep response' behavior of the tissue. Similarly, stress relaxation occurs when a step strain is applied. When the load is removed, the expelled fluid rehydrates the tissue because the negatively charged GAGs attract the water back into the matrix. As a consequence of this fluid transfer, important processes like nutrient transportation, waste removal and joint lubrication occur [Fithian et. al., 1989; Athanasiou et. al., 2004]. It should be understood that apart from taking part in resisting compressive forces exerted on menisci, GAGs play a crucial role in the general well being of the tissue.

1.1.4.1. Influence of Collagen Orientation on Meniscus Biomechanics

The triple helical structure of collagen imparts important functions in different tissues in the body and it is especially present in tissues where tensile stress is dominant. The presence of the high amounts of collagen Type I and its orientation play a profound role in the load carrying capability of the meniscus. Type I collagen one of the fibril forming collagens and it can form large fibrils (up to 300 nm in diameter) from individual collagen molecules. Another crucial characteristic of collagen is its fibril orientation, the degree of which varies among tissues. Variation of the organization extent and direction can be observed (*Fig. 4*).



Figure 4. Collagen structure and fiber orientation in a meniscus tissue. (1) Superficial layer, (2) lamellar layer and (3) central main layer [Hansen et. al., 2006].

As we go from the surface to the inside of a meniscus, the fiber orientation in the superficial layer is random. In the lamellar layer, just beneath the superficial layer, fibers are also randomly oriented, except at the periphery of the anterior and posterior horns where fibers are oriented radially. In contrast to these, the deep zone is composed of fibers organized circumferentially with a few radially oriented [Sweigart *et. al.*, 2001]. This special orientation of fibers has been shown to be very important for the functions of the meniscus because it is now known that circumferential fibers resist tensile forces and function as a load transmitter across the knee joint, whereas the radially oriented ones provide rigidity to the tissue and help to resist compressive forces and splitting [Sweigart *et. al.*, 2001; Hansen *et. al.*, 2006].

1.1.4.2. Mechanical Evaluation of the Tissue; Compression, Tension and Shear Tests

As it was mentioned in the previous sections, anatomy and structure of the meniscus are very important, thus, the mechanical properties are among the main issues that should be taken into consideration when designing a tissue engineered scaffold.

Mechanical properties of the natural meniscus have been studied by many authors and a baseline data is available related to these properties. All the studies have demonstrated that meniscus biomechanical properties vary depending on the location where the force is applied, on the force direction and whether the load is applied on the femoral or tibial surface. Most of the studies performed on meniscus are tensile [Proctor *et. al.*, 1989; Fithian *et. al.*, 1990; Skaggs *et. al.*, 1994; Tissakht *et. al.*, 1995; Goertzen *et. al.*, 1996, 1997; Lechner *et. al.*, 2000; Holloway *et. al.*, 2010; Nerurkar *et. al.*, 2010], with a few ones being compressive [Proctor *et. al.*, 1989; Hacker *et. al.*, 1992; Leslie *et. al.*, 2000; Sweigart *et. al.*, 2004; Holloway *et. al.*, 2010; Nerurkar *et. al.*, 2010] and shear studies [Fithian *et. al.*, 1990; Anderson *et. al.*, 1991; Zhu *et. al.*, 1994]. Due to variation in the orientation of collagen, tests have been performed in different orientations and positions

of the meniscus tissue, both circumferentially and radially and also in the superficial and deep regions of the tissue. *In vivo* tests are mainly performed on animals such as pig, cow or sheep due to the lack of human tissue but there also exists some data related to human meniscus properties also [Fithian *et. al.*, 1990; Sweigart *et. al.*, 2004].

Confined, unconfined compression and creep indentation tests are the main compressive tests done on meniscal tissues. Variations in different regions of meniscus have been observed due to the changes in orientation and makeup [Athanasiou *et. al.*, 2009]. Creep indentation test has shown that the highest aggregate modulus value is that of anterior meniscus (150 kPa) [Sweigart *et. al.*, 2004]. On the other hand, Young's modulus was found to be two fold higher in the axial direction than that in the circumferential and radial directions, which has been thought to be due to proteoglycan presence [Leslie *et. al.*, 2000]. Proctor *et. al.* (1989) showed that while aggregate modulus does not vary significantly among the samples taken from the superficial zone of bovine meniscus, it was stiffer in the posterior than in the anterior regions of deeper zones [Proctor *et. al.*, 1989]. The same behavior was observed by Hacker *et al.*, 1992]. Variation among the compressive properties of meniscus of different animals was reported by Joshi and coworkers [1995]. Ovine aggregate modulus showed the closest behavior to the human meniscus [Joshi *et. al.*, 1995].

Tensile properties of meniscus are also crucial. While doing the tensile testing it is important to have sample consistency with respect to direction and position because meniscus has been shown to be anisotropic [Gabrion *et. al.*, 2005]. Lateral meniscus has higher tensile properties compared to the medial one. Medial meniscus has the highest stiffness in the anterior region whereas the lateral one is stiffer in the posterior region [Fithian *et. al.*, 1990]. Tensile tests on meniscus have revealed that meniscus is isotropic in its surface and becomes anisotropic in the deeper layers. It can be said that the tensile properties of meniscus are 10-fold higher in the circumferential direction compared to

the radial one and it is in the range of 100-300 MPa [Sweigart *et. al.*, 2004; Gabrion *et. al.*, 2005; Athanasiou *et. al.*, 2009].

A third important force exerted on meniscus is shear. Shear modulus is the main property measured to compare the shear properties of the natural meniscus. Either creep indentation or dynamic oscillatory strain is the approach used to do this testing. Dynamic shear modulus is found to be anisotropic and frequency dependent, the former being due to collagen organization and the latter due to the viscoelasticity of meniscus tissue [Zhu *et. al.*, 1994]. According to Fithian *et. al.* (1990) a normal human meniscus has a shear modulus of 120 kPa.

1.2. Meniscal Injuries and Repair Techniques

1.2.1. Meniscal Injuries

One of the most common problems or injuries that a knee can experience is meniscal tear. Medial meniscal tears are two times more common than the lateral damages [McDermott *et. al.*, 2006]. Meniscal tears can appear in both young and old patients. In old patients a tear generally occurs as a result of degenerative processes or due to minor injuries such as after the up and down motion of squatting, whereas in young people it can be mainly because of sporting activities or heavy lifting. Various tears on different parts of the meniscus are shown in *Fig. 5*. The main symptoms of a meniscal injury are swelling, pain, restricted movement, knee locking and inability to move. All of these reduce the quality of life of a person and therefore, many researchers have focused on the treatment of this defect.

Spontaneous healing is expected to occur in the peripheral zone-related tears due to the presence of blood supply, however, when the inner parts of the meniscus are damaged

the healing capability by itself is almost none or very limited [Sweigart *et. al.*, 2001; Buma *et. al.*, 2004; McDermott *et. al.*, 2006; Hoben *et. al.*, 2006; Angele *et. al.*, 2007; Schoenfeld *et. al.*, 2007; van der Bracht *et. al.*, 2007; Drengk *et. al.*, 2008;]. Newman *et. al.* (1989) have shown that the type of tear also influences the degree of healing. It was shown that even when the radial tears heal, the tissue does not recover its full mechanical function since such a tear disrupts the structure of collagen. On the other hand, tissues may recover their full mechanical functions after longitudinal tears [Newman *et. al.*, 1989].



Figure 5. A healthy meniscus (a) and various meniscal tears; (b) jagged-edge, (c) oblique, (d) radial, (e) horizontal, and (f and g) vertical longitudinal tears [Athanasiou *et. al.*, 2009].

1.2.2. Meniscal Repair Techniques

A variety of repair methods have been developed to increase the level of healing. Treatments can be nonsurgical and surgical. Nonsurgical treatments include application of certain solutions inside the joint like addition of synovial fluid or chondroitin sulphate-hyaluronic acid combinations are carried out. The surgical method includes procedures such as meniscectomy, meniscal repair, meniscus replacement and tissue engineering as will be mentioned in the sections below:

1.2.2.1. Meniscectomy

Meniscectomy is total removal or removal of torn meniscus and was the first and most common method used in clinical applications. Since meniscus was initially believed to be a functionless part of the leg, meniscus was removed when complications or injuries occurred. However, it was later shown that total meniscectomy leads to osteoarthritic degeneration of the knee joint due to abnormal stresses applied on the articular cartilage. It is now known that even partial meniscectomy results in osteoarthritic degeneration but it still remains an option in case of irreparable tears [Schoenfeld *et. al.*, 2007]. Compared to total meniscectomy, partial meniscectomy has, however, been demonstrated to result in a lesser degree of cartilage degeneration since the remaining meniscal tissue can still absorb some of the stress exerted on the tissue. In some cases, a fibrin clot might form at the surgery site and this may act as a kind of site for cell ingrowth [Arnoczky *et. al.*, 1985]. Removal of lateral meniscus results in a higher degree of osteoarthritis than removal of medial meniscus [McDermott *et. al.*, 2006; van Tienen *et. al.*, 2009].

1.2.2.2. Meniscal Repair

Meniscal repair is another approach to repair torn menisci and avoid the problems of partial or total meniscectomy. Sutures, fibrin sealants, meniscal arrows are common methods utilized in case of tears in the vascular zone. Whenever possible, meniscal tears are repaired by suturing or with bioabsorbable fixation devices. Fibrin sealants are inferior to sutures but they can be used together with suturing to obtain better results [Drengk *et. al.*, 2008]. The avascular zone requires vascular induction and this can be achieved by either trephination or creation of vascular access channels [Schoenfeld *et. al.*, 2007]. In the case of vascular access channels some large channels are created from the meniscus periphery to the tear site but when this is done the collagen structure is destroyed. Collagen structure disruption is prevented or significanlty reduced by trephination process which requires a small-sized needle to create the channels [Sweigart *et. al.*, 2001]. All these methods have been shown to solve the problem only when the tears are not so complex and the meniscal tissues are not degenerated to a great extent.

Shelbourne *et. al.* (2004) compared meniscal repair with partial meniscectomy in patients who underwent anterior cruciate ligament reconstruction and had a buckethandle type tear in their lateral menisci. They reported that there was no significant difference between these groups except that pain level was reduced in the patients whose menisci were repaired. It was also stated that a further follow-up is required to decide on a final recommendation [Shelbourne *et. al.*, 2004].

1.2.2.3. Meniscus Replacement

The third method used to treat meniscus injuries is to replace the damaged tissue with either a natural or synthetic material. Meniscal allografts [Arnoczky *et. al.*, 1992; Jackson *et. al.*, 1992], meniscal prostheses or substitutes [Messner *et. al.*, 1993 and 1994; Sommerlath *et. al.*, 1993; Goble *et. al.*, 1999; Johnson *et. al.*, 2000; Kobayashi *et.*

al., 2003] are the three main approaches utilized to date. Results obtained from meniscal allografts seem to be promising but there are no long term results yet. Moreover, there are certain constraints like paucity of donor tissue, complexity of the surgery, risk of disease transmission, and the occurrence of articular degeneration after transplantation, which limit meniscal transplantations.

An unseeded Teflon-net was tested in 1983 as a replacement material and followed up for a period of 12 months in a canine model, and articular degeneration was observed [Toyonaga *et. al.*, 1983]. Then in 1986 it was followed by combinations of carbon fibers (CF), poly(L-lactic acid) (PLLA) and polyurethane (PU) on canine models, again without cells, which resulted in fibrous tissue ingrowth but still degeneration of articular cartilage was present [Veth *et. al.*, 1986; Leenslag *et. al.*, 1986]. Teflon, (polytetrafluoroethylene, PTFE) [Messner *et. al.*, 1994], polyvinyl alcohol hydrogel (PVA) [Kobayashi *et. al.*, 2003 and 2005; Holloway *et. al.*, 2010], tendon grafts [Johnson *et. al.*, 2000], and Dacron [Messner *et. al.*, 1993] have also been used but the main important problem with these materials was the low mechanical properties they exhibited.

Regarding the use of natural materials, it can be said that so far, collagen-based sponges have produced promising results and a well known product is collagen-GAG scaffold developed by Stone *et al.* (1990; 1992; 1997) called the Collagen Meniscus Implant (CMI). It is already in phase II clinical trials and is being evaluated in a multicenter clinical trial in the USA. CMI was used in many partial medial meniscal replacement applications by its inventors. Rodkey *et. al.* (1997) reported an improvement in the pain and activity scores, increase in the amount of meniscal tissue and inhibition of cartilage degeneration upon using these collagen based implants. These collagen-based constructs are not suitable for large tears because they need an outer rim for fixation and also it is difficult to suture. Moreover, CMI implants were shown to shrink after a period of time, the mechanical properties are inferior to those of the native meniscus and are not
suitable for severe meniscal injury treatment or for total meniscectomy [Buma *et. al.*, 2004; Schoenfeld *et. al.*, 2007].

Another choice is the use of an autograft from a tendon to replace the meniscus, expecting the graft to be converted into fibrocartilage [Sweigart *et. al.*, 2001; Schoenfeld *et. al.*, 2007]. The success level of meniscal transplants is 80%; however, efficiency problems after 7-8 years of transplantation and articular degeneration occurrence are not yet overcome.

1.3. Tissue Engineering of the Knee Meniscus

Tissue engineering of knee meniscus seems to be a viable alternative for the treatment of meniscal complications. There exists a variety of definitions for tissue engineering; one of the earliest and most commonly referred to is that of Langer and Vacanti (1993) "tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ". The main components of a tissue engineered product in general are the cells, bioactive molecules such as growth factors that guide the differentiation of stem cells and the scaffold material on which the cells are seeded.

The principle of tissue engineering of meniscus is that first appropriate cells are obtained from a biopsy, isolated and proliferated *in vitro* to obtain sufficiently high 10^5 - 10^6 cell number. Meanwhile, the scaffold is prepared in the desired shape and the material of which is chosen depending on the properties needed (degradation duration, mechanical strength, processability). The cells are then seeded onto the scaffold and cultured for a certain duration *in vitro*. In general, long cell cultures are preferred to obtain a mature tissue, which is ready for implantation in the human body (*Fig. 6*). In addition to selection of cell type and material, addition of growth factors, use of bioreactors and dynamic vs static incubation are the choices that need to be made. These are especially important for load-bearing tissues, thus, it is crucial for engineering of meniscal tissues of the avascular zone.



Figure 6. A schematic representation of tissue engineering of knee meniscus.

