ÇUKUROVA UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES

MSc THESIS

Hana BAHRIA

DISCUSSION OF UNCONVENTIONAL COLLAGEN SCAFFOLDS FOR REPAIRING MENISCUS AVASCULAR AREA TEARS USING ELECTROSPINNING

DEPARTMENT OF TEXTILE ENGINEERING

ADANA- 2017

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We certify that the thesis titled above was reviewed and approved for the award of degree of the Master of Science by the board of jury on 26/10/2017

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ABSTRACT

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Until now menisectomy is the most used solution for the illness of meniscus tears in the avascular area. To avoid using this surgery and over their researches, researchers found that electrospun collagen type I scaffold is the most suitable solution. In this Thesis, we give a comprehensive explanation of the step of formation of a collagen I electrospun scaffold and methods of characterization. And also we discuss some factors which influence the diameter of a collagen I electrospun nanofibers.

Keywords: Collagen type I, Electrospinning, meniscus scaffold, Fiber diameter

ÖZ

YÜKSEK LİSANS TEZİ

MENISKÜS AVASKÜLER BÖLGE YIRTIKLARININ TEDAVİSİ İÇİN ELECTROSPINNING İLE ÜRETİLEN KONVANSİYONEL OLMAYAN KOLAJEN İSKELELERİNİN TARTIŞILMASI

Hana BAHRIA

ÇUKUROVA ÜNİVERSİTESİ
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: Yrd. Doç. Dr. İlhami İLHAN

Menisküs avasküler bölge yırtığı hastalıkları için bugüne kadar en çok kullanılan çözüm menisektomi olmuştur. Bu ameliyattan kaçınmak için araştırmacılar daha uygun bir çözüm bulmaya çalışmışlardır. Yapılan araştırmalar sırasında, elektrospun kolajen tip I iskeletlerinin en uygun cevap olduğu buldundu. Normal olarak, Kolajen tip I çözülmesi HFIP (1,1,1,3,3,3-Heksafluoro-2-propanol) ile olmaktadır. Bu çözücünun toksik hali, oluşan iskele biyouyumluluk derecesini etkileyebilir. Bu tezde, çeşitli kaynaklardan elde edelen ve HFIP veya diğer "Yeşil" çözücüler ile çözülen Kollajen tip I kullanılmış ve elektrospun nanofiberlerin çapı üzerinde bu iki faktörün etkisi tartışılmıştır.

Anahtar Kelimeler: Kolajen tip I, Elektrospinning, Menisküs iskelesi, Lif çapı

GENIŞLETİLMİŞ ÖZET

Menüsküs, genel olarak memelilerdeki ve özellikle insandaki diz eklemi yapısında önemli bir parçadır. Yüklerin iletilmesinde ve yağlamada kritik bir rol oynar. Bu organizmada yapılan her hangi bir değişiklik normal işleyen eklem yapısını değiştirir. Yaşa göre veya spor yaralanmalarına bağlı olarak meniscus yırtıkları görünebilir. Bu yırtıklar iki gruplar toplanabilir:

- Kendiliğinden iyileşebilir yırtıklar
- Müdahaleye ihtiyaç duyan yırtıklar

Bu durum özellikle kan damarlarının varlığı veya eksikliğine bağlı bir durumdur. Günümüzde halen, menüsküs yırtıklarının ikinci türü için, kısmi veya total menisektomi en popüler çözümdür. Gözlemlere göre, bu operasyonun dezavantajı diz Osteoporozunun (OA) gelişimine sebep olmasıdır. Bu yetersiz çözüm tibial ile femur kemikleri arasındaki temas alanını azaltır ve bitişik yüzeyin stress yoğunluğunu arttırır. Bu amaçla, avasküler bölgede yırtılan bir menüsküsü rehabilite etmek için birçok teknik bulunmaktadır. Örneğin, dikişler, vidalar, oklar, ve dartlar. Kısa vadede, bu onarımın prosedürleri ağrı, kilitleme ve dengesizliğe sebep olabilmektedir. Uzun vadede ise önemli bir faydası doğrulanamamıştır. Onarımı geliştirmek veya yaralı meniskal dokuyu değiştirmek için başka yaklaşım çeşitleri ayrıca denenmiştir. Örneğin, meniskal allograftlerin transplantasyonu verilebilir. Bu operasyon acı çekmeye sebep olmakta ve buna rağmen sadece kısa bir dönem için diz fonksiyonunu düzeltebilmektedir. Diğer araştırmacılar, doğal biyomalzemeler kullanarak meniskal dokuyu değiştirmeyi düşünmüşlerdir. Örneğin, periosteal doku, ince bağırsak, submukoza, acellular domuz meniskal doku, perikondrial doku ve bakteriyel selüloz denenmiştir. Henüz, bu yaklaşımlar organizasyonun yapısal ve biyolojik ve meniskal doku mekanik özelliklerinin

replikasyonun eksikliği nedeniyle klinik uygulamalarda büyük oranda uygulanmamıştır.

Hücresel mikro çevreyi taklit etmek için Elektrospinning metodu kullanılmıştır. Bu metod, polilaktik asit (PLA) ve polikaprolakton (PCL) gibi sentetik malzemelerden ve kolajen, jelatin ve kitozan gibi doğal malzemelerden oluşan nanoküre fiberlerinin üretilmesini gerçekleştirilebilir hale getirmektedir. Aslında, Elektrospun (ES) fiber matrislerle ilaç verme başka biyomedikal uygulamalara ile beraber yara iyileşmesinde başarıyla kullanılmıştır. Bu üretilen nano-fibrous iskeleleri, özellikle büyük biomimetik yüzey alanı ve uygun mekanik mukavemet avantajlarına sahiptir: İskelenin gözenekli yapısı ve yüksek yüzey-hacim oranı, hücre bağını hızlandırmada, hücre çoğalması ve besinlerin Electrospun (ES) lif matrisi vasıtasıyla taşınmasına yardımcı olmaktadır. Biyolojik olarak uyumlu olmasına rağmen, sentetik polimerlerin yerleştirildikleri zaman, enflamasyona ve yabancı cisim tepkimesine sebep olduğu bilinmektedir. Doğal polimerler bu komplikasyonları önleme yeteneğine sahiptir. Özellikle kolajenden oluşan bir iskele için, Kolajen en iyi seçimdir çünkü kolajen menisküsün ana fibriler bileşeni olmaktadır. Üstelik kolajen biyopolimeri biyouyumludur ve klinik olarak onaylanmış / temizlenmiş cisim olarak kullanılır.

Elektrospun-lanmış kolajen liflerinin çaplarını etkileyen parametreler ile ilgilenen öncelikle çalışmalar, elektrospinning makinesi ile alakalı kontrollü değişkenlerin bulunduğunu doğrulamıştır. Bu parametreler:

- Akış hızı
- Elektrik alan kuvveti
- Uç ve kollektör arasındaki mesafe
- Kollektör kompozisyonu ve geometrisi

ve ortam parametreleri:

- Sıcaklık
-Nem

Normal olarak, Kolajen tip I'in çözülmesi 1,1,1,3,3,3-Heksafluoro- 2-propanol (HFIP) ile olmaktadır. Bu çözücünün toksik hali, elde edilen iskelenin biyouyumluluk derecesini etkileyebilir. Bu bağlamda, kolajen tip I "Yeşil Elektrospinning" 'i elde etmek için başka iyi huylu hibrit çözücü kombinasyonları kullanılmıştır. Bu tezde, çeşitli kaynaklardan elde edilen ve HFIP veya diğer ''Yeşil'' çözücüler ile çözülen Kollajen Tip-I'den elde edilen elektrospun nanofiberlerin çapı üzerinde iki faktörün (çözücü ve kolajen kaynağı) etkisi araştırılmıştır.

Elektrospun kollajen iskeletinin uygulanmasına bir örnek olarak, menüsküste bulunmuş yırtıkları seçilmiştir. Bu tezde, menisküs yırtıklarını iyileştirilmek için, elektrospun kollajen tip I iskelenin oluşturmasının farklı adımları tanıtılmıştır:

- Öncelikle, insan menisküsünün morfolojisinden, yırtıklarından ve tedavi yöntemlerinden bahsedilmiştir.
- Ardından, kollajen tip I'in çözülme türleri, Elektrospinning prosesi, Kollajen I ve diğer polimerlerle olası kombinasyonlarından bahsedilmiştir.
- Devamında, elektrospinning sonrasında gelen ve elektrospun iskelelerin mekanik özelliklerinin geliştirilmesini çapraz bağlama prosesinin farklı yöntemlerinden bahsedilmiştir.
- Dördüncü olarak, oluşan iskele kalitesini değerlendirmek için kullanılan farklı teknikler gösterilmiştir.
- Beşinci olarak, kolajen ile tedavi amaçlı birlikte kullanılan diğer maddelerin etkileşimi ve bunların kullanım alanlarına değinilmiştir.
- Altıncı olarak menisküs yırtıklarının tedavisinde kullanılmak üzere iskele oluşturulmasıyla ilgilenen farklı çalışmalar sunulmuştur.

- Son olarak, çözücü tipi ve collagen I kaynağının elektrospinning ile elde edilen nanoliflerin çapları üzerine etkisi yapılmış çalışmalara göre tartışılmıştır.

Önceki araştırmalara göre farklı kaynaklardan elde edilen kollajen tip I'in çözülmesi için 3 çözücü tipi kullanılagelmektedir. Bu çözücülerden HFIP, en iyi çözücü olarak tespit edilmiştir ancak toksik karakteri ve aşındırıcı yapısı nedeniyle araştırmacılar alternatifler bulmaya çalışmaktadırlar. Bu bağlamda, PBS (Phosfate Buffered Saline) ve Asetik Asit collagen tip I için yeşil (doğa dostu) çözücüler olarak gündeme gelmiştir. Farklı kaynaklardan elde edilmiş Collagen I'i çözmek için farklı çözücüler kullanmış olan araştırmacılar, collagen I elektrospin nanolifleri için farklı lif çapları elde etmişlerdir. Bu çalışmada mevcut literatür tartışılarak gelinen noktada menisküs yırtıklarının tedavisi için en uygun lif çapını (insan menisküsündeki colajen lif çaplarının değiştiği aralık olan 50 nm ile 200 nm arası kalınlıkları) veren yeşil çözücü ve collagen tip I biopolimer kaynağı kombinasyonu belirlenmeye çalışılmıştır.

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To my Brothers. You were always there for me through hard times. Thank you for believing in me.

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1. INTRODUCTION

Meniscus is an important component in the knee structure of mammalian in general and human especially. It plays a critical role in transmitting loads and joining lubrication. Any modification in this organism alters the normal functioning in the knee. By age or due to sports injuries, tears could appear in the meniscus. Those tears are assembled in two groups: Tears that could heal spontaneously and others that need some intervention. This is related especially to the presence or lack of blood vessels. Until now, for the second kind of Meniscus tears, partial or total meniscectomy are the most popular solution. This operation causes the decrease of the contact area between the tibial and the femur which causes an increase in the stress concentration of the adjacent surface and carries the appearance of knee Osteoporosis (OA). So, finding alternative solutions is a critical necessity in order to rehabilitate the good functioning of an altered meniscus. Several techniques that involved sutures, screws, arrows, and darts to repair meniscal tears, have been developed. The problem with those interventions is that after a short time from the surgery, they relieve pain, locking, and instability. Some researchers also tried the transplantation of meniscal allografts, others considered replacing meniscal tissue using natural biomaterials, as periosteal tissue, small intestine submucosa, acellular porcine meniscal tissue, perichondrial tissue, and bacterial cellulose. Yet, these approaches have not been applied in clinical application largely because they still reveal pain.

As a continuation of the previous researches and in order to mimic the cellular microenvironment of the meniscus, Electrospinning process was involved. This method makes it realizable to produce nanoscale fibers composed of synthetic materials such as polycaprolactone (PCL) and polylactic acid (PLA) and of natural materials such as collagen, chitosan, and gelatin. In fact, Electrospun (ES) fiber matrices have been used successfully in several biomedical applications such as drug delivery and wound healing. The produced nanofibrous scaffolds possess

special characteristics that reunite having both an appropriate mechanical strength and a large biomimetic surface area. This tissue is described as a porous structure that have high surface-to-volume ratio which helps in accelerating cell attachment, cell proliferation, and transport of nutrients through the Electrospun (ES) fiber matrice. Although being biocompatible, synthetic polymers presents the risk of causing serious inflammation and foreign body reaction when implanted in human body. In the other hand, natural polymers have the ability to avoid these complications especially for a scaffold composed of collagen. This biopolymer is the best choice since it is the main fibrillar component of the meniscus, in addition of being biocompatible and used in clinically approved devices.