1.3.1. Cell Type and Source

In the tissue engineering of meniscus, either mature cells that are differentiated or progenitor cells that can differentiate *in vivo* to the phenotype wanted can be used [van der Bracht *et. al.*, 2007]. Autologous fibrochondrocytes, are the most widely used cells in order to avoid the problems associated with allogenic and xenogenic sources. These cells are generally obtained from the contralateral meniscus or the healthy regions of the damaged tissue of the patient. It is important that the cells should easily expand *in vitro* and reach large numbers for *in vitro* tests. It has been reported that in order to produce a tissue of 1 cm³ aproximately 25 x 10^6 to 37 x 10^6 cells are needed [Hoben *et. al.*, 2006]. Another problem is that the cells should be isolated from adult tissues since these cells have shown reduction in their synthetic profile as the age increases [Webber *et. al.*, 1986].

Another cell type that is commonly used is the chondrocyte. In comparison to the fibrochondrocytes, they are easier to obtain, have a higher capability of increasing in number in cell culture conditions and have a better synthetic profile for collagen and GAG synthesis. Their source can be cartilageneous tissues like articular, auricular or nasal one [Hoben *et. al.*, 2006; Marsano *et. al.*, 2007; Drengk *et. al.*, 2008].

Dermal fibroblasts were also tested in meniscus tissue engineering as they can be easily isolated from biopsies and easily proliferated [Mizuna *et. al.*, 1996].

1.3.2. Growth Factors

Stem cells have the potential to differentiate into different tissues and self renew if necessary conditions are provided. The main challenge of working with stem cells is that an appropriate method should be found to induce them into fibrocartilage differentiation. In order to achieve this, compounds like dexamethasone, ascorbic acid, insulin, transferrin, selenous acid, L-proline and sodium pyruvate are added to the culture medium [Mackay *et. al.*, 1998]. Moreover, various growth factors such as the transforming growth factor β (TGF- β), platelet derived growth factor bb (PDGF bb), insulin growth factor-I (IGF-I), fibroblast growth factor 2 (FGF2) and bone morphogenetic proteins 2 and 6 (BMP2 and BMP6) were also added [Webber *et. al.*, 1986; Pangborn *et. al.*, 2005; Hoben *et. al.*, 2006; van der Bracht *et. al.*, 2007; Drengk *et. al.*, 2008]. Each growth factor influences different processes such as proliferation, GAG production, cell migration, DNA synthesis, proline, sulphate uptake or differentiation [Pangborn *et. al.*, 2005], and therefore, the choice of the growth factor is done based on the cell type and the target tissue.

1.3.3. Scaffold Types: Synthetic and Natural

The most important ingredient of tissue engineering of meniscus is the material used to prepare the scaffold. It can be said that to date a variety of scaffold materials, both synthetic and natural, have been utilized. Synthetic polymers are commonly used by the researchers and the most popular ones are Teflon, CF, PU, PLLA, poly(L-lactic acid-co-glycolic acid) (PLGA), poly(ε -caprolactone) (PCL), polyesters, polyvinyl alcohol (PVA) hydrogel or their combinations [Toyonaga *et. al.*, 1986; Veth *et. al.*, 1986; Leenslag *et. al.*, 1986; Webber *et. al.*, 1990; Ibarra *et. al.*, 2000; Kobayashi *et. al.*, 2003; Buma *et. al.*, 2004; Schoenfeld *et. al.*, 2007]. In addition to some being biodegradable, polymeric scaffolds have the advantage that their properties such as degradation rates, porosity and strength can be controlled. Moreover, they are often cheaper thus can be tested in higher amounts, they have a larger variety and are easier to manipulate. A number of studies have been carried out *in vitro* and *in vivo*. A group studying meniscal tissue engineering is Ibarra and colleagues, whose scaffolds were seeded with autologous meniscus fibrochondrocytes and the cell carrier was PGA or PLGA. New tissue formation and the presence of collagen and GAG produced by the cells were reported histologically. Their

results were promising even though they claimed that more work need to be held to optimize the conditions [Ibarra *et. al.*, 2000]. Another study carried out by Kang *et. al.* (2006) attempted regeneration of a whole meniscus in a rabbit model where polyglycolic acid (PGA) scaffolds were seeded with rabbit meniscal chondrocytes. They have shown that the regenerated new meniscus was similar to the natural one in terms of histological appearance but no long term *in vivo* results were given and no mechanical testing was done on the scaffolds [Kang *et. al.*, 2006]. Webber *et al.* (1990) in another study used RGD sequences on their fibrochondrocyte-seeded scaffolds and they concluded that RGD sequences enhance cell attachment on scaffold surfaces and make it a good candidate for meniscus tissue engineering applications [Webber *et. al.*, 1990]. Promising results in the treatment of cartilage degeneration was obtained by Kobayashi and colleagues who used PVA hydrogels as a cell carrier in a rabbit model; their results however, were short term (1.5 years) [Kobayashi² *et. al.*, 2003].

Nerurkar *et. al.* (2011) have used poly(ε -caprolactone) (PCL) nanofibers seeded with mesenchymal stem cells to understand the interplay between the material composition and the mechanical properties of the scaffold since it is important to design a functional engineered tissue. The main aim of the study was to observe the influence of GAG and collagen depletion on both compressive and tensile properties of the scaffolds. The inner annulus fibrosis, which is similar in composition with meniscus regarding the amount of collagen and GAG, was the model they used to understand the role of GAG in tensile behavior of the natural tissue. What they report is that GAG removal by chondroitinase resulted in a significant decrease in the compressive properties of the scaffolds to a significant extent. On the other hand, while GAG removal was observed to increase the tensile properties of both aligned PCL nanofibers and the native annulus fibrosus tissue, collagen removal decreased significantly the tensile properties. All these findings showed that while GAG presence is important for resisting the compressive stresses that

meniscus tissue undergoes, while presence of collagen and absence of GAG increases the stiffness of the tissue under tensile forces [Nerurkar *et. al.*, 2011].

Among the natural materials, small intestine submucosa [Cook *et. al.*, 1999; Gastel *et. al.*, 2001], periosteal tissue [Walsh *et. al.*, 1999], perichondral tissue [Bruns *et. al.*, 1998] and collagen [Stone *et. al.*, 1996 and 1997] were also used. One of the earliest trials that were done to engineer a meniscus tissue used fibrin clot which was seeded with bone marrow mesenchymal stem cells or fibrochondrocytes but did not end with successful results [Isoda *et. al.*, 1998]. Mueller *et al.* who investigated *in vitro* contractile behavior of bovine meniscal cells seeded on collagen type I and type II-GAG-based scaffolds reported that while after three weeks, the collagen type I-GAG scaffolds showed a 50% shrinkage while those based on collagen type II did not. It was stated that meniscal cells can express myofibroblast phenotype due to the presence of α -smooth muscle actin positive cells [Mueller *et. al.*, 1999].

One of the few relatively successful implants is CMI, which is used without cells and therefore, is not a tissue engineered product. A few studies were carried out on cell-seeded CMI but as a research application rather than a commercial product. Martinek *et. al.* (2006) seeded CMI with autologous sheep fibrochondrocytes and observed that the macroscopic and histologic performance of the implant was improved. Compared to the unseeded CMI, the cell seeded implant showed enhanced vascularization, a higher amount of ECM, accelerated scaffold remodeling; however, further studies were needed to show its feasibility for human applications [Martinek *et. al.*, 2006].

It can be concluded that even though many natural and synthetic materials were studied for use in meniscus regeneration, none of them was found to be totally satisfactory. An ideal meniscal implant should be biodegradable, have an appropriate degradation rate, ability to promote cell proliferation and differentiation, and have appropriate mechanical properties.

1.4. Aim and Novelty of the Study

The goal of this study was to design a meniscus substitute to be constructed using tissue engineering. The design involved a novel multilayered structure from collagen type I. The main approach was to prepare a construct based on a natural material which would be biocompatible and have optimum mechanical properties in terms of tension, compression and shear. The 3D construct was formed by combining three different foams with different composition, pore size and mechanical properties and placing electrospun mats in between these layers of foams. The foam in the upper layer was composed of Coll-CS-HA prepared by freezing at -20°C followed by lyophilization. The CS:HA ratio was chosen to be similar to that of the native tissue. On the other hand, the foams in the middle and bottom layers were made only of collagen and they were prepared at -20°C and -80°C, respectively. Aligned Coll-PLGA nano/microfibers obtained by electrospinning process were placed in between the layers of these three foams. They were incorporated into the 3D construct in order to mimic the structure of the native meniscus tissue and to enhance the mechanical properties of the whole construct.

For the unique design of the 3D construct, a patent application was made. The design is modelled after the medial meniscus; however, we believe that it will be suitable for both (medial and lateral) menisci applications.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Collagen type I from bovine Achilles' tendon (BACT I), chondroitin sulfate A (CS) sodium salt from bovine trachea, hyaluronic acid (HA) potassium salt from human umbilical cord, amphotericin B, hematoxylin, phloxine, saffron, Fast Green FCF, Collagenase type I and II from Clostridium Histolyticum, trypsin from bovine pancreas, hyaluronidase, Thiazolyl Blue Tetrazolium Bromide (MTT), glutaraldehyde (GLU), glycine, N-hydroxysuccinimide (NHS), trizma base, coomassie brilliant blue, normal goat serum (NGS), sodium azide, propidium iodide (PI), 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), FITC-labelled phalloidin, paraformaldehyde (37%), neutral buffered formalin (10%), silanized-prep slides and sodium cacodylate trihydrate were obtained from Sigma-Aldrich (USA and Germany). 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), Safranin-O, bovine serum albumin (BSA) and penicillin/streptomycin were purchased from Fluka (Switzerland). Rabbit antihuman collagen type I and II were obtained from Novotec (France) whereas fetal bovine serum (FBS), 1-ethyl-3-(3dimethylaminopropyl) carbodiimide (EDC), trypsin-EDTA (0.25%), SnakeSkin pleated dialysis tubing and freezing microtome blades were bought from HyClone, Thermo Scientific (USA).

Liquid diaminobenzidine (DAB) + substrate chromogen system, Dako EnVision System-HRP labeled polymer antirabbit and Tris buffer (TBS) were obtained from DAKO (USA). Ethanol, acetic acid (HAc), sodium dihydrogen phosphate dihydrate, disodium sodium chloride. hydrogen phosphate heptahydrate, acetone, methylcyclohexane, lithium carbonate and Tween-20 were from Merck (Germany). In addition to these, SpectraTM Multicolor broad range protein ladder, poly(L-lactic acidco-glycolic acid; PLGA (50:50), Resomer® RG503H) and Triton-X 100 were supplied by Fermentas (Thermo Scientific, USA), Boehringer-Ingelheim (Germany) and AppliChem (USA), respectively. Trypan blue (0.4%) and DMEM/F12 (1:1) were purchased from GIBCO Invitrogen Inc. (USA) whereas genipin (GP) was bought from Wako Pure Chemical Industries (USA).

2.2. Methods

2.2.1. Collagen Type I Isolation from Rat Tail

Collagen Type I was isolated from Sprague-Dawley rat tails. Tails were dissected by a full incision and then stripped. All the tendons were dissolved in 0.5 M acetic acid (2-3 days) at 4°C. The suspension was filtered through glass wool and then dialyzed at 4°C against phosphate buffer (pH 7.2). When the collagen was precipitated and a milky solution formed, it was centrifuged (Sigma 3K30, Germany) (16000 g, 10 min) at 4°C. After that it was maintained overnight in 0.15 M acetic acid, precipitated by addition of 25 g NaCl and centrifuged. A second dialysis was performed followed by centrifugation. The collagen pellet obtained was sterilized in 70% alcohol for 2 days. After centrifugation the product was lyophilized (Labconco Freezone 6, USA).

2.2.2. Collagen Characterization

Collagen purity was assessed by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, collagen isolated from Sprague-Dawley rat tail was treated at 95°C for 5 min in mercaptoethanol. Then collagen solution was loaded on 10% (w/v) gels and visualized by staining with 0.2% (w/v) Coomassie Brilliant Blue solution.

2.2.3. Scaffold Preparation

2.2.3.1. Collagen Foam Preparation

A suspension of 2% (w/v) bovine Achilles' tendon collagen type I in 0.5 M acetic acid was prepared. Then the slurry was homogenized (Sartorius Homogenizer, BBI-8542104, Potter S, Germany) for 5 min at a speed of 1000 rpm until the solution was homogeneous, which was then poured into an appropriate container and frozen either at -20°C or -80°C for 24 h. Next, the frozen suspension was lyophilized under vacuum for 15 h. The lyophilized matrix was then physically crosslinked with dehydrothermal (DHT) treatment by keeping the samples under vacuum at 150°C for 48 h (vacuum oven Model 281A, Cole-Parmer, USA).

2.2.3.2. Collagen-Chondroitin Sulfate-Hyaluronic Acid (Coll-CS-HA) Foam Production

Collagen suspension was prepared as mentioned in the section above. Meanwhile, CS and HA were mixed together and dissolved in 0.5 M acetic acid. Then both the liquids were mixed and homogenized at 1000 rpm for 5 min in order to obtain a homogeneous

suspension. The final Coll:CS:HA ratio was arranged to be 33:15:1 (w/w) in order to mimic the natural meniscus composition. The suspension was frozen at -20°C for 24 h followed by lyophilization as above.

2.2.3.3. Collagen-PLGA Fiber Production by Electrospinning

Collagen type I isolated from Sprague-Dawley rat tails was used in preparing collagenbased fibers. A solution of 10% (w/v) RTC I was prepared in 1,1,1,3,3,3-hexafluoro isopropanol (HFIP). Meanwhile a solution of 15% (w/v) poly(L-lactic acid-co-glycolic acid) (PLGA) (50:50) polymer in HFIP was prepared. Then a blend of Coll-PLGA was prepared at a ratio of (1:1, v:v). The solution was then put into a 10 mL syringe with a blunt ended needle (22 Ga x 1.25''; 0.70 mm ID x 32 mm) and connected to a syringe pump (New Era Pump Systems Inc., UK). The needle tip was connected to a high voltage source (Gamma High Voltage Research, USA). After that the solution was electrospun at 21 kV, the flow rate and the distance between the collector and the syringe tip were set as 10 μ L/min and 14 cm, respectively. Depending on the collector type, fibers were prepared in both aligned and random form.

2.2.3.4. Preparation of the 3D Construct

The three different foams, namely the Coll foams prepared after freezing at -20°C and -80°C and the Coll-CS-HA foam were prepared as mentioned above. Then they were physically crosslinked with dehydrothermal (DHT) treatment at 150°C for 48 h. These foams were packed one on top of the other with Coll-PLGA fibrous layers in between. The organization was as follows: on top of Coll foam prepared by freezing at -80°C, 5 layers of Coll-PLGA fibers were attached by adding some collagen solution (15%, w/v) prepared with rat tail collagen dissolved in HFIP. The solution was added only at the periphery by means of a syringe. Then, the foam prepared by freezing at -20°C was placed on top. Again 5 fibrous layers were attached on the foam surface followed by the final layer, the Coll-CS-HA-based foam (top layer).