Previous studies that were interested in studying the parameters that affects the diameters of electrospun collagen fibers affirmed that there are controlled variables related on the Electrospinning machine such as: flow rate, electric field strength, distance between tip and collector and collector composition and geometry, and ambient parameters such as temperature and humidity, which can affect the measure of the fiber diameter.

In the first part of this thesis, we will explain the different steps of formation of an electrospun meniscus scaffold starting with an explanation of the meniscus and its important role in the good functioning of the knee. Then, we will continue with an explanation of the mechanism of Electrospinning in general and especially that of Collagen. After that, previous studies about the meniscus scaffold in general are going to be mentioned. The second part is our study will be a discussion about new factors that could influence the measure of a collagen I electrospun nanofiber.

2. ELECTROSPUN MENISCUS SCAFFOLD

2.1. Introduction of The Meniscus

Menisci are located in the tibial plateau in the knee. They are characterized by their semilunar disc-shaped fibrocartilaginous tissues which facilitate transmitting loads and contribute in joining lubrication. This component is composed principally of two parts: the vascular region and the avascular region. By age or due to sports injuries, Meniscus tears could take place. Those kinds of attacks have the ability to heal completely if they occur in the vascular region while spontaneous healing in the avascular region is not possible due to the lack of blood vessels there (Baek et al., 2015).

In the United States, the mean frequency of the meniscal injury is 0.066% which makes the meniscus the most commonly damaged area in the knee joint. Removing the meniscus completely was the major treatment since 1889 and it prevailed for almost 80 years. However, in the period between 1960 and 1980, follow up radiographic studies reported a high incidence of knee Osteoporosis (OA) after the removal of a part or the whole meniscus. This operation is known by the name menisectomy (Guo et al., 2015).

In order to preserve the meniscus structure and function, several techniques were used such as polymer-based arrows, darts, screws, staples, and other suture devices, while the non-satisfying obtained results make the partial or total meniscectomy more prevalent. In this context another approach appears. It involves the production of tissue engineering directed to use in repairing and replacing damaged meniscus (Baek et al., 2015).

First of all, we will start by explaining the structure of the meniscus.

2.2. Structure and Properties of The Meniscus

2.2.1. Meniscal Anatomy

The knee meniscus is described as fibrocartilaginous since it shares characteristics of both fibrous tissues (tendon and ligament) and cartilaginous tissues (articular cartilage). Its wedge-shaped architecture permits it to transfers the related loads from the femur to the tibia , absorbs the corresponding shock, and enhances joint stability (Silverman, 2011). These semi-lunar tissues dwell between the femur and tibia within the medial and lateral parts of the knee (Figure 2.1). About 80% of the tibial plateau zone is covered by the lateral meniscus, whereas the medial meniscus only covers ~60% of this area (figure 2.2): Lateral meniscus is presented in “O” shape, whereas the medial meniscus has a “C” appearance. Additional to the good adaptation of the geometry of the meniscus to the founded shape of the femoral condyle and tibial plateau, the anterior and posterior insertional ligament help in attaching the menisci tightly and ensure their best fixation to the tibial. Blood vessels and nerves issued from the surrounding joint capsule and synovial tissues could only penetrate 10–25% of the outside of the adult meniscus. For this reason, the meniscus is typically classified into three zones according to vascular and nervous distribution: the outer vascular/neural area (red-red zone), the inner entirely avascular/neural area (white-white zone), and the junctional area (red-white zone) between the former two regions. Note here that the white-white zone is the one that cannot self-heal well when damaged (Guo et al., 2015).

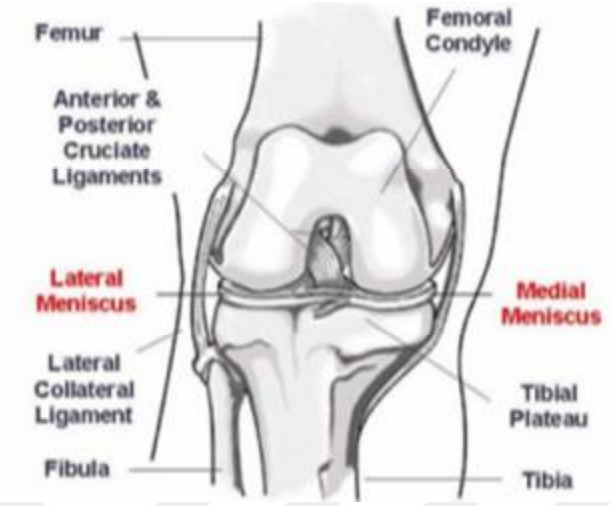

Figure 2.1. Anatomic location of the meniscus (Guo et al., 2015)

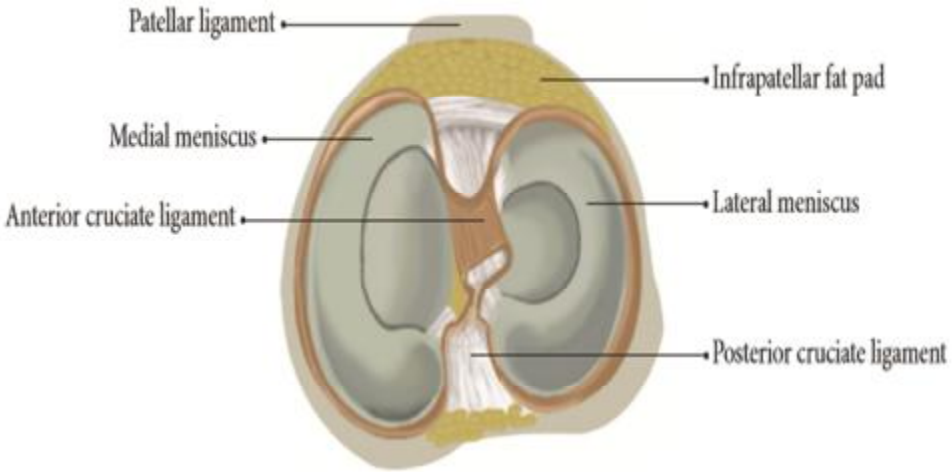

Figure2.2. Top view of the anatomical healthy meniscus (Guo et al., 2015)

2.2.2. Meniscal Chemical Composition and Cell characterization

The meniscal extracellular matrix (ECM) fibril compositions are composed mainly in collagen with a minority of Elastin (< 0.6%). The collagen type distribution is as fellow (Guo et al., 2015):

- Outer region: - >90% collagen I
  -<10% other collagen: type II, III, IV, VI, and XVIII
- Inner region: - 60% collagen type II
  - 40% collagen type I

Moving to the meniscal cells population which is classified into three types referring to the location and the morphology : The outer one-third of the meniscal area comprises “fibroblast-like cells” which are characterized by their elongated morphology, whereas, the inner two-thirds of the meniscal region mainly contains “fibrochondrocytes” (MFCs) which are characterized by their oval to round morphology. Their function consists of maintaining and remodeling the extra cellular matrix. Also, others type of cells named “Fusiform cells’’ are found in the superficial zone and are aligned and parallel to the meniscus surface (Guo et al., 2015).

2.2.3. Mechanical Properties of The Meniscus

The unique architecture of the meniscus consists of circumferentially oriented collagen fibers interspersed with radial collagen “tie” fibers. Thanks to its architecture as fiber-reinforced matrix, the mechanical properties of this tissue are highly anisotropic and highly dependent on the fiber direction.

The tensile properties of the human meniscus range from:

- 48-259 MPa (489,464- 2641,06 kg/cm^2) in the circumferential direction
- 3- 70 MPa (30,5915- 713,801 kg/cm^2) in the radial direction

Those measures depend on anatomic location and species. Also, we mention that the compressive properties of the meniscus range between 50 to 400 MPa. Those properties are combined in order to enhance congruency, load distribution, and shock absorption across the joint (Baker, 2010).

2.2.4. Meniscal Biomechanical Properties

The anatomic geometry of the meniscus, which occupies 60% of the contact area between the femoral condyles and tibial plateau, is highly related to its biomechanical properties: it is described by its perfect fitting to the shape where it is found. So, it could supply a significant expansion in the contact area of the knee joint. When the knee carries an axial load, a tensile hoop stress took place around the circumference which causes the extension of the meniscus that could lead to its extrusion out of the knee joint. Here comes the role of the firm attachment at the anterior and posterior insertional ligaments to ensure its stability. Then, both the anterior/ posterior insertional ligaments and meniscus give the protection to the tibial cartilage. Hence, the damage of one or both of them means changing the load transmission mechanism which leads to its damage (Paletta et al., 1997).

Figure 2.3 shows that the axial load force (F) perpendicular to the meniscus surface and horizontal force (fr) are created by compressing the femur (Ff). F rebounds due to the tibial upgrade force (Ft), whereas the fr leads to meniscal extrusion radially, which is countered by the pulling force from the anterior and posterior insertional ligaments. Consequently, tensile hoop stress is created along the circumferential directions during axial compression (Guo et al., 2015).

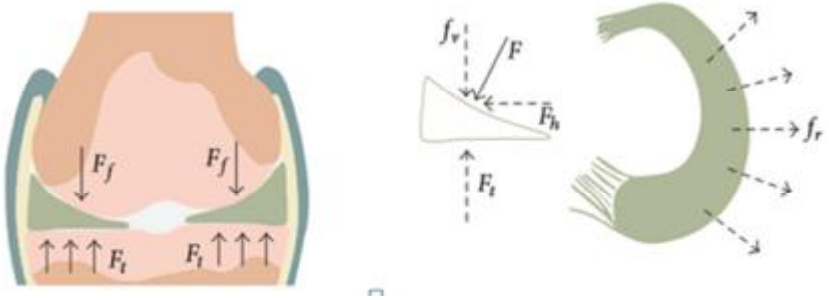

Figure2.3. Schematic diagram of the meniscus force bearing mechanism compression (Guo et al., 2015)

2.3. Meniscal Lesions and Development of Knee O

Meniscal lesions and the development of knee (Osteoporosis) OA are related one to the other: This is due to the fact that Meniscal lesions can cause the knee OA and also a knee OA could lead to a Meniscal tears. So, a patient with a knee OA has usually an abnormal configured meniscus. An injured meniscus launches the synovium to liberate various inflammatory, which lead to the destruction of the meniscal matrix and further the tibial cartilage (Englund et al., 2009).

2.4. Classification of Meniscal Lesions and Therapeutic Strategies

The damage of the meniscus is a very common disease in the knee joint. Therefore, Meniscal lesions are categorized into groups depending on age: for younger patients (<40years), Meniscal injuries are normally caused by truma or congenital meniscal disease due to sports activities, while for older patients (>40years), those troubles are mostly related to degenerative tears (McDermott, 2011). Meniscal injury takes place mostly in the medial meniscus rather than the lateral, at a ratio of about 2:1 and this can be explained by the fact that the medial meniscus fixation is steadier. Meniscal damage could occur in several forms involving circumferential or longitudinal tears: in this case, the fracture occurs between collagen bundles itself, or radial tears where collagen bundles are disrupted. According to Casscells classification, meniscal lesions are grouped into eight different types (Figure 2.4) although, meniscal injuries are simply classified clinically into peripheral meniscal lesions and avascular meniscal lesions (Guo et al., 2015).

As a dense connective tissue, the meniscus is adaptable to have a healing capacity suitable with its mechanical functionality. With age, the vasculature of the meniscus retreats to the periphery, the cellularity decreases and the collagenous ECM gets denser. For a juvenile meniscus, injuries heal easily, but for adults complete healing could be observed only in the vascularized periphery (Petrosini et

al., 1996). The first strategy to ensure the healing of the avascular region was the drilling of channels to promote blood vessel invasion. Unfortunately, this method causes fibro vascular scars with disorganized collagen and inferior mechanical properties (Newman et al., 1989).