Figure 7. A schematic presentation of the 3D construct.

2.2.3.5. Final Stabilization of the 3D Construct

The 3D construct consisting of foams and fibers was crosslinked in order to stabilize the wholw structure, and to increase its mechanical properties. Therefore, a chemical crosslinking was performed. The crosslinker used was EDC/NHS at a ratio of 170 mM/217 mM. The 5 layered construct was immersed in a 50 mM NaH₂PO₄ solution (pH, 5.5) containing EDC/NHS for 2 h. Then the pH was stabilized with 0.1 M Na₂HPO₄ (pH, 9.1) for 1 h. The following steps were washing with 1 M NaCl for 2 h, with 2 M NaCl for 1 day with several changes of solution and finally several times with distilled water. The final construct was then lyophilized for 8 h after freezing at -20°C.

2.2.4. Measurement of Matrix Pore Size Distribution

Pore size distribution of the foams was determined using a mercury porosimetry (Quantachrome Corporation, Poremaster 60, USA) under low pressure conditions (50 psi).

2.2.5. Measurement of Fiber Diameter

In order to examine the electrospun nanomat structure, scanning electron microscopy (SEM) was used. Samples were sputter coated with gold-palladium (10 nm) and viewed using a Quanta FEI SEM (USA). Fiber diameter was measured at 3 random fields by using Image J program (NIH, USA). The average size of fibers was measured from the micrographs at 2000 fold magnifications. The image of each sample was divided into four regions by applying a grid and the mean diameter of fibers was calculated using the results of 25-30 measurements from three randomly selected fields.

2.2.6. Mechanical Characterization of Foams and Selection of the Best Crosslinker

A mechanical testing system (Lloyd LRX 5K, Lloyd Instruments Limited, UK) with a load cell of 100 N was used by applying tension, compression and shear forces.

2.2.6.1. Uniaxial Tensile Test of the Foams

Foams (2%, w/v) were prepared from BATC I in a rectangular-shaped plastic container. Then samples (35 mm long, 10-12 mm wide, and 3-5 mm high) were cut. Some samples (n = 5) were first dehydrothermally (DHT) treated at 150°C for 48 h and then further crosslinked with the following crosslinkers: glutaraldehyde (0.5%, w/v) (GLU), genipin (GP) (0.1%) and EDC/NHS (170 mM/217 mM) and for 30 min, 2 h and 48 h, respectively. Another set of foams (n = 5 for each case) was only crosslinked with GP, EDC/NHS, GLU, or DHT at the same concentrations. The control set (n = 5) was uncrosslinked (UXL). The tensile testing speed was set as 0.5 mm/min according to ASTM D882-00 and the gauge length was 10 mm. Tensile strength (MPa) and elastic modulus (MPa) were calculated as described in the ASTM standard using the load-displacement graphs (*Fig. 8*).



Figure 8. A typical load-displacement curve obtained from tensile test of collagen foams.

2.2.6.2. Unconfined Compression Test

Unconfined compression test was performed on BATC I-based foams (2%, w/v) using a displacement rate of 0.5 mm/min. Samples were prepared and crosslinked as described in section 2.2.6.1. The resultant load vs displacement graphs were used to calculate the linear elastic modulus (E*), elastic collapse stress and strain ($\sigma_{el}^*, \epsilon_{el}^*$) and the slope of the collapse plateau ($\Delta\sigma/\Delta\epsilon$) for each sample (n \geq 5). E* was determined as the slope of the initial linear part of the load–displacement curve. $\Delta\sigma/\Delta\epsilon$ was determined as the slope of the second linear region.



Figure 9. A representative load vs displacement curve of the collagen-based scaffolds under unconfined compression. Load and displacement values were divided by cross sectional area and initial length, respectively, to obtain the corresponding stress and strain.

Elastic collapse stress (σ_{el}^*) and elastic collapse strain (ϵ_{el}^*) were determined from the intersection of the first and second linear regions of the load vs displacement graphs (*Fig. 9*) [Harley *et. al.*, 2007].

2.2.6.3. Shear Test on Collagen Foams

After deciding on best crosslinker using the compressive and tensile property data of the foams crosslinked as described in sections 2.2.6.1 and 2.2.6.2, shear testing of the foams was performed. The test was done by placing the sample between two grips at a gauge length of 12 mm and while one of the grips was kept static the other one applied a load in the z direction as shown in *Figure 10*. Samples were prepared with the same dimensions as in the case of tensile testing (1 cm x 3 cm) and the strain rate was set to 0.5 mm/min. The test was done on UXL, EDC/NHS crosslinked and DHT+EDC/NHS crosslinked -20°C and -80°C collagen foams and on Coll-CS-HA foams. Shear modulus (kPa) was calculated from the linear elastic region of the load vs displacement graphs.



Figure 10. The shear test set up used with collagen-based foams.

2.2.7. Degradation Profile of Foams In Situ

Initially, both uncrosslinked (UXL) and crosslinked (EDC/NHS and DHT+EDC/NHS) foams were placed in sterile PBS in a shaking incubator at 37°C for a total period of 3 months; while the samples were removed once every 2 weeks, washed several times with distilled water, lyophilized to complete dryness and weighed. The test was performed according to ASTM F 1635-04a ASTM procedure ('Standard test method for *in vitro* degradation testing of hydrolytically degradable polymer resins and fabricated forms for surgical implants'). The same degradation procedure was applied to Coll-CS-HA foams and 3D constructs for a period of 1.5 months where the samples were weighed weekly.

2.2.8. Surface Modification of Foams with Oxygen Plasma Treatment

Oxygen plasma was applied using a plasma device (Advanced Plasma Systems Inc., USA) expecting to increase the surface porosity of the -20°C and -80°C collagen foams. The plasma instrument is shown in *Figure 11*. Oxygen exposure time and RF power were set as 3 min and 200 W, respectively.



Figure 11. Plasma treatment instrument.

2.2.9. In Vitro Studies

2.2.9.1. Fibrochondrocyte Isolation

After surgical removal from the patients following their written consent (Hacettepe University Department of Orthopaedics) meniscus biopsies were placed in sterile PBS containing 0.1% antibiotics (penicillin-streptomycin). Cells were isolated as described by Webber *et al.* with some modifications. Briefly, the tissue sample was cut into very small pieces (1 mm x 1 mm) and then the minced tissue was treated with 0.3% collagenase type II at 37°C for 24 h in order to free the cells from the tissue. The cells were then washed three times with 10% FBS and the suspension was centrifuged at 3000 rpm for 5 min. Finally, the cells were resuspended in 50:50 DMEM/F12 medium containing 10% FBS, 1% penicillin-streptomycin, L-glutamine and 15 mM Hepes, cultured in 25 cm² tissue culture flasks and then grown under incubator conditions of 37°C and 5% CO₂. Cells were then trypsinized, separated, washed, and seeded into new 75 cm² tissue culture flasks to obtain a sufficient number of cells.

2.2.9.2. Cell Characterization

2.2.9.2.1. RT-PCR for Detection of Collagen Type I and Type II

The isolated cell phenotype was checked by RT-PCR through isolating total RNA, converting it to cDNA and finally checking for collagen type I and II expression by reverse PCR.

2.2.9.2.2. Flow Cytometry Analysis

Microscopic particles like various cell types can be measured and analyzed by flow cytometry, a technique that involves passage of a cell suspension in a fluid stream by an electronic detection apparatus. A beam of single wavelength light is passed through a hydrodynamically-focused fluid stream. It is composed of several detectors namely forward scatter, side scatter and some other fluorescent detectors and has been used in the diagnosis of different health disorders; but it can also be applied to separate cell populations or sort particles according to their properties (ex. cell size). Since the cells used in this study were isolated from human meniscus biopsies, cell viability and cell phenotype had to be checked to make sure that no other cell populations were present.

Flow cytometry analysis, which was carried out at Kocaeli University KÖGEM (Center for Stem Cell and Gene Therapies Research and Practice), was used to determine the phenotypic characteristics of cells isolated from human meniscus the cells. The cells were harvested and resuspended in their own culture medium at a concentration of 5 x 10^5 cells/mL. Hundred microliters of this suspension was incubated with 2 μ L of the appropriate mAb during 45 min at 4°C. After washing with CellWash buffer (BD Biosciences, USA) cells were analyzed on a FACSCalibur (BD Biosciences, San Diego, USA). For each sample approximately 10,000 events were analyzed. Dead cells were excluded using propidium iodide (PI) staining. The data were analyzed with Cell Quest software (BD Biosciences, USA) and the forward and side scatter profiles were gated out of debris and dead cells. Immunophenotyping of human meniscus cells was performed with antibodies against the following human antigens: CD14 (lipopolysaccharide (LPS) receptor, monocyte differentiation antigen; BD Biosciences, USA, 342408), CD15 (3-fucosyl-N-acetyl lactosamine; BD Biosciences, USA, 333172), CD31 (PECAM-1, expression limited to endothelial cells, platelets, leukocytes, and their precursors; BD Biosciences, USA, 555445), CD34 (Hematopoietic Progenitor Cell Antigen; BD Biosciences, USA, 333172), CD44 (Hyaluronate/lymphocyte homingassociated cell adhesion molecule-HCAM; BD Biosciences, USA, 555479), CD45 (Protein tyrosine phosphatase, receptor type, C/ PTPRC/ leukocyte common antigen/cell marker of hematopoietic origin; BD Biosciences, USA, 342408), and CD105 (Endoglin, expressed on many cell types including endothelial cells, articular chondrocytes, bone marrow derived mesenchymal stem cells, monocytes etc.; Serotec MCA 1557F, 0.1 mg). CD15, CD31, CD45 and CD105 were fluorescein isothiocyanate (FITC)-conjugated, whereas CD14, CD34 and CD44 were phycoerythrin (PE)-conjugated. An isotype control was included in each experiment, to calculate the specific staining.

2.2.9.3. Cell Culture and Cell Seeding on Scaffolds

Cells were passaged until passage 6. The cell culture medium used was DMEM/F12 (1:1) containing 10%, v/v fetal bovine serum, 1% antibiotics (penicillin+streptomycin) and 1 μ L/mL amphotericin B. Cells were stored frozen, with their appropriate medium and 10% DMSO, in a liquid nitrogen tank at -196°C until use. Following thawing, the cells were used after reaching confluency. During culturing the cells were incubated in a CO₂ incubator (Sanyo MCO-17 AIC, Sanyo Electric Co. Ltd., JAPAN) at 5% CO₂ and 37°C. The *in vitro* experiments were conducted under standard culture conditions.

For cell seeding, the medium was discarded from the flask, the cells were detached with Trypsin (0.25%)-EDTA treatment for 2 min at 37°C. After detachment, trypsin was deactivated with serum and the cells were collected by centrifugation at 3000 rpm for 5 min. The supernatant was discarded and the cell pellet was resuspended in 3 mL fresh medium. After that, the cells were stained with Trypan blue and counted with a hemocytometer. After determining the live cells, 50 μ L of fibrochondrocyte cell suspension was seeded onto collagen foams at a cell density of 1x10⁵ cells/cm². The scaffolds were then incubated in the CO₂ incubator for 2 h in order to allow the cells to

attach to the scaffold. Finally, 1 mL of medium was added into each well and the medium was daily changed.

2.2.9.4. Cell Proliferation on Scaffolds

Cell proliferation on collagen foams and 3-D construct was assessed by using MTT assay. Cells were seeded onto scaffolds at a density of 1×10^5 cells/sample. Scaffolds were rinsed with PBS before adding MTT solution (1 mg/mL). Then, scaffolds were incubated in MTT solution in a CO₂ incubator at 37°C for 2.5 h. Formazan crystals produced by live cells were then extracted using acidified isopropanol (2 mL) by transferring the cell seeded scaffolds into 15 mL centrifuge tubes. The samples were stirred well until all the crystals were dissolved. After that, 150 µL of solution was taken and transferred into 96-well plates and the optical density (OD) was measured spectrophotometrically at 550 nm. MTT testing was done on days 1, 7, 14 and 21 in triplicate.

2.2.9.5. Flurorescence Microscopy

Fluorescence microscope was utilized to study cell morphology and cytoskeleton organization. Cells were seeded on the collagen foams as was described above. At the end of 1, 21 and 45 days the samples were stained and prepared for microscopic observation as will be presented in the following sections and studied.

2.2.9.5.1. Staining with FITC-Labeled Phalloidin and DAPI

Staining with FITC-labeled Phalloidin was performed in order to observe the orientation of cytoskeletal actin filaments of meniscus cells. Samples were fixed with 3.7%

formaldehyde for 30 min and then washed with PBS (pH 7.4). Cell membranes were permeabilized by immersing in a 1% Triton X-100 solution for 5 min at room temperature. After washing with PBS, samples were incubated at 37°C for 30 min in 1% BSA containing PBS solution in order to block nonspecific binding. After washing, FITC-labeled Phalloidin (1:100 dilution in 0.1% PBS-BSA) was added and samples were incubated for another 1 h. After that the specimens were rinsed with PBS, DAPI solution (diluted 1:1000 in PBS solution) was applied onto the scaffolds which were then incubated at 37°C in dark for 45 min. Finally, the samples were washed with 1% PBS-BSA solution, transferred to a microscope slide and observed under a fluorescence microscope (Olympus IX 70, Japan).

Confocal microscopy (Leica DM2500, Germany) was used to assess the distribution of cells within the fibrous structure of the scaffolds. Specimens were stained with FITC-labeled Phalloidin and DAPI and an argon laser was used to excite the dyes and examine the specimens at wavelengths of 488 nm and 532 nm.

2.2.9.6. HPS Staining

Five micrometer thick sections of foam samples that were cut using freezing microtome (Leica CM1510 S, Germany) were washed twice with methylcyclohexane, once with 100% alcohol and then with running water for 4 min, 4 min and 2 min, respectively. After that, sections were stained with hematoxylin for 5 min, followed by rinsing with running water for 2 min, 10 s wash with acid alcohol and then 2 min with running water. The slides were then put into lithium carbonate for 15 s and in tap water for 3 min. Cell sytoskeleton was stained with phloxine for 5 min and rinsed with running water for 4 min. Washing with 70% and 100% alcohol for 10 s and 1 min was followed by staining with saffron dye for 4 min, twice wash with 100% alcohol for 10 s and three times with methylcyclohexane for 2 min. The sections were then mounted onto microscope slides and examined under light microscope (Olympus IX 70, Japan).

2.2.9.7. Safranin O-Fast Green Staining

Sections of samples with 5 μ m thicknesses were taken with freezing microtome. The sections were fixed with cold acetone at -20°C for 10 min, air dried and stained with hematoxylin for 10 min. After washing for 10 min under running water, the sections were stained for 5 min with Fast Green followed by a quick rinse with acetic acid solution (1%, v/v). Finally, samples were stained with 0.1% (w/v) Safranin O for 5 min, mounted and examined under light microscope.

2.2.9.8. Immunohistochemistry of the Samples

Sections of samples with 5 µm thicknesses were transferred to silanized slides and fixed with cold acetone for 10 min. The slides were air dried at room temperature, treated with hyaluronidase (0.2%, w/v in TBS) for 15 min at 37°C and washed with TBS (S3001, DAKO, USA) for 5 min. Anti-human collagen types I and II primary antibodies (Novotec: 20111 and 20211) (diluted in TBS + 3% BSA + 1% NGS) was added onto the sections and allowed to stay overnight. After washing with TBS + 0.2% Tween 20 for 10 min, the unspecific activity was blocked by immersing in TBS + 3% BSA + 0.9% NaCl solution for 20 min. This was followed by inhibition of endogeneous peroxidases by immersing in TBS + 3% BSA + 500 μ L H₂O₂ at 30 vol. + 10 mg NaN₃ for 20 min. Labeling was done with Envision + System-HRP labeled Polymer anti-rabbit (K4002, DAKO, USA) for 45 min. It was rinsed and washed with TBS for 10 min after each of the above steps. Staining with DAB chromogen (K3467, DAKO, USA) (1.5 mL TBS + 0.5 mL kit buffer + 2 drops of DAB) was performed for 2-5 min followed by a 10 min rinse under running water. Sections were counterstained with hematoxylin for 5 min, washed for 5 min with running water and differentiated with aqueous ammonia solution for 2 min. Finally sections were mounted in aqueous mounting medium (S3025, DAKO, USA).

2.2.9.9. SEM Examination

Fibrochondrocyte seeded foams or the 3D multilayer construct were fixed after 1, 21 and 45 days in 2.5% glutaraldehyde in 0.1M, pH 7.4 sodium cacodylate buffer for 2 h and then washed with cacodylate buffer and distilled water several times. After lyophilization, the scaffolds were sputter coated with gold-palladium (10 nm) and studied with a scanning electron microscope (FEI Quanta 200F, USA) at METU Central Laboratoty and UNAM (Bilkent University).

2.2.10. *In vivo* studies

Six months old, New Zealand male rabbits were used to perform in vivo experiments in this study. The experiments were performed at Kırıkkale University, Faculty of Veterinary Medicine. Before the surgical operation, animals were kept in quarantine for 1 week in order to make sure that they did not have any health and especially orthopedics-related problems. Rabbits were anesthetized with intramuscular injection of a combination of 35-40 mg/kg im ketamine and 5 mg/kg im xylazine. The hair on the knee joint was cut and the animals were made ready for operation under aseptic conditions (Fig. 12A). A parapatellar incision was created in the medial part of the knee joint. First, dermal skin and subcutis were incised and then incision was done medial collateral ligament to make possible entrance in the medial compartment of the knee joint. Then, total meniscectomy was carried out (Fig. 12B) by cutting the medial meniscus both at the anterior and posterior positions, and the space created was fitted with the 3D collagen-based artificial meniscus prepared with appropriate dimensions and form mimicking that of the natural medial meniscus of a rabbit (Fig. 12C). After the implantation, starting with medial collateral ligament and the knee joint capsule, all the created incisions were closed. The animals were allowed to be free in their cages and all

the care and handling was done as required. The animals were sacrificed 3 and 10 weeks post operation.



Figure 12. In vivo implantation of 3D construct in the medial compartment of the knee of New Zealand rabbits (6-months old males). (A) Preparation for surgery under aseptic conditions, (B) total meniscectomy of the medial meniscus, and (C) implantation of the costruct.

The final multilayered 3D construct that was used for implantation was seeded with White New Zealand rabbit meniscal cells (fibrochondrocytes) isolated from medial meniscus and incubated for 10 days in culture medium at 37° C under 5% CO₂ in a carbon dioxide incubator. Cell passage numbers used were P2-P4. Three different sample groups were used: (1) control rabbits whose medial meniscus were removed and left as is (n = 7), (2) animals whose medial menisci were replaced with unseeded 3D construct (n = 7) and (3) animals whose medial menisci were replaced with cell-seeded 3D construct (n = 7).

2.2.11. Statistical Analysis

The statistical analysis of the data was carried out using Student's *t-test*. All the results were expressed as mean \pm standard deviation.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Collagen Characterization by SDS-PAGE

Collagen type I was isolated from Sprague-Dawley rat tail, as it was mentioned in Section 2.2.1. After isolation, collagen characterization was done by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in order to make sure that the obtained collagen was collagen type I. The typical SDS-PAGE band pattern for collagen Type I is a doublet at apparent molecular weights of 115 and 130 kDa and another doublet at 215 and 235 kDa (data sheet from Sigma Aldrich Co.). Three collagen batches isolated at different times were used in the test to show the reproducibility of the used procedure. The two doublet-pattern was obtained with all the collagen samples isolated from rat tail, which is a good confirmation of collagen purity (*Fig. 13*) and was referred to as rat tail collagen Type I (RTC I) throughout this study.



Figure 13. SDS-PAGE of collagen Type I isolated in this study from Sprague-Dawley rat tails.

3.2. Scaffold Preparation

3.2.1. Collagen Foam Preparation and Physical and Microscopical Characterization

Commercial, insoluble collagen type I isolated from bovine Achilles' tendon (BATC I) was used to prepare the foams by lyophilizing a suspension of BATC I. Since BATC I does not completely dissolve in acetic acid, it was maintained for a period of 7 days in a shaking incubator at 37°C and then homogenized to obtain finer particles and thus smoother-surfaced foams. Another reason for this homogenization was if large particles would remain, they would integrate with each other less and therefore result in foams with lower mechanical properties, which is unsuitable for meniscus.

Different polymer concentrations (1, 1.5 and 2%, w/v) were tried in order to study the influence of polymer concentration on foam mechanical properties and to choose optimum conditions required for foam production. Foams prepared by freezing at -20°C were initially crosslinked with genipin (GP), a natural crosslinker derived from geniposide present in Gardenia jasminoides fruit, since it is a good crosslinker for proteins and then tested for the influence of crosslinker on the compressive properties of the foam. As it was expected, the higher the solution concentration used to prepare the foams, the higher were the foam mechanical properties (*Table 1*); the highest compressive modulus was obtained with 2%, w/v solution (781.6 \pm 94.6 kPa). A 3-fold increase in the mechanical properties of foams was observed with double crosslinking, with DHT and GP. A similar observation was reported by Tierney *et. al.*, (2009) who stated that doubling concentration of collagen from 0.5% to 1% and increasing the DHT crosslinking temperature and duration from 105°C (24 h) to 150 °C (48 h) resulted in 3-4 fold increase in the compressive modulus of their foams [Tierney *et. al.*, 2009].

Table 1. Compression test results of BATC I-based foams crosslinked with different methods.

Foam Concentration [#] (%, w/v)	Crosslinker Type	E [*] (kPa)
1.0	GP	41.9 ± 8.9
	DHT + GP	223.3 ± 37.4
1.5	GP	123.7 ± 21.7
	DHT + GP	392.6 ± 82.7
2.0	GP	196.7 ± 39.5
	DHT + GP	781.6 ± 94.6

GP: Genipin; DHT: dehydrothermal treatment; [#]: All foams were prepared by freezing at -20°C.

The final goal of this study was to obtain a foam with high mechanical properties and appropriate porosity and interconnectivity. The final collagen solution concentration that was chosen for use in this study was 2%, w/v both because of the mechanical properties and also it was difficult to prepare BATC I solutions with a higher concentration.



Figure 14. Scanning electron micrographs (SEM) of a collagen foam (2%, w/v). (A) Middle surface (magnification: x250) and (B) cross section in vertical direction (Magnification: x150).

Figure 14 shows that the size of pores of a typical 2% (w/v) foam was between 50-200 μ m (*Fig. 14A*) and the foam crosssection was highly porous throughout (*Fig. 14B*) even though the pores were slightly longitudinally oriented and they had a good pore interconnectivity.

3.2.2. Coll-CS-HA Foam Production

A combination of collagen type I (BATC I), chondroitin sulfate (CS) and hyaluronic acid (HA) was used to prepare a softer foam than obtained with BATC I alone. A homogeneous solution was obtained after a 5 min homogenization at 1000 rpm. The CS and HA constituted 15% and 1%, w/w of total weight, respectively, which mimics the composition of the natural tissue; the total amount of collagen used was the same with that used to prepare pure collagen foams.

A skin layer was observed on the top surface of the foam (*Figs. 15A and 15B*), which was also seen in the pure collagen foams. As seen in *Figure 15B*, some fibrous structures were formed and the fiber surface seems to be not smooth but rather needle-like protrusions were seen. Moreover, compared to the pure collagen foams, these foams were more porous ($\sim 150 \ \mu m$) and the interconnectivity was much higher both in the middle layer (*Fig. 15C*) and in the cross section (*Fig. 15D*). These pores would help cell penetration towards the core of the scaffold.



Figure 15. Scanning electron micrographs (SEM) of Coll–CS–HA uncrosslinked sample. (A) Top surface (x500), (B) a higher magnification of top surface (x15000), (C) horizontal cross section and (D) vertical cross section.

3.2.3. Influence of Freezing Temperature on Foam Physical Properties

Three different foams with varying physical properties were produced in this study. Coll-CS-HA-based foam was prepared after freezing at -20°C, whereas two other pure collagen foams were prepared after freezing at -20°C and -80°C, respectively in order to obtain foams with different pores sizes and mechanical properties. It was shown that freezing rate has a significant influence on the pore size of foams prepared by lyophilization [O'Brien *et. al.*, 2005; Harley *et. al.*, 2007]. At lower freezing temperatures, a lower pore size is expected to form due to the small size of ice crystals that form at lower temperature. As it can be observed in *Fig. 16*, the pore size and porosity of foams is higher in the COLL-CS-HA (-20°C) case (*Fig. 16A*), followed by BATC I foams prepared at -20°C (*Fig. 16B*) and -80°C (*Fig. 16C*). These 3 foams were later used as the top, middle and bottom layers of the final 3D tissue engineered construct.

After the lyophilization process, a skin layer was formed on the surfaces of foams (*Figs. 16B and 16C*). The same observation was made by other researchers in our group who prepared collagen foams for constructing an artificial corneal stroma. They suggested that skin layer formation could be an advantage for the production of full-thickness cornea where a top layer of different cell type was to be used [Vrana *et. al.*, 2007]. However, in this study, foams produced at -20°C and -80°C were to be the middle and lower layers of the 3D construct and an open surface was required to allow cell infiltration throughout the whole scaffold. Therefore, both of these foams were treated with oxygen plasma, which is an appropriate method used to slightly modify various polymeric surfaces by adding chemical groups and/or by ablation of a thin surface layer which helps open closed pores on the surface.



Figure 16. Scanning electron micrograph of surfaces of collagen-based foams prepared by lyophilization after freezing at different temperatures. (A) COLL-CS-HA, -20°C; (B) BATC I, -20°C and (C) BATC I, -80°C (Magnification: x500).



Figure 17. Scanning electron micrograph of collagen foams prepared by lyophilization after freezing at -20°C. (A) Untreated and (B) treated with oxygen plasma at 200W for 3 min (Magnification: x500).

The aim of using oxygen plasma treatment in this study was to open the closed pores on the foam surface so that cells can penetrate easily in the layers. As it was expected, plasma treatment increased the porosity on the foam surface (*Figure 17*) and created some bridge-like structures that could help cell-to-cell interaction during cell growth.

3.2.4. Fiber Production by Electrospinning

Several techniques have been used to fabricate nano/microfibers, among which are drawing, template synthesis, wet spinning, phase separation and electrospinning. All these methods have their own advantages and disadvantages and they give rise to fibrous structures with varying characteristics. Electrospinning was the procedure used in this study to obtain random and aligned nano/microfibers. It is a widely used fiber fabrication method in biomedical fields and the main principle is the application of high electrostatic forces to eject continuous fibers from a polymeric solution. It is made up of three basic compartments: a syringe or capillary tube with a needle at the tip, a high voltage source and a metallic conductive collector. The main parameters that influence the process are the potential used, the polymer concentration, the needle diameter and the distance between the needle tip and the collector. Moreover, the resultant fiber characteristics are highly dependent on the polymer type and the solvent used [Deitzel *et.al.*, 2001; Dersch *et. al.*, 2003; Dzenis *et. al.*, 2004; Kim *et. al.*, 2005; Ndreu *et. al.*, 2008]. In this research nanofibers were prepared by using collagen type I, a natural polymer and PLGA, a synthetic polymer, both biodegradable.

It was extremely difficult to prepare BATC I suspensions more concentrated than 3%. In addition to this, since the material that is used in preparing solutions to be electrospun has to be well dissolved and because BATC I is not a totally soluble polymer, RTC I was preferred in the production of electrospun nano/microfibers due to its higher solubility. 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) was used to dissolve RTC I and it is a common solvent used in electrospinning different types of collagens due to its low
boiling point (61°C) and good conductivity, which are very important properties in electrospinning [Mathews *et. al.*, 2002; Liu *et. al.*, 2010]. This composition yielded fine nanofibrous structures (*Fig. 18*). The nanofibrous mats were dried under vacuum for 1 day to remove any trace amount of HFIP and then kept in desiccator until use.

As mentioned in Section 1.1.4.2, it is well known that menisci show different collagen orientations at different layers of the tissue. In the superficial layer and in the lamellar zone (just beneath the superficial layer) a random collagen organization is observed. However, at lower layers a highly circumferential organization of fibers is observed. To mimic this, RTC I-based nanofibers were produced in random (*Fig. 18A*) and parallel oriented form (*Fig. 18B*) by using different collector designs. *Figure 18B* shows that the parallel alignment of the fibers in the mat is quite good and the fiber diameter is more homogeneous compared to the random ones. While the fiber diameter for random collagen fibers was 860 ± 230 nm, the diameter of aligned fibers was lower (535 ± 132 nm), even though the operation conditions were same in both cases.