Until now, the most widespread orthopedic surgeons induce a partial meniscectomy in cases of unrepairable or degenerative meniscal injuries: This procedure looks for preserving as much healthy, functional tissue as possible while impeding further tear propagation. The complete meniscectomy is used where tears are complex or when no healthy tissue remains. The use of meniscectomy, whatever it was partial or total, could not prevent the development of knee OA since using this method contribute in decreasing the contact area between the femoral condyle and tibial platform and increasing stress concentrations on the adjacent articular surfaces (Englund et al., 2008). Therefore, a lot of meniscal repair and reconstruction techniques have been processed such as surgical methods that include suturing and stabilizing the meniscal tears, meniscal allografts, small intestinal submucosa (SIS) implants, and autogenous tendon grafts. In 1984, Milachowski and Wirth patterned their first free meniscal allograft transplantation. The use of this device in younger patients improves knee function and minimizes pain after a short follow-up. Unfortunately, despite providing long-term protective benefits to the cartilage, meniscal allograft remains not effective enough. This is due to the probability of causing a disease transmission, reducing material properties and the shrinkness of the allograft itself (Samitier et al., 2015). Concerning, SIS and autogenous tendon grafts, they also yielded bad results (Cook et al., 2006).

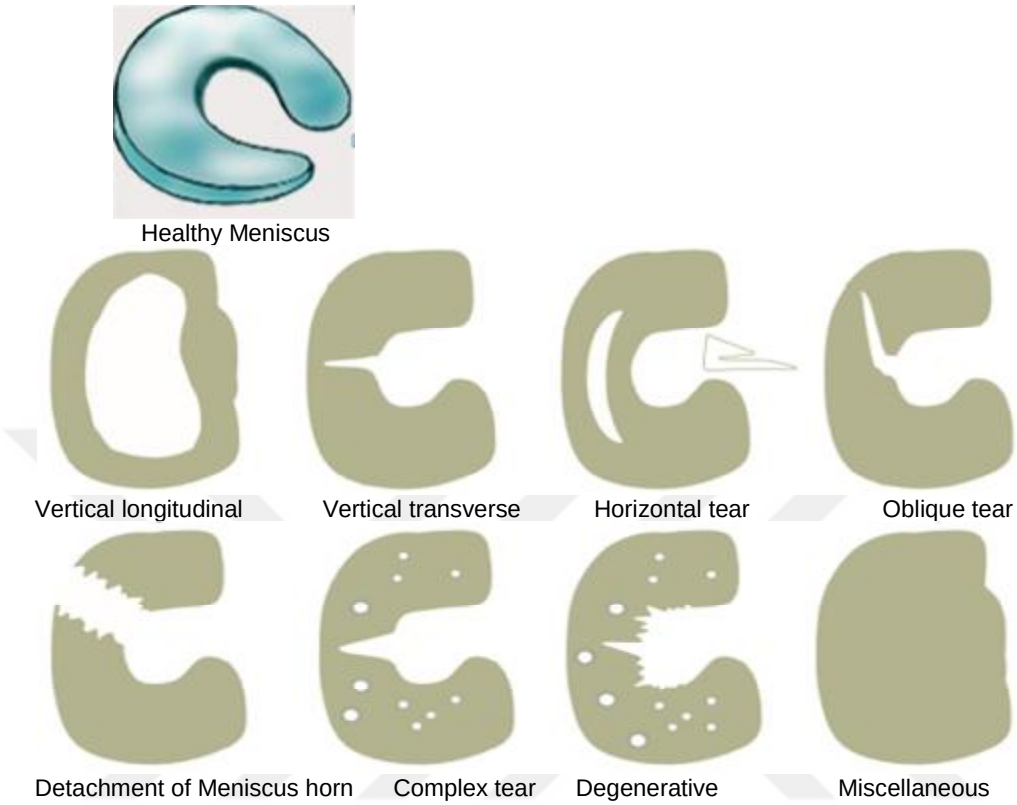

Figure 2.4. A comparison between a normal meniscus and Casscells classification for different types of meniscal lesions (Guo et al., 2015)

2.5. Advances in Meniscal Scaffold

Tissue engineering is known as a multi disciplinary field that aims to restore and regenerate tissue or organ function. In this context, the scaffold serves as a ground for cell delivery and support the tissue remodeling. A good scaffold has to be biocompatible, biodegradable, support cell growth and tissue regeneration and assist similar mechanical and physical properties as the original material that it intends to replace. A scaffold could be prepared either of natural or synthetic polymers (Law et al., 2016).

2.5.1. Absorbable Scaffolds Derived from Biological Components

Absorbable scaffold derived from biological component are defined as the extracellular matrix (ECM) related scaffold and biological scaffolds. Experiences show that implanting a collagen based scaffold in the meniscus without cell seed gives good results regarding meniscal like tissue regeneration and its integration in the host meniscal rim. This kind of scaffold which was defined as" Collagen meniscus implant" (CMI) helped patient to regain more mobility compared to patients who experience the partial meniscectomy (Rodkey et al., 2008).

Other studies reported that after 10 years from implanting CMI, most of patient reported remarkable pain relief and functional improvement without any degenerative knee joint diseases (Zaffagnini et al., 2011).

Silk is also another fibrous protein. As it is known by its performed biomechanical capacities, Multi-purpose usage, accepted biocompatibility and good degradability, it was considered in producing tissue engineering for chondrogenesis, osteogenesis, ligament engineering and others views. Silk fibroin extracted from *Bombyx mori silk worm cocoon* was used also to produce scaffolds. Those scafolds are characterized by being multilayered and multi porous in order to mimic native meniscal morphology and architecture. Results show that this kind of scaffold provides a good growth rate of cellules. Compressive and tensile modules are also high but they do not reach those in the native meniscus (Mandal et al., 2011).

Also, the use of Bacterial cellulose BC provides a good biomechanical capacity, superior hygroscopicity and crystallinity, and accepted biocompatibility, which enable this polysaccharide to be applied to blood, vessel, cartilage, and bone tissue engineering, as well as to treat burns. Experiences done on BC prove that its compressive module is lower than the one in the pig meniscus although being able to produce aligned collagen fibers (Martinez et al., 2012).

2.5.2. Hydrogel Scaffolds

Thanks to their non-cytotoxicity and insoluble features, hydrogels were used in producing meniscal scaffolds. Those materials are known by being able to absorb a large quantity of water (>90%) and owing a great versatility which permit them to uniformly mix seed cell, to load growth factors and to create an appropriate morphology. Only, the problem of poor tensile capacity and bioactivity are presented here (Soppimath et al., 2002).

Researchers done on Polyvinyl alcohol-hydrogel (PVA-H), methacrylated gelatin (GelMA) meniscal scaffold, gelatin (G)/chitosan (Cs) scaffolds, platelet-rich plasma (PRP) combined with gelatin hydrogel scaffold, proved that those meniscal scaffolds enhance meniscal regeneration (Guo et al., 2015).

2.5.3. Decellularized Meniscal Scaffolds

Decellularized Meniscal Scaffolds are characterized by providing a good nest for cell growth and keeping suitable meniscal architecture. However, this kind of meniscus makes the penetration of the seed cells difficult. Some approaches were used to overcome this drawback using rh BMO-2, sodium dodecyl sulfate (SDS), self-developed enzymatic process to treat ovine menisci, concomitant decellularization and oxidation processes, neoteric sonication decellularization system. Those methods help in ameliorating porosity in uneven ways (Guo et al., 2015).

The use of bioabsorbable synthetic polymer is widely induced in forming meniscal scaffold. Polymer such as polyurethane (PU), polyglycolic acid (PGA), polylactic acid, and poly (ε caprolactone) (PCL) provide various benefits like versatility, availability and good biomechanical properties. Unfortunately, those kinds of polymer present hydrophobic properties, shortage in bioactivity, and generation of aseptic inflammation or an immune response (Guo et al., 2015). In this context, Natural polymers seem to be the best materials that have the ability to avoid these complications and especially, the collagen, which is known by its uses

in the development of biomaterials due to its biocompatibility, low toxicity to most tissues, and well-documented structural, physical, chemical, and immunological properties (Aktürk and Keskin., 2016).

In order to mimic meniscus nano and microstructure with the same mechanical properties and high cell compatibility, the process of Electrospinning was adopted for repairing meniscus tears: the Electrospinning process has the ability to produce nanofiber that mimic the nanofibers found in the meniscus. Due to their high surface to volume ratio and their porosity, Electrospun fibers ameliorate cell attachment and cell proliferation through the scaffold (Baek et al., 2015). The figure 2.5 shows a high-resolution SEM image of electrospun collagen fibers with a smooth surface. This characteristic enables the fiber to have a bigger surface compared to his volume.

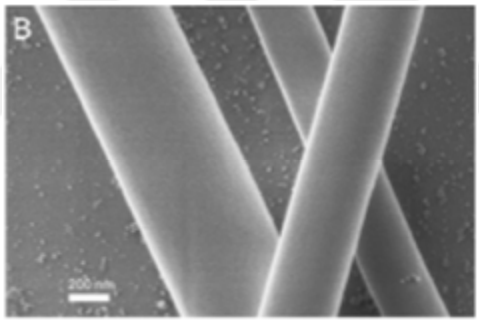

Figure 2.5. SEM image of an electrospun collagen fibers (Yang et al., 2008)

2.6. Electrospinning of Collagen: General Presentation

In our days, the study of material at nano level acquires a lot of interest. In fact, it is one of the rapidly growing scientific disciplines due to its enormous potential in creating novel materials. This technology that is named "Nanotechnology", has hugely impacted many different sciences and engineering fields, such as electronics, materials science, and polymer engineering. Indeed, when the diameters of polymer fiber materials are contracted from micrometers to nanometers, several amazing characteristics loom up such as: smaller pores and

higher surface area, flexibility in surface functionalities and superior mechanical performance compared with any other known form of material.

A number of techniques such as drawing, phase separation, template synthesis, self-assembly, Electrospinning, etc. have been recently used to prepare polymer nanofibers. Only “Electrospinning” has gained the most interest as it produces nanofibers which can be collected to make a nonwoven scaffold. In addition, Electrospinning is adopted to produce ultra-fine fibrous scaffolds using natural and synthetic polymers to imitate the microcellular organism (Garg and Bowlin, 2011).

The benefit of using collagen scaffold over normal scaffolds resides on the fact that Collagen substance has a lot of special characteristics as the ability to be reabsorbed into the body and the non-toxicity. Also, it is a premium for connection and biological interaction along cells. Collagen could also be treated into several formats as porous sponges, gels, and sheets, in addition to being capable to be cross-linked with chemicals in order to make it stronger or to ameliorate its degradation rate .Collagen has been utilized in multiple biomedical applications such as various types of surgery, cosmetics, drug delivery, bioprosthetic implants and tissue-engineering of multiple organs. Moreover, for the duplication of natural tissue, collagen is the best polymer (Oliveira et al., 2010).

2.6.1. Presentation of Collagen

Collagen is the most abundant protein in mammalian (including the human body). It is found specifically in the extracellular matrix (ECM) and it constitutes 30 % of all vertebrate body protein. In general, Collagen is considered as a tissue scaffold and a drug release material. Owing to be the major structural protein, it conferees strength to tissues such as dermis, bone, cartilage, tendon, ligament, and internal organs. In addition, thanks to its biocompatibility, low antigenicity, promotion of cell attachment and growth, and being relatively non-immunogenic, it is the best material for biomedical devices (Friess, 1998).

Collagen, as a biopolymer, is defined by its unique structure, size, and amino acid sequence. Its molecules are characterized by being reunited in three polypeptide chains twined around one another as in a three-stranded rope. Those cordages are held together primarily by hydrogen bonds between adjacent (-CO) and (-NH) groups, and secondly by covalent bonds. Figure 2.6 presents (a) Primary amino acid sequence, (b) secondary left handed helix and tertiary right handed triple-helix structure (Friess, 1998). These collagen triple helices gathered to form collagen fibrils with an average diameter between 50-200 nm .The basic collagen molecule is rod-shaped with an average length of 300 nm, a diameter of 150 nm and has an approximate molecular weight of 300 kD (Carlisle et al., 2010).