Collagen nanofibers were reported to fuse or melt after certain treatments like crosslinking in water-based systems [Barnes *et. al.*, 2007; Kinikoglu *et. al.*, 2011]. Therefore, an appropriate crosslinking method had to be found in order to maintain the integrity of the fibrous structure. The fibers of uncrosslinked pure collagen nanofibers were not fused or did not have beads and the fiber surface was smooth (*Fig. 19A*). In order to preserve this structure in aqueous solutions, they were dehydrothermally (DHT) crosslinked and then placed in water for 24 h for a change in the fiber morphology. A slight increase in the diameter and bead-like formations appeared on the fiber surfaces (*Fig. 19C*) especially after water treatment (*Fig. 19E*).



Figure 18. SEM of electrospun RTC I fibers (10%, w/v) obtained with different collector designs. (A) Random and (B) parallel (x2000). Arrow indicates orientation direction

In one application DHT-treated fibers were treated with EDC/NHS to chemically crosslink it. Fiber fusion was observed but the structure was still quite porous (*Fig. 19G*). Barnes *et. al.*, (2007) reported similar results regarding the preservation of fibrous structure and morphology of collagen type II-based products after a treatment with EDC in ethanol. However, even though the structure of their fibers was best maintained by crosslinking with EDC compared to the traditional glutaraldehyde-based crosslinking, still some fiber fusion and gel formation was observed [Barnes *et. al.*, 2007]. In the present study, a lower extent of fiber fusion was observed and this is probably due to the pre-treatment of fibers with DHT.

A common approach used to improve the properties of collagen nanofibers is to blend it with other polymers. In this study, collagen (10%, w/v) was blended with PLGA (15%, w/v) was blended with PLGA (15%, w/v) was blended with PLGA (15%, w/v) was blended with PLGA (15%, w/v) was blended with PLGA (15%, w/v) was blended with PLGA (15%, w/v) was blended with PLGA (15%, w/v) was blended with PLGA (15%, w/v) was blended with PLGA (15%, w/v) was blended with PLGA (15%, w/v) was blended with placed wi

w/v) at a ratio of 1:1 (v/v) and the treatments applied to pure collagen nanofibers were also applied to these Coll-PLGA fibers. When UXL Coll-PLGA fibers were incubated in water for 24 h, the mat became less porous possibly due to the gellation of collagen (*Fig. 19F*). *Figure 19* shows that the best preservation of the fibrous structure was Coll-PLGA fibers that were crosslinked with EDC/NHS (*Fig. 19H*). In the case of DHT, the fibrous integrity seems to be maintained quite well but the pores seen on the surface of fibers might be due to PLGA melting as a result of the high temperature used in DHT (*Fig. 19D*).

The alignment of Coll-PLGA nano-microfibers was not as good as that of pure collagen fibers but still the majority of them were oriented parallel to each other (*Fig. 20*). The alignment level observed by Jose *et. al.*, (2009) was ca. 30° with respect to alignment axis who proposed that this was because the fibers repelled each other due to electrostatic repulsion. Interestingly, the authors considered this deviation as an advantage since it increased the mat pore size and interconnectivity, which is expected to increase cellular penetration [Jose *et. al.*, 2009].

The average Coll-PLGA fiber diameter produced in the present study was in the range of 460 nm and 1.2 μ m. As in this case, a decrease in average fiber diameter has been reported upon increase of collagen content in different collagen/PLGA blends [Liu *et. al.*, 2010].



Figure 19. SEM of collagen-based fibers after various treatments to preserve their fibrous structure. (A) Coll (10%, w/v), UXL, untreated, (B) Coll (10%, w/v)-PLGA (15%, w/v), UXL, untreated, (C) Coll (10%, w/v), DHT (150°C, 48 h), (D) Coll (10%, w/v)-PLGA (15%, w/v), DHT (150°C, 48 h).



Figure 19. (continued) (E) Coll, DHT, 24 h water treatment, (F) Coll-PLGA, UXL, 24 h water treatment, (G) Coll, DHT+EDC/NHS crosslinked and (H) Coll-PLGA, EDC/NHS crosslinked only. Collagen and PLGA concentrations were 10 and 15%, respectively.



Figure 20. SEMs of aligned Coll-PLGA nano-microfibers produced by electrospinning. Arrow shows the main fiber alignment direction.

3.2.5. Preparation of the 3D Construct

3-D artificial meniscus design consisted of three different collagen-based foams with different mechanical properties separated by fibers. The natural meniscus (*Fig. 1*) is softer and jelly-like on the surface and it becomes stronger deeper in the tissue. In order to mimic this, the upper layer of our design was made with a COLL-CS-HA foam and the CS-HA ratio was chosen to mimic the natural human meniscus. As mentioned in section 3.2.3, the middle and lower layers were made with BATC I where the former was prepared by lyophilizing the solution after freezing at -20°C and the latter was prepared by freezing at -80°C.



Figure 21. SEM of the 3D final construct. (A) Cross section (x100), (B) a higher magnification (x300) of A, and (C) image showing presence and alignment of fibers inside the 3D construct (Magnification: x1000). Arrows point to fibrous layers and their alignment. T: Top; M: Middle; B: Bottom; F: Fibers.

After oxygen plasma treatment of foam surfaces, the multilayered 3D construct was prepared according to *Figure 7* as explained in Section 2.2.3.4. The layers were attached to each other by applying a viscous collagen solution (10%, w/v) at the corners of each layer. At least 5 layers of Coll-PLGA fibers were combined by slightly compression to obtain a thick fibrous mat and the layers were attached to maintain the alignment. *Figure 21* is an SEM of the whole construct showing the various foam layers and the fibers in between (*Fig. 21A*).

An important point that should be mentioned here is that through the 3D construct preparation process it was possible to preserve the fibrous structure and integrity even after the final crosslinking treatment with EDC/NHS (*Fig. 21C*). This is very important because in previous studies collagen was shown to dissolve after application of a variety of crosslinking procedures as a result of interaction with water. Eventually, Kinikoglu *et. al.* (2011) found that DHT at high temperatures (150°C for a period of 48 h) was the best approach [Kinikoglu *et. al.*, 2011]. In the present study, the reason for being able to maintain the fibrous structure is the presence of PLGA in the fiber composition; PLGA is not dissolved in aqueous media and EDC/NHS cannot crosslink it.

3.3. Mechanical Characterization of Collagen Foams and Choice of Crosslinker

Collagen-based scaffolds are crosslinked in order to maintain their structural integrity in aqueous media and the most common chemical crosslinkers used are glutaraldehyde (GLU), 1-ethyl-3-(3-dimethyl aminopropyl carbodiimide hydrochloride/N-hydroxy succinimide (EDC/NHS) and a recently popular natural crosslinker, genipin (GP). Genipin crosslinks free amino groups, including those in the lysine, hydroxylysine and arginine amino acids present in collagen forming intramolecular and intermolecular crosslinks (see Appendix B). It has been shown to be 5000 times less cytotoxic than the

other crosslinkers [Cauich-Rodriguez *et. al.*, 1996; Sung *et. al.*, 1999]. Glutaraldehyde also reacts with free amine groups of the polypeptide chains (see Appendix C); however, in many studies it was reported to be cytotoxic [Jayakrishnan *et. al.*, 1996]. Therefore, it was used in low concentrations (0.5%, v/v) when needed in our experiments. The third crosslinker tested in this study was EDC/NHS (in a ratio of 170 mM / 217 mM). The ratio between EDC, NHS and collagen needs to be optimized to have a high extent of crosslinking. EDC is a zero-length crosslinker, which means that it does not remain in the structure after crosslinking. The detailed crosslinking mechanism of proteins with EDC is given in Appendix D.

A physical crosslinking method also, dehydrothermal treatment (DHT), was used in combination with all these 3 chemical crosslinking methods. It involves subjecting collagen molecules under vaccuum at temperatures higher than 90°C, which removes water molecules of the structure and results in the formation of intermolecular crosslinks. All the crosslinkers were used at their optimum conditions, to study their influence on the mechanical properties (both tensile and compression) of foams and based on these properties the optimum crosslinker was found.

The tensile test performed on pure collagen foams prepared by freezing at -20°C showed that the stiffest materials under tension were obtained in the case of double crosslinking, DHT+GP and DHT+GLU, except in the the case of DHT+EDC/NHS; the increase in mechanical stiffness was 2-3 fold. The highest tensile modulus was around 4 MPa and it was obtained with only EDC/NHS (without DHT) treatment followed by DHT+GP, DHT+EDC/NHS and DHT+GLU (*Fig.22*). The uncrosslinked samples had the lowest stiffness showing the important contribution that crosslinking made on foam stiffness. Tensile strength values (*Fig. 23*) almost exactly mimicked the trends observed in the stiffness data and are therefore similarly interpreted.



Figure 22. Tensile modulus values of foams crosslinked with different crosslinkers and tested under tension.



Figure 23. Tensile strength values of foams crosslinked with different crosslinkers.

Unconfined compression test also was performed on uncrosslinked and crosslinked collagen foams prepared with different concentrations and crosslinked as shown above. Calculations were done according to Harley *et al.* (2007) who state that the stress–strain curve for low-density, elastomeric open-cell foams in compression is characterized by three regions: *a linear elastic region*, *a collapse plateau region* and *a densification region* [Harley *et. al.*, 2007]. A similar behavior was observed by collagen-based foams (*Fig. 9*) and all the calculations were done as explained in Section 2.2.6.2.

As in the case of tension test, the highest compressive properties were mostly obtained with foams crosslinked first with DHT and then EDC/NHS. The elastic compressive stress value for the EDC/NHS treated foams was higher than that of all the double crosslinked ones as in the tension studies but the difference was not as significant as earlier. On the other hand, elastic compressive moduli were significantly higher in all cases of double crosslinking compared to single chemical crosslinker use. Interestingly, DHT treated samples had very low compressive moduli (almost the same or slightly higher than the UXL) but when the DHT method was combined with a chemical crosslinker (including EDC/NHS this time) the results were 2-4 folds higher (*Fig. 24*). The difference between the DHT+GP, DHT+EDC/NHS and DHT+GLU in both σ^*_{el} and E were not significant. As a consequence, after taking into consideration both the tensile and compressive results obtained and also the fact that EDC is a non-toxic crosslinker, DHT+EDC/NHS crosslinking method was chosen as optimum and was applied to all foams in the later experiments.

Figure 24. Elastic compressive strengths and moduli of BATC I-based foams crosslinked with different crosslinkers. Note that all foams were prepared after freezing at -20°C and the numerical data is presented in Appendix E.

3.4. Mechanical Properties of Separate Layers of 3D Construct

After the decision on the best crosslinker, all the foams that were to be used in the 3D construct were characterized separately under compression, tension and shear.

3.4.1. Unconfined Compression Test

The upper layer of the 3D construct (Coll-CS-HA, -20°C), when tested for its compressive properties (*Table 2*), a decrease was observed upon treatment with DHT; however, the increase with double crosslinking was significant especially for linear elastic collapse stress (σ_{el}^{*}). With DHT+EDC/NHS crosslinking foam stiffness increased 2-fold. In a study carried out by Harley *et. al.* (2007) collagen-GAG foams with varying pore sizes were produced and the influence of pore size (96-151 µm) on both compressive and tensile properties was determined. No significant change in elastic collapse stress and linear elastic modulus with pore size change was observed. However, a higher compressive modulus of foams with the largest mean pore size was observed but this was claimed to be due to the structural anisotropy of this particular foam.

Table	2.	Compressive	properties	of	COLL-CS-HA-based	foams	prepared	at	-20°C
(upper	lay	ver).							

Samples	Compressive Properties					
	$\sigma_{el}^{\ \ *}(kPa)$	ε _{el} *	E _{el} * (kPa)	$\Delta\sigma/\Delta\epsilon$ (kPa)		
UXL	10.04 ± 1.15	0.14 ± 0.02	85.85 ± 9.04	59.31 ± 8.56		
DHT	5.97 ± 1.43	0.11 ± 0.02	68.42 ± 9.72	36.24 ± 7.85		
DHT+EDC/NHS	23.85 ± 3.93	0.21 ± 0.04	118.31 ± 4.87	88.30 ± 27.50		

 σ_{el}^* : elastic collapse stress; ϵ_{el}^* : elastic collapse strain; \mathbf{E}_{el}^* : linear elastic modulus; $\Delta\sigma/\Delta\epsilon$: collapse plateau modulus.

In a similar study, the influence of different crosslinkers like DHT and various ratios of collagen to EDC and NHS (1:1:5 and 5:2:1) were studied. Foams were tested in the wet state and the highest compressive modulus was obtained in the case of 5:2:1 ratio of Coll:EDC:NHS with a value of 7.2 ± 0.1 Pa. therefore, as the ratio of collagen to EDC was increased the compressive properties were higher. DHT crosslinking on its own was observed to be around 1 Pa [Harley *et. al.*, 2007]. These values are very low compared to what were obtained with Coll-CS-HA foams produced in the present study but the foams in this case were tested in the dry state and they were double crosslinked (*Table 2*).

In the case of foams prepared at different temperatures the difference between them was not so distinct (*Tables 3 and 4*) and again the highest lineat elastic moduli were obtained with DHT+EDC/NHS treated samples. While Coll-CS-HA foams and -20°C collagen foams had similar linear elastic collapse stress and strain values when uncrosslinked, those of -80°C collagen foams were almost 2-fold higher. However an inverse relationship was observed in their compressive modulus values (87.76 ± 14.09 and 85.85 ± 9.04 vs 55.49 ± 2.25 kPa). No significant difference of -20°C and -80°C collagen foams was seen in the case of only DHT, EDC/NHS or double crosslinked samples. However, the difference in the compressive moduli of double crosslinked Coll-CS-HA foams and those prepared by freezing either at -20°C or -80°C was two-fold and this is critically important for the mechanical properties of the whole 3D construct. It should be stated that even on their own in the dry state, both -20°C and -80°C BATC I foams had properties similar to that of natural meniscus (~ 240 kPa compared to 150-220 kPa).

	Compressive Properties					
Samples	$\sigma_{el}^{\ *}(\mathbf{kPa})$	* E _{el}	$\mathbf{E_{el}}^{*}$	Δσ/Δε (kPa)		
UXL	10.34 ± 3.09	0.13 ± 0.06	87.76 ± 14.09	62.96 ± 3.90		
DHT	23.24 ± 2.61	0.24 ± 0.06	94.53 ± 16.36	58.56 ± 8.71		
EDC/NHS	45.65 ± 15.59	0.41 ± 0.08	158.69 ± 32.37	116.77 ±16.84		
DHT+EDC/NHS	82.88 ± 14.35	0.42 ± 0.09	228.31 ± 30.94	114.80 ±44.93		

Table 3. Compressive properties of collagen foams prepared at -20°C (middle layer).

Table 4. Compressive properties of collagen foams prepared at -80°C (lower layer).

Controlog	Compressive Properties					
Samples	σ _{el} *(kPa)	ε _{el} *	E _{el} *(kPa)	$\Delta \sigma / \Delta \epsilon$ (kPa)		
UXL	16.19 ± 1.44	0.36 ± 0.06	55.49 ± 2.25	38.03 ± 2.99		
DHT	34.68 ± 3.72	0.51 ± 0.08	100.88 ± 8.72	38.46 ± 15.13		
EDC/NHS	31.04 ± 7.05	0.24 ± 0.05	151.26 ± 22.96	98.54 ± 56.22		
DHT+EDC/NHS	68.05 ± 5.34	0.31 ± 0.02	242.03 ± 9.85	129.13 ±47.54		

The influence of layer combination in the mechanical properties of the 3D construct was also studied. Testing was carried out with foams in dry state and as shown in *Figure 25*, a significant increase (2-fold) was observed in its compressive modulus (~ 240 kPa compared to 440 kPa of the 3D structure). This is very good espcially since the properties were also twice higher than those of the natural tissue.

Figure 25. Comparison of compressive moduli of dry foams in separate and in the 3D form as crosslinked with DHT+EDC/NHS.

Various studies have been carried out to improve mechanical properties of scaffolds for the replacement of meniscus or repair of meniscus tears. Among these, Holloway and *et. al.* (2010) have obtained promising results. They reinforced poly(vinyl alcohol) (PVA) with non-degradable materials like ultrahigh molecular weight polyethylene (UHMWPE) and poly(propylene) (PP) and an increase in the compressive properties was observed. The highest compressive modulus, 0.24 ± 0.02 MPa, was obtained with 20% PVA after six cycles of freeze-thaw [Holloway *et. al.*, 2010]. This value is quite close to those of our collagen foams but 2-fold lower than the 3D construct. Promising but not adequate results were reported in rabbits with the same material [Kobayashi *et. al.*, 2005].

3.4.2. Uniaxial Tensile Test

Most of the studies that were carried out give information about the tensile mechanical properties of the scaffolds. In addition to compressive properties, Holloway *et. al.* (2010) studied the tensile properties of PVA reinforced either with PP or UHMWPE. In the former (PP) case, the highest tensile modulus obtained was around 8 MPa, however, in the latter (UHMWPE) a significant increase was observed (from 90.6 \pm 21.6 MPa to 258.1 \pm 40.1 MPa). The value was close to that of the natural tissue, and based on these results they reported the suitability of this composite for meniscus replacement; however, it should not be forgotten that this composite material is nondegradable and its integration with the neighbouring tissue will be limited.

In the tensile testing of foams of the 3-layers of the 3D construct, an increase with crosslinking was observed for both Coll-CS-HA and the two collagen foams (*Fig. 26*). The lowest properties were obtained with the UXL foams, the lowest of the uncrosslinkeds was Coll-CS-HA foam and the highest was the -80°C collagen foam. A small increase was seen upon DHT crosslinking and the highest among all was obtained with DHT+EDC/NHS treated foams, except the -80°C collagen foam with which the EDC/NHS treated structures showed higher tensile properties compared to DHT+EDC/NHS crosslinked ones. Probably this difference is due to the longitudinal direction of pores which has been observed to be higher for -20°C collagen foams. Thus,

-80°C collagen foams crosslinked with EDC/NHS were more resistant to higher tensile strengths compared to samples treated with DHT+EDC/NHS. The treatment with DHT did not have a great influence on foam tensile properties.

Figure 26. Young's moduli of tensile tested Coll-CS-HA and Coll foams (-20°C and -80°C) crosslinked with various crosslinkers.

In summary, a significant 8-fold increase in the tensile modulus was obtained with DHT+EDC/NHS crosslinked Coll-CS-HA foams compared to UXL ones; a 2-fold increase occurred to DHT+EDC/NHS treated -20°C collagen foams while almost a 2-fold decrease was shown by -80°C collagen foams (*Fig. 26*).

The influence of the combination of layers on the tensile properties of the 3D construct can be observed in Figure 27. The foams crosslinked with DHT+EDC/NHS were compared since the final construct was crosslinked in that way. A tensile modulus of 2.97 ± 0.33 MPa was obtained with the 3D construct which was significantly higher than individual foams prepared by freezing at -80°C (1.34 ± 0.38 MPa) (P = 0.03), and Coll-CS-HA-based foams $(1.20 \pm 0.37 \text{ MPa})$ (P = 0.002). It should not be forgotten that the 3D construct is made up three different layers with different mechanical properties and with fibrous layers in between, which might have imparted a crucial increase in the mechanical properties of the multilayered structure. Aligned fibers have been shown to increase the tensile strength and modulus of fibrous mats, which is believed to be because of uniaxial direction of fibers and the alignment of molecular chains in the fiber along fiber axis [Hung et. al., 2006]. The more uniform is the fiber structure and morphology the higher are the tensile properties of the fibrous mats. In addition, it has been observed that fiber diameter also affects mechanical properties of the samples since a higher orientation of molecular chains and an increase in the fibrous crystallinity occurs [Wu et. al., 2007; Baji et. al., 2010].

Comparing the tensile properties of the natural meniscus (2-300 MPa) with those obtained in this study, it can be said that the values of the current study are lower for use in total meniscectomy but it could be suitable for partial meniscus replacements or replacement for radial tears. In any case the data presented here is of cell-free foams and one must remember that ECM deposition by the cells make the cell carrier stronger.

Figure 27. Comparison of Young's Modulus of tensile tested foams, individually and as 3D construct. Crosslinkage with DHT+EDC/NHS.

3.4.3. Shear Test

Either dynamic oscillatory or constant shear strain can be applied to meniscus to measure the dynamic or transient shear modulus values. A human meniscus tissue has been shown to have a shear modulus of 120 kPa even though this changes depending on the region where shear stress is applied. Shear modulus in the anterior region has been found to be higher compared to that of posterior and central regions [Buma *et. al.*, 2004].

Shear test was performed in this study on foams crosslinked with different crosslinkers as in the case of compression and tension. To be consistent with the literature results, the shear load was applied up to approximately a 10% strain. The highest shear modulus was obtained from the foams crosslinked with EDC/NHS in the case of -80°C collagen foams (230 ± 22 kPa), followed by DHT+EDC/NHS for -20°C collagen foams (186 ± 71 kPa) and Coll-CS-HA (160 ± 19 kPa), which follows the trends observed in tensile testing; that is, a decrease in the values for -80°C foams upon double crosslinking (*Fig.* 28). The difference between the UXL and DHT+EDC/NHS treated foams in all cases was significant.

Figure 28. Shear modulus (G) values of collagen-based foams prepared and crosslinked in different ways.

The 3D construct showed similar results with DHT+EDC/NHS crosslinked Coll-CS-HA and -20°C foams; its shear modulus was even slightly higher (194 \pm 20 kPa) (*Fig. 29*). According to the student t-test done on these results, the most significant differences

were between the 3D construct and the -80°C foams (P = 0.004). Overall, it can be said that all these constructs possessed higher shear properties than the natural tissue (~ 120 kPa), therefore, they are expected to be strong enough to resist shear stresses that could be applied on them *in vivo*. To our knowledge, this is the first study reporting the shear properties of scaffolds prepared with the aim of meniscus tissue replacement.

Figure 29. Comparison of shear properties of dry individual foams and the 3D construct all crosslinked with DHT+EDC/NHS.

3.5. Matrix Pore Size Distribution

There is believed to exist an optimum pore size range for cell penetration which may vary with the tissue and the cell type. In addition to being important for cellular attachment, pore size was shown to be crucial for nutrient and waste transportation. Earlier, it has been stated that pore size of a scaffold should be large enough to allow cell migration and small enough to have an appropriate surface for a minimal ligand density, expressed by the materials used, to which cells will bind [Yannas et. al., 2001]. O'Brien et al. have reported that MC3T3 cell attachment and viability on collagen-GAG scaffolds was the highest in the pore size range 95.9-150.5 µm [O'Brien et. al., 2005]. In another study, where skin regeneration was studied, it was concluded that skin regeneration does not occur in scaffolds with pore sizes lower than 20 µm and higher than 120 µm [Yannas et. al., 1989]. Vascular smooth muscle cells were found to prefer binding scaffolds with a pore size range of 63-150 µm, whereas fibroblasts preferred a broader range (38-150) µm on PLLA-based scaffolds [Zeltinger et. al., 2001]. All these findings show that in addition to the type of material used, the scaffold pore size is of utmost importance and cellular performance is highly dependent on the cell type. Cells that were used in this study were human fibrochondrocytes (meniscus cells) isolated from human meniscus biopsies. As can be seen in the following figure (Fig. 30), meniscus cell sizes vary between $50 - 150 \,\mu\text{m}$. Thus, the pore size of the scaffold that will be used for cell culture studies should be distinctly larger than this range.

Figure 30. Phase contrast image of fibrochondrocytes isolated from surgically removed meniscus biopsies (Magnification: x10).

Only a few studies report on the influence of pore size on meniscus cells. Implants with four different pore sizes (50-90, 90-150, 150-250, 250-500 μ m) were studied by Klompmaker and coworkers (1993), and they found that optimal ingrowth and incorporation of cells occur in the case of microporous implants [Klompmaker *et. al.*, 1993]. Another study by the same group has shown that microporous implants with a pore size of 100-300 μ m allowed fibrocartilage formation [Klompmaker *et. al.*, 1991]. Tissue ingrowth in vivo and degradation in vitro of two different foams, one with higher porosity and pore size interconnectivity (30 μ m) and the other with lower macroporosity and interconnectivity (10-15 μ m) were studied by Tienen *et. al.* (2002). In the former case, good tissue ingrowth but low compressive properties were obtained, whereas in the latter one full tissue ingrowth did not occur since the implant degradation profile was

faster). Therefore, both foam types were found to be inappropriate for meniscus reconstruction [Tienen *et. al.*, 2002].

Pore size distribution of dry individual foams and of the 3D construct was determined in this study by mercury porosimeter at low pressures (50 psi). Pore size of uncrosslinked collagen foams (2%, w/v) varied between 5 and 200 μ m with the greatest fraction between 10-60 μ m and that of UXL Coll-CS-HA was between 100-200 μ m. With crosslinking, a reduction in the large pore fraction was observed but still the range was the same (data not shown). As it was shown before, Coll-CS-HA foams, were more porous than the collagen foams. The range varied between 50-200 μ m with the highest distribution being between 150-200 μ m (*Fig. 31A*).

In the case of DHT+EDC/NHS-crosslinked -20°C and -80°C collagen foams prepared at both, the main pore size range was 10 – 60 μ m but there were also pores in the size of 100-200 μ m (*Figs. 31B and C*). Interestingly no significant difference was observed between the pore sizes and the ranges of -20°C and -80°C foams. In the -20°C foams the distribution peak was sharper whereas -80°C foams showed a broader and more homogeneous distribution of pores and a slightly higher percentage of large pores. In both cases, presence of large pores is good because they would allow the cells to populate the foam whereas the smaller ones would allow interconnectivity. Finally, since the 3D construct is a combination of the three foam layers a broader distribution of pores was obtained with an increase in the fraction of large pores (100-200 μ m) due to the presence of the highly porous Coll-CS-HA foam and also of middle-sized pores as a result of -20°C and -80°C collagen foams (*Fig. 31D*). As a conclusion, it can be said that the final 3D construct is suitable for use as a scaffold in meniscus tissue engineering.

Figure 31. Pore size distribution of collagen-based foam. (A) Coll-CS-HA, (B) -20°C, (C) -80°C foam, and (D) the 3D construct (All the structures were DHT+EDC/NHS treated).

Figure 31. (Continued).

3.6. Degradation Profile of Foams *In Situ*

Another crucial point that should be considered while designing a tissue engineered scaffold is the degradation rate of the material used, in order to understand how long the implant will stay in the body. Obviously, the presence of ECM-secreting cells or their absence, influence this rate significantly. Depending on the application, the material is chosen to be nondegradable or degradable. The latter are more preferred in tissue engineered constructs, since the material is designed to serve as a temporary cell carrier while the new tissue is formed. The material should, therefore, neither degrade too fast nor too slow because in both cases the implant integration with the surrounding tissue is limited.

Different crosslinking methods were used in this research and their influence on the degradation profile of collagen-based foams was studied for a period of 3 months. The results showed that $83.1 \pm 3.6\%$ of the uncrosslinked foams degraded within 3 months whereas the corresponding values for DHT+EDC/NHS and EDC/NHS-crosslinked foams were 28.1 ± 0.9 and $77.9 \pm 0.6\%$. Interestingly, foams crosslinked only with EDC-NHS showed almost a similar behavior with the UXL after 4 weeks till the end of test (*Fig. 32*). This finding is consistent with what Vrana *et. al.* (2007) have reported. In their study EDC/NHS treated collagen foams showed a rapid degradation of $73.6 \pm 1.1\%$ after a period of 1 month [Vrana *et. al.*, 2007]. In this study, double crosslinking with DHT+EDC/NHS reduced the degradation rate of the foams, by 3-fold.

Figure 32. Degradation profile of uncrosslinked and foams (2%, w/v) crosslinked with EDC/NHS and DHT+EDC/NHS in PBS at 37°C.

The same procedure was applied to the Coll-CS-HA foams and to the 3D construct. As shown in *Figure 33*, Coll-CS-HA foams showed a very rapid weight loss in the first week ($42.7 \pm 0.3\%$) and then the rate of loss was quite low (from $42.7 \pm 0.3\%$ to $49.9 \pm 0.6\%$) in 5 weeks. The final weight that remained after 6 weeks was about 50%. A similar behavior was observed with the 3D construct. The rapid degradation in the first two weeks ($46.5 \pm 2.1\%$) probably occurred because of the rapidly degrading Coll-CS-HA foam. The final weight of the 3D construct that remained at the end of 6 weeks was found to be $38.4 \pm 3.1\%$, which is much lower than the weight of BATC I collagen foams crosslinked with DHT+EDC/NHS ($82.3 \pm 0.7\%$).

Figure 33. Degradation profile of Coll-CS-HA foams and the 3D construct in PBS at 37°C.