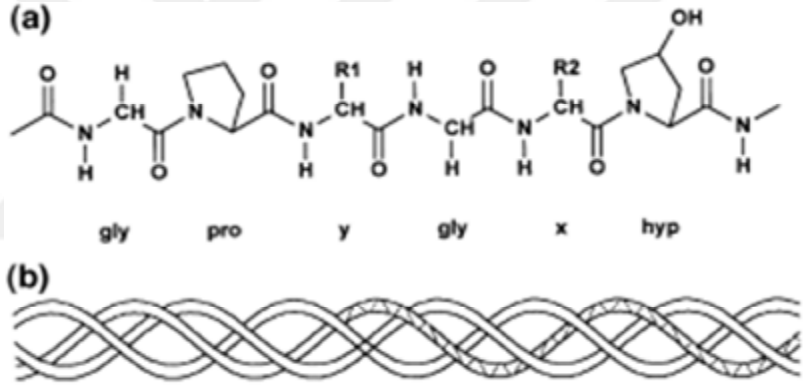

Figure 2.6. Chemical structure of collagen (Friess, 1998)

The collagen family is made of a high variety of molecular combination, supramolecular structure, and function. Basing on its structural organization and sequence homologies, Collagens are grouped into different categories. As an example, we could cite the human body, which comprehends 29 types of collagen. Those proteins can be distributed into different subfamilies such as fibrillar collagens, fibril-associated and related collagens, beaded filament-forming collagen, basement membrane and associated collagens, transmembrane collagens, and hexagonal network collagens (See Table2.1) (Balasubramanian et al., 2012).

Table 2.1. Classification of Collagen types

Categories of collagen	Type of collagen	Diameter of fibers	Arrangement of fibers	Placement of fibers
Fibrillar collagens	I, II, III, V, XI, XXIV, XXVII.	50 to few hundred nm	-Parallel bundles (in tendons and ligaments), -Orthogonal lattices (in the cornea) -Concentric weaves (in bone).	in the ECM of connective tissues such as tendon, skin, and bone
Fibril-associated and related collagens	IX, XII, XIV, XVI, XIX, XX, XXI,XXII	Non-fibrillar collagens as they do not form fibrils by themselves, but they are associated with the surface of collagen fibrils: they are found in the basement membrane junctions between tissues.		
Network-Forming Collagens	IV, VI, VIII, X	It makes linear and lateral associations to form open networks rather than fibrils: Collagen networks act as supporting structures for cells and tissues, serve as selective molecular filters and barriers, function as anchorage for neighboring cells and contain and protect the developing embryos.		
Multiplexins	IV, VII, XV, XVIII	These collagens occur in the endothelial and epithelial basement membrane zones of a wide variety of tissues.		
Transmembrane collagens	XIII, XVII, XXIII, XXV	The transmembrane collagens are widely expressed and participate in cell adhesion, epithelial–mesenchymal interactions during morphogenesis, neuromuscular signaling, and host defense against microbial agents.		

2.2.6. Electrospinning Theory

2.6.2.1. History of Electrospinning

The origin of electrospinning dated back to the early twentieth century: In 1934, Formhals invented his first invention. Though this method had not gained any importance because of some problems related to the technical difficulties of the spinning method such as fiber drying and collection. In his second patent, Formhals revised his earlier approach and increased the distance 'feeding nozzle- fiber collecting device' to give more chance to the electrospun fibers to dry. After that in 1940, Formhals introduced another method to produce composite fiber from multiple polymers by electrostatically spinning. In the 1960s, Taylor started his studies on the jet forming process and in 1969 he examined the shape of the polymer droplet that took place at the tip of the needle when an electric field is applied: This conical shape of the jet took later his name and referred as the "Taylor Cone". The importance of this trick resides on defining the onset of the extensional velocity gradients in the fiber forming process. So, after examining different kinds of viscous fluids, he found that an angle of 49.3 degrees is required to keep the balance between the surface tension of the polymer and the electrostatic forces. (Subbiah et al., 2005)

In subsequent years, focuses shifted to studying the structural morphology of nanofibers. Researchers went over the structural characterization of fibers and its relations with the process parameters. Scanning Electron Microscopy (SEM), Wide-angle X-ray Diffraction (WAXD), Differential Scanning Calorimetry (DSC) and Transmission Electron Microscopy (TEM) have been used in order to characterize electrospun nanofibers. In 1971, Baumgarten reported the Electrospinning of acrylic nanofibers with diameters ranging from 500 to 1100 nm. In addition to that, he detected a specific dependence of fiber diameter with the viscosity of the solution. He also proved that after an initial increase in the applied field, the diameter of the jet shrunk to a minimum value then it increases as the electric fields increases. Larrondo and Mandley made polyethylene and

polypropylene fibers from the melt. They found that the use of this method produces fibers with a larger diameter than the process using solvent. Consequently, they were interested in studying the relationship between the fiber diameter and the melt temperature and they found that the diameter decreases as the melt temperature increases. Moreover, according to them, fiber diameter decreases by 50% when the applied voltage doubled which also shows the importance of applied voltage on fiber characteristics. In 1987, Hayati et al. found that liquid conductivity has a major role in the electrostatic disruption of liquid surfaces. In fact, their results showed that the combination of highly conducting fluids with a highly applied voltage produced a highly unstable jet that whipped around in different directions. Relatively, the production of stable jet requires the use of semi conducting liquids. After a break up of a more than a decade, a major renaissance in research on Electrospinning raised due to the increased knowledge of the powerful application of nanofibers in different fields, such as catalyst substrates, protective clothing, high-efficiency filter media, and adsorbent materials. Research on nanofibers gained a big attention due to the work of Doshi and Reneker who studied the characteristics of polyethylene oxide (PEO) nanofibers. They observed that to decrease the jet diameter an increase in distance needle- collector is required. In this context, Jaeger et al. studied the process of the PEO electrospun fibers thinning: They noticed that the diameter of the flowing jet was shrunk to 19 μm after traveling 1 cm from the orifice, 11 μm after traveling 2 cm, and 9 μm after 3.5 cm (Subbiah et al., 2005).

2.6.2.2. Principle of Electrospinning in General

The technique of Electrospinning is composed of two major divisions: Melt Electrospinning and Solution Electrospinning: We will introduce just the solution Electrospinning since it is the most popular and is the one used in collagen Electrospinning. In general, the process of “Solution Electrospinning” technique consists of electrically charging the polymer solution using a high voltage in order

to produce ultra-fine fibers. The left part of the figure 2.7 shows the basic Electrospinning setup which especially consists of:

- A pipette or a syringe filled with polymer solution/melt
- A high voltage source
- A grounded conductive collector screen

The right part of the figure presents the inset SEM micrograph that shows the randomly arranged fibers obtained.

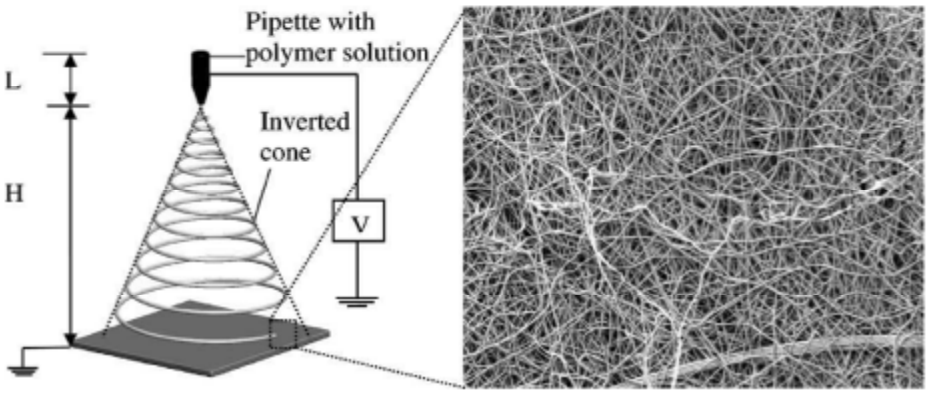

Figure 2.7. Schematic of the Static metal plate (Baji et al., 2010)

The explication of the process is as follow: Firstly, the polymer solution is placed in the syringe pump. After that, it is forced to the tip in order to form a pendant drop. Secondly, free charges are induced inside the polymer as a result of the application of the voltage potential using the immersed electrode in the syringe. Thirdly, the formed pendant elongates due to the presence of the electric field and forms ‘’The Taylor cone’’. Finally, when the applied potential reaches the critical value that allows it to overcome the surface tension of the liquid, a jet of liquid is ejected from the cone tip toward the charged collector: During its trip, the jet undergoes a chaotic motion or bending instability which allows it to evaporate the

solvent and to leave behind just a dry fiber situated in the collector device (Baji et al., 2010).

2.6.2.3. Methods of Electrospinning

Aligned fibers have found interest in several engineering applications, such as nanocomposites, tissue engineering, sensors, filters, electronic devices... In this context and in order to better control the Electrospinning process, researchers have looked to get highly ordered and aligned fibers using mechanical and electrostatic methods. They found that the collector nature influences fundamentally the spun fibers morphology, their physical characteristics and their arrangement and packing density: 4 kinds of collectors are founded:

- Static collector (mentioned before)
- Rotating drum collector
- Rotating disk collector
- Static parallel electrodes (Baji et al., 2010)

Rotating drum collector: This kind of collector is generally used to produce aligned arrays of fibers. Figure 2.8 shows in its above part the electrospinning setup using a rotating drum and in its bellow part, the inset SEM micrograph that shows the aligned fibers obtained.

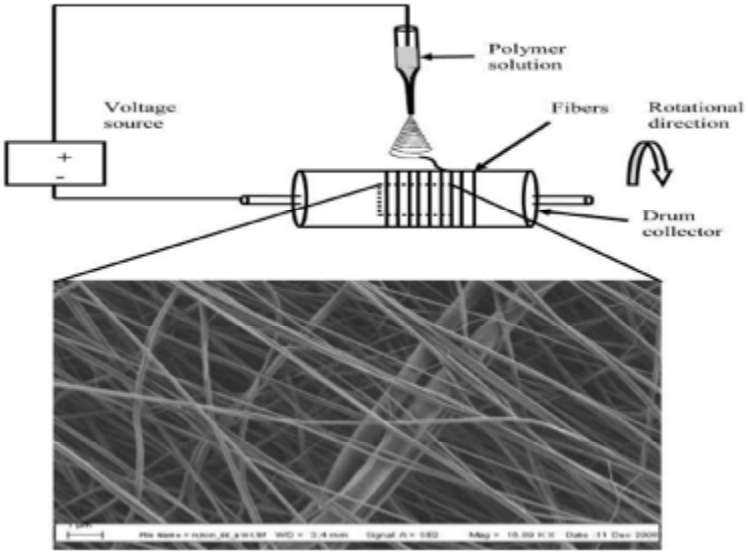

Figure 2.8. Schematic of the rotating drum (Baji et al., 2010)

It is fundamental that the linear speed of the rotating drum matches with the evaporation rate of the solvent: In fact, when the rotational speed is slower than the fiber take-up speed, the fibers collected on the durum will be randomly oriented. Applying a suitable rotational speed develops centrifugal force near the vicinity of the circumference of the rotating drum, which provokes the elongation of the fibers so a decrease in their diameters before being placed on the drum. A continuous increase of the speed to much higher level induces the take-up velocity which drags to break the depositing fiber jet so no continuous fibers will be collected (Baji et al., 2010).

Rotating disk collector: The rotating disk collector can be presented as a derivative of the rotating drum collector. It is used to obtain uniaxially aligned fibers. The advantage of using this kind of collector over the use of the normal drum collector is that the fibers are presented on the sharp-edged disk then are collected as aligned patterned nanofibers. This method consists of applying an electric field which is concentrated on the tapered edge of the disk so the charged

polymer jet is attracted toward this locality. Comparing the quality of the fibers obtained using this method and the others using the rotating drum, the ones obtained by the Rotating disk collector are much better, but the only problem here that a few aligned fibers could be produced since there is only a small area at the tip of the disk (Baji et al., 2010). Figure 2.9 shows in its left part the Electrospinning setup using a disk collector and in its right part, the inset SEM micrograph that shows the aligned fibers obtained.

Static parallel electrodes: The benefits of using this technique are related to the simplicity of its setup and the facility of collecting single fibers for mechanical testing: The air gap found between the two electrodes contributes of creating a residual electrostatic repulsion between the spun fibers which helps their alignment. Figure 2.10 shows in its left part the Electrospinning setup uses static electrodes and in its right part, the optical micrograph that shows the aligned fibers collected using this technique (Baji et al., 2010).

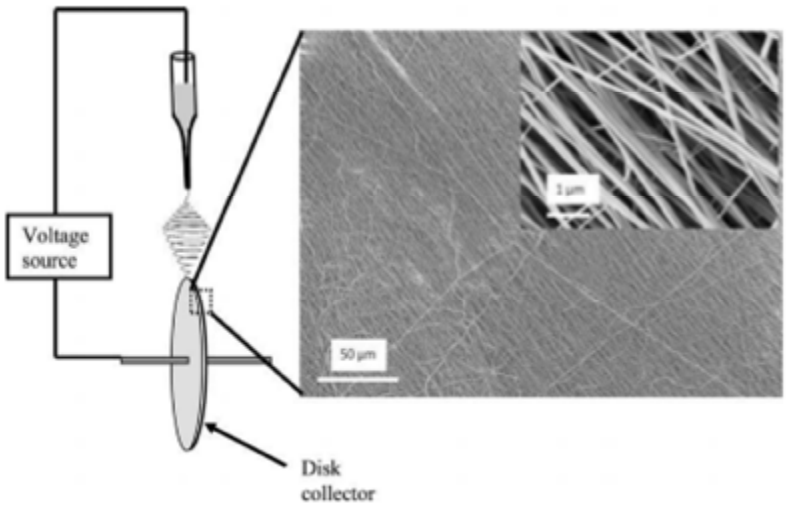

Figure 2.9. Schematic of the disk collector (Baji et al.,2010)

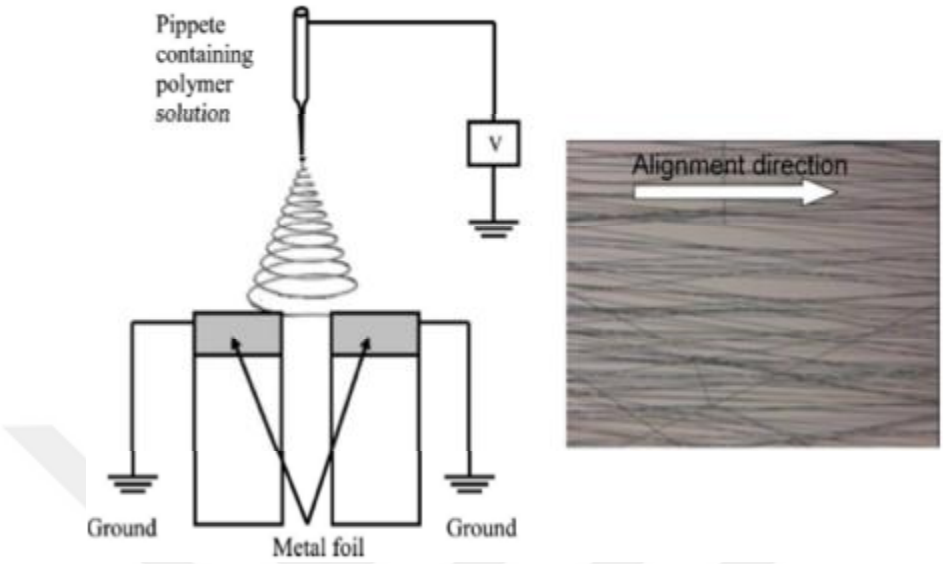

Figure 2.10. Schematic of parallel static electrodes (Baji et al., 2010)

2.6.2.4. Functionalization of fiber strategies

Electrospinning process has normally the ability to functionalize the produced fibers and allows the introduction or the deliberation of the bioactive molecule to the cell over a long duration. But, sometime Electrospinning could have a problem in the functionalization of fibers produced using organic solvents which are incompatible with their bioactive molecules. To solve this malfunctioning, several strategies are used.

Surface functionalization: This method uses physical or chemical immobilization of the bioactive molecule post Electrospinning. It is composed essentially of adsorbing biologically functional nanoparticles or drug encapsulated polymeric nanoparticles layer by layer assembly of polyelectrolyte or chemical immobilization of bioactive molecules on the fiber surface. An example of chemical functionalization of fiber surface is presented in figure 11.a.

Blend Electrospinning: Generally, this technique is used for drug released functionality. It consists of mixing the target bioactive molecule with the polymer solution prior to Electrospinning. (See figure 11.b).

Emulsion Electrospinning: Emulsion Electrospinning could be considered as an extension of blend Electrospinning since it demands the same basic set up. This technique requires the spinning of two immiscible solutions simultaneously. The fiber forming polymer involves an organic solvent and makes the continuous phase whereas the bioactive molecules are dissolved in aqueous solution and formulate the dispersed phase. This method was used in producing functionalized fibers with different morphologies for regenerative therapies. (See figure 11.c).

Coaxial Electrospinning: This technique consists of Electrospinning fibers issued from two different solutions: The process here involves pumping separately those solutions through two nozzles connected to a high voltage source (See figure 11.d), which results in a core- sheath fiber morphology. Hence, coaxial Electrospinning provides functionalizing fibers with bioactive molecule (Szentivanyi et al., 2011).

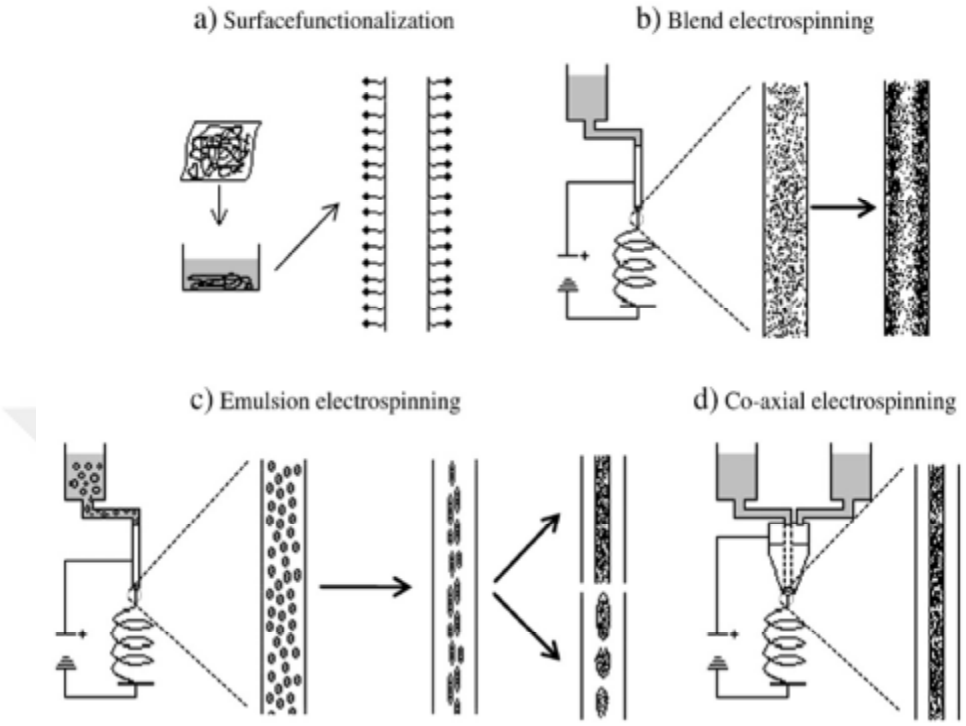

Figure 2.11. The different strategies for functionalization of electrospun fibers (Szentivanyi et al, 2011)

2.6.3. Electrospinninng of Collagen

As a historical view, we could shortly admit that the first experiences of Electrospinning collagen-based scaffolds were executed by Huang et al. in 2001. Those tests consist of combining the Electrospinning of collagen and polyethylene oxide (PEO) to form nanofibers. The diameter range of those descendant products was between 100 and150 nm (Huang et al., 2001). In 2002, the Electrospinning of pure collagen was successfully observed using type I and type III collagen. The solving of those proteins was provided by 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) which is considered as a very suitable solvent for those types of collagen. It is considered as an organic and volatile solvent which has a boiling point at 61°C. This low value permits it to initiate the evaporation of the solvent under normal

atmospheric conditions so facilitating the deposition of the fiber in the required dry state. The perfect concentration of collagen was found at 0,083g/ml. Matthews et al. announced that the average diameter of electrospun fibers collected ranged between 100 and 730 nm. (Matthew et al., 2002)

In addition to using 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) as a solving agent, 2,2,2-trifluoroethanol (TFE) is also commonly used to solve collagen for Electrospinning. However HFIP and TFE, due to their corrosive nature, can decrease the water stability of collagen scaffolds and transforms collagen to gelatin (Chakrapani et al., 2012). This drawback pressed researchers to find more suitable solvents which could mimic results obtained using 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) without denaturing or altering the biologically properties of collagen. They found that the combination of phosphate buffered saline (PBS) and ethanol contributes in facilitating the formation of a stable Taylor cone. In this context, it was noticed that increasing the salt concentration in buffer contributes in decreasing the average fiber diameter from 540 to 210 nm (Dong et al.,2009). Also, we can cite the experience marked by Liu et al. which consists of developing an acetic acid aqueous solvent system. The average of the electrospun fiber diameter is 149.1 6 ±20.6 nm. (Liu et al., 2010).

2.7. Collagen cross-linking

Cross-linking of collagen scaffolds is the next step after Electrospinning. It consists on a lateral linking where two or more adjacent molecules of a polymer join to form a bigger molecule. This operation contributes on raising the melting point of the polymer in addition to making it stronger which ameliorates the mechanical properties of a scaffold (Dong and Lv, 2016). There are three major approaches for cross-linking collagen: Covalent chemical cross-linking of neighboring fibrils, Physiochemical cross-linking methods and Intermolecular cross-linking of amino acid side chains.

Covalent chemical cross-linking of neighboring fibrils: This method consists of covalently coupling neighboring collagen fibrils using the combination of reactive groups in the collagen fibrils and the cross-linking molecules. As an example of those agents, Glutaraldehyde, epoxy compounds, and isocyanates could be cited. The disadvantage to these compounds consists on being included within the cross-link as strange chemical species toward biological tissues, and as a consequence of that, several kinds of unfavorable conditions, such as inflammation, foreign body response, immunogenic reactions can occur. As being the most common used cross-linking agent, Glutaraldehyde can be mentioned here: it is characterized by its low cost, availability, rapid reactivity, and solubility in aqueous solution. The cross-linking of collagen-based tissues using glutaraldehyde decreases especially the biodegradation rate which makes them biocompatible while conserving anatomic integrity, scaffold strength, and flexibility (Dong et al., 2009). Although, it has received criticism as a crosslinking agent for implants since it promotes calcification and releases toxic molecules from the implant (Liu et al., 2010). The reaction of Crosslinking collagen with glutaraldehyde is presented in figure 2.12.

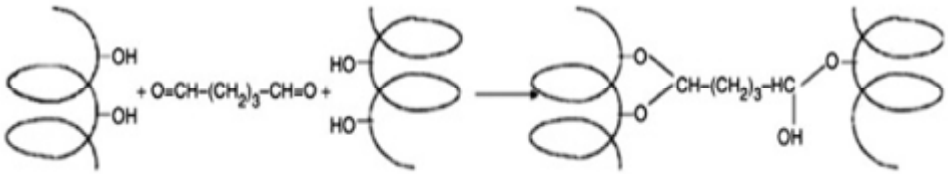

Figure 2.12. Crosslinking reaction of collagen with glutaraldehyde (Liu et al., 2010)

Physiochemical cross-linking methods: The basic principle of these methods consists of exciting the reactive amino acid side chain found already in the collagen fibrils in order to create a covalent cross-link without the addition of foreign chemical substances into the scaffold. These processes include microwave

irradiation, photooxidation, dehydration, and dehydrothermal treatment. The use of this method is preferred over covalent chemical crosslinking agents such as Glutaraldehyde since no chemicals are required and the risk of producing a toxic species decreases. As an example, we cite the exposition of collagen scaffold to UV irradiation with a wavelength light around 254 or 514 nm. The UV treatment is capable to improve the mechanical integrity of the scaffolds (Liu et al., 2010). However, overexposing the collagen has damaging effects on fibril structure (See figure 2.13).

Intermolecular cross-linking of amino acid side chains: Intermolecular cross-linking of the reactive amino acid side chains uses a catalyst, such as a carbodiimide or azyl acid which will not be a part of the cross-link like glutaraldehyde, epoxies, and isocyanates. After cross-linking the catalyst and its byproducts can be easily washed out of the scaffold. In recent days, Carbodiimide cross-linking becomes preferred over glutaraldehyde owing to its increased biocompatibility. The most common carbodiimide compound is 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide. It reacts with the carboxyl groups of side chains in collagen which generates the amide cross-link and ejects made wastes of the carbodiimide. The produced isopeptide bond imitates the native peptide bond present in proteins. The carbodiimide reaction was found to be optimized at pH 5 (Liu et al., 2010). Figure 2.14 presents the crosslinking of collagen using 1-Ethyl-3-diaminopropyl-carbodiimide (EDC) and N-hydroxysuccinimide (NHS).