A significant improvement in the biological stability of collagen in biological medium was achieved by Ma *et. al.* (2004). The degradation rate of collagen-based foams was reduced by crosslinking with EDC/NHS in the presence of a basic amino acid (lysine), a neutral one (glycine) and an acidic amino acid (glutamic acid). A dependence on amino acid type on degradation rate was observed; while the glycine had less effect, glutamic acid significantly increased the biodegradability. On the other hand and surprisingly, addition of a basic amino acid like lysine resulted in a decrease in the rate of degradation. The authors claim that in EDC/NHS crosslinking mechanisms, to have improvements in the stability of collagen scaffolds, the ratio of NH₂/COOH should be in the range of 2-14 and out of this range the degree of weight loss becomes higher. According to them, two mechanisms can take place: (1) creation of crosslinks between collagen molecules in the presence of amino acids or (2) blocking of functional groups

as a result of amino acid addition. Which reaction will take place is shown to be highly dependent on the NH₂/COOH ratio [Ma *et. al.*, 2004]. In the present study, the EDC/NHS ratio was 9 to 1; therefore, an optimum stability of the foams was obtained.

3.7. In Vitro Studies

3.7.1. Meniscus Cell Shape and Phenotype Expression

Four distinct cell shapes are identified in a human meniscus depending on the location in the meniscus. In the superficial zone of the meniscus, spindle-shaped cell morphology is observed whereas red zone and red-white zone cells have more processes on them and they are known as fibroblast-like cells. These two types of cells are attached to each other by gap junctions and they are divided into two groups depending on the cell processes they have. In the white zone of meniscus where no blood vessels are present, cells are more round or chondrocyte-like. A fourth cell morphology with no cytoplasmic projections and fusiform in shape is observed in the superficial region of the meniscal tissue [Hellio Le Graverand et. al., 2001; Athanasiou et. al., 2009]. All these cell shapes (Figs. 34A and B) were detected with the cells isolated in this study. However, the majority of the cells seem to be fibroblast-like cells after 2 weeks of cell culture (Fig. 34A). This was also confirmed by Wildey et. al., (1998), who investigated the protein mRNA levels in vitro in the dog meniscus. Three types of cells were compared: articular chondrocytes, tendon fibroblasts and meniscus cells, isolated from the same animal. In regards the cell morphology, the researchers indicated that meniscus cells similar to tendon fibroblasts were more spindle-like in shape, reached confluence and increased in number 2 to 3-fold after a week of culture. On the other hand, articular chondrocytes were seen to be more polygonal in shape and had a lower rate of proliferation [Wildey et. al., 1998].

Figure 34. Phase contrast images of fibrochondrocytes isolated from meniscus biopsies. (A) A general view of meniscus cells (HPS stained), and (B) presence of different cell shapes indicating that these cells are probably fibrochondrocytes (Magnification: x20).

A meniscal tissue is predominantly made up of collagen type I, thus, fibroblast-like cells are expected to populate it. However, in addition to their fibroblast-like characteristics, meniscus cells show chondrocyte-like behavior too, and produce collagen type II. This is the reason why these cells are called fibrochondrocytes; they are able to exhibit both chondrocytic and fibroblastic characteristics.

In addition to cell morphology, which was studied microscopically, cell phenotype was also checked by RT-PCR. As shown in *Figure 35*, the isolated cells maintained their phenotype by expressing both collagen type I and type II. Even though the cells were isolated from two different persons of different ages, they showed similar behavior.

1: Col I (600bp) 2: Col II (500bp) 3: β2 microglobulin (300 bp)

Figure 35. Results of the RT-PCR for collagen type I and II from meniscus biopsies of patients 1 (56 years old) and 2 (30 years old).

This is expected because cell proliferation and proteoglycan synthesis is also not too much age and sex dependent as shown by Webber et. al. (1986). Influence of age (6, 12 and 24 month-old New Zealand rabbits) and sex on fibrochondrocyte functions was studied both in primary and secondary cultures. While cells from young males (6 months old) were reported to produce more proteoglycans than young females, a reverse trend was observed with 2 year old rabbits. Therefore, it was concluded that cell from both skeletally immature and mature rabbits were able to synthesize proteoglycans and proliferated well independent of age and sex of the animals [Webber et. al., 1986].

Research done to understand the similarity of meniscus cells with the cells of the surrounding tissue, revealed that in the first day of culture after isolation, meniscus cells expressed very low or undetectable levels of COL1A1, COL2A1 and aggrecan mRNA transcripts. In time (Day 7), the level of COL1A1 transcript was significantly increased. However, articular chondrocytes expressed high levels of COL2A1 and aggrecan since Day 0 of monolayer culture. Trace amounts of COL2A1 were expressed even on Day 0 by meniscus cells but on Day 7 the level was undetectable. The authors claim that this could be related to the fact that cells that produce more Type II collagen are present in the inner third of the meniscus and due to the wedge shape of meniscus the area that was used to isolate cells was smaller compared to the one containing more collagen Type I producing cells. Furthermore, the behavior of meniscus cells was found to be more like that of patellar tendon fibroblasts, which might prove that meniscus cells have more a fibroblastic character [Wildey *et. al.*, 1998]. In the present study, both cell sources were found to produce collagen Type I and II but it should be mentioned that the test was done after 2 weeks of culture, which might be a sufficient period for cells to increase their mRNA levels of both COL1A1 and COL2A1.

3.7.2. Flow Cytometry

Cell viability was checked by propidium iodide staining using flow cytometry and it was observed that 97% of the cells in the chosen region were alive. Some PI-negative cell debris, only a few PI-positive dead cells and mainly PI-negative viable cells were seen. In addition to this, a cellular protein, Annexin V, was used as a probe to detect cell apoptosis. In this study, all the cells were found to be Annexin V negative (*Fig. 36*). Based on the results shown in *Figure 36*, it can be said that fibrochondrocytes used in this study have a large size and are cells with a lot of internal complexity.

Figure 36. Propidium iodide (PI)/Annexin V-FITC plot according to cell size and PI staining intensity (PI, y-axis) of meniscus cells at passage 2.

Morever, the cells were mainly accumulated in the same region, which shows that only one cell phenotype was present (*Fig. 37*). This is a good proof of the fact that the cells that were used in cell culture procedures were only fibrochondrocytes and no endothelial cells were present, thus there was no need for cell population separations.

Figure 37. SSC/FSC and SSC/CD45 FITC plot of meniscus cells according to cells size. Note that the cells are accumulated mainly in one region.

After a period of 3 weeks of culture, the cells were analyzed with respect to the presence of certain cell membrane markers also. The cell markers used were: (a) CD31-expressed only in endothelial cells, leukocytes and their precursors; (b) CD34-found in lymphohematopoietic stem and progenitor cells, embryonic fibroblasts and small-vessel endothelial cells; (c) CD15-associated with human myeloid differentiation; (d) CD44-expressed in many cell types and is a hyaluronan receptor; (e) CD105-responsible for cellular response modulation and is seen in articular chondrocytes, endothelial cells and many other cells; and (f) CD45-expressed in all hematopoietic cells. As shown in *Figure 39*, the isolated cells were 92.72% CD105 and 98.54% CD44 positive. The other markers, that is, CD45, CD15, CD31, CD34 and CD14 were present in very low amounts (0.59, 1.33, 0.72, 0.93 and 0.37%) so it can be said that cells are almost negative with respect to these cell markers (*Fig. 38*).


Figure 38. Expression of cell membrane markers by fibrochondrocytes after a period of 3 weeks by cells gated in the region where the cells were viable.

These findings are consistent with the results reported by Verdonk *et al.* (2005) who stated that meniscus cells were CD105 and CD44 positive after a period of two weeks and the presence of the other markers decreased in time to a very low level. However, the authors have also indicated the presence of CD34 positive-CD31 negative cells both in the synovial sublining part and in the vascular region of meniscus, that are said to be endothelial progenitor cells responsible for vascular formation [Verdonk *et. al.*, 2005]. These were not seen in the present case and this may indicate that the biopsies used in

this study were mostly from the avascular region of the meniscus or because the cells were checked quite late (after 3 weeks) CD34 (+) - CD31 (-) cells might have lost their phenotype.

3.7.3. Fibrochondrocyte Seeding on Collagen-Based Scaffolds

Human fibrochondrocytes were seeded on the collagen-based foams and also the 3D construct to study the influence of foam type and the pore size on cell behavior. Cell seeding was initially done on Coll-CS-HA foam and -20°C and -80°C collagen foams. In the case of collagen foams, after 10 days of incubation the cells were observed to adhere and spread well mostly on the foam surface (*Fig. 39*).



Figure 39. Light microscopy of fibrochondrocyte cell distribution throughout the collagen scaffold after 10 days in the culture medium. Cells were stained with Hematoxylin-eosine (Magnification: x10).

Probably the cells could not penetrate the foam easily in the first days. It is expected that as the scaffolds are degraded a higher degree of infiltration will take place.

FITC-labeled Phalloidin and DAPI staining were performed in order to observe both the cytoskeleton and the nucleui of the cells. *Figure 40* presents the meniscus cell seeded foams after 45 days of cell culture on -20°C and -80°C collagen foams. A very good cell adhesion and spreading was observed on both scaffold types. The cytoskeleton of cells was more clearly seen on -80°C foams and this might be probably due its thinner pore walls. Moreover, on this foam the pore size distribution was broader and the pore size was slightly smaller and this might have influenced cell penetration into the scaffolds. However, fluorescent microscopy revealed no significant difference in cell-to-cell and cell-to-matrix interaction for both cases. A similar behavior in terms of cell spreading was observed with Coll-CS-HA foams also.



Figure 40. Fluorescence micrographs of fibrochondrocytes on collagen foams. Stain (A, B) FITC-Labeled Phalloidin and (C, D) DAPI. Collagen (A and C) -20°C foams, and (B and D) -80°C foams. Cells were cultured for 45 days (Magnification: x20 and each bar represents 50 μ m).

Distribution of cells within the scaffolds was observed with CLSM (*Fig. 41*). As mentioned above, initially the cell number was low and the cells into the collagen foams

preferred to stay on the surface instead of penetrating (*Figs. 41A and 41C*). However, as the materials degraded in time, a higher cell penetration and increased cell numbers were observed (*Figs. 41B and 41D*). The most uniform distribution was seen with Coll-CS-HA foams but collagen foams also showed a satisfactory distribution after 6 weeks of incubation.



Figure 41. CLSM image of fibrochondrocyte cells seeded on a -80°C collagen foam incubated in cell culture. Time of incubation: (A) 10 days and (B) 45 days. (C and D) Z-stacks of A and B.

SEM analysis also revealed that fibrochondrocytes attached and spread well on all scaffold types (*Fig. 42*). The cell seeded foams were cultured for a period of 45 days to obtain a mature tissue.



Figure 42. Fibrochondrocyte attachment and spreading on various foams. (A) Coll-CS-HA, (B) -20°C collagen and (C) -80°C collagen foam after 45 days of culture. (D) Cell sheet formed on the surface of -20°C collagen foam. Insets present the surfaces of unseeded foams and the arrow indicates the cell sheet on the foam surface.

The highest cell penetration was observed to occur in the case of Coll-CS-HA foams possibly due to the higher porosity it possessed and the higher number might be because of the presence of molecules like CS and HA, which might have affected the cell proliferation rate [Suzuki *et. al.*, 1998; Kon *et. al.*, 2008; Keogh *et. al.*, 2010]. On the other hand, cell penetration throughout the scaffolds was seen in both collagen-based foams but to a lower extent compared to Coll-CS-HA foams. It was observed that the cells covered totally the surface forming a cell sheet (*Fig. 42D*). Moreover, a faster cellular migration was observed laterally than vertically. This might have occurred due to the fact that the influence of the increase in solution concentration influence on pore width on the edges of collagen foams was to a small extent compared to its effect on the pore size throughout the foam, which was significantly reduced.

3.7.4. Cell Proliferation on the Foams and 3D Construct

In addition to microscopical observation, proliferation of human fibrochondrocyes in collagen-based foams was assessed by MTT assay for up to 21 days of incubation (*Fig. 43*). The results showed that cells in all scaffolds continued to increase in number for 21 days and the highest cell number was observed on Coll-CS-HA foams. Even though the seeding density was the same in all cases $(1x10^5 \text{ cells/cm}^2)$ cell adhesion extent in the first day was different. This was probably due to differences in the surfaces and porosity of foams. Furthermore, both collagen-based foams were treated with oxygen plasma before seeding in order to eliminate the skin layer formed during the lyophilization process and this might have made the surface more hydrophilic than the cells would prefer. In addition to this, again because of a process-dependent parameter, the surface of foams especially the surface of -20°C collagen foams was wedge shaped when viewed in z direction. This probably has resulted in the flow of cell suspension downward to the TCPS surface. As a consequence, low level attachment occurred in the first day of culture; however, after the cells were attached on the surface of the foams they increased

in number. Meniscus cells were shown to have a population doubling time of 17-19 hours and their attachment to surfaces occurs in less than 24 h [Webber *et. al.*, 1986], which was corroborated with the behavior of cells used in this research also. Regarding cell proliferation, fibrochondrocytes showed an increase in proliferation in all cases from Day1 to Day7; however after this point the cell behavior changed depending on foam type. While cells on Coll-CS-HA based foams kept increasing in number, cells seeded on -20°C collagen foams proliferated until Day14 and then the increase was not that significant till Day21. On the other hand, cells that were present on -80°C collagen foams showed a slight increase from Day7 to Day14 and then a significant increase from Day14 to Day21 (*Fig. 43*).



Figure 43. Meniscus cell proliferation on different collagen-based foams determined by the MTT assay.

Probably in both cases where a slight increase was observed, the cells reached confluency on the foam surface and this might have resulted in cell death. Meanwhile the remaining cells continued increasing in cell number. The highest cell proliferation was observed on the 3D constructs. This time the cell number after one day of incubation was the highest and the reason for this could be the fact that the upper layer was Coll-CS-HA foam, which was shown to be a good substrate for the cells due to its chemical composition and higher porosity. In Coll-CS-HA foams a good attachment occurred but still some of the cells were lost since they might have gone through the scaffold and leak onto the TCPS because of the larger pores. In the case of 3D construct no cells could go down to the TCPS surface due to the presence of fibrous mats and then -20°C foam was underneath the Coll-CS-HA foam, preventing higher cell loss. The cells kept increasing in number very satisfactorily in the 3D construct.