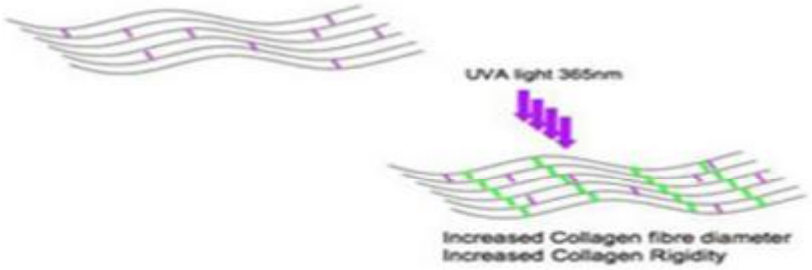

Figure 2.13. Crosslinking of collagen using UV radiation (Liu et al., 2010)

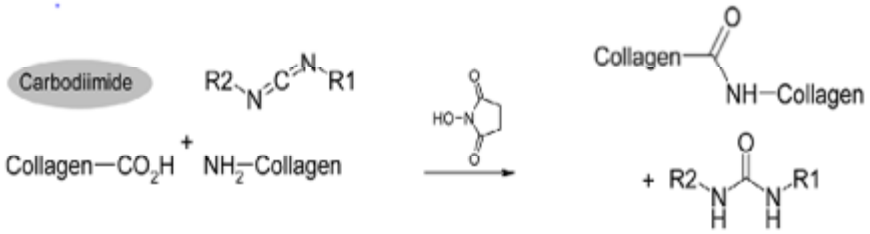

Figure 2.14. Collagen crosslinking using (EDC) and (NHS) (Liu et al., 2010)

A summary table that resumes the advantage and the disadvantage of each process of Crosslinking is presented below:

Table 2.2. Advantages and disadvantages of different methods of crosslinking

Methods of Crosslinking	**Advantages**	**Disadvantages**
Covalent chemical cross-linking of neighboring fibrils	Low cost of the used chemical products.	Including crosslinking molecules within the scaffold as strange substance.
Physiochemical cross-linking methods	No addition of foreign chemical substances into the scaffold.	Over exposing the collagen to UV radiation can damage the fibril structure.
Intermolecular cross-linking of amino acid side chains	Using catalyst that could be washed out after the crosslinking operation	**********

2.8. Characterization of Electrospun Collagen Fibers and Scaffold

2.8.1. Geometrical Characterization

Scaffold microstructure is described by its fiber diameter, fiber orientation, porosity and pore size: fiber diameter and fiber orientation can be estimated directly from SEM image. Contrary, porosity and pore size are frequently evaluated by a various type of techniques:

Electron Microscopy : To evaluate the morphology of the collagen electrospun fiber, both Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) are used (Dong et al., 2009). SEM has the advantages of being a quick method for observing the produced fibers and using small samples size for its operations (See figures 2.7, 2.8, 2.9, and 2.10).

Porosity Measurement: Scaffold porosity is the void volume related to the total volume of a scaffold. Therefore, it represents the part of the scaffold available for cell infiltration and tissue formation. The ideal porosity values for electrospun scaffold range from 70 to 95% and may be determined gravimetrically. (Chakrapani et al., 2012). Scaffold porosity can be calculated using those equations:

$$\mathbf{d_s} = \frac{scaffold\ mass}{scaffold\ thikness \times scaffold\ area}\ ;\ \mathbf{P_s} = 1 - \frac{scaffold\ density}{bulk\ material\ density} \quad (2.1)$$

ds: scaffold density

P_s: scaffold porosity

For unknown bulk density

$$\mathbf{V_{pt}} = \frac{wet\ weight \times dry\ weight}{liquid\ density}\ ;\ \mathbf{P_s} = \frac{Total\ pore\ volume}{scaffold\ thikness \times scaffold\ area} \quad (2.2)$$

V_{pt}: Total pore volume

The porosity of the collagen scaffold was also tested using the SEM instrument. The image issued from this last is examined using Image J software (Punnose et al., 2015). This program transforms the gray scale level of 256 into binary images by calculating the threshold. The equation used in this measurement is:

$$P = (1 - n/N) \times 100 \quad (2.3)$$

P is the porosity percentage of the binary image

n is the number of white pixels

N is the total number of pixels in the binary image

Determination of pore size: Three principle methods are used to characterize the pore size of an electrospun scaffold: Capillary flow porometry: It is a characterization technique which provides the measure of pore size with respect to gas flow on the scaffold. It is broadly used to measure minimum, maximum and mean flow pore sizes in addition to pore size distribution. This method is usually used in filtration application but it proves its usability also in perfusion seeding of cells (Bagherzadeh et al., 2013). A typical dry curve and wet curve for a fibros scaffold are given in figure 2.15. The pore size and distribution are calculated using the following equation:

$$D = \frac{4\,\gamma cos\theta}{P} \quad (2.4)$$

D: is pore diameter

γ: is surface tension of the wetting liquid

θ: is the contact angle of the wetting liquid

P: is the differential pressure

The second technique is SEM base analysis (Tomba et al., 2010). It uses a small representative sample of the scaffold where all fibers are projected in the same plane and the void area between them is measured. The pore diameter distribution is estimated from the area distribution.

The third technique is Liquid instruction\extrusion porosimetry (Manickam and McCutcheon, 2012). This technique provides the measure of the pore diameter and volume which is more relevant to cell scaffold interaction. Also, pore

characterization is realized by introducing mercury or excluding the wetting liquid out of pore while a pressure application. Figure 2.16 shows an example of the obtained curve data.

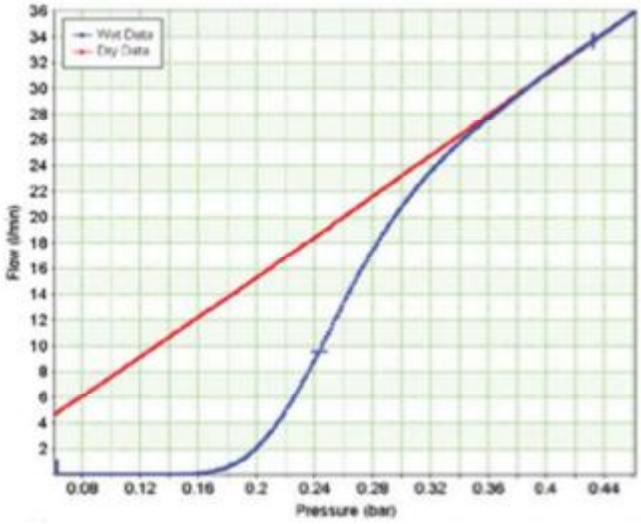

Figure 2.15. Typical dry curve and wet curve for a fibrous scaffold (Bagherzadeh et al., 2013)

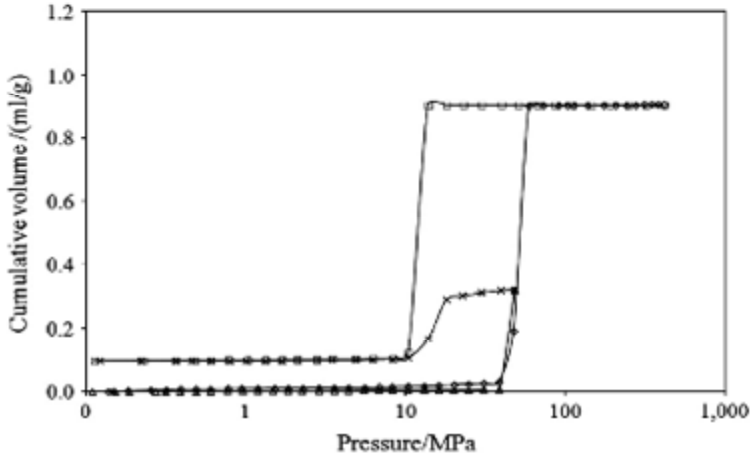

Figure 2.16. Mercury intrusion and extrusion curves for porosimetry experiments (Bafarawa et al, 2014)

Surface contact measurement: Wettability is an important factor to characterize biomedical tissue engineering since they are exposed to blood and other body fluids. This test is generally done by estimating the contact angle of a liquid applied on the surface of the scaffold or tissue using a contact angle meter (Chakrapani et al., 2012).

Figure 2.17. Images of water droplets on collagen scaffolds (a) uncrossliked, (b,c) crosslinked captured at different time after droplet deposition (Kitsara et al., 2015)

2.8.2. Chemical Characterization

To determine the surface chemistry of the collagen electrospun nanofibers, FTIR analysis is used (Erdem et al., 2017). This test is critical for tissue engineering scaffold which are supposed to provide a cellular growth, physiological function and keep normal states of cellular differentiation. (Bürck et al., 2013). An exemple of FTIR spectra is given bellow.

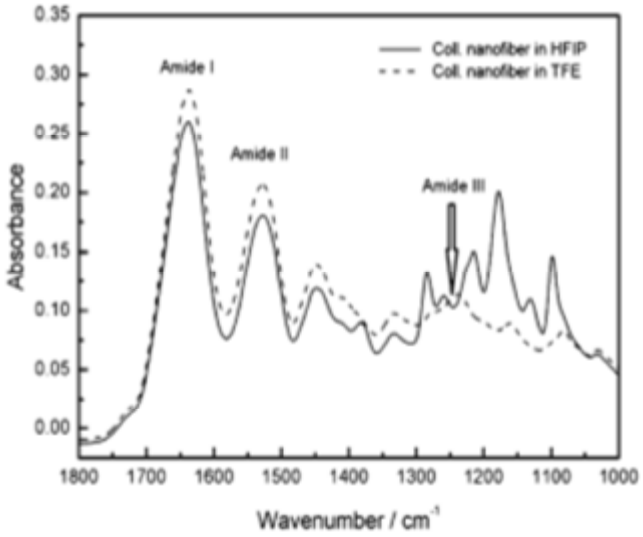

Figure 2.18. FTIR spectra of electrospun collagen nanofibers obtained from HFIP and TFE (Bürck et al., 2013).

2.8.3. Mechanical Characterization

The mechanical properties of scaffolds destined to be used in biomedical fields are very important. Firstly, researchers reported that cell differentiation is affected by the mechanical properties. Secondly, to perform the tissue function, the scaffold and the native tissue that it intends to replace must have the same properties. Finally, during implantation, the scaffold must have a high mechanical stability and support tissue regeneration and structure degradation (Carlisle et al., 2010). The use of the produced collagen electrospun nanofiber scaffold is directed toward the biomedical application that is why the precise measurement of the mechanical properties is vital. Punnose et al reported the measurement of the Young's modulus, the tensile strength and the elongation at break of the nanofibers scaffolds (Punnose et al., 2015).

According to literature, samples which are prepared to be measured were cut into different shapes. Some researchers reported that they used strips in rectangular shapes (Punnose et al., 2015). Others reported that their samples were

cut into a dog bone shaped specimens (Baek et al., 2016; Matthews et al., 2002). Other reported that their samples were cut into a dumbbell shape (Rho et al., 2006).

2.9. Collagen-based scaffold: Possible combinations

In order to enhance mechanical and structural properties of the collagen-based scaffold, a mixture of natural or\ and synthetic polymer are used.

2.9.1. Combination of Collagen with natural polymer

Blending collagen with a natural polymer is widely used in tissue engineering. In this thesis the combinations with chitosan and fibroin are taken as examples:

Chitosan: As owing a low toxicity, being non-immunogenic and biodegradable, chitosan is considered a good choice for the biomedical application. Also, being a positively charged polymer makes its combination with collagen, which is an anionic polymer, perfect to form a two component scaffold with ameliorated mechanical and biological properties. This kind of scaffold, which is characterized by its satisfying porosity, provides a good grow to 'Mesenchymal stem cells' (MSCs) with pseudopodia spreading into the scaffold also shows the good cytocompatibility between (MSCs) and the scaffold (Yan et al., 2015).

Silk fibroin: Silk fibroin shows a good biocompatibility, biodegradability, and mechanical properties which make it a suitable biomaterial for scaffold fabrication. Experiences show that the combination Collagen-Silk fibroin is beneficial in corneal tissue engineering application (Long et al., 2015).

2.9.2. Combination of Collagen with synthetic polymer

As like blending with a natural polymer, blending collagen with synthetic collagen has been largely used for tissue engineering. Examples of these synthetic polymers are; poly (ε-caprolactone) (PCL), polylactic acid (PLA), poly (ethylene

glycol) (PEG), polyglycolide (PGA), poly (lactide-co-glycolide) (PLGA) and polyvinyl alcohol (PVA).

In addition to being a non-toxic, low cost, bioresorbable polymer which has a good mechanical properties and slow degradation rate, the combination of PCL with collagen fibrous scaffold is characterized by a low crystallinity, a small size and an ameliorated dehydration temperature (50 to 60°C) comparing to 32,5°C for collagen. This kind of scaffold proves its efficacy in repairing large skeletal muscle tissue defects (Choi et al., 2008).

The blended collagen\PLA scaffolds own open pores throughout the scaffold and better stiffness rate comparing to collagen scaffold. These characteristics permit it to be used in cartilage tissue engineering (Haaparanta et al., 2014).

Besides, the multi-component scaffolds, that use the combination of natural and\or synthetic polymers such as; collagen\chitosan\PLA, hyaluronic acid\collagen\PEG and PCL\PLGA\collagen, prove their potential application in vascular tissue engineering, repair of central or peripheral nervous system and regeneration of bone or liver tissue (Hardy et al., 2015).

2.9.3. Combination of collagen with inorganic hybrid scaffold

The examination of PLLA\collagen\ HA scaffold shows a good cytocompatibility and superior osteoinductivity. This scaffold proves also its efficacy as a supportive for steam cell-based therapies for bone repair and reconstruction. Besides, Shin et al. showed that GO\PLGA\Collagen hybrid fibrous scaffold has a good attachment, proliferation and myogenic differentiation of C2C12 skeletal myoblasts (Shin et al., 2015).

2.10. Applications of collagen scaffolds

As being the major component of ECM in many tissue and organs, multiple typical applications of collagen scaffold are used.

2.10.1. Nerve tissue

The use of collagen-based scaffold facilitates the regeneration of injured nerves. This is thanks to their good platform for nerve regeneration and repair. Liu et al. showed the possibility of fabricating 3D scaffolds utilizing collagen fibers and their related application potential for SCI repair (Liu et al., 2012). Boecker et al. proved that this method improves axonal regeneration by linking seeding cells with scaffolds (Boecker et al, 2015).

2.10.2. Cartilage tissue

A recent technology named ‘Matrix-Induced-Autologous chondrocyte implantation’ (MACI) has been developed. It is composed of three surgical processes: Extraction, Purification, and Expansion in vitro of chondrocytes; Seeding of chondrocytes on a 3D matrix, which can later be re-implanted. This method was evaluated by Basad et al who proved its safety, efficacy, and usefulness in surgical operations for treating damaged cartilage (Basad et al., 2015).

2.10.3. Tendon\Ligament Tissue

The electrospun fibers can mimic the fibrous structure of tendon and ligament tissue repair. A study done by Cardwell et al. showed that the use of large diameter fiber is preferred in tendon\ligament tissue engineering (Cardwell et al., 2014). Another study (Yunoki et al., 2015) proved that the use of oriented structure can promote the repairing efficacy of tendon\ligament due to the anisotropy of native tissue.

2.10.4. Vascular Graft

Lee et al. showed that PCL\Collagen scaffold has the ability to resist high degrees of pressure and flow for a long time in addition to providing a suitable environment for the growth of the vascular cell (Lee et al., 2008). The

characteristics of this kind of fibrous scaffolds were: Elasticity: 2.7 ±1.2 MPa, Tensile strength: 4.0 ±0.4 MPa and Burst pressure: 4912 ± 155 mmHg, which were considered appropriate to use. Tests of biocompatibility give also satisfying results.

2.10.5. Skin

Ma et al. presented GA-treated collagen/chitosan scaffolds as an owner of a good cytocompatibility and able to promote a good cell infiltration and effective proliferation (Ma et al., 2003). Moreover, the scaffolds are capable of pushing along the infiltration of the fibroblasts from the surrounding tissue in vivo, which marks their ability in dermal repair. Rho et al. showed that in open wound healing tests, the collagen scaffold speeded up the disappearance of the surface tissue debris, proliferation of fibroblasts and young capillaries in the early stage of wound healing (Rho et al., 2006).

3. PREVIOUS WORKS

Several researchers were interested in fabricating scaffold destined to be used in repairing meniscus tears.

Chiari et al. (2006), fabricated a porous composite scaffold using hyaluronic acid and PCL in order to repair total and partial meniscal lesion in sheep. The properties of the implant material were evaluated regarding its size, its biomechanical stability, tissue in growth, and integration: Eight sheep were treated with three total and three partial meniscus replacements (right joint was treated) while two meniscectomies served as empty controls. The animals were euthanized after 6 weeks. The specimens were assessed by gross inspection and histology, and compared with the nonoperated left joints. The used scaffolds here were implanted without being seeded with cell. For 6 weeks, the implant maintains its morphology and kept its position. According to the historical results, the implant has been integrated in the native menisci and a wide number of blood vessels were created to tightly bond the capsule that was wrapped by synovial-like tissue. The problem in this case was about the body weight which trains the extrusion and the degeneration of the cartilage in both the total and partial transplantation (Chiari et al., 2006).

Orteq Sports Medicine in London- UK (2006), invented a novel biodegradable Actifit implant which is composed of PU (20%) and PCL (80%). This acellular meniscal scaffold is designed to provide a template for tissue in-growth rather than providing mechanical support. Moreover, it is characterized by its interconnected pore structure which improves vessel in-growth and restoration of the meniscus: In a canine model, the scaffold was found to act as a template for the organization and proliferation of cell in the interconnected segments of the porous scaffold. Biopsy was conducted for a 12 months post implantation in human meniscus demonstrated fibrochondroblast-like cells integrating into the scaffold. Following initial laboratory studies, scaffold was shown to improve contact areas

and pressures in a sheep model. A study comparing this scaffold to partial meniscectomy in a canine model showed tissue in-growth onto the scaffold and integration with the surrounding capsule after 6 months post intervention (Spencer et al., 2012). The figure 3.1 shows a Polymer scaffold where from left to right: microscopic view, medial and lateral implants, and schematic of insertion are presented.

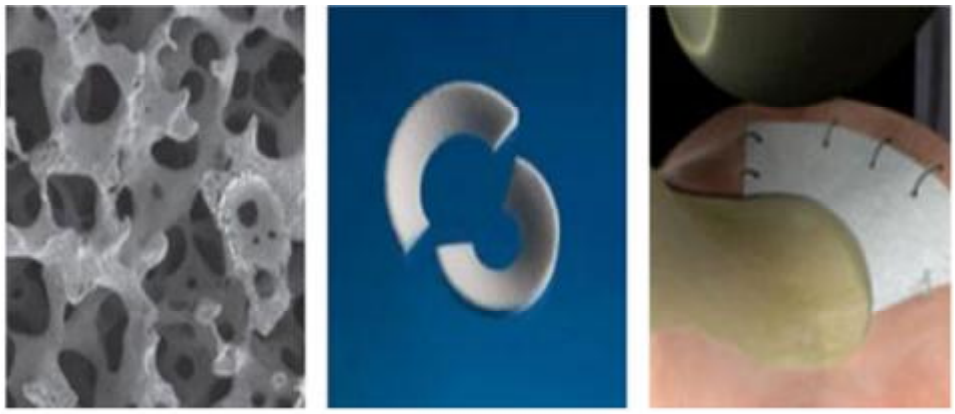

Figure 3.1. Polymer scaffold (Spencer et al., 2012)

Baker and Mauck (2007), employed Poly(ε-caprolactone) (PCL) nanofibrous scaffolds for the tissue engineering of the meniscus. Non-aligned (NA) and fiber-aligned (AL) nanofibrous scaffolds, which were seeded with meniscus cells, were used to test the hypothesis that fiber-alignment would improve the mechanical properties. As result, a significantly larger increase in mechanical properties was observed for AL nanofiber scaffold compared to the NA constructs. Cell-seeded NA constructs increased in modulus by ~1 MPa in 10 weeks while cell-seeded AL construct increased by >7 MPa in the same period. Also, they found that cells in the AL group were presented in an aligned morphology, while the ones formed in the nonaligned (NA) group were assembled in a polygonal shape. In addition to that, researches prove that the biomechanical properties performed in the AL group are better than those in the NA scaffolds which

demonstrate that AL scaffold help in directing the growth of cells and ameliorated biomechanical capacity (Baker and Mauck, 2007).

Koller et al (2012), attempted to enhance the bioactivity of synthetic scaffolds by adding polyethylene terephthalate (PET) to hyaluronic acid/PCL scaffolds: The produced scaffolds were tested after being covered by $2x10^6$ ovine meniscus cells. The specimens were harvested in triplets at 12 hours, 7, 14, 21 and 28 days. Results showed that the addition of PET to a hyaluronic acid/polycaprolactone scaffold enhances a cell viability and increased type II collagen secretion (Koller et al., 2012).

Fisher et al. (2013), developed a novel electrospinning method to produce scaffolds composed of circumferentially aligned (CircAl) nanofibers in order to mimic the structure of the meniscus, and quantified their structure and mechanical properties in order to compare them with traditional linearly aligned (LinAl) scaffolds. Nanofibrous scaffolds here were created using poly (e-caprolactone) (PCL) dissolved in dimethylformamide and tetrahydrofuran. The produced fibers were locally oriented in CircAl scaffolds, but their orientation varied considerably as a function of position, whereas LinAl fibers did not change in orientation over a similar length scale. Mechanical analysis of CircAl scaffolds revealed significant interactions between scaffold length and region: the tensile modulus near the edge of the scaffolds decreases with the increase of the scaffold length. Overall, the results obtained, following the seeding of juvenile bovine mesenchymal stem cells (MSCs) in the scaffolds, showed a circumferential cellular alignment in the CircAl scaffolds that resemble the one seen in the native meniscus (Fisher et al., 2013).

Baek et al (2015,) investigated a novel approach that consists of building layers of aligned polylactic acid (PLA) electrospun (ES) scaffolds with human meniscus cells embedded in the extracellular matrix (ECM) in order to form neotissues that resemble the meniscus tissue. In this work, PLA ES scaffolds with randomly oriented and aligned fibers were seeded with human meniscus cells derived from vascular or avascular regions. The evaluation of results focused

essentially on pursuing cell viability, cell morphology, and gene expression profiles using confocal microscopy, scanning electron microscopy (SEM), and real-time polymerase chain reaction (PCR). They found that the morphology and the mechanical properties of PLA scaffolds (with and without cells) were influenced by the fibers direction of the scaffolds. Also they found that ES materials, which possess the same mechanical strength as the native meniscus and can support neotissue formation, show potential to be used in cell-based meniscus regeneration strategies (Baek et al., 2015).

Baek et al (2016), as a continuity of their previous work, have recently studied the use of human meniscus cell-seeded electrospun (ES) collagen type I scaffolds in order to know the possibility of implanting these cell-seeded scaffolds to repair defects created in meniscal avascular tissue explants. Experiences here were held using human meniscal cells (derived from vascular and avascular meniscal tissue) which were seeded and cultured on aligned ES collagen scaffolds for 4 weeks before implantation. Results were evaluated for cell viability, gene expression, and mechanical properties. Surgical defects resembling ‘‘longitudinal tears’’ were created in the avascular zone of bovine meniscus and implanted with cell-seeded collagen scaffolds and cultured for 3 weeks. Tissue regeneration and integration were evaluated by histology, mechanical testing, and magnetic resonance imaging. Results show that cell-seeded collagen scaffolds was integrated in the native tissue better than acellular collagen scaffolds. Human meniscal cell-seeded ES collagen scaffolds may therefore be useful in facilitating meniscal repair of avascular meniscus tears. It is worth to mention that in this work, the successful solving of collagen I here was using 20× phosphate buffered saline (PBS) and ethanol (Baek et al., 2016).