In general, it was observed that the cells started proliferating faster after a period of 10 days of culture and it should be mentioned that no extra component like growth factors, which have been shown to have influence on cell adhesion, migration and proliferation, were used in this study [Pangborn *et. al.*, 2005; Marsano *et. al.*, 2007; Gruber *et.al.*, 2008; Pabbruwe *et. al.*, 2010]. Moreover, to obtain a better profile, other cell sources also could be tried to observe whether a better fibrocartilage-like tissue could be formed. Marsano *et. al.*, (2007) reported that the best imitation of the inner and outer native meniscus was obtained with articular chondrocyte-generated tissues compared to those generated by fat pad and meniscus cells that resembled more the outer meniscus region. According to the researchers, chondrocyte cell de-differentiation occurs when cultured in a monolayer. A decrease in collagen type II production accompanied by an increase in collagen type I was reported; thus, resembling the natural meniscus tissue more [Marsano *et. al.*, 2007].

The advantage of pre-seeding scaffolds before implantation for both tissue maturation and preservation of the cell phenotype has been proved by many researchers. CMI, a collagen-based scaffold, was seeded with meniscus cells by Martinek *et. al.* (2006) and improvements were observed compared to the non-seeded one.

3.7.5. Mechanical Testing of Wet Cell Seeded Foams

As it was mentioned before, the knee meniscus is a fibrocartilage tissue that is subjected to both compressive and tensile forces. The large amount of collagen in the tissue helps resist tensile stresses and the GAGs are needed for responding to compressive forces. A number of studies have been held to observe the presence and accumulation of collagen and GAGs produced by cells *in vitro*. For load-bearing tissues like tendon, ligament and meniscus, where mechanical properties are important, research has been done in order to evaluate the mechanical properties of the scaffold (mainly tensile and compressive) in collaboration with cell activity. However, only a few studies have been carried out to understand the relationship between the composition, organization and mechanical function of the native tissues or the tissue substitutes, which is a crucial prerequisite while designing new tissues.

Unconfined compression was performed on all collagen-based foams that were used in this study after 1, 21 and 45 days of culture and they were compared with the unseeded control scaffolds to study the effects of cell presence on the mechanical properties of the tissue. *Fig. 44* shows that even after one day of culture the cells present in the scaffolds increased the compressive properties of the scaffold even though not to a significant extent. A decline was observed on Day21 of culture for all scaffolds (except the 3D construct) and this might be due to the degradation of the scaffold but later the values increased significantly possibly due to the increased cell number and they deposited their own GAGs and collagen which had a significant role in increasing the compressive

properties of the scaffold. These findings were in agreement with those reported by Nerurkar *et. al.* (2011), Basalo *et. al.* (2004), Wilson *et. al.* (2009). As expected, the highest mechanical properties were obtained with the 3D constructs especially after 45 days of culture. Regarding the compressive properties of different foams on their own, it can be said that the highest values were shown by -80° C foams as was expected due to its lower and broader distribution of its pore size and the lowest compressive moduli corresponded to Coll-CS-HA foams (*Fig. 44*). It should be mentioned that the increase of compressive properties due to ECM production by cells in time was crucial.



Figure 44. Compressive modulus values of wet foams after 1, 21 and 45 days of culture.

A similar finding was reported by Zorlutuna *et. al.*, (2009), who reported a significant increase in the mechanical properties of nanopatterned collagen films prepared for

vascular graft application. While the tensile properties of all films were reduced by Day 45, a significant increase occurred by 72 days and this was as a result of ECM produced by the smooth muscle cells [Zorlutuna *et. al.*, 2009].

Pabbruwe et. al. (2010) reported a similar design to that used in this study; it was a multilayered structure produced with the aim of repairing meniscus injuries in the avascular zone. The similarity is in that the three layers were combined with each other. They used a well known collagen membrane product, Chondroguide, designed for cartilage regeneration and it was placed between two discs of ovine meniscal cartilage. The membrane was seeded with bone marrow derived stem cells (BMSCs) and the integration of these cells with the meniscal cartilages was studied. It was found that while differentiated BMSCs showed a lower integration rate, the undifferentiated cells presented a significant level of integration (almost 50%), showing that undifferentiated cells have a higher integration capacity. Another important observation was that BMSC integrated more through the rough surface with a more open structure than the smooth one [Pabbruwe et. al., 2010]. In agreement with these findings, it has been shown that stiffness of the material used has a great influence on cell behavior, especially on anchorage-dependent cells. DHT, EDC/NHS and GLU crosslinking mechanisms were applied to collagen-GAG scaffolds to obtain foams with varying stiffness [Geogh et. al., 2010]. A higher cell number but a lower differentiation was obtained with stiff structures whereas the opposite occurred with softer DHT treated scaffolds. Moreover, the less stiff ones showed a significant cell-mediated scaffold contraction

In the present study, the importance of presence of GAGs in determining the scaffold compressive properties was shown by the abundance of cells after 45 days in culture medium. Safranin O was used to microscopically show the production of GAGs by the meniscus cells in all constructs. An intensive staining and good distribution of GAGs was observed after 45 days *in vitro* (*Fig. 45*).



Figure 45. Safranin O staining of meniscus cell-seeded collagen-based scaffold after 45 days of culture. Cell nuclei are stained in dark blue by hematoxylin whereas GAGs are stained orange by Safranin O.

3.7.6. Immunohistochemical Studies

Immunohistochemical staining was done specifically for collagen type I and type II on all the scaffolds. In order to obtain a mature tissue, scaffolds were maintained in culture for 45 days. *Figure 46* shows the production of both collagen types by fibrochondrocytes on Coll-CS-HA compared to the negative control were none of the collagen types was seen as expected. Similar results were shown by the collagen-based foams and the 3D construct also. Both collagen types were found to be distributed throughout the foams along with fibrochondrocytes.



Figure 46. Immunohistochemistry of newly synthesized collagens in 45 days cultured scaffolds. (A) Collagen type I and (B) collagen type II produced in Coll-CS-HA scaffolds. (C) Negative control for both collagen types. (D) and (E) Newly synthesized collagen type I in the 3D construct. Cell nuclei were stained in dark blue with hematoxylin.

3.7.7. In Vivo Studies

The 3D collagen-based construct, prepared with appropriate dimensions (10 mm in length and 2-2.5 mm in width) and form mimicking that of the natural medial meniscus of a rabbit, was implanted into the medial compartment of the knee joint of the New Zealand rabbits (*Fig. 47*). There were three groups: (1) control rabbits whose medial meniscus were removed and left as is (n = 7), (2) animals whose medial menisci were replaced with unseeded 3D construct (n = 7) and (3) animals whose medial menisci were replaced with cell-seeded 3D construct (n = 7).



Figure 47. Implantation of 3D construct in the medial knee meniscus of 6 months old female New Zealand rabbits. Arrow points to the implant.

The experiments were terminated at the end of 3^{rd} and 10^{th} weeks post implantation. Histological, microscopical and immunohistochemical characterization to evaluate the biocompatibility of the implant are ongoing; however, it should be mentioned that in the initial stages no inflammatory signs like redness, swelling, heat and pain was recorded.

CHAPTER 4

CONCLUSION

Menisci are fibrocartilaginous tissues that play a crucial role in the knee biomechanics. The most common problems that can occur to the knee joint are meniscal tears, which make the knee unfunctional, cause pain and more importantly lead to knee osteoarthritis after some time. The healing of these tears by tissue regeneration is possible only if the injury is in the outer portion of the tissue where vascularization is present. On the other hand, if the tear is in the avascular region of meniscus, repair is almost impossible. Therefore, whenever possible meniscal tears should be repaired. Among the common treatments are partial and complete meniscectomy, meniscal repair, and meniscus transplantation take place. Each of these procedures has it own advantages and disadvantages but the most important problem, which is not yet overcome with the current methods is osteoarthritis. Tissue engineering seems to be a promising method for finding solutions to these meniscus problems. Various materials and approaches are proposed for use in meniscus regeneration but the results were not very satisfactory especially in terms of the scaffold mechanical properties.

A collagen type I-based 3D construct, with a unique multilayered sponge-nanofiber design, was produced in this study. The construct was composed of three different foam layers with varying pore sizes and mechanical properties. To mimic the nature of the meniscus tissue and to increase the mechanical properties of the 3D construct, 5 layers of aligned Coll-PLGA nano-microfibers produced by electrospinning process were

added between the layers. The upper layer was Coll-CS-HA-based whereas the middle and the lower layers were collagen based. The difference between the middle and lower layers was preparation conditions.

To increase the mechanical properties the best crosslinker to be used in all further studies was chosen to be EDC/NHS in combination with DHT.

Mechanical properties (compressive, tensile and shear) of all individual dry foams and the 3D construct were evaluated. Coll-CS-HA showed the lowest properties and the highest compressive modulus was obtained with double crosslinked -80°C foams. As was expected, the highest compressive and tensile properties were obtained with the 3D construct. In the dry state the 3D construct showed hgiher compressive and shear properties than the natural tissue but the tensile properties were much lower.

Regarding the pore size distribution results, the highest porosity and pore size distribution was seen with Coll-CS-HA foams. Concerning the 3D construct, since it is a combination of the three foam layers, a broader distribution of pores was obtained with an increase in the fraction of large pores (100-200 μ m) due to the presence of the highly porous Coll-CS-HA foam and also of middle-sized pores as a result of -20°C and -80°C collagen foams. With these properties it seemed to be suitable for meniscus tissue engineering applications.

All the individual foams and the 3D construct were seeded with human fibrochondrocytes and the highest cell proliferation was obtained with the 3D constructs followed by Coll-CS-HA foams and then -20°C and -80°C collagen foams. Immunohistochemical studies showed that cells produced both collagen types I and II and also they produced new GAGs after 45 days of incubation in cell culture medium.

The influence of cell seeding on the compressive properties of wet individual foams and the 3D construct was also observed. The compressive properties of the foams were seen to improve upon cell seeding. Upon implantation into New Zealand rabbits for 3 and 10 weeks, no inflammatory response has been recorded and the microscopic observations indicate that the 3D construct blends in with the neighbouring tissue.

It can thus be stated that the 3D construct has proper compressive mechanical properties, although the tensile properties are lower, they get better with cell seeding and these cells produce both collagen type I and II and also GAG as needed for a meniscus. These show that this meniscus substitute has a significant potential for meniscectomy patients.

Ongoing and Future Work

The collagen-based 3D construct used in this study showed a significant potential as a meniscus replacement. Some modifications, however, need to be made in order to improve its mechanical performance.

In the future studies, some other cell sources, like mesenchymal stem cells, in combination with growth factors are planned to be used to check the possibility of obtaining better *in vitro* performance such as higher cell penetration and proliferation. In addition, dynamic cultures instead of static ones as used in this study are planned to obtain a higher ECM and GAG production, thus, to better mimic the native tissue.

In vivo studies on the knee of New Zealand rabbits are in progress and the preliminary histological studies have shown appropriate integration of the scaffold with the surrounding tissue. In case the *in vivo* results are promising, implantation of the final 3D

construct into sheeps, which have meniscus with properties and size similar to that of the humans, is being considered.

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APPENDIX A

CALIBRATION CURVE FOR THE CONVERSION OF ABSORBANCE TO CELL NUMBER



Calibration curve for determination of human meniscus cells (Patient 1, P3)
APPENDIX B

CROSSLINKING MECHANISM OF PROTEINS WITH GENIPIN (GP)





Crosslinking mechanism of proteins with genipin. NH_2 group of the protein can bind to the (A) ester group (outside the ring structure), or (B) ring [Butler *et. al.*, 2003]. Note that p denotes to protein.

APPENDIX C

CROSSLINKING MECHANISM OF PROTEINS WITH GLUTARALDEHYDE (GLU)



 $-R-NH_2$: Protein

Crosslinking mechanism with glutaraldehyde. (A) Glutaraldehyde activated protein, and (B) Glutaraldeyde crosslinked protein [Hasirci *et. al.*, in press].

APPENDIX D

CROSSLINKING MECHANISM OF PROTEINS WITH 1-ETHYL-3-(3-DIMETHYLAMINOPROPYL) CARBODIIMIDE (EDC)



Crosslinking mechanism of proteins with EDC [Hasirci et. al., in press].

APPENDIX E

COMPRESSIVE PROPERTIES OF -20°C COLLAGEN FOAMS

Elastic compressive strength and moduli of -20°C collagen foams crosslinked with various crosslinkers.

	Compressive Properties		
Crosslinker type	Elastic collapse stress (σ^*_{el}) (kPa)	Linear elastic modulus (E) (kPa)	
UXL	29.8 ± 4.2	277.6 ± 62.9	
DHT	30.4 ± 4.6	296.9 ± 96.7	
DP	17.6 ± 6.3	131.9 ± 59.5	
DHT+GP	41.9 ± 6.7	502.9 ± 92.6	
EDC/NHS	43.7 ± 3.4	248.4 ± 80.4	
DHT+EDC/NHS	112.3 ± 21.1	545.4 ± 109.8	
GLU	51.8 ± 10.0	277.7 ± 101.5	
DHT+GLU	114.9 ± 4.9	536.8 ± 136.9	

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2003-2007	METU, Biotechnology Research Unit Ankara, Turkey	Full time Researcher
2000 (40 days)	FAKO Pharmaceutical Factory, Istanbul/Turkey	Summer Practice

AWARDS

- ✓ 2 Silver medals from International Mathematics Project Competition held in Turkey in 1997.
- ✓ 'Best thesis of the year' award for M.Sc. Thesis given by Middle East Technical University (May 2008).
- ✓ Rudolf Cimdins award for Best Poster Presentation given by ESB2010, Tampere, Finland.

FOREIGN LANGUAGES

Albanian - Native language

English - Advanced

Turkish - Advanced

Italian - Intermediate

PUBLICATIONS

- Nikkola L., Seppälä J., Harlin A., Ndreu A., Ashammakhi N., 'Electrospun diclofenac sodium releasing nanoscaffold', J Nanosci. Nanotechnol., 6(9-10): 3290-5 (2006).
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ORAL PRESENTATIONS

- 1. Halili-Ndreu A., Bahcecioglu G., Hasırcı N., Hasırcı V., 'Creating a mechanically functional design for partial meniscus replacement', Tissue Engineering and Regenerative Medicine Society (TERMIS), 07-10 June, Granada, Spain (2011).
- Bahcecioglu G., Ndreu A., Hasırcı N., Hasırcı V., 'Characterization of alginic acidcoated PLLA-PLGA foams for potential use in tissue engineering of the meniscus', Tissue Engineering and Regenerative Medicine Society (TERMIS), 07-10 June, Granada, Spain (2011).
- 3. Halili-Ndreu A., Hasirci N., Hasirci V., 'Novel scaffolds for meniscus tissue engineering', 24th European Conference on Biomaterials, September 4-8, Dublin, Ireland (2011) (to be presented).

PATENTS

Hasırcı V. and Ndreu A., 'Collagen-based meniscus tissue engineering' (Kollajen temelli yapay meniscus); Patent No: 2011/03662.