Guthold et al (2010), characterized the mechanical properties of individual electrospun collagen type I fibers using a combined atomic force microscopy (AFM) and inverted optical microscope system. The measured fibers were electrospun from a 0.080 g/ml collagen solution in 1,1,1,3,3,3-hexafluro-2-

propanol. Results showed that the ratio of fibers ranged from 160 nm to 783nm with an average of 320 ±126 nm and that the strain bending modulus of the fibers had a strong dependence with the radius: As the radius of the fiber decreased the modulus increased. The average modulus of the electrospun fibers was 2.8 ± 0.4 GPa. Figure 3.2 shows the typical electrospun collagen type I fibers stress strain curve.

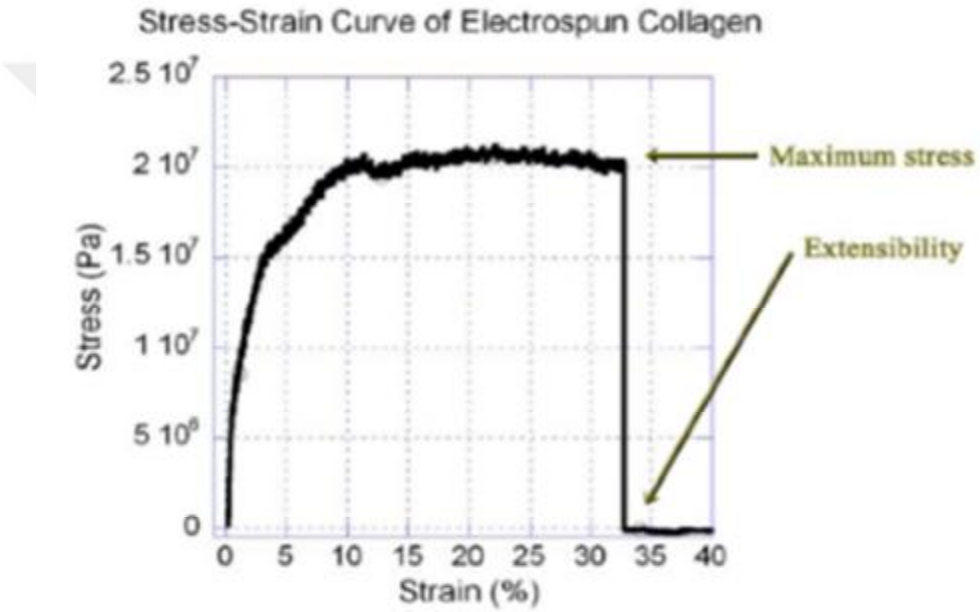

Figure 3.2. Typical stress-strain curve acquired during a fiber manipulation

The average extensibility of fibers was 33%±3% and do not depend on the fiber radius which means that an individual electrospun collagen type I fiber can be stretched 1.33 times their initial length before rupture. The maximum stress or the peak stress, which is the highest stress value reached before the fiber rupture was also measured (figure 3.2): As the strain value increased from zero to 12% the stress increased. At 12% strain, the stress applied to the fiber reached a plateau where it kept the same value until the fiber ruptured. As the modulus, the maximum stress depends on the radius of the fiber: As the radius increased, the

maximum stress decreased. This study also showed that mechanical properties of a collagen mat could be controlled by the fiber diameter which depends on the solvent concentration and other spinning factors (Carlisle et al., 2010).

4. DISCUSSION

In this section it will be discussed about parameters that influence the diameter measure of an electrospun nanofiber produced from a biopolymer source of collagen type I. Firstly, the general design of Electrospinning process of the collagen I will be explained. After this general explanation, a discussion will be held about the collagen I fiber diameter which is the focus of this study.

4.1. The General Design of Electrospinning Process of the Collagen I

The general model of the process of collagen I Electrospinning is shown as bellow:

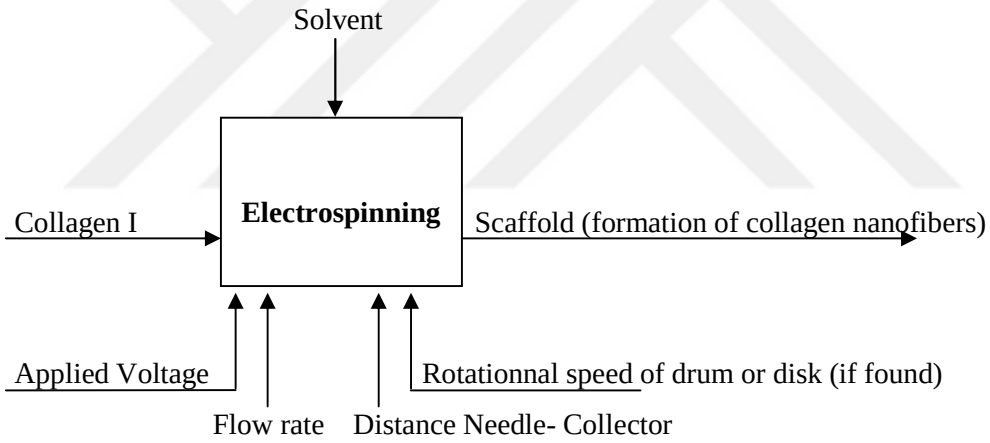

Figure 4.1. The general model of the process of Electrospinning

4.2. Discussion about The Collagen I Fiber Diameter

The collagen fiber is the elementary composition of a meniscus scaffold. As mentioned before the diameter of the collagen fiber in the meniscus range from 50 200 nm. So, the goal of researchers is to produce collagen scaffold that mimic the one in the human body, studying the diameter of the electrospun nanofiber and the factors that influence its measure is very important. Previous studies that were

interested in studying the parameters that affects the diameters of electrospun collagen fibers affirmed that there are controlled variables related to the electrospinning machine such as: flow rate, electric field strength, distance between tip and collector and collector composition and geometry, solution characteristics such as viscosity, surface tension, conductivity and ambient parameters such as temperature and humidity, which can affect the measure of the fiber diameter. The table bellow resumes all work done in this field. The first column is the solvent type. In our table, we have 3 types of solvents: 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), PBS 20× Buffer: potassium chloride and Acetic acid aqueous solvent
The second column is the source of collagen type I. in our table, there are 7 different sources: Calf skin, Bovine skin/ tendon, Human placental, Fish skin, Rat tail, Porcine skin, Atelocollagen.

The following table presented the optimized values that researchers can find after varying the conventional parameters such as concentration of collagen and solvents, flow rate, electric field strength, distance between tip and collector and ambient parameters such as temperature and humidity.

Table 4.1. The different collected measure of collagen type I Electrospun fibers

Solvent	Source of collagen type I	Average Fiber diameter (nm)	References
HFIP	Calf skin	460	(Rho et al., 2006)
		269	(Yang et al., 2008)
		275	(Teng et al., 2008)
		462	(Boland et al., 2004)
		250	(Zong et al., 2006)
	Bovine dermis	187	(Matthews et al., 2002)
		100	(Li et al., 2005)
		175	(Bürck et al., 2013)
		120	(Song et al., 2008)
		125	(Shih et al., 2006)
	Human placental	390	(Matthews et al., 2002)
		415	(Matthews et al., 2002)
	Fish Skin	310	(T. Zhou et al., 2016)
	Rat tail	569	(Liu et al., 2010)
		600	(Thomas et al., 2007)
PBS	Calf skin	630	(Y. Zhou et al., 2015)
	Bovine dermis	496	(Baek et al., 2016)
		467	(Baek et al., 2016)
		350	(Dong, Wu, & Clark., 2011)
		420	(Meng et al., 2012)
	Porcine skin	105	(Bak et al., 2016)
	Atelocollagen	100	(Kitsara et al., 2015)
Acetic acid	Bovine tendon	142	(Castilla-Casadiego et al., 2016)
		272	(Meimandi-Parizi et al., 2013)
	Fish skin	550	(Punnoose et al., 2015)
	Rat tail	149	(Liu et al., 2010)

In this table, we notice that the measure of a collagen I Electrospun fiber is different in term of solvent type and collagen I source: For HFPI, PBS and acetic acid as solvents, we notice that the use of different sources of collagen I influences the measure of the electrospun nanofiber.

For HFIP, we notice that using different sources of collagen I influence the measure of the produced nanofiber diameter. For this kind of solvents, the best measures where found for the nanofibers produced using collagen I extracted from bovine dermis: All measures are between 100 to 187 nm. Since the measure of collagen fiber in the meniscus range from 50 to 200 nm, the combination of HFIP as solvent and Bovine dermis as source of collagen I is considered suitable: In this context, the smaller value, which is 100 nm, was found by (Li et al., 2005). Whereas, the bigger value was found by (Thomas et al., 2007) who produced nanofibers using Rat tail as source of collagen I. The measure of the nanofiber diameter is 600nm.

Moving to the use of green solvents, we firstly mention PBS where the two best values were found by Kitsara et al. (2015) and Bak et al. (2016). Kitsara et al. have found a measure of collagen I equal to 100 nm, using Atellocollagen as source of collagen I. And Bak et al. have found the measure of 105 nm using porcine skin as source of collagen I. Whereas, the bigger diameter measure, which is 630 nm, was found by Y. Zhou et al. (2015) who used calf skin as source of collagen I. Moving to the other type of green solvent which is Acetic Acid. For this solvent, we found that the two best measures were obtained by Castilla-Casadiego et al. (2016) and Lui et al. (2010). Castilla-Casadiego et al. have obtained a measure of 124 nm where the used collagen I was extracted from Bovine tendon and the measure found by Lui et al was 149 nm where Rat tail is used as source of collagen I. Whereas, the bigger diameter which is 550 nm was found by Punnoose et al. who used fish skin as source of collagen I.

As a conclusion, we affirm that the combination of HFIP as solvent and Bovine dermis as source of collagen I, and the combination of PBS as solvent and Atelocollagen as source of collagen I give the same measure of nanofiber which is 100 nm. So, as HFIP is considered as toxic solvent, the best green combination is found using PBS as solvent and Atellocollagen as source of collagen I.

5. CONCLUSIONS AND RECOMMENDATIONS

In this thesis, we introduced the different steps of formation of an electrospun collagen type I scaffold prepared to be used in healing meniscus tears.

Firstly, we introduced the morphology of the native human meniscus, the different types of Meniscus tears and their treatment methods. Secondly, we presented the process of Electrospinning collagen type I. In this section we started with presenting the collagen. After that, we explained the theory of Electrospinning and its different methods. Then, we moved to presenting the different solvents used until now in solving collagen I. Thirdly, we mentioned the different methods of Crosslinking as it is the technique that comes after Electrospinning and enable the enhancement of the mechanical properties of the electrospun scaffold. Fourthly, we cited the different techniques used to evaluate the quality of the formed scaffold. Fifthly, we mentioned the different combination of collagen with other substances and their application fields. Sixthly, we presented different works that are interested in forming scaffold dedicated to be used in healing meniscus tears. Finally, we discussed the influence of the solvent type and the source of collagen I on electrospun nanofiber diameter measures.

According to previous researches to solve collagen type I, 3 types of solvents were used. HFIP was considered as the best solvent but due to its toxicity and corrosive nature researchers tried to find other alternatives. In this context, PBS and Acetic acid were considered as green solvents for the collagen type I. Researchers, who used different type of solvents to solve collagen I extracted from different sources, found different measures of the electrospun collagen type I diameter. So, we conclude firstly that both the solvent type and the source of collagen I influence the measure of the electrospun fiber diameter and secondly according to our previous discussion, that the best green combination of solvent type and source of collagen I is found using PBS as solvent and Atellocollagen as source of collagen I. This combination gives a fiber diameter equal to 100nm. This

measure is very suitable since the collagen I fiber in the meniscus range from 50 to 200 nm and it is found using with a green solvent.

We suggest to researchers who are studying at this same subject that more experiences should be made using the green solvents (PBS or acetic acid) and Atellocollagen and porcine skin as source of collagen I.

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RESUME

Hana BAHRIA was born in 08/01/1990 in Monastir- TUNISIA. She studied her primary and secondary school in Moknine- Monastir. In 2008, she started her university studies by stduying 2 years preparatory in the Preparatory Institute for Engineering Studies of Monastir (IPEIM)-TUNISIA, specialty Mathematic- Physics. From 2010 to 2013, she studied in the National School of Engineering of Monastir (ENIM)-TUNISIA specialty Textile engineering. In 2014, she Worked in the laboratory of research '' Chimie appliquée et environnement UR 13 Es 63''. In 2015, she starts studying Master in Textile engineering in Cukurova University